On a reef far, far away: Offshore transport of floodwaters following extreme storms impacts sponge health and associated microbial communities

Running title: Offshore sponge microbiomes after extreme storms

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Originality-Significance Statement

Stressors associated with terrestrial runoff have contributed to substantial population declines in nearshore marine ecosystems worldwide over the last three decades. It has been assumed that offshore marine ecosystems (>100 km from land) are largely unaffected by terrestrial runoff. Our findings, however, suggest that flooding events can significantly impact offshore marine organisms, based on the detection of shifted microbiomes and human pathogens in offshore sponges after extreme storm events across two separate years, and lack of detection in a non-flooding year.

Summary

Terrestrial runoff can negatively impact marine ecosystems through stressors including excess nutrients, freshwater, and contaminants. Severe storms, which are increasing with global climate change, generate massive inputs of runoff over short timescales (hours to days); such runoff impacted offshore reefs in the northwest Gulf of Mexico (NW GoM) following severe storms in 2016 and 2017. Several weeks after coastal flooding from these events, NW GoM reefs experienced mortality (2016 only) and/or sub-lethal stress (both years). To assess the impact of storm-derived runoff on reef filter feeders, we characterized the microbiomes of two sponges, *Agelas clathrodes* and *Xestospongia muta*, during periods of lethal stress, sub-lethal stress, and no stress over a three-year period (2016-2018). Increased anaerobes during lethal stress indicate hypoxic conditions were associated with the 2016 mortality event. Additionally, we found evidence of wastewater contamination (based on 16S libraries and quantitative PCR) in sponges 185 km offshore following storms (2016 and 2017), but not during the non-flooding year (2018). We show that water quality changes following severe storms can impact offshore benthic organisms, highlighting the need for molecular and microbial time series from near- and offshore reef ecosystems, and for the continued mitigation of stormwater runoff and climate change impacts.

INTRODUCTION

Tropical coral reef ecosystems have evolved in the context of nutrient-poor (oligotrophic) waters. Thus, when nutrient-laden (eutrophic) terrestrial runoff mixes with reef-associated waters, this can directly or indirectly stress or kill reef organisms (Knight and Fell, 1987; Kerswell and Jones, 2003; Fabricius, 2005; Humphrey *et al.*, 2008). Terrestrial runoff can also expose reef organisms to decreased salinity, and increased levels of turbidity and contaminants (e.g., microbial pathogens, chemical pollutants). Terrestrial runoff can constitute a chronic stress in areas with developed coastlines or at river outflows, or an acute stress when floodwaters generated by extreme storms move offshore. Storm-derived floodwaters originating in urban areas may constitute a greater threat, containing high nutrient and contaminant loads due, in part, to overflows of municipal and industrial wastewater management systems (Chen *et al.*, 2019; Humphrey *et al.*, 2019). Storm-associated runoff is increasingly recognized as a threat to reefs since the intensity and precipitation associated with tropical storms is increasing with climate change (Knutson *et al.*, 2010; Emanuel, 2017).

Terrestrial runoff can ultimately reduce dissolved oxygen levels and generate hypoxia in reef-associated waters through several mechanisms (reviewed in Nelson and Altieri, 2019). Increased nutrient levels can trigger bacterial, phytoplankton, and zooplankton blooms in the proximity of reefs. As these blooms die off, they are consumed by heterotrophic bacteria, whose respiration draws down oxygen within the water column, causing reef organisms to suffocate (Nelson and Altieri, 2019). Turbidity increases can reduce light penetration over reefs, limiting the ability of benthic phototrophs (or photosynthetic microbial symbionts within benthic hosts) to produce oxygen via photosynthesis (Fabricius, 2005). Hyposaline runoff can also stratify the water column above reefs, generating a freshwater lens at the air-sea interface that prevents reoxygenation of bottom waters, as oxygen below this layer is consumed by respiring benthic reef organisms (e.g., Kealoha *et al.*, 2020). The increasing potential for hypoxic conditions on reefs has recently been recognized (Nelson and Altieri, 2019; Hughes *et al.*, 2020) yet, relatively little is known regarding how often low dissolved oxygen contributes to reef decline and over what spatiotemporal scales this occurs.

Although various studies document the impacts of terrestrial runoff on coral reefs, few works specifically address runoff derived from floodwaters; all of these latter works focus on nearshore, shallow water reefs (Ostrander *et al.*, 2008; Lapointe *et al.*, 2010). It has been assumed that offshore or deep (e.g., mesophotic) reefs are unlikely to interact with terrestrial runoff (e.g., Szmant, 2002). For example, reefs of the Flower Garden Banks National Marine Sanctuary (FGBNMS, northwest Gulf of Mexico, Figure 1a) occur ~185 km offshore in relatively deep water (20-30 m), and boast some of the highest coral cover (~55%) in the wider Caribbean (Johnston *et al.*, 2016). Since 2015, the Texas coast has experienced several flooding events related to extreme storms: Memorial Day Flood of 2015; Tax Day Flood of 2016, Hurricane Harvey in 2017, and Hurricane Imelda in 2019. Each of these floods impacted Central Texas nearshore ecosystems, including salt marshes (Congdon *et al.*, 2019; Oakley and Guillen, 2019) and oyster beds (Kiaghadi and Rifai, 2019). FGBNMS reefs were not surveyed for floodwater impacts following the Memorial Day Flood of 2015 because it was assumed that any floodwaters that moved into Galveston Bay (Texas, USA) or the adjacent continental shelf would dissipate before reaching the reef system.

