

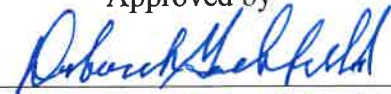
Variability in Antibacterial Chemical Defenses in Caribbean Sponges  
of the Genus *Aplysina*

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.

Oxford  
May 2016


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## ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the help and support of many amazing people. I would like to express my gratitude to all of my committee members: Dr. Deborah Gochfeld, Dr. Marc Slattery, and Dr. Kristine Willett for all of their help throughout this project. To Deb, thank you so much for your mentorship, patience, and constant support. While working with you, I have gained, not only scientific knowledge, but also, an appreciation for research. Thank you so much for this opportunity and for sharing your passion for marine ecology with me. To Marc, thank you for your encouragement throughout this project, and for allowing me to use your laboratory and equipment. I am grateful for all of the expert advice that you have given me.

I would also like to express my gratitude to Dr. Sridevi Ankisetty and Melinda Ansley for their leadership in the lab. To Sridevi, thank you for your guidance, and for patiently answering all of my chemistry questions. I am thankful to have had the chance to work with you. To Mindi, thank you for all of your help with the bacterial assays and the many other procedures in lab. I am grateful for your all of your advice, and I am so happy to have gained your friendship through this experience.

I would also like to thank two other Honors students for their help and company while working in the lab. Thank you Claire Vickers and Sarah Criddle.

Lastly, I would like to thank my family and friends for their constant support. To Mom, Dad, and Seth, I am so grateful for all of your love and encouragement throughout my entire life. Thank you for inspiring me to achieve great things, and for always believing in me. To my closest friends, Scotlynn Farmer, Abby Smith, Sosa Adah, Suman Ali, Ashley Singleton, and Macie Sheffield, thank you for the laughs, love, encouragement, and for keeping me sane throughout the years.

Portions of this research were funded by grants from the National Science Foundation to Dr. Gochfeld and from the National Oceanic and Atmospheric Association/National Institute of Undersea Science and Technology to Drs. Gochfeld and Slattery.

Samples were collected under Bahamas Department of Marine Resources Scientific Research Permit MAF/FIS1,12,46<sup>A</sup>,79 and US Virgin Islands Division of Fish and Wildlife Scientific Research, Retention and Export Permit DFW14046T. The staffs of the Bahamas Marine Ecocentre and the Center for Marine and Environmental Studies at the University of the Virgin Islands helped with sample collection.

## Abstract

Coral reefs represent one of the most diverse ecosystems on earth, providing habitat for at least 25% of all marine species. Coral ecosystems are faced with many stressors, both physical and biological, that are causing their continuing decline. Understanding the impacts of these stressors, and the resilience of coral reefs, is important to the long-term survival of these critical ecosystems.

As a major constituent of marine communities, sponges are an important phylum contributing to the ecology of coral reefs. Like other components of coral reef habitats, sponges also face a wide variety of biological stressors including pathogens, predators, and competitors. Chemical defenses serve as a key source of protection against these ecological threats. *Aplysina* is one of the most abundant genera of sponges found in the Caribbean, and these sponges are particularly susceptible to a disease that differs in prevalence among geographic locations, suggesting variability in resistance. Unfortunately, to date, the pathogen(s) responsible for causing this sponge disease remain elusive, but to determine whether chemical variation associated with different morphotypes and geographic locations translates into variability in bioactivity, this study evaluated the general antibacterial activity of sponge extracts against known coral pathogens and human enteric bacteria.

This study assessed the chemical variability among healthy individuals of three distinct sponge morphotypes, thin and thick *Aplysina cauliformis* and *A. fulva*, found over

various geographic regions in the Caribbean, including a pristine site in the Bahamas and sites in St. Thomas, US Virgin Islands, that vary in their amount of human impact. These sponges produce different chemical profiles between morphotypes and locations. This suggests possible genetic differences between morphotypes and supports using chemotaxonomic methods for classifying them as species. This study also assessed the antibacterial activity of organic extracts from each morphotype against four strains of bacteria. The bacteria selected included known coral pathogens and potential marine pathogens found in human waste. Antibacterial activity for all three morphotypes was highly selective against the four bacterial strains, and varied among morphotypes and their locations of origin. This variability in bioactivity may explain some of their differential susceptibility to disease.

## Table of Contents

<b>ACKNOWLEDGEMENTS</b>	<b>III</b>
<b>ABSTRACT</b>	<b>IV</b>
<b>LIST OF FIGURES</b>	<b>VII</b>
<b>LIST OF TABLES</b>	<b>VII</b>
<b>INTRODUCTION</b>	<b>1</b>
<i>CORAL REEFS' GLOBAL IMPORTANCE AND DECLINE</i>	1
<i>THE SIGNIFICANCE OF SPONGES ON THE CORAL REEF</i>	4
<i>CHEMICAL DEFENSES</i>	5
<i>VARIATION IN CHEMICAL DEFENSES</i>	6
<i>SPONGES IN THE GENUS APLYSINA</i>	7
<i>THIS STUDY</i>	9
<b>METHODS</b>	<b>11</b>
<i>SAMPLE COLLECTION</i>	11
<i>EXTRACT PREPARATION</i>	13
<i>CHEMICAL FINGERPRINTING</i>	14
<i>BACTERIAL GROWTH ASSAYS</i>	15
<b>RESULTS</b>	<b>18</b>
<i>CHEMICAL FINGERPRINTS</i>	18
<i>CONCENTRATIONS OF EXTRACTS</i>	22
<i>BACTERIAL GROWTH ASSAYS</i>	24
<b>DISCUSSION</b>	<b>31</b>
<i>VARIABILITY AMONG MORPHOTYPES</i>	31
<i>GEOGRAPHIC VARIABILITY IN CHEMICAL PROFILES</i>	32
<i>ANTIBACTERIAL ACTIVITY</i>	34
<b>CONCLUSION</b>	<b>38</b>
<b>REFERENCES</b>	<b>40</b>

## List of Figures

Figure 1. Thin <i>Aplysina cauliformis</i> , thick <i>A. cauliformis</i> and <i>Aplysina fulva</i> .....	11
Figure 2. Locations of collection sites used in this study.....	12
Figure 3. Representative HPLC chromatograms of thin and thick <i>Aplysina cauliformis</i> , and <i>Aplysina fulva</i> extracts.....	18
Figure 4. Non-metric multidimensional scaling plot showing the similarity of chemical constituents of all <i>Aplysina</i> morphotypes from all sites in St. Thomas and the Bahamas.....	19
Figure 5. Non-metric multidimensional scaling plots showing the similarity of the chemical constituents in thin <i>Aplysina cauliformis</i> , thick <i>Aplysina cauliformis</i> and <i>Aplysina fulva</i> among the four sites in St. Thomas. ....	21
Figure 6. Extract concentrations (mean $\pm$ SE) for thin <i>Aplysina cauliformis</i> , thick <i>Aplysina cauliformis</i> , and <i>Aplysina fulva</i> from four sites in St. Thomas and for thin <i>Aplysina cauliformis</i> , thick <i>Aplysina cauliformis</i> , and <i>Aplysina fulva</i> from all sites in St. Thomas combined and from the Bahamas. ....	23
Figure 7. Slopes (mean $\pm$ SE) of the bacterial growth curves for four bacterial strains (AC= <i>Aurantimonas coralicida</i> , SM= <i>Serratia marcescens</i> , VC= <i>Vibrio coralliilyticus</i> , YE= <i>Yersinia enterocolitica</i> ) exposed to extracts from thin <i>Aplysina cauliformis</i> , thick <i>Aplysina cauliformis</i> , and <i>Aplysina fulva</i> from four sites in St. Thomas.....	26
Figure 8. Slopes (mean $\pm$ SE) of the bacterial growth curves for four bacterial strains (AC= <i>Aurantimonas coralicida</i> , SM= <i>Serratia marcescens</i> , VC= <i>Vibrio coralliilyticus</i> , YE= <i>Yersinia enterocolitica</i> ) exposed to extracts from thin <i>Aplysina cauliformis</i> , thick <i>Aplysina cauliformis</i> , and <i>Aplysina fulva</i> from the Bahamas and four sites in St. Thomas.....	28
Figure 9. Mean ( $\pm$ SE) slopes of the bacterial growth curves for four bacterial strains (AC= <i>Aurantimonas coralicida</i> , SM= <i>Serratia marcescens</i> , VC= <i>Vibrio coralliilyticus</i> , YE= <i>Yersinia enterocolitica</i> ) exposed to extracts from thin <i>Aplysina cauliformis</i> , thick <i>Aplysina cauliformis</i> , and <i>Aplysina fulva</i> from all sites in St. Thomas combined and from the Bahamas.....	30

## List of Tables

Table 1. Sample sizes of each sponge morphotypes from each site that was extracted for chemical fingerprinting. ....	13
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## **Introduction**

### *Coral Reefs' Global Importance and Decline*

Coral reefs are one of the most diverse ecosystems on earth, providing habitat for a variety of all marine species (Spalding et al., 2001). Coral reefs are found in tropical regions around the globe. They are also present along the coasts of more than one hundred countries, including the United States (NOAA Coral Reef Conservation Program, 2015a). The annual global economic value of coral reefs is approximately \$30 billion, as they offer multiple goods and services for humans, such as fisheries, tourism, coastline protection, possible pharmaceutical leads, and aesthetic values (Cesar, 2003). The economic and intrinsic value of coral reefs worldwide is at risk due to major declines in coral populations. There are approximately 500 million people worldwide that rely on coral reefs for the many benefits they provide (NOAA Coral Reef Conservation Program, 2015a). These people will be dramatically affected by coral reef declines. It has been estimated that 19% of the existing area of coral reefs has already been lost, with 15-20% at a threat for loss within the next 10-40 years (Wilkinson, 2008). However, recent climatic events have accelerated this rate of decline (NOAA Coral Reef Conservation Program, 2015b). Because of this alarming rate of degradation, it is important to study the causes of coral reef decline and determine how to optimize coral reef resiliency.