However, three months after the 2016 flood, a localized mortality event (LME) occurred on a portion of the East Flower Garden Bank, an offshore, relatively deep (~20 m) ecosystem. During the LME, approximately 82% of corals in a 0.06km^2 area, experienced partial or full mortality (Johnston *et al.*, 2019), and declines in many other benthic invertebrates, such as sponges, were also observed. Although data on abiotic conditions at East Bank during the 2016 LME are not available, salinity and temperature measurements from nearby sites suggest that poor water quality from floodwaters moving offshore and subsequent low dissolved oxygen levels played a critical role in the LME (Le Hénaff *et al.*, 2019; Kealoha *et al.*, 2020). Then, in late August of 2017, Hurricane Harvey released over one meter of rainfall over the course of six days in some areas of southeastern Texas (Blake and Zelinsky, 2018). Given that a smaller scale event (Tax Day Flood) triggered reef mortality, there was concern regarding the impacts that the Hurricane Harvey-derived water mass would have on FGBNMS ecosystem health. Although salinity was depressed near the FGBNMS following Hurricane Harvey, much of the water mass diverted southwest along the coast (Roffer *et al.*, 2018), and no mass mortality was observed on the reef (Wright *et al.*, 2019).

In association with declines in coral cover worldwide, the relative abundance of sponges has increased on some reefs, especially in the Caribbean, as has their overall contribution to reef ecology, structure, and biogeochemistry (Bell *et al.*, 2013; Pawlik and McMurray, 2020). Sponges harbor a diversity of microbial symbionts (e.g., bacteria, archaea, protists, fungi and viruses) that contribute to their health and nutrition but can be disrupted by environmental stress (Slaby *et al.*, 2019). Although relatively few reports document the sub-lethal effects of environmental stress on sponge microbial communities, thermal stress is known to disrupt the bacterial communities associated with many sponge species (Lesser *et al.*, 2016; Pita *et al.*, 2018). Sponge-associated bacterial communities have been also shown under experimental conditions to be resistant to some flood-associated stressors, such as elevated nutrients (Simister *et al.*, 2012; Luter *et al.*, 2014) and reduced salinity (Glasl, Smith, *et al.*, 2018). Understanding how and when environmental stressors influence sponge-microbe associations, and the subsequent implications for sponge health and function, is increasingly important as sponges increase in abundance on reefs.

This study assesses whether storm-derived coastal flooding events can perturb the microbial symbioses of offshore reef sponges. We leverage the Tax Day Flood (2016) and Hurricane Harvey (2017) as natural 'experimental treatments' applied to two sponge species (*Xestospongia muta* and *Agelas clathrodes*; Figure 1b-c) at the East Bank (EB) and West Bank (WB) of the FGBNMS. Bacterial communities were sampled from sponges at five time points: in July 2016 (at detection of the mortality event), one month after the mortality event (August 2016), immediately after Hurricane Harvey (September 2017), one month after Hurricane Harvey (October 2017), and approximately one year following Hurricane Harvey (October 2018) (Figure 1d). No flooding occurred in southeast central Texas during 2018, and thus samples from this time point function as an 'experimental baseline'. We hypothesized that: (1) sponge-associated bacterial communities shift during flood years (relative to the non-flood year); (2) flood year bacterial communities contain genetic signatures of terrestrial-derived bacteria; and (3) anaerobes have a higher relative abundance in July 2016 LME samples, reflecting hypoxic conditions that likely occurred during the LME.

RESULTS

Reduced surface salinity at the FGBNMS following floods

In the vicinity of FGBNMS, mean surface salinity is generally variable between mid-May through mid-August (shaded grey in Supplemental Figure S1). In early spring and in fall, however, salinity is more consistent (~ 35 ppt). Approximately 30 days after the 2016 Tax Day Flood impacted Texas, a period of depressed mean salinity (relative to the mean salinity for a six year period: 2013-2018) was recorded from 15 May 2016 to 15 August 2016 at Texas Automated Buoy System (TABS) Real Time Ocean Observations, Buoy V (Supplemental Figure S1b). On average, salinity was 1.2 ppt lower than the six-year mean during this period; the most significant deviation occurred around 2 July 2016 when mean salinity reached a minimum of 29.1 ppt (3.2 ppt below the six-year mean). In 2017, salinity remained > 35 ppt until early June. TABS buoy V data are unavailable for much of June to August 2017, so it is unclear how salinity changed in the months preceding Hurricane Harvey. Two abrupt reductions in salinity were recorded near FGBNMS during mid-to-late September 2017 following Hurricane Harvey, before salinity returned to 35 ppt in October (Supplemental Figure S1c). In contrast, no significant influence of freshwater was recorded in the northwest Gulf of Mexico coast in 2018 and salinity remained approximately 35 ppt after mid-June (Supplemental Figure S1d).

After the 2016 LME was discovered at EB, opportunistic sampling of debris from dead organisms, hereafter referred to as ‘dead invertebrate tissue’ (DIT), occurred on 27 July 2016; the mean salinity was 31.7 ppt, which was 2.1 ppt below the six-year mean for the area (Figure 1d). On this date, mean salinity had already been lower than the six-year mean for 44 days. Additional samples were collected on 6 August 2016, approximately four days after salinity had returned to 35 ppt (Figure 1d). Sampling immediately post-Hurricane Harvey on 16 September 2017 occurred when mean salinity was 34.6 ppt, or 1.3 ppt lower than the six-year mean (Figure 1b). During sampling that occurred approximately one-month post-hurricane (on 21 October 2017), mean salinity had been > 35 ppt for 19 days prior to sampling (Figure 1d). Samples for the non-flood year were collected on 23 October 2018, which had a mean salinity of 35.7 ppt; salinity had been > 35 ppt for 94 days (Figure 1d).

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157 Acute shifts in sponge-associated bacterial communities following flood events

158 Paired-end Illumina MiSeq sequencing of the V4 region of the bacterial 16S rRNA gene yielded
159 5,975,358 high quality sequences from samples of DIT (associated with the 2016 LME, n=8) and two
160 sponge species (*A. clathrodes*, n=54; *X. muta*, n=44) (Table 1). After removal of Mitochondrial,
161 Chloroplast, and Unassigned reads, the total pool of 5,034,341 bacterial sequences were clustered into 9,221
162 Operational Taxonomic Units (OTUs). All samples clustered into 3 distinct groups, which were all
163 significantly different from each other (ANOSIM: $p < 0.01$; Supplementary Table S1). These groups were:
164 1) DIT (July 2016) and diseased sponges of both species (Aug. 2016); 2) visually healthy *A. clathrodes*
165 (Aug. 2016, Oct. 2017, Oct. 2018); and 3) visually healthy *X. muta* (Aug. 2016, Sept. 2017, Oct. 2017, Oct.
166 2018; Figure 2). Because healthy sponges had species-specific bacterial communities (ANOSIM: Global R
167 = 0.688, $p = 0.01$), subsequent analyses were conducted on each sponge species individually. Additionally,
168 within each sponge species, there were no differences between EB and WB sites (ANOSIM: $p > 0.05$)
169 (Supplemental Table S2), therefore, sites were grouped for subsequent analyses.

170 Bacterial communities of *X. muta* were shifted significantly during all collection dates associated
171 with flood events (Aug. 2016, Sept. 2017, Oct. 2017), relative to samples collected during the same season
172 in a no flood, baseline year (Oct. 2018) (ANOSIM: $p < 0.05$, Supplemental Table S3). Bacterial
173 communities of *X. muta* in Aug. 2016 and Sept. 2017 showed the greatest shift (Figure 3a) and had
174 significantly higher variability (mean pairwise dissimilarity) compared to Oct. 2018 communities (ANOVA
175 with Tukey's comparisons: $p < 0.001$) (Figure 3c). In Oct. 2017, *X. muta* bacterial community structure
176 was still significantly different than bacterial communities under ambient conditions (Oct. 2018,
177 Supplemental Table S3). However, Oct. 2017 communities clustered more closely to Oct. 2018 than to
178 Sept. 2017 communities, and variability of bacterial communities in Oct. 2017 was similar to that in Oct.
179 2018 (ANOVA with Tukey's comparisons: $p = 0.982$, Figure 3c). Bacterial communities of *X. muta* had
180 similar levels of Shannon Diversity across time points (ANOVA: $F = 0.83$ $p = 0.490$). However, there was
181 a significant difference in OTU richness over time (ANOVA: $F = 6.07$ $p = 0.002$), with communities from

Sept. 2017 having lower OTU richness than communities from Oct. 2018. *X. muta* bacterial communities during Oct. 2018 of the no flood year were dominated by Gammaproteobacteria (19.9 ±0.9%), Chloroflexi (13.6 ±0.7%), Actinobacteria (13.5 ±1.2%), and Acidobacteria (11.1 ±1.0%) (Supplemental Figure S2a). Four bacterial Families in *X. muta* communities showed significant differences in abundance across sampling time points (Supplemental Table S4). Rhodobacteraceae and Nitrosopumilaceae had higher abundances in Oct. 2018; Synechococcaceae had higher abundance in Aug. 2016 and Sept. 2017; and Anaerolineaceae and Chloroflexaceae had higher abundances in Oct. 2017 (Kruskal-Wallis: $p < 0.002$).