Climate change has been identified as a key anthropogenic stressor to marine



communities on a global scale. Due to the burning of fossil fuels, there is an abundance of carbon dioxide in the atmosphere that is not only causing a rise in ocean temperature, but also changing the chemistry of the water. Over the past 100 years, the average temperature of the upper layers of the ocean has increased by 0.6 °C (Hoegh-Guldberg & Bruno, 2010). This rise in seawater temperature poses a huge threat to reefs, as it causes coral bleaching (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007). Coral bleaching refers to the loss of the corals' essential photosynthetic symbionts, known as zooxanthellae. Furthermore, an increase in temperature may also contribute to a higher frequency of marine diseases by increasing the growth and virulence of aquatic pathogens, or by decreasing the ability of marine species to produce immune defenses (Slattery & Gochfeld, 2012). Another consequence of climate change is ocean acidification. A decrease in the ocean's pH is caused by the carbon dioxide in the atmosphere dissolving into the ocean, making it more acidic (Hoegh-Guldberg & Bruno, 2010). This decrease in pH causes the shells and skeletons of many marine creatures to degrade, including the calcium carbonate skeletons of corals (Pandolfi et al., 2011).

Some more local anthropogenic factors that can be attributed to coral reef degradation include sedimentation from terrestrial runoff, land based pollution, and overfishing (Jackson et. al. 2014). These various forms of human impact are stimulated by coastal population growth and development and can increase the input of toxic substances to the marine environment, thereby reducing the quality of coastal waters and causing coral reef degradation (Smith et al., 2008). For example, organic pollutants can alter species diversity in the sponge community (Powell et al., 2014), and high levels of dissolved inorganic nitrogen and phosphate reduce coral calcification by up to 50%

(Fabricius, 2005). Marine diseases are frequently, but not exclusively, found in areas that are highly impacted by humans. For certain coral diseases, there is a correlation between disease prevalence and proximity to the shore, which is affected by many human derived stressors, especially sedimentation (Smith et al., 2008). Some coral diseases, such as yellow band disease, are positively correlated with nutrient enrichment originating from human activity (Bruno et al., 2003).

Coral disease is now recognized as a major cause of reef degradation and a severe threat to coral reefs around the world (Weil et al., 2006; Harvell et al., 2007). Many coral diseases have been identified and characterized morphologically (Sheridan et al., 2013); however, little is known about the etiology and pathology of these diseases. The best studied coral disease, Black Band Disease (BBD), is a polymicrobial disease characterized by complete tissue degradation (Sekar et al., 2008). BBD has been identified in most Caribbean coral species and at least 46 coral species in the Indo-Pacific, Red Sea and Indian Ocean (Woodley & Downs, 2016a). White band disease (WBD) has also had a damaging effect on the Caribbean reefs. *Acropora palmata* and *Acropora cervicornis*, two of the most important species for maintaining the structure of Caribbean reefs, were greatly affected by WBD (Woodley & Downs, 2016a), and are now listed as endangered species (Aronson et al., 2008a, b). Although coral diseases are most well studied, other coral reef organisms besides corals are affected by disease, including soft corals (Slattery et al., 2013), gorgonians (Woodley & Downs, 2016b), algae (Slattery & Gochfeld, 2012), and sponges (Webster, 2007), and diseases are affecting increasing numbers of species at increasing numbers of sites worldwide.

Although the future of coral reefs seems bleak, people around the globe are taking action to preserve these aquatic ecosystems. Many conservation programs have been initiated. Moreover, multiple reefs have been listed by the United Nations Education, Scientific and Cultural Organization (UNESCO) as World Heritage Sites (UNESCO World Heritage Centre, 2016). Since it is impossible to identify a single cause for the decline in coral reef populations, it is clear that monitoring the aforementioned stressors and the resiliency of coral reefs is important to the long-term survival of this underwater ecosystem (Bellwood, 2004), but understanding the causes and effects of these stressors is even more crucial.

### *The Significance of Sponges on Coral Reefs*

As one of the major constituents of benthic populations, sponges represent a leading phylum in the study of new chemical compounds found in the marine environment (Blunt et al., 2015). Now considered major habitat-forming members on Caribbean coral reefs, sponges serve significant roles in their environment. They provide food (Pawlik, 2011), and are a source of shelter (Westinga & Hoetjes, 1981) for other organisms on the reef. Furthermore, sponges increase water clarity (Strimaitis, 2012) and assist in regeneration of reefs by anchoring living corals to the reef frame (Wulff & Buss, 1979). In a study of the diversity and abundance of Caribbean coral reef sponges, when sponges were removed from corals, the corals experienced 40% mortality in six months, vs. 4% mortality when the sponges were present (Wulff & Buss, 1979). This evidence suggests that sponges could be essential to promoting coral reef resilience.

Conversely, it has also been suggested that sponges are aiding in the decline of corals. One of the major negative effects sponges have on corals is competition for space. Interactions between the Caribbean sponge, *Plakortis halichondrioides* and several coral species resulted in overgrowth of the coral by the sponge (Porter & Targett, 1988). Furthermore, the allelopathic activity of some sponge species has been shown to affect the symbiotic algae known as zooxanthellae found in corals, and result in a decrease in photosynthetic activity (Engel & Pawlik, 2000; Pawlik et al., 2007a).

### *Chemical Defenses*

Marine sponges, and other sessile species, face a wide variety of biological stressors, including pathogens, predators, and competitors. Because of their inability to move away from stressors, it is necessary that they possess alternative defensive capabilities. Some sponge species, such as *Tetilla* sp., possess sharp spicules to deter predators, while other species use camouflage as their defense by living in holes or crevices (Braekman and Daloz, 1986). Although sponges are the least evolutionarily advanced members of the animal kingdom, they are able to produce a wide variety of secondary metabolites, including alkaloids, terpenoids, and glycosides, for defense against herbivores, predators, competitors, and pathogens (Slattery & Gochfeld, 2012). Chemical defenses serve as a key source of protection against the ecological threats that affect sponges (Slattery & Gochfeld, 2012; Gochfeld et al., 2012a). It is possible that the abundance of sponges found on coral reefs can be attributed to their effective defense mechanisms (Braekman and Daloz, 1986; Pawlik, 2011; Loh & Pawlik, 2014).

### *Variation in Chemical Defenses*

The production of biologically active secondary metabolites is believed to impose a significant cost to the producing organism. The optimal defense theory (ODT) explains that the production of chemical defenses can be beneficial or costly to an organism depending upon the presence or absence of a biological stressor (Tollrian & Harvell 1999). Therefore, organisms must allocate their resources with respect to primary biological functions (Loh & Pawlik, 2014). Inducible defenses, which are only produced when faced with a specific biological threat, such as a predator, pathogen or competitor, have been suggested as a cost-saving alternative to constant production of defensive metabolites (Paul & Van Alstyne, 1992; Hay, 1996). In addition, when faced with additional extrinsic stressors, such as pollution or climate-associated impacts, the cost of defense may be even higher than under unstressful conditions (Slattery et al., 2008).

Understanding the variation in chemical defenses is important in order to begin to understand factors that control their production (Sacristán-Soriano et al., 2011a). There are multiple explanations for variations in chemical defenses among organisms.

Variation in the secondary metabolites of hard corals (Gochfeld et al., 2006; Pappas, 2010), octocorals (Slattery, 1999), and sponges (Webster et al., 2008; Gochfeld et al. 2012a) has been observed in response to colony condition, specifically in response to a pathogen or other stressor. In studies assessing differences in the chemical profiles of healthy versus diseased sponges, researchers found quantitative and qualitative differences in their chemical constituents (Webster et al., 2008; Gochfeld et al., 2012a). This may occur if the compounds found in high concentrations in the healthy sponges

defend them against the pathogens that cause disease, or if an inducible response to pathogenesis results in the presence of unique compounds in the diseased sponges. Differences in the chemical profiles could also be a result of trade-offs in defense production as a result of stress, or changes in the associated microbial community, which are believed to play a role in the production of secondary metabolites in sponges (Sacristán-Soriano et al., 2011b).

The production of chemical defenses in sponges also varies significantly within species over geographic regions. Because different geographical regions offer diverse conditions to the species that live there, variation in the chemical defenses of those species is expected. Geographical differences on various scales, from meters to hundreds of kilometers, result in variation in chemistry within the sponge species, *Stylissa massa* (Rohde et al., 2012). Variable chemical defense was also identified in the sponge *Plakortis angulospiculatus* over a depth gradient (Slattery et al., 2015).

### *Sponges in the Genus Aplysina*

*Aplysina* is one of the most prominent sponge genera in the Caribbean (Loh and Pawlik, 2014) and there is an extensive body of literature regarding its production of diverse secondary metabolites and its chemical ecology (Ciminiello et al., 1999; Sacristán-Soriano et al., 2011a, 2012; Gochfeld et al., 2012 a, b). This genus produces brominated alkaloids (BAs), which are found to vary within species (Nuñez et al., 2008) and at different geographic locations (Sacristán-Soriano et al., 2011a), and they are known to serve many ecological functions. Secondary metabolites in *Aplysina cauliformis* exhibited feeding deterrence against the bluehead wrasse, *Thalassoma*

*bifasciatum* (Pawlik et al., 1995) and the omnivorous sharpnose pufferfish, *Canthigaster rostrata* (Gochfeld et al., 2012a), as well as antibacterial activity against known coral pathogens and human enteric bacteria (Gochfeld et al., 2012a). These compounds also have allelopathic effects on corals and other sponges (Engel and Pawlik, 2000; Pawlik et al., 2007). Other brominated compounds are thought to play a role in deterring surface fouling in *Aplysina fistularis* (Walker et al., 1985).