Similar to *X. muta*-associated bacterial communities, bacterial communities of *A. clathrodes* also shifted significantly during all collection dates, relative to 'baseline' samples collected during a no flood year (Oct. 2018). *A. clathrodes* communities also differed between flooding events (Aug. 2016 vs. Oct. 2017, Figure 3b, Supplemental Table S3). Like in *X. muta*, bacterial communities in *A. clathrodes* displayed higher variability in response to the flood events (Figure 3d). *A. clathrodes* bacterial communities displayed differences in both OTU richness (ANOVA: $F = 57.13$ $p = 0.0001$) and Shannon Diversity (ANOVA: $F = 40.76$, $p = 0.0001$) across time points, with Oct. 2017 having significantly lower OTU richness and diversity. *A. clathrodes* bacterial communities during Oct. 2018 were also dominated by Gammaproteobacteria (21.2 ±0.4%), Chloroflexi (20.4 ±0.2%), Actinobacteria (16.7 ±0.9%), and Acidobacteria (13.6 ±0.8%, Supplemental Figure S2b). Eight bacterial Families in *A. clathrodes* communities showed significant differences in abundance across time points (Supplemental Table S4). Solibacteriaceae and Nitrosopumilaceae had higher abundance in Oct. 2018; Vicinamibacteraceae and Rhodospirillaceae had higher abundance in Oct. 2017; Chromatiaceae, Ectothiorhodospiraceae, and Nitrospiraceae had lower abundance in Oct. 2017; and Desulfovibrionaceae had a higher abundance in Aug. 2016 (Kruskal-Wallis: $p < 0.002$).

Sponge microbiomes during the July 2016 Localized Mortality Event were dominated by anaerobes

Bacterial communities from visually healthy and diseased sponges sampled immediately after the LME were dominated by taxa classified as aerobes (mean = 13.5 ± 0.6% and range = 0.95 - 27.6%, Figure

4). However, bacterial communities from DIT sampled during the LME had a significantly higher proportion of taxa classified as anaerobes (40.0 ± 8.0%), compared to bacterial communities sampled from sponges at all other time points (Mann-Whitney multiple comparisons; $p < 0.0125$, Figure 4). Within bacterial communities of DIT, there were 19 Families at > 0.1% relative abundance. Fourteen of these Families were characterized as obligate, facultative, or aerotolerant anaerobes, including several members of the Class Bacteroidetes, several members of the Class Clostridia, sulfate-reducers (Families Desulfovibrionaceae and Desulfuromonadaceae), and sulfur-oxidizers (Family Thiothrichaceae, Supplemental Figure S3a). Sponge bacterial communities in diseased individuals of both host species were primarily represented by bacterial Families categorized as 'various' (i.e. Families with Genera that have different oxygen requirements). For example, Family Rhodobacteraceae, which contains members with different oxygen requirements, dominated samples from diseased individuals of both sponge species, representing an average of 36.9 (± 4.3)% of bacterial communities in these samples (Supplemental Figure S3b). Some of the same taxa that were abundant in DIT samples (July 2016), such as Flavobacteriaceae, Peptococcaceae, and Desulfovibrionaceae, were also abundant in diseased sponge samples (Aug. 2016), but were absent (or were in minor taxa, < 0.1%) in visually healthy sponges.

Sponge microbiomes show signs of wastewater contamination after flooding

Bacterial OTUs classified as Family Enterobacteriaeceae were recovered from the majority of samples of both sponge species. *Xestospongia muta* bacterial communities had low abundances (< 0.1%) of Enterobacteriaceae (Figure 5a), displaying no significant differences across dates (Kruskal-Wallis: $H = 6.80$, $p = 0.146$). However, *A. clathrodes* bacterial communities in 2016 (diseased and visually healthy samples) had a significantly higher abundance of Enterobacteriaceae (Kruskal-Wallis: $H = 33.67$, $p = 0001$), relative to samples from this host in other years. Bacterial communities of *A. clathrodes* in Aug. 2016 displayed 7.92 (± 1.97)% and 1.61 (± 1.03)% abundance of Enterobacteriaceae for diseased and visually healthy samples, respectively (Figure 5b), whereas in Oct. 2017 and Oct. 2018, samples had a low abundance (< 0.1%) of Enterobacteriaceae.

To test the hypothesis that FGB sponges were exposed to wastewater-derived bacteria from storm generated floodwaters, samples were screened for seven human pathogens using quantitative PCR. Diseased and visually healthy sponge samples collected in 2016 and 2017 yielded positive detection for 2 out of 7 human pathogens screened: *Escherichia coli* and *Klebsiella pneumoniae* (Figure 5c,d). No human pathogens were detected from sponges sampled during the no flood year (Figure 5c,d). In *X. muta*, *E. coli* abundance was highest in visually healthy samples from Aug. 2016, with a mean of 1.96×10^3 ($\pm 1.40 \times 10^3$) gene copies per g tissue, compared to a mean of 8.96×10^1 ($\pm 8.54 \times 10^1$) and 6.90×10^1 ($\pm 2.18 \times 10^1$) gene copies per g tissue for diseased samples from Aug. 2016 and visually healthy sponges from Sept. 2017, respectively (Figure 5c). In *X. muta*, *K. pneumoniae* abundance was similar across groups, averaging 1.08×10^2 ($\pm 5.78 \times 10^1$), 7.84×10^1 ($\pm 7.27 \times 10^1$), and 1.06×10^2 ($\pm 6.74 \times 10^1$) gene copies per g tissue for diseased samples from Aug. 2016, visually healthy sponges from Aug. 2016, and visually healthy sponges from Sept. 2017, respectively (Figure 5c). In *A. clathrodes*, *E. coli* was more abundant in samples from Aug. 2016, with means of 6.47×10^4 ($\pm 5.76 \times 10^4$) and 2.85×10^4 ($\pm 1.79 \times 10^4$) gene copies per g tissue for diseased and visually health sponges, respectively, compared to Oct. 2017, with a mean of 6.26×10^2 ($\pm 4.17 \times 10^2$) gene copies per g tissue (Figure 5d). In *A. clathrodes*, *K. pneumoniae* was less abundant (> 2 orders of magnitude difference) compared to *E. coli*, displaying similar abundance across groups where it was detected, averaging 8.22×10^1 ($\pm 7.65 \times 10^1$), 8.36×10^1 ($\pm 5.68 \times 10^1$), and 4.61×10^1 ($\pm 3.29 \times 10^1$) gene copies per g tissue for diseased samples from Aug. 2016, visually healthy sponges from Aug. 2016, and visually healthy sponges from Oct. 2017, respectively (Figure 5d). No samples tested positive for *Enterococcus* spp., *Pseudomonas aeruginosa*, *Salmonella enterica*, *Serratia marcescens*, and *Staphylococcus aureus* (data not shown).