*Aplysina* is susceptible to *Aplysina* Red Band Syndrome (ARBS), an infectious disease that is characterized by an advancing red band on the branch of the sponge that leaves behind necrotic tissue to be colonized by algae (Olson et al., 2006). While the etiologic agent of this disease is currently unknown, transmission studies suggest that it is contagious (Olson et al., 2006). The cyanobacterium responsible for the red color has been identified, but this bacterium alone is not sufficient to infect a healthy sponge, suggesting that ARBS may be a polymicrobial disease (Olson et al., 2013). ARBS is very prevalent in the Bahamas, affecting up to 15% of *Aplysina cauliformis* populations (Olson et al., 2006; Easson et al., 2013), whereas in St. Thomas, US Virgin Islands, it only affects an average of 1.5% of *A. cauliformis*, even though *A. cauliformis* is considerably more abundant in St. Thomas (Gochfeld, personal communication). In a study on the long-term effects of ARBS on *A. cauliformis*, sponges affected with ARBS were more likely to die and disappear from the reef (Easson et al., 2013). Sponges affected by ARBS have been found adjacent to healthy sponges of the same species, suggesting variability in their resistance to infection (Olson et al., 2006). In an antimicrobial assay using extracts from healthy and diseased *A. cauliformis*, the extracts presented similar antibacterial activity, suggesting that diseased sponges are not entirely

immunocompromised, even though their chemical constituents differ (Gochfeld et al., 2012a).

### *This Study*

This study assessed the chemical variability among healthy sponges of three distinct *Aplysina* spp. morphotypes found over wide geographic regions in the Caribbean. To date, thin and thick morphotypes of *Aplysina cauliformis* are considered conspecifics, whereas *Aplysina fulva* differs from *A. cauliformis* by only a few base pairs, depending on the gene sequenced (Sperling et al., 2011). The convention has been to consider *A. fulva* a distinct species, but this has been questioned by scientists using molecular techniques. However, these three branching *Aplysina* morphotypes co-occur on many Caribbean reefs, and they vary in color, texture, size and the production of a bright blue exudate upon handling (Gochfeld, personal communication); thus, it is possible that these three morphotypes possess differences in chemistry that can be used to distinguish them and endow them with different ecological virtues. In addition, any geographic variability in chemical compounds that these sponges produce may result in variability in their defensive characteristics, which could play a role in their protection from pathogens and confer differential levels of host resistance to disease (Gochfeld & Aeby, 2008) that could help explain differences in the prevalence of ARBS across the Caribbean (Gochfeld, personal communication).

To determine whether different morphotypes possess different chemical profiles, multiple major metabolites were measured in extracts from each sponge morphotype collected from the Bahamas, where ARBS is common (Olson et al., 2006; Easson et al.,



2013), and from several sites in St. Thomas, U.S. Virgin Islands, where ARBS is rare (Gochfeld, personal communication). In addition, to assess geographic variability over a finer spatial scale, chemical variability was assessed among different sites within St. Thomas that vary in their relative amount of human impact. Along with variation in chemical profiles, this study also evaluated the antibacterial activity of these sponge extracts against known coral pathogens and human enteric bacteria, to determine whether chemical variation associated with different morphotypes and geographic locations translates into variability in antibacterial activity that might provide differential protection against pathogens.

## Methods

### *Sample Collection*

Healthy individuals of the three *Aplysina* morphotypes (thin and thick *A. cauliformis* and *A. fulva*; Figure 1) were collected from Brewer's Reef, Saba Island, Flat Cay, and Savana Cay in St. Thomas, US Virgin Islands, as well as from Lee Stocking Island, Exuma Cays, Bahamas (Figure 2). Sponges were collected underwater into separate resealable plastic bags and taken to the lab, where they were promptly frozen. The frozen sponges were then transferred to the National Center for Natural Products Research (NCNPR) at the University of Mississippi.

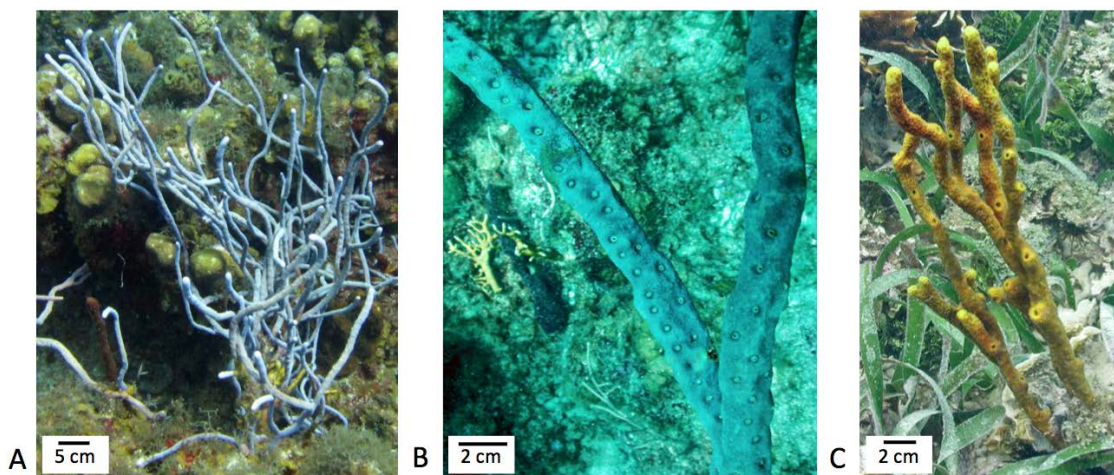


Figure 1. (A) Thin *Aplysina cauliformis*, (B) thick *Aplysina cauliformis*, (C) *Aplysina fulva* (Photos by D. Gochfeld).



Figure 2. Locations of collection sites used in this study. (A) Locations on the country scale: 1= St. Thomas, U.S. Virgin Islands; 2= The Bahamas. (B) Locations within St. Thomas: 1=Brewer's Reef; 2= Flat Cay; 3= Saba Island; 4= Savana Island.

### *Extract Preparation*

Table 1 shows the number of each sponge morphotype from each site that was extracted for chemical fingerprinting. The samples were removed from the freezer and a 5.0 cm piece was cut from each sponge, placed in a pre-weighed Whirl-Pak® bag. The sponges were then lyophilized for 24 hours. After freeze-drying, sponge dry weight was recorded. The sponge samples were then crushed into a fine powder.

Table 1. Sample sizes of each sponge morphotypes from each site that was extracted for chemical fingerprinting.

	Bahamas	ST-Brewer's	ST-Flat Cay	ST-Saba	ST-Savana
Thin <i>A. cauliformis</i>	10	10 <sup>1</sup>	10 <sup>1</sup>	10	10
Thick <i>A. cauliformis</i>	10	10	10	4	10
<i>A. fulva</i>	10	10	10	10	10

<sup>1</sup>One sample was used for assay method development so only n=9 were used in the antibacterial assays.

Organic extractions were performed on each sample by placing 0.3 grams of each lyophilized sponge into a 50 ml centrifuge tube and submerging each sample in 15 mL of high performance liquid chromatography (HPLC)-grade methanol. The tubes were then sonicated for 15 minutes and placed into a centrifuge for 10 minutes. The extract was decanted into a pre-weighed vial and the solvent was removed under vacuum. Extractions were repeated twice for a total of three times. The final weights of the combined extracts were recorded.

To determine the natural environmental concentrations of the extracts, the displacement volume was determined for a sub-sample of three sponges from each

collection. The average displacement volume of the three samples and the dry weight of each extract were used to calculate the natural extract concentrations for each sponge. Overall extract concentrations (g/mL) were compared using a two-way ANOVA with country and morph as fixed factors. One-way ANOVAs were performed to compare extract concentrations for each morphotype between the St. Thomas sites.

### *Chemical Fingerprinting*

For chemical fingerprinting, each of the organic extracts was dissolved in HPLC-grade methanol to a concentration of 5 mg/mL. Each vial was sonicated to ensure that the sample was dissolved. The vials were then placed into a centrifuge for 10 minutes so that any insoluble particles would be forced to the bottom, and avoid being pipetted into the HPLC vials. The extracts were then transferred into HPLC vials and 20  $\mu$ l of each extract was injected into a Waters Alliance 2685 HPLC system. A Phenomenex 5  $\mu$ m Luna C-18 250 x 4.6 mm column was used with a gradient elution of solvent mixtures (0-5 minutes: 90% solvent A [HPLC-grade water with 0.05% trifluoroacetic acid (TFA)], 10% solvent B [HPLC-grade acetonitrile with 0.05% TFA]; 5-25 minutes: A=50% solvent A; 25-40 minutes: 30% solvent A). Chromatograms for each sample at wavelength 254 nm were integrated to quantify areas under the curve for 18 peaks selected from a combination of representative spectra from all three morphotypes. The overall chemical profiles for each of the three morphotypes in St. Thomas and the Bahamas were compared using analysis of similarity (ANOSIM) in PRIMER v6 (Clarke & Gorley, 2006). A one-way analysis of similarity percentages (SIMPER) using

morphotype as the fixed factor was used to determine which peaks showed the greatest dissimilarity among the morphotypes and sites and was also performed in PRIMER v6.

### *Bacterial Growth Assays*

#### Bacterial selection and culture conditions

To date, a single specific pathogen responsible for ARBS has not been cultured (Gochfeld, personal communication); therefore, the strains of bacteria selected for the antibacterial assays included known coral pathogens and terrestrial pathogens that can cause disease in humans and can survive in the ocean, and therefore have the potential to become pathogenic to sponges. *Aurantimonas coralicida* and *Vibrio coralliilyticus* have both been identified as coral pathogens (Ben-Haim et al., 2003; Denner et al., 2003). *Serratia marcescens* and *Yersinia enterocolitica* are human pathogens that can enter the marine ecosystem via human waste and contamination (Gochfeld and Aeby, 2008), and *S. marcescens* has also been reported to cause coral disease (Patterson et al., 2002). The bacteria were cultured under conditions provided by the American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), from which they were purchased (Gochfeld and Aeby 2008). These included marine broth at 28°C for *A. coralicida* and *V. coralliilyticus*, Trypticase Soy Broth media at 28°C for *S. marcescens*, and Tryptose media at 32°C for *Y. enterocolitica*.

#### Assay Procedures

The antibacterial activity of *A. cauliformis* and *A. fulva* extracts was assessed using 96-well plate assays. Each assay was run in triplicate. For each morphotype at each site, 10 replicate sponge extracts (except as noted in Table 1) were tested. Sponge extracts were dissolved in dimethyl sulfoxide (DMSO) to a standard concentration of 100 mg/mL. Each bacterial strain was cultured for 24 hours and then diluted to an optical density (OD<sub>600</sub>) absorbance reading of between 0.09 and 0.11 on an Eppendorf BioPhotometer. For each sponge extract, the triplicate experimental wells contained 190  $\mu$ l of the bacterial culture and 10  $\mu$ l of the sponge extract. Three wells of each of the following were used as controls: 200  $\mu$ l of media only, 200  $\mu$ l of the bacterial culture alone, and 195  $\mu$ l of the bacterial culture plus 5  $\mu$ l of 1mg/ml ciprofloxacin. Two wells containing 190  $\mu$ l of the media plus 10  $\mu$ l of each sponge extract were also used as controls for the color of the extract. The total volume of each well was 200  $\mu$ L. Optical density (absorbance) readings were taken initially (0 hours) and at 24 hours on a BioTek Synergy HT Multi-Detection Microplate Reader. Between readings, the plates were placed on a shaker in an incubator at the appropriate temperature for that particular bacterial strain. To compare the slopes of the 24 hour growth curves from assays performed at different times and on different plates, the mean optical density of the three control wells (bacterial culture alone) was subtracted from the optical density of each well containing an extract in that assay, in order to account for natural bacterial growth in each assay. Additionally, the average optical density of the two wells containing extract + media was also subtracted from those wells in order to account for the dark color of the extracts. The positive control, ciprofloxacin, had a significant inhibitory effect on bacterial growth in all of the assays, but those data were not used in any further analyses.

To assess whether extracts from different morphotypes and locations exhibited significant activity against the four bacterial strains, the mean slope of the growth curves for each extract against each bacterial strain was calculated. T-tests were performed to determine whether the mean slopes from each morphotype at each location differed significantly from zero, indicating significant inhibitory (negative value) or stimulatory (positive value) activity. For each bacterial strain, one-way ANOVAs were performed on the mean slopes of the growth curves for extracts from St. Thomas, using both site and morph as the fixed factor. Sites and morphs from significant ANOVAs were compared using Fisher's Partial Least-Squares Difference post-hoc tests. Two-way ANOVAs were performed to compare levels of antibacterial activity among morphotypes and country, using country and morphotype as fixed factors, followed by Tukey's pair-wise comparisons for significant interactions.



## Results

### *Chemical Fingerprints*

To compare chemical constituents of the three morphotypes, 18 peaks were quantified from the HPLC chemical fingerprints. The chemical profiles differed significantly between morphotypes. Obvious qualitative differences are evident when comparing the HPLC chromatograms (Fig. 3), and significant quantitative differences were identified by ANOSIM ( $R=0.953$ ,  $P=0.001$ ).

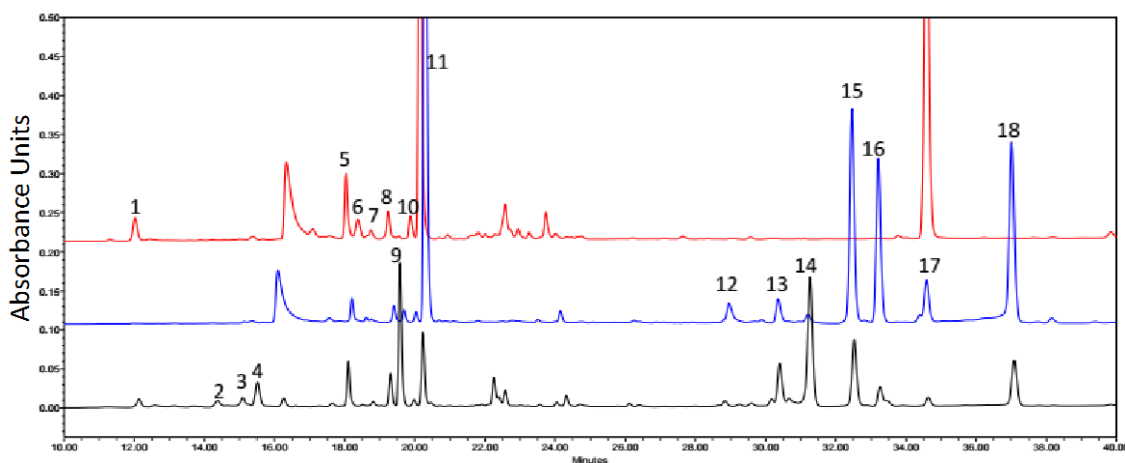


Figure 3. Representative HPLC chromatograms of thin (top, red) and thick (middle, blue) *Aplysina cauliformis*, and *Aplysina fulva* (bottom, black) extracts at 254 nm.

When the overall chemical profiles of all of the samples are considered together in an nMDS plot, the three morphotypes separate discretely, but sponges from the Bahamas and St. Thomas had overlapping distributions (Fig. 4).

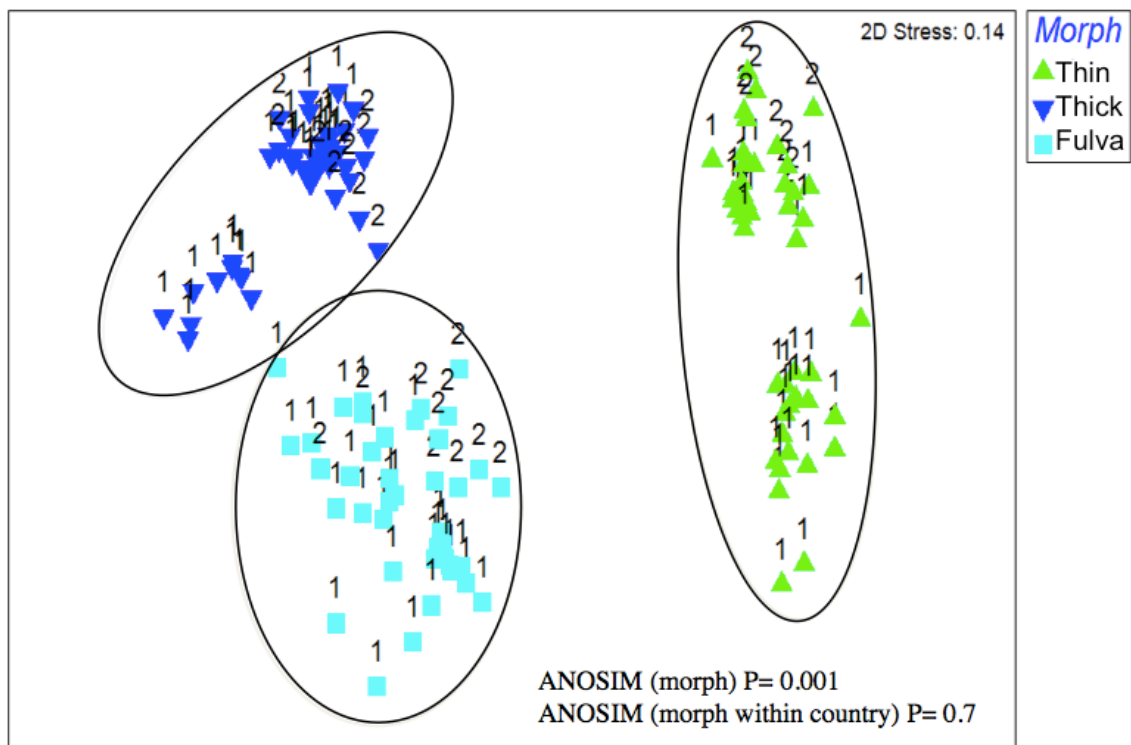


Figure 4. Non-metric multidimensional scaling plot showing the similarity of chemical constituents of all *Aplysina* morphotypes from all sites in St. Thomas (1) and the Bahamas (2). Circles represent significant groupings by ANOSIM.

Some compounds were only present in either one or two of the extract types (e.g. peaks 13, 14, 15, 16, and 18 were absent in thin *A. cauliformis*, peaks 3 and 4 were only found in *A. fulva*), indicating qualitative differences in the chemical profiles. Quantitative differences are indicated by the difference in the size of the peaks for compounds found in all three extracts (e.g. peaks 6, 9, 11 and 17) (Fig. 3).

SIMPER analysis indicated that 80% of the dissimilarity between thin and thick *A. cauliformis* morphotypes was due to peaks 15, 17, 1, 11, 10, 18, and 16, respectively.

Peaks 1, 17, 15, 14, 11, 10, 16, 9, 13, and 8 were responsible for 74% of the dissimilarity between thin *A. cauliformis* and *A. fulva*. Lastly, peaks 15, 11, 14, 10, 17, 18, 9, 16, 8, and 13 accounted for 59% of the dissimilarity between thick *A. cauliformis* and *A. fulva*.