DISCUSSION

Flooding disrupts microbiomes of offshore reef sponges

It has been assumed that remote marine ecosystems (>100 km from land) are not affected by terrestrial runoff. Our findings, however, suggest that flooding events can significantly impact offshore

benthic reef organisms, based on the detection of shifted bacterial communities and human pathogens in sponges after extreme storm events across two separate years. Flooding events altered the bacterial communities of both *X. muta* and *A. clathrodes*, as shown by disruptions to their community structure in 2016 and 2017, relative to sponge bacterial communities collected during the same season in a no flood year in Oct. 2018. Furthermore, we quantified an increased relative abundance of the Enterobacteriaceae and two known human pathogens in post-flood sponge samples, indicating that floodwaters of terrestrial origin can reach and interact with offshore reefs following extreme storm events.

Bacterial communities associated with the two sponge species exhibited some differences in the strength and duration of their response to flood water stress, likely due to the fact that they harbor distinctive bacterial communities even under ambient conditions (e.g., in 2018). Larger shifts in *X. muta* bacterial communities were associated with the 2016 Tax Day Flood, which caused mortality at EB. *X. muta* bacterial communities were relatively resilient following the sub-lethal stress associated with Hurricane Harvey and were similar to baseline by Oct. 2017. Interestingly, *A. clathrodes* sponges exhibited larger shifts in its microbiota following Hurricane Harvey (sub-lethal stress) than it did following the 2016 Tax Day Flood, and *A. clathrodes* microbiomes remained disrupted in Oct 2017. Wright *et al*. (2019) similarly found that interspecific differences were the strongest driver of gene expression changes in two coral following Hurricane Harvey. Differences in host function or ability to regulate its bacterial community may explain why *X. muta* showed earlier changes (and more dispersion) in response to the 2017 flood, whereas shifts in *A. clathrodes* bacterial communities lingered.

What mechanism(s) could have produced the observed changes in sponge microbiomes during flood years? Invasion of floodwater-derived bacteria into host tissues, invasion of seawater-derived microbes into host tissues and/or shifts in the abundance of sponge-associated taxa already present on hosts all could have played a role; it may not be possible to confirm the mechanism(s) underlying natural events in the field. For example, increases in the abundance of marine cyanobacteria in the Family Synechococcaceae were a key driver of differences observed in *X. muta* bacterial communities after flooding. Synechococcaceae were enriched over EB reefs following the 2016 Tax Day Flood (Kealoha *et*

al., 2020) and were associated with higher ammonium concentrations and therefore associated with terrestrially-derived flood waters. Filtration of the water column by *X. muta* individuals could have concentrated Synechococcaceae within sponges during this period. However, observed increases in Synechococcaceae could also have been driven by the proliferation of the resident sponge-associated populations due to an increase in nutrients in the sea water column. Yet, the abundance of sponge-associated *Synechococcus* decreases in *X. muta* following some environmental stresses, such as disease and thermal stress (Angermeier *et al.*, 2011; Lesser *et al.*, 2016). Reduced capacity of the animal host to regulate its microbiome under stress could be a contributing factor for any of these mechanisms (e.g., (Zaneveld *et al.*, 2017; Pita *et al.*, 2018). Thus, it is highly plausible that two (invasion of seawater-derived microbes, shifts in sponge-associated microbes) of the three mechanisms above contributed, albeit to different degrees, to observed sponge microbiome species following each flood.

Wastewater contamination after severe flooding reaches offshore marine ecosystems

The increased abundance of Enterobacteriaceae in reef sponges 185 km offshore after flooding in 2016 and, particularly, the detection of two fecal coliforms (*E. coli* and *K. pneumoniae*) during and after flood exposure strongly suggest that these reefs were exposed to wastewater contamination after severe storms. Although the Family Enterobacteriaceae is ubiquitous in nature, occupying terrestrial, aquatic, and marine habitats, especially the intestine of homeothermic animals (Whitman, 2015), members of this group are not common or abundant members of the microbiome of sponges (Moitinho-Silva *et al.*, 2017; Cleary *et al.*, 2019). It is possible that other sources of Enterobacteriaceae, such as excreted waste from reef fish and marine mammals (Wallace *et al.*, 2013) or wastewater disposal from nearby ships, explain the presence of this bacterial family across sponge samples. However, given that no human pathogens were detectable from October 2018 (no flood) samples, the most parsimonious explanation for the Enterobacteriaceae detections in this study is that FGBNMS reefs were exposed to land-based human wastewater via terrestrial runoff following the 2016 and 2017 floods.