Chemical fingerprints for all three morphotypes among the four sites in St. Thomas differed significantly (ANOSIM:  $R=0.994$ ,  $P=0.001$ ). Chemical fingerprints for the thin *A. cauliformis* varied significantly among sites (ANOSIM:  $R=0.597$ ,  $P=0.001$ ), and pairwise tests showed significant differences between samples from all four sites except between Flat Cay and Savana (Pairwise Test:  $R=0.118$ ,  $P=0.089$ ) (Fig. 5A). Thick *A. cauliformis* also had significantly different chemical fingerprints (ANOSIM:  $R=0.56$ ,  $P=0.001$ ). Pairwise tests for the thick morphotypes from Savana differed significantly when compared to thick samples at other St. Thomas sites, Brewer's Reef (Pairwise Test:  $R=0.942$ ,  $P=0.001$ ), Flat Cay ( $R=0.946$ ,  $P=0.001$ ), and Saba Island ( $R=0.872$ ,  $P=0.003$ ) (Fig. 5B). *Aplysina fulva* also exhibited distinct chemical profiles compared to thin and thick *A. cauliformis* (ANOSIM:  $R=0.583$ ,  $P=0.001$ ), and pairwise tests showed a significant difference between all three sites ( $P=0.001$  for all pairwise comparisons) (Fig. 5C). Data for *A. fulva* from Savana is not available because *A. fulva* was not found at that site.

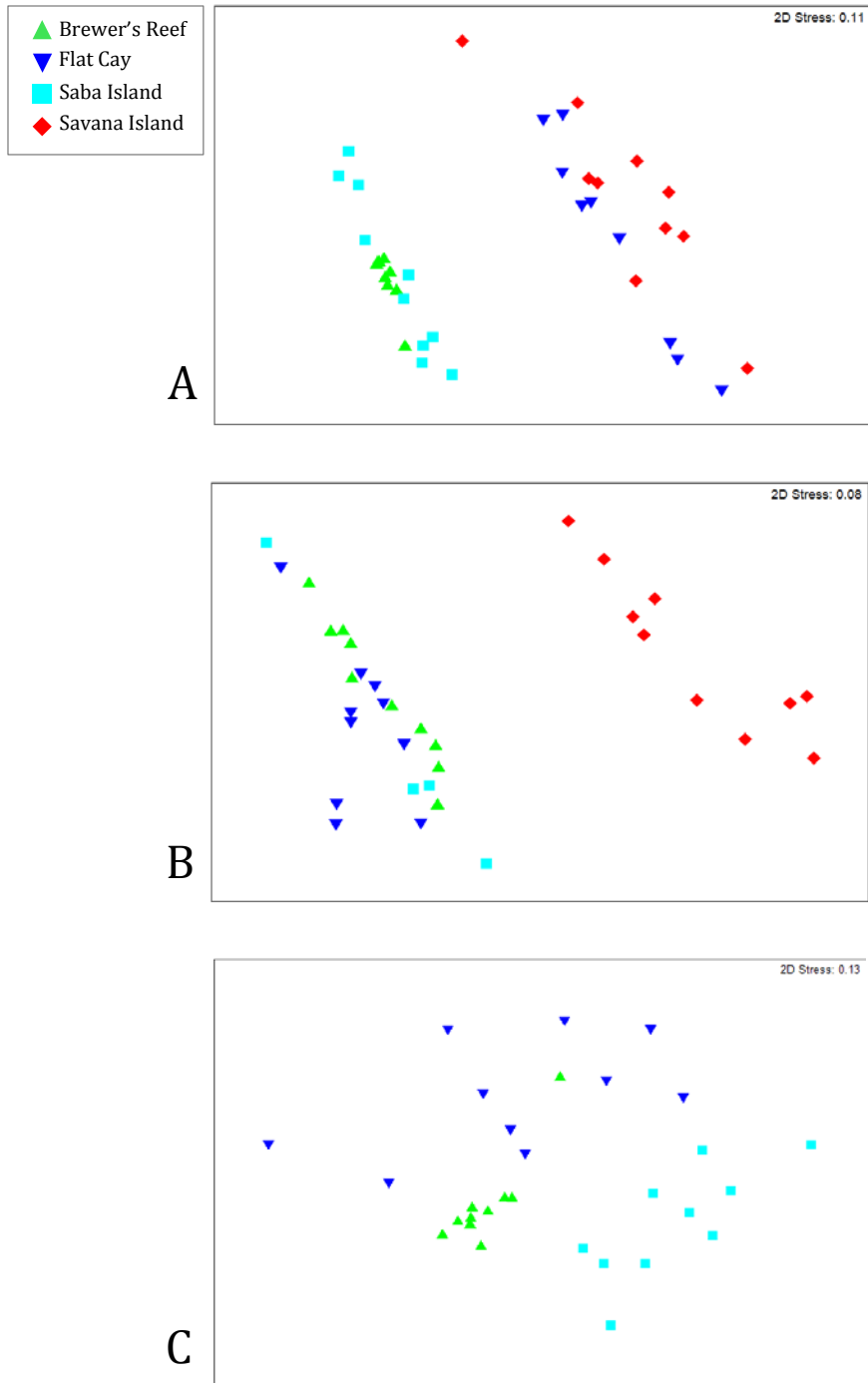


Figure 5. Non-metric multidimensional scaling plots showing the similarity of the chemical constituents in (A) thin *Aplysina cauliformis*, (B) thick *Aplysina cauliformis* and (C) *Aplysina fulva* among the four sites in St. Thomas.

### *Concentrations of Extracts*

The concentrations of the extracts from *Aplysina* were highly variable between the three morphotypes. Figure 6A shows the mean extract concentrations for each morphotype by site. On a local scale, only *A. fulva* produced significantly different concentrations of extracts from the three different reefs in St. Thomas (Brewer's Reef, Flat Cay, and Savana Island; One-way ANOVA:  $P=0.0046$ ). For *A. fulva*, metabolite concentration in the samples from Brewer's Reef differed significantly from those from Flat Cay and Saba Island (One-way ANOVA:  $P= 0.0013$ ,  $P= 0.0307$ , respectively). Extract concentrations did not vary by site for either thin or thick *A. cauliformis* samples from St. Thomas (One-way ANOVA:  $P=0.16$  for both morphotypes). When extract concentrations from all morphotypes at all sites within St. Thomas were combined and compared to those from the Bahamas, a significant country x morphotype (Two-way ANOVA,  $p < 0.05$ ) was observed. Figure 6B shows the average extract concentrations from each morphotype for all of the St. Thomas sponges alongside those from the Bahamas sponges. Concentration of the extracts differed significantly between morphotypes (Two-way ANOVA, Morphotype:  $df= 2$ ,  $F= 21.5$   $P< 0.001$ ), but not between countries (Two-way ANOVA, Country:  $df= 1$ ,  $F= 3.7$   $P= 0.0555$ ).

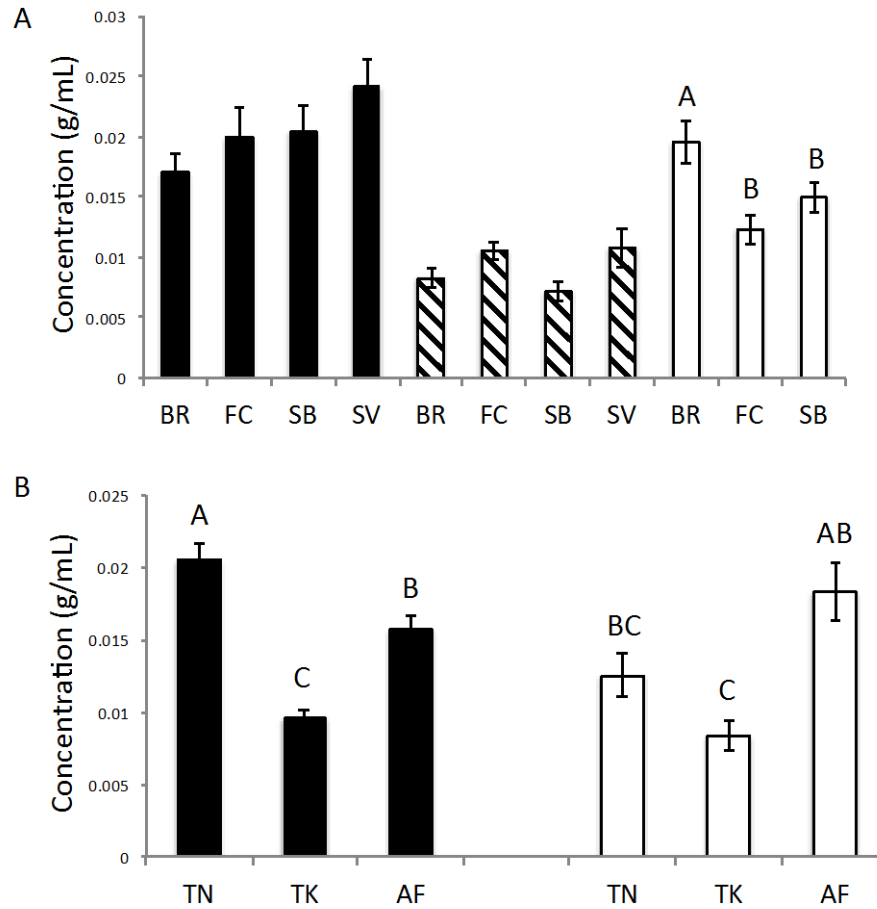


Figure 6. (A) Extract concentrations (mean  $\pm$  SE) for thin *Aplysina cauliformis* (black bars), thick *Aplysina cauliformis* (hatched bars), and *Aplysina fulva* (white bars) from four sites in St. Thomas (BR=Brewer's Reef, FC=Flat Cay, SB=Saba Island, and SV=Savana Island). Letters indicate sites that differ from each other significantly by Fisher's PLSD post-hoc test. (B) Extract concentrations (mean  $\pm$  SE) for thin *Aplysina cauliformis* (TN), thick *Aplysina cauliformis* (TK), and *Aplysina fulva* (AF) from all sites in St. Thomas combined (black bars), and from the Bahamas (white bars). Letters indicate groups that differ from each other significantly by Tukey's post-hoc tests.

## *Bacterial Growth Assays*

In all of the bacterial growth assays, the positive control ciprofloxacin inhibited the growth of all four bacterial strains tested. Overall, out of 56 assays (Two *A. cauliformis* morphotypes from five locations, and *A. fulva* from four locations, tested against four bacterial strains), 38 (67.9%) exhibited significant inhibitory and nine (16.1%) exhibited significant stimulatory activity, whereas nine (16.1%) showed no significant activity. Figures 7 and 8 show the slopes of the growth curves for each morphotype at each site against each bacterial strain, with asterisks representing significant antibacterial activity based on t tests ( $P < 0.05$ ).

Although all three morphotypes exhibited antibacterial activity, they exhibited a high degree of selectivity against the four different bacterial strains (Fig. 7). Results from t-tests show inhibitory activity for most of the extracts against *Aurantimonas coralicida*, *Serratia marcescens*, and *Vibrio coralliilyticus*, whereas, thin *A. cauliformis* showed significant stimulatory activity in all locations against *Yersinia enterocolitica*.

There were also significant differences in antibacterial activity for all three morphotypes between sites in St. Thomas for the four bacterial strains (Fig. 7). For the thin morphotypes, there were significant differences in antibacterial activity among sites for the assays using *A. coralicida* (One-way ANOVA:  $P < 0.001$ ), *S. marcescens* ( $P = 0.0127$ ), and *V. coralliilyticus* ( $P < 0.001$ ). For the thick morphotype, there were significant differences among sites for all four bacterial strains (One-way ANOVA:  $P < 0.001$  for *A. coralicida*, *S. marcescens*, and *V. coralliilyticus*;  $P = 0.23$  for *Y. enterocolitica*). Assays using *A. fulva* resulted in significant differences in antibacterial activity only for *V. coralliilyticus* (One-way ANOVA:  $P < 0.001$ ). The greatest variability

was seen in the *Y. enterocolitica* assays, in which all three morphotypes from Saba Island stimulated *Y. enterocolitica* growth, while activity against this bacterium was more variable or not significant at the other sites. Likewise, all three morphotypes from the Bahamas inhibited the growth of all three bacterial strains except *Y. enterocolitica*, whose growth was stimulated by all three morphotypes from the Bahamas (Fig. 7).



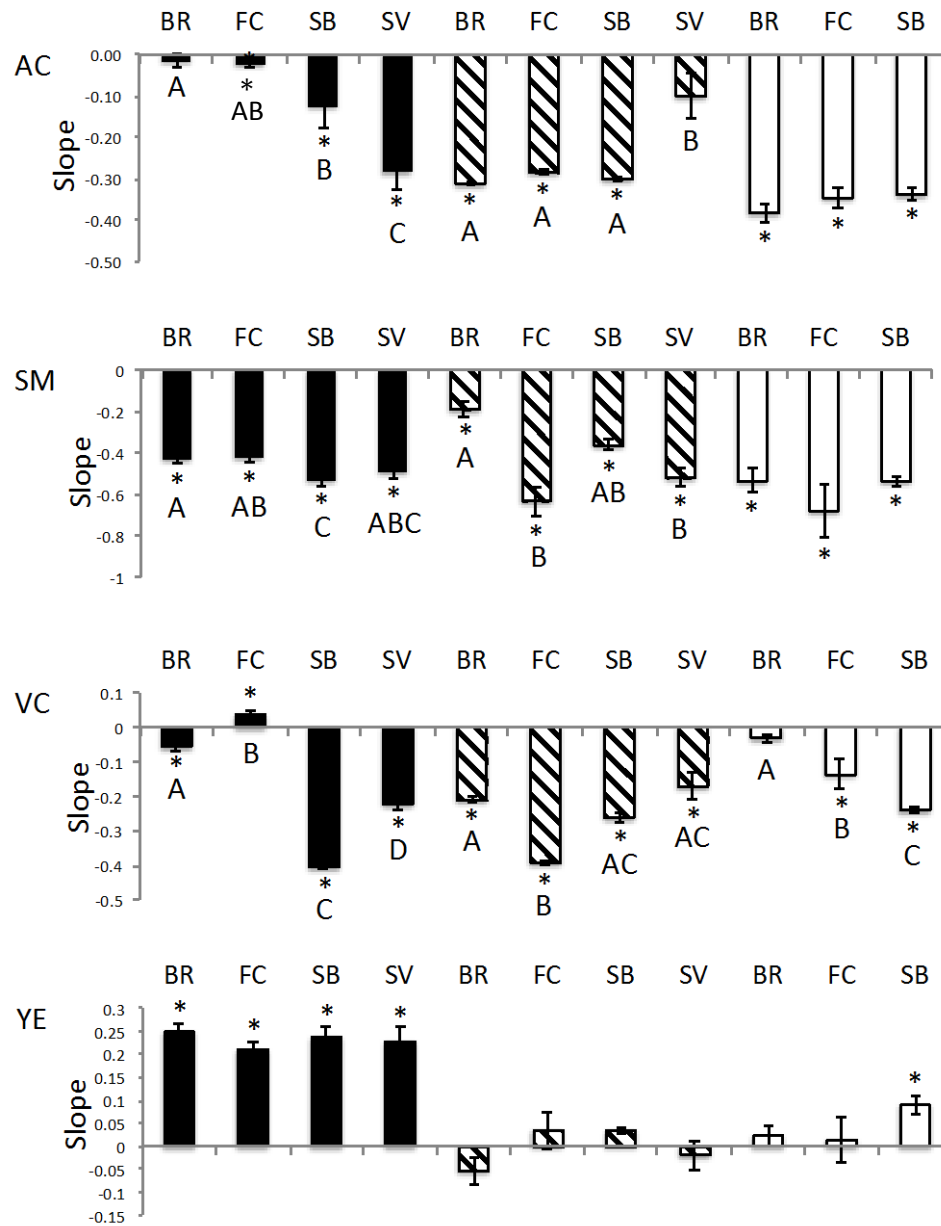


Figure 7. Slopes (mean  $\pm$  SE) of the bacterial growth curves for four bacterial strains (AC=*Aurantimonas coralicida*, SM=*Serratia marcescens*, VC=*Vibrio coralliilyticus*, YE=*Yersinia enterocolitica*) exposed to extracts from thin *Aplysina cauliformis* (black bars), thick *Aplysina cauliformis* (hatched bars), and *Aplysina fulva* (white bars) from four sites in St. Thomas (BR=Brewer's Reef, FC=Flat Cay, SB=Saba Island, and SV=Savana Island). Greater negative values indicate more inhibitory extracts. \*Slope is significantly different from 0 at  $p < 0.05$  (t-tests). Within each morphotype, letters indicate sites that differ from each other significantly by Fisher's PLSD.

Within the four sites on St. Thomas, and within the Bahamas, antibacterial activity varied significantly by morphotype for certain bacterial strains (Fig. 8). At Brewer's Reef and Saba Island, there were significant differences among morphotypes for all four bacterial strains (Brewer's Reef: One-way ANOVA:  $P < 0.0001$  for all four strains; Saba Island:  $P = 0.0009$  for *A. coralicida*,  $P = 0.0023$  for *S. marcescens*, and  $P < 0.0001$  for *V. coralliilyticus* and *Y. enterocolitica*; Fig. 8). At Flat Cay, there were significant differences in bioactivity among morphotypes for *A. coralicida* (One-way ANOVA:  $P < 0.001$ ), *V. coralliilyticus* ( $P < 0.005$ ), and *Y. enterocolitica* ( $P = 0.0027$ ), but not for *S. marcescens* ( $P = 0.12$ ). At Savana, only assays using *A. coralicida* and *Y. enterocolitica* resulted in significant differences in bioactivity among morphotypes (One-way ANOVA:  $P = 0.014$  and  $P < 0.001$ , respectively). All bacterial strains tested against the Bahamas extracts resulted in significant antibacterial activity among morphotypes (One-way ANOVA,  $P \leq 0.019$ ), except for *Y. enterocolitica*, which exhibited a trend toward significant activity ( $P = 0.0532$ ).