It is unclear whether wastewater-derived bacteria contributed to mortality at EB in July of 2016, but detection of wastewater contamination at FGBNMS raises the question: do fecal coliforms pose health risks to offshore reefs? Human or animal wastewater contamination is linked to negative impacts on coral communities, particularly based on the input of excess nutrients, but chemical, bacterial, and pharmaceutical contamination are also potential issues (reviewed in Wear and Thurber, 2015). Generally, there is little information on the impacts of fecal-coliform bacteria on coral reef ecosystems. The wastewater-derived bacteria, *Serratia marcescens*, is a pathogen of *Acropora palmata* coral in the Caribbean (Sutherland *et al.*, 2010, 2011), and in Hawaii, fecal coliforms colonized coral tissues after exposure, potentially contributing to a major disease outbreak (Beurmann *et al.*, 2018). There is little information on the effect of fecal coliform exposure on marine sponge health, but some sponges may be relatively tolerant, using bacterial cells as a source of nutrition (Chaves-Fonnegra *et al.*, 2007). The surprisingly far reach of contaminated floodwaters observed here underscores the urgent need to understand how floodwaters impact the health and function of reef environments. A key question to address is whether detection of *E. coli* and *K. pneumoniae* represent detection of DNA from dead wastewater-derived bacterial cells or whether living wastewater-derived bacteria are potentially interacting with sponges (and other marine life) following extreme storms. If wastewater-derived bacteria contaminating sponges are metabolically active, then we must determine how long they persist within the sponge microbiome and the extent to which these microbes impact sponge physiology and function. A better understanding of the interactions between benthic reef hosts and terrestrial-derived bacteria will support effective management and protection of offshore reef ecosystems, such as the FGBNMS.

Previous work has shown the utility of using sponges as monitoring and potential bioremediation tools for fecal-coliform contamination in near-shore environments (Longo *et al.*, 2010; Maldonado *et al.*, 2010). This work demonstrates that sponge species can be effective tools for monitoring wastewater contamination in offshore marine ecosystems, and that the species selected for monitoring requires consideration. *E. coli* and *K. pneumoniae* were detected in both *A. clathrodes* and *X. muta* samples, but *A. clathrodes* had a higher frequency of detection and higher abundance of *E. coli*. Sponge size is a key driver

of filtering rate in marine sponges (Morganti *et al.*, 2019). *X. muta* (often exceeding 1m diameter, McMurray *et al.*, 2008) can achieve larger sizes than *A. clathrodes* (typically up to 100 cm wide, Parravelandia *et al.*, 2014), and thus the former species could theoretically accumulate more terrestrial-derived bacteria, compared to *A. clathrodes*. However, other interspecific differences, such as rates of digestion of filtered material, may be important in selecting sponge species as biomarkers. We detected more *E. coli* and *K. pnemoniae* in *A. clathrodes*; thus, this species, as compared to *X. muta*, shows promise as a biomarker for wastewater contamination at offshore reefs, such as FGBNMS, and should be further explored.

Anaerobic taxa implicate hypoxia in the 2016 Localized Mortality Event at East Bank, FGBNMS

Although there are no direct measurements of dissolved oxygen available from the EB FGBNMS at the time of the July 2016 LME, hypoxic conditions are thought to have contributed to the mortality observed (Kealoha *et al.*, 2020). In corals, hypoxic micro-environments generated by high microbial respiration at sites of coral-algal interaction cause increased microbiome variation (community dispersion), as well as increased abundance of bacteria classified as strict anaerobes (Barott *et al.*, 2012). We report a similar pattern of increased abundance of strict anaerobes in sponge microbiomes collected in July 2016. Our data support the hypothesis that low oxygen conditions, likely generated by increased microbial and/or reef respiration, contributed to the 2016 LME at the EB of the FGBNMS.

Some anaerobes detected in DIT (July 2016) samples may be of terrestrial origin. *Cloacamonas* is found primarily in anaerobic digesters of municipal wastewater treatment plants (Pelletier *et al.*, 2008), and some Genera within the Bacteroides, Clostridiaceae, Christensenallaceae, and Ruminococcacaeae Families are abundant members of mammalian gut microbiota (Arumugam *et al.*, 2011; Waters and Ley, 2019), and are thus potential indicators of wastewater contamination on FGBNMS reefs. However, other anaerobes and microaerophiles present in DIT samples may be marine in origin. Desulfovibrionaceae and Thiotrichaceae contain sulfate-reducing and sulfur-oxidizing bacteria, respectively (Castro H F *et al.*, 2000; Garrity *et al.*, 2015), and Genera within both Families are both commonly found in anoxic and microaerophilic layers of marine sediments (Postgate and Campbell, 1966; Mußmann *et al.*, 2003; Zouch