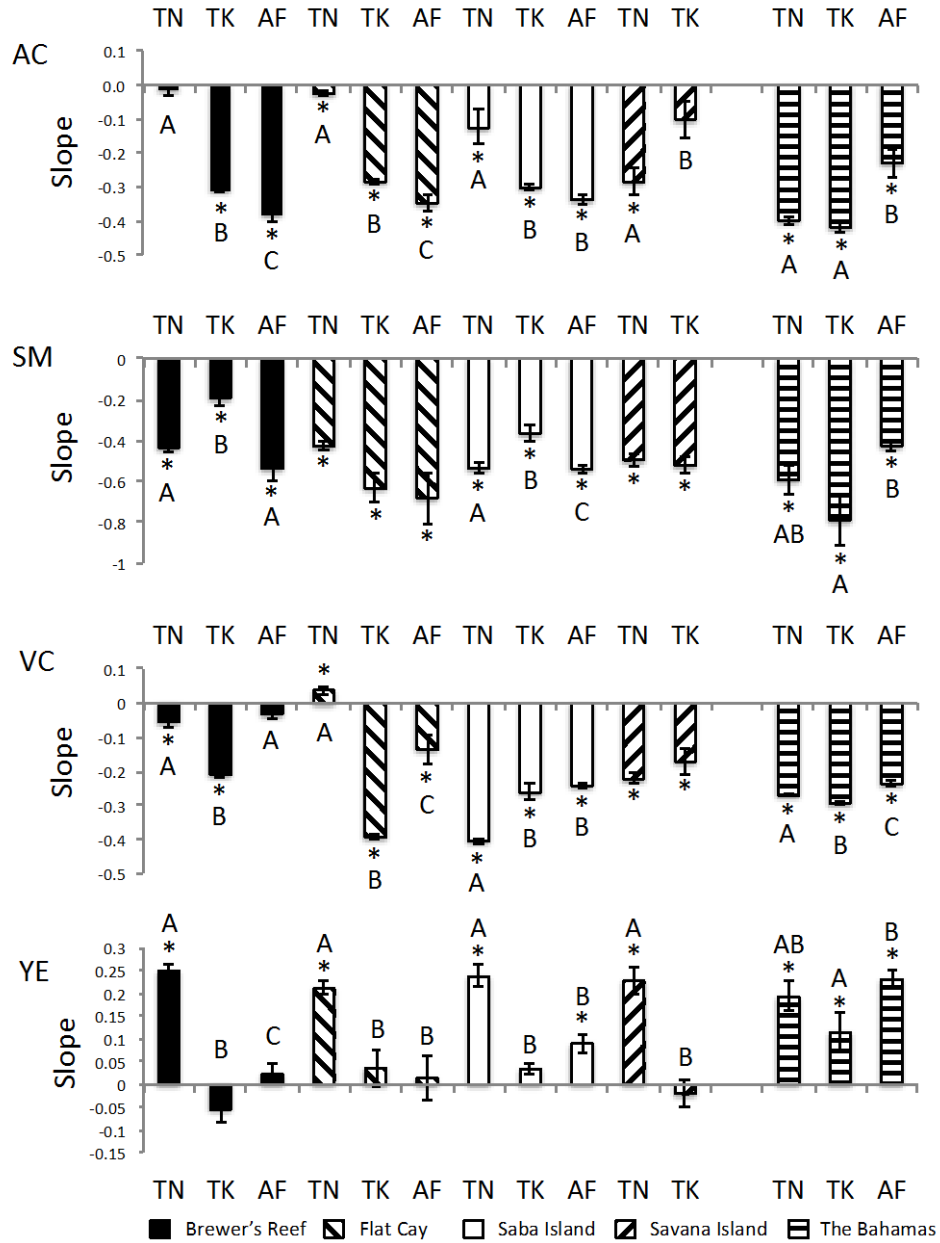


Figure 8. Slopes (mean  $\pm$  SE) of the bacterial growth curves for four bacterial strains (AC=*Aurantimonas coralicida*, SM=*Serratia marcescens*, VC=*Vibrio coralliilyticus*, YE=*Yersinia enterocolitica*) exposed to extracts from thin *Aplysina cauliformis* (TN), thick *Aplysina cauliformis* (TK), and *Aplysina fulva* (AF) from the Bahamas and four sites in St. Thomas. Greater negative values indicate more inhibitory extracts. \*Slope is significantly different from 0 at  $p < 0.05$  (t-tests). Within each site, letters indicate morphotypes that differ from each other significantly by Fisher's PLSD.

When all sites within St. Thomas were combined, significant country x morphotype interactions were observed for all bacterial strains (Two-way ANOVA,  $p < 0.05$ ) except *V. coralliilyticus* ( $P=0.455$ ). Figure 9 shows the average slopes of the growth curves for extracts from each morph for all of the St. Thomas sponges alongside those from the Bahamas sponges. Antibacterial activity in the extracts from St. Thomas differed significantly from the Bahamas extracts against all four bacteria (Two-way ANOVA, Country:  $df= 1$   $F= 20.20$   $P< 0.001$  for *A. coralicida*;  $df= 1$ ,  $F= 5.33$ ,  $P= 0.0226$  for *S. marcescens*;  $df= 1$ ,  $F= 8.98$ ,  $P= 0.0033$  for *V. coralliilyticus*;  $df= 1$ ,  $F= 20.61$ ,  $P< 0.001$  for *Y. enterocolitica*). There were also significant differences in antibacterial activity based on sponge morphotype for two of the bacterial strains (Two-way ANOVA, Morphotype:  $df= 2$ ,  $F= 4.35$ ,  $P= 0.0149$  for *V. coralliilyticus*;  $df= 2$ ,  $F= 21.18$ ,  $P< 0.001$  for *Y. enterocolitica*), but not for the other two ( $df= 2$ ,  $F= 2.83$ ,  $P= 0.063$  for *A. coralicida*;  $df= 2$ ,  $F= 2.22$ ,  $P= 0.11$  for *S. marcescens*).

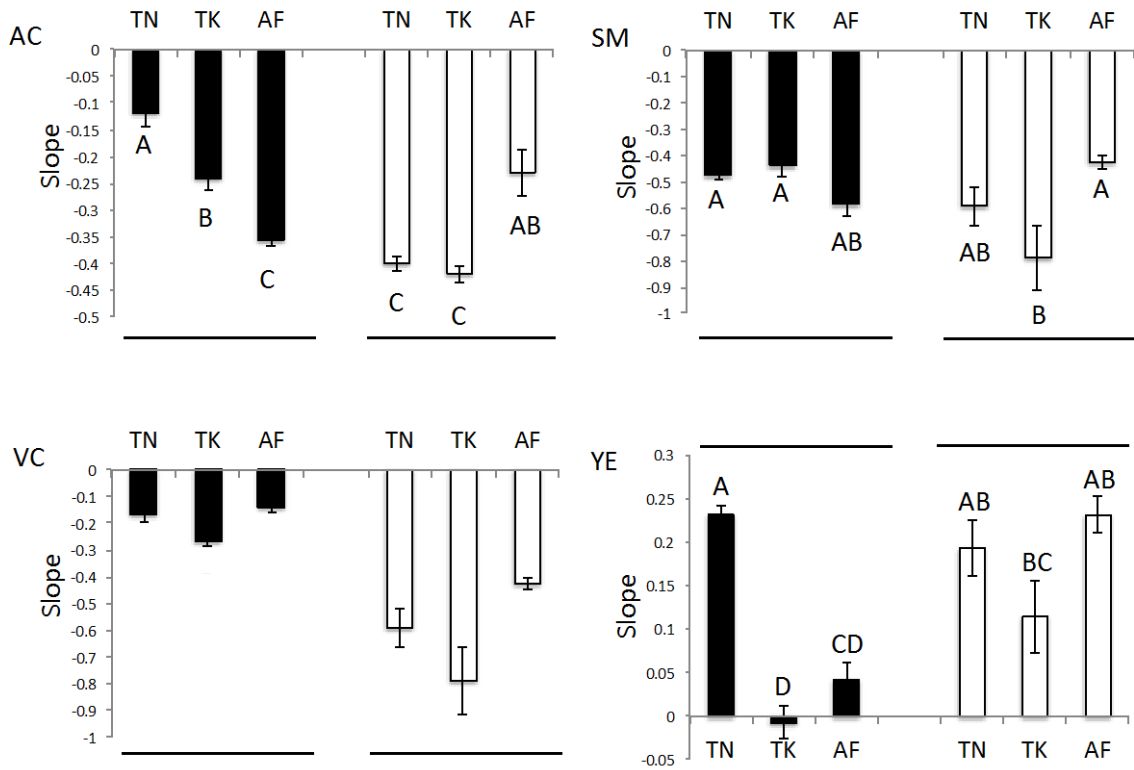


Figure 9. Mean ( $\pm$  SE) slopes of the bacterial growth curves for four bacterial strains (AC=*Aurantimonas coralicida*, SM=*Serratia marcescens*, VC=*Vibrio coralliilyticus*, YE=*Yersinia enterocolitica*) exposed to extracts from thin *Aplysina cauliformis* (TN), thick *Aplysina cauliformis* (TK), and *Aplysina fulva* (AF) from all sites in St. Thomas combined (black bars) and from the Bahamas (white bars). Larger bars indicate more inhibitory or stimulatory extracts. Lines indicate that the countries differ from each other significantly by least square means post-hoc tests. Letters indicate groups that differ from each other significantly by Tukey's post-hoc tests.

## Discussion

### *Variability Among Morphotypes*

Sponges of the genus *Aplysina* on Caribbean reefs show morphological and ecological variation. In addition to other tubular species not evaluated in this study, this genus occurs in three branching morphotypes, currently called thin *Aplysina cauliformis*, thick *Aplysina cauliformis*, and *Aplysina fulva*, which differ in their physical appearance. The thin morphotype branches extensively, does not produce an exudate when handled, and its diameter is about half that of the thick morphotype, which forms denser straight branches, and produces a blue exudate when handled. These are currently considered morphotypes rather than separate species, because genetic markers used to date have not been able to differentiate them (Gochfeld, personal communication). *Aplysina fulva* appears branching, thin, is yellow in color and also produces a blue exudate when handled. *Aplysina fulva* is considered a different, but genetically very similar species. In a comparison of the mitochondrial genome of *A. cauliformis* and *A. fulva*, the genomes were found to be the same length and only differed by six nucleotides, and authors questioned whether this is a sufficient difference for characterization at the species level (Sperling et al., 2011). Traditional taxonomic techniques for marine sponges (e.g., spicule composition and skeletal morphology) may not correctly determine true evolutionary relationships, because they are based primarily on morphological

characteristics that can vary in response to environmental factors. In a study done on the morphotypes of another marine sponge, *Suberites ficus*, three subspecies could not be morphologically differentiated (Sole-Cava & Thorpe, 1986). *Aplysina* spp. are particularly difficult to characterize using traditional taxonomy since they lack spicules and their internal fiber morphology is not distinctive (Gochfeld, personal communication). However, this study has demonstrated that the chemical profiles of each *Aplysina* morphotype can be used to differentiate them chemotaxonomically. Chemotaxonomy is not a new concept (Castellanos et al., 2003; Erpenbeck et al., 2012), but is not widely used, and could prove to be a more reliable method of classifying polymorphic marine taxa.