363 *et al.*, 2017). Interestingly, *Beggiatoa* (Family Thiotrichaceae) and *Desulfovibrio* (Family
364 Desulfovibrionaceae) are both essential components of the microbial consortium that causes black band
365 disease in corals (Sato *et al.*, 2016). *Beggiatoa* is considered an indicator of low DO conditions and thick,
366 white, filamentous mats *in situ* can be presumptively identified as this genus (Rosenberg and Diaz, 1993;
367 Rabalais *et al.*, 2001; Altieri *et al.*, 2017). White, filamentous mats were also observed during the LME in
368 July 2016 (Johnston *et al.*, 2019), and thus, members of the Thiotrichaceae may be the source of the 'white
369 bacterial mat' described by divers during DIT sample collection. Desulfovibrionaceae and Thiothrichaceae
370 together may be opportunistically contributing to mortality initially caused by hypoxic conditions on EB in
371 July 2016.
372
373 Comparisons of FGBNMS sponge microbiomes to those in the wider Caribbean
374 This study is the first to characterize sponge-associated microbial communities from the northwest
375 Gulf of Mexico (and FGBNMS), offering the opportunity for comparisons of sponge microbial
376 communities across regions of the GoM and the Caribbean. *X. muta* associated bacterial communities at
377 the FGBNMS in 2018 (no storm condition) were similar between EB and WB and were dominated by Phyla
378 also commonly reported from *X. muta* in the Florida Keys, Bahamas, and greater Caribbean (i.e.
379 Proteobacteria, Chloroflexi, Cyanobacteria, Poribacteria, and to a lesser extent, Acidobacteria,
380 Actinobacteria, and Thaumarchaeota (Schmitt *et al.*, 2012; Olson and Gao, 2013; Montalvo *et al.*, 2014;
381 Fiore *et al.*, 2015; Morrow *et al.*, 2016; Villegas-Plazas *et al.*, 2019). These previous studies report regional
382 differences due to changes in relative abundance of these shared Phyla (Fiore *et al.*, 2015; Morrow *et al.*,
383 2016). *X. muta* bacterial communities from the FGBNMS may also be regionally distinct, in particular
384 containing a high abundance of Thaumarchaeota archaea (~10%) compared to what has been reported from
385 other regions (<5%; Fiore et al 2013). Members of Thaumarchaeota, such as Nitrosomopumilaceae, play
386 an important role in nitrogen cycling in *X. muta* via ammonia oxidation (López-Legentil *et al.*, 2010).
387 Ammonia-oxidizing archaea are outcompeted in environments with higher levels of ammonium (Erguder

388 *et al.*, 2009), so the greater abundance of Nitrosomopumilaceae likely reflects the oligotrophic environment
389 of the offshore FGBNMS reefs during no storm conditions.

390 This study is also the first to describe bacterial communities associated with *A. clathrodes* using
391 next-generation sequencing technology. Bacterial communities of *A. clathrodes* at the FGBNMS contained
392 Phyla (i.e. Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi, Thaumarchaeota) also present in other
393 *Agelas spp.* from the Florida Keys, Belize, and Central Amazon Shelf (Olson and Gao, 2013; Deignan *et*
394 *al.*, 2018; Rua *et al.*, 2018; Gantt *et al.*, 2019). However, higher abundances of Archaea, especially
395 Euryarchaeota and Crenarchaeota, and Firmicutes were found in other *Agelas* spp. (Deignan *et al.*, 2018;
396 Rua *et al.*, 2018). Diseased sponges (both *A. clathrodes* and *X. muta*) sampled after the 2016 LME were
397 dominated by Alphaproteobacteria, especially Rhodobacteraceae. Alphaproteobacteria were also enriched
398 in sponges affected by *Agelas* Wasting Syndrome (Deignan *et al.*, 2018), suggesting that this group of
399 bacteria could play a role in pathogenesis and/or serve as a biomarker of disease risk for FGBNMS sponge
400 communities.

401

402 Mitigating the Impacts of Future Storms on Offshore Reefs

403 This study demonstrates that transport of floodwaters following extreme storms can contribute to
404 shifts in the microbiomes of benthic reef organisms 185 km offshore, promoting bacterial communities that
405 are more variable. Detection of bacteria typically associated with wastewater within these sponge samples
406 illustrates that marine-terrestrial interactions, and thus, the potential impacts of human land and waste
407 management practices, extend far beyond the shoreline. The GoM is regularly impacted by hurricanes, and
408 thus marine communities in the region have evolved in a disturbance regime that includes bursts of storm-
409 generated terrestrial runoff. However, the ongoing expansion of impermeable surfaces (e.g., concrete,
410 pavement) in Texas and other coastal areas, as well as changing extreme storm patterns (e.g., slower moving
411 hurricanes with greater precipitation) are increasing the frequency and intensity of floodwater influx into
412 marine environments.

This study of the potential impacts of the 2016 and 2017 floods was catalyzed because an LME affected the East Bank following the Tax Day Flood. We hypothesize that flood waters associated with other recent extreme storm events (e.g., 2015 Memorial Day Flood, flooding generated by Hurricane Imelda in September 2019) in the region likely also caused sub-lethal stress at FGBNMS. However, targeted sampling of FGBNMS did not occur following these storms. Flood waters reaching the offshore ecosystems of the FGBNMS have other implications beyond disruptions of benthic host-associated microbial communities. For example, excess nutrients, especially nitrogen, promote growth of macroalgae (Lapointe, 1997). Long term monitoring of benthic cover at FGBNMS has documented an increase in macroalgae, without a corresponding loss in corals, over the last two decades (Johnston *et al.*, 2016) and suggests nutrient input may have been affecting these offshore reefs since 1999. Growth of macroalgae on coral reefs is generally detrimental as macroalgae outcompete corals for light and space and promote coral disease (McCook *et al.*, 2001; Barott and Rohwer, 2012; Haas *et al.*, 2016). If unchecked, continued 'pulses' of nutrients from storm-derived flooding events may underlie or exacerbate increased coral-algal interactions and trigger declines at this 'reef of hope', which currently boasts coral cover greater than 50%.