### *Geographic Variability in Chemical Profiles*

Sponges have the ability to modify their production of secondary metabolites in response to various stressors (Turon & Becerro, 1996; Gochfeld et al., 2012a). Since sessile marine organisms cannot move away from environmental stressors, these stressors can impact the ability of these organisms to produce secondary metabolites (Rohde et al., 2012). Determining how anthropogenic factors affect the chemical production of these sponges could help determine the relative ability of sessile organisms to protect themselves from biological stressors.

The four St. Thomas sites used in this study differ significantly in their levels of human impact. Savana Cay is the most pristine of the four sites, being furthest offshore (with lowest exposure to watershed development), while the others suffer from various levels of human derived stressors. Nearshore sites, such as Brewer's Reef, are heavily

impacted by sedimentation (Smith et al., 2008) and have higher levels of total nitrogen and total phosphorus, relative to offshore sites, such as Flat Cay, Saba Island, and Savana Cay (Sabine et al., 2015). Brewer's Reef is adjacent to a beach that is frequently visited and is associated with high vehicle traffic (Sabine et al., 2015). Offshore sites are less exposed to land-based pollution, which degrades the health of nearshore reefs (Sabine et al., 2015). Measurements of turbidity showed higher levels at Brewer's Reef than at Flat Cay or Saba Island (Sabine et al., 2015). However, a busy commercial port and sewage outflow is located upstream of Flat Cay (Smith et al., 2012).

There were significant variations in the chemical constituents produced by the *Aplysina* samples collected from four sites in St. Thomas. Thin and thick *A. cauliformis* collected from Savana are chemically distinct from those of Brewer's Reef and Saba Island (for thin; Fig. 5A) and Flat Cay (for thick, Fig. 5B), indicating that there is a significant difference in the metabolites produced by the sponges at Savana Cay. *Aplysina fulva* chemical profiles differ among the three sites at which they were collected (Brewer's Reef, Flat Cay, and Saba Island), likely indicating some differences in environmental conditions among those sites. However, *A. fulva* did not occur at Savana, suggesting that it may prefer habitats with greater exposure to anthropogenic stressors (e.g., runoff from land, which might provide nutrients for the sponges; Gochfeld et al., 2007). Extract concentrations for *A. fulva* samples from St. Thomas were significantly different between the three locations, with the highest concentration found in the samples from Brewer's Reef. It is possible that sponges from Brewer's Reef are producing more secondary metabolites as a defense against the relatively high levels of potentially pathogenic bacteria present in the sewage run-off.



### *Antibacterial Activity*

There are several links between the variability in chemical profiles and the antimicrobial activity of the sponges. For thin *A. cauliformis*, the chemical profiles of those from Brewer's Reef were significantly different than those from Saba Island. This corresponds with the antibacterial data where there were significant differences in antibacterial activity for the extracts from Brewer's Reef and Saba Island against all of the bacterial strains tested, except for *Y. enterocolitica*. For thick and thin *A. cauliformis*, samples from Savana Cay are chemically distinct from all other sites, and also differ in their antibacterial activity against *A. coralicida*. Furthermore, antibacterial activity for the thick extracts from Savana Island against both *A. coralicida* and *V. coralliilyticus* was significantly different from those of Flat Cay, which also correlates with the chemical profile data. This could be due to the higher abundance of stressors in Flat Cay associated with its relative proximity to shore. Lastly, the antibacterial activity of *A. fulva* against *V. coralliilyticus* was significantly different at all three sites, congruent with differences in chemical profiles. Site-specific variations in antibacterial activity may be attributed differences in the sponge-associated microbial communities, which could be related to differences in the water quality or levels of environmental stress. These variations in chemical profiles and bioactivity could also be explained by many biotic and abiotic factors such as pathogens (Slattery, 1999), predation pressures (Slattery & Paul, 2008) and water quality (Gochfeld et. al., 2012b).

Of the three morphotypes within St. Thomas, thin *A. cauliformis* extracts were the least active, inhibiting bacterial growth significantly in only 56.3% of the assays as compared to 68.8, and 66,7% for thick *A. cauliformis* and *A. fulva*, respectfully. This

suggests a possible genetic component to defense against the bacterial strains tested, and supports the contention that the thin and thick morphotypes are indeed genetically distinct. In addition, thin *A. cauliformis* tend to be more susceptible to disease, which may be associated with an overall lower level of antibacterial activity (Gochfeld & Aeby, 2008).

Sponges rely on their ability to produce secondary metabolites for defense against several biotic stressors, including predators, competitors, and pathogens. This study attempts to determine the extent to which three *Aplysina* morphotypes inhibit the growth of potentially harmful bacteria. A previous study on the antibacterial activity of *A. cauliformis* used disk diffusion assays (Gochfeld et al., 2012a); however, this study used 96-well plate liquid growth assays because they require less extract and offer better quantitative analysis. In the previous study, natural volumetric concentrations of healthy sponge extracts inhibited the growth of *Aurantimonas coralicida* and *Vibrio coralliilyticus*, which are two of the bacterial strains used in this study (Gochfeld et. al., 2012a). This is consistent with the results from the 96-well plate assays used in this experiment. Conversely, another study found that *A. cauliformis* extracts were inactive against a panel of eight strains of marine bacteria (Newbold et. al., 1999). An experiment by Rohde et al. (2012) showed inhibition of *Y. enterocolitica*, but not *V. corallyticus*, *S. marcescens*, or *A. coralicida* by crude extracts of the sponge *Stylissa massa*. These data indicate that sponge extracts are highly selective in their antibacterial activity, which is beneficial, as sponges may rely, at least in part, on their associated microbial communities to produce compounds that inhibit the growth of a broader spectrum of potential pathogens (Ritchie, 2006). Using resources to inhibit the growth of all bacteria

would not be advantageous to the sponge, when its resources could instead be used for other primary functions, such as growth or reproduction (Tollrian & Harvell 1999; Loh & Pawlik 2014). It would be more favorable for the sponge to inhibit only the organisms that may be detrimental to its health. Consistent with this, several of the extracts stimulated the growth of *Y. enterocolitica*. Extracts from thin *A. cauliformis* from St. Thomas, as well as all of the extracts from the Bahamas, stimulated the growth of *Y. enterocolitica*. It is possible that this bacterial strain is beneficial to the health of these sponges, possibly serving as a source of food for these filter feeders since it is not known to be a marine pathogen like the other three bacterial strains tested.

Extracts of thin *A. cauliformis* from Brewer's Reef and Flat Cay were not significantly inhibitory against *A. coralicida*, but thin extracts from Saba Island and Savana Cay were. Both Saba Island and Savana Cay are located farther offshore than Brewer's Reef and Flat Cay. Since the nearshore sites are more heavily impacted by anthropogenic stressors, sponges from these sites may be too stressed to put energy into chemically defending themselves against this pathogen. Conversely, the thick *A. cauliformis* extracts from Savana Cay were not significantly inhibitory towards *A. coralicida*, but the thick extracts from the other sites were. Results from the nMDS plot show that thick *A. cauliformis* extracts from Savana are chemically distinct from all other sites. It is possible that the *A. coralicida* is not present in Savana Cay, so thick extracts have not been exposed to it, and have therefore not developed resistance against it.

It is important to note that the assays in this experiment tested sponge extracts at 10X natural concentrations; therefore, the results of these assays may show more antibacterial activity than would occur at natural concentrations. However, the object of

this study was to test the relative differences between the morphotypes and sites, and since there are only minor differences in natural concentrations among those, the results are still relevant for addressing those questions.

## Conclusion

The results of this study showed significant differences in chemical profiles among all three morphotypes, which can be used to distinguish them chemotaxonomically. These differences translate into differences in antibacterial activity among the morphotypes and among geographic regions both within and between countries. *Aplysina* spp. in this study exhibited selectivity in antibacterial activity. These variations in chemical profiles and bioactivity between locations suggest that anthropogenic factors such as coastal development, sewage and agricultural runoff, and other sources of pollution, may affect sponges and other coral reef organisms directly or indirectly by exposing the reefs to sedimentation and toxic pollutants. The prevalence of sponges affected by *Aplysina* Red Band Syndrome differs among locations, with thin *A. cauliformis* exhibiting lower levels of ARBS in St. Thomas than in the Bahamas in spite of the low levels of anthropogenic impact in the Bahamas. Since the bacterial strains tested in this study are not directly associated with ARBS, it is possible that sponges could still have specific antibacterial activity against the pathogens that are responsible for ARBS. While the overall differences in chemical profiles of the sponges in this study may not be able to entirely explain the differences in antibacterial activity or disease prevalence between different locations, there are a few other possible explanations. It is possible that one or a few specific constituents from within the overall chemical profile are responsible for the biological activity, and therefore the patterns of chemistry and

antibacterial activity do not match exactly. Alternatively, rather than depending on chemical defenses, sponges could use alternative defense mechanisms against ARBS-associated pathogens. Differences in antibacterial activity could also be explained by the fact that pathogens may possess site-specific differences in type, abundance, and virulence. Furthermore, the differences in the levels of human impact between sites could affect not only the stress on the sponge, but also the types of pathogens in the water to which the sponges would be exposed.

Coral reef communities are constantly changing in response to local and regional stressors. Maintaining coral reef biodiversity is important, as humans receive many benefits from this natural environment. In order to preserve this biodiversity, constant observation of the effects of both natural and anthropogenic stressors on marine organisms is necessary. As a major component of coral reef ecosystems, sponges contribute to the diversity and complexity of the reef structure. Because of the array of secondary metabolites that sponges produce, they serve as a model organism for the study of marine ecology. Studying the variation in the chemical defenses of sponges at various scales provides insight into which stressors might be responsible for their production. Understanding the causes and effects of both biotic and abiotic stressors on the reef is essential to finding approaches to improve coral reef resilience.

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