This study demonstrates how water quality changes following severe storms can influence microbiomes of offshore benthic organisms, potentially impacting their physiology and ecosystem functions such as water filtration. Our findings clearly demonstrate the urgent need for: 1) continued mitigation of stormwater runoff and climate change impacts; and 2) establishment of microbial and water quality time series for near- and offshore reef using standardized protocols. This latter program will generate baseline data on the microbiomes of key benthic reef taxa under normal conditions, providing critical context (Glasl, Bourne, *et al.*, 2018) in which to detect and mitigate floodwater-derived stress on reefs.

EXPERIMENTAL PROCEDURES

Pelagic water properties during sample collection periods

The Flower Garden Banks National Marine Sanctuary (FGBNMS, northwest Gulf of Mexico) is currently comprised of three banks: East Bank (EB), Stetson Bank and West Bank (WB) (Figure 1a). To characterize local surface salinity (sensor depth of 2 m) in parts per thousand (ppt) and temperature (°C) representative for the FGBNMS before, during, and after each sampling period, water property data collected each half hour for April - October for the years 2013 - 2018 were downloaded from the Texas Automated Buoy System Real Time Ocean Observations, Buoy V archives (http://tabs.gerg.tamu.edu). Buoy V (27° 53.7960'N, 93° 35.8380'W) is located approximately 3 km from EB and 25 km from WB. Data were filtered to remove all timepoints containing no data and to exclude outlier data (i.e., measurements where salinity abruptly changed to <1 ppt or >70 ppt from 35 ppt). Data were not available (due to instrumentation failure) between the dates of 6 June 2017 through 30 August 2017. The remaining data were then plotted using the ggplot2 package version 3.2.1 using code from https://github.com/rachelwright8/TagSeq_FGB_HurricaneHarvey. Data were summarized to determine means (black line) and daily ranges (grey shading) for April to October over the six-year period. Daily means for each sampling date were also calculated and plotted on top of the six-year summary data. (Figure 1d). For sampling campaigns that spanned more than one day, only the first day of sampling was plotted (the maximum length of a sampling campaign was 5 days). To assess the lags between continental flooding (associated with an extreme storm) and changes in water quality at the FGBNMS, daily salinity mean and range data for April to October of individual years 2015, 2016, 2017 and 2018 (red lines and shading, Supplemental Figure S1a-d) were overlaid on surface salinity averages for the six-year summary data.

Sponge Sample Collections

Samples were collected at five timepoints spanning 2016-2018 from two locations within the FGBNMS; East Bank (27° 52'54.84”, 93° 37'41.84") and West Bank (27°54'28.8", 93°36'0.72", Figure 1a, Table 1, Supplemental Table S1). Samples from July 2016 were samples of dead invertebrate tissue (DIT) collected opportunistically on the day that the EB LME was discovered; sampling capabilities at this time point were extremely limited in terms of available sterile collection materials and dive time, relative to

subsequent sample time points. Thus, the July 2016 DIT samples are a composite representation of the organic mat that had formed on the tops of dying sponges, corals, and other organisms including brittle stars and urchins (Supplemental Table S5). At all other sampling timepoints, fragments were collected from individual *A. clathrodes* and *X. muta* sponges (Figure 1b,c). The same individual sponges were not repeatedly sampled across timepoints due to time constraints in available ship and dive time. In August 2016, samples were collected from 'diseased sponges' that were exhibiting progressive tissue loss, as well as from visually healthy sponges. For all other timepoints, samples were collected from visually healthy sponges as diseased sponges were not observed. In total, 109 samples, collected from depths ranging 18 - 27 m, are analyzed in this study (Table 1, individual sample metadata provided in Supplemental Table S5). Samples were clipped from colonies using health status and species-specific cutting shears that were wiped clean between samples. Each sample was placed in an individual sterile bag for transport to the surface. Once topside, each sample was immediately transferred to liquid nitrogen and stored at -20°C until further processing.

Bacterial Community Analysis

DNA was extracted from 250 mg of sponge sample using the Nucleospin Soil DNA extraction kit (Takara Bio); whereas DNA from DIT samples was extracted using the DNeasy PowerSoil DNA extraction kit (QIAGEN). Clustering of samples from ill health states (i.e. DIT samples, some of which were collected from *X. muta*, and diseased sponge samples) suggests that there was no significant bias from extraction method. Extracted DNA was submitted for high-throughput sequencing of the V4 hypervariable region of the 16S gene using the Illumina Mi-Seq (Paired-end 2x 300 read) platform. High quality sequences were analyzed through the QIIME 1.8 software package to generate Operational taxonomic units (OTUs) clustered at 97% identity (Caporaso *et al.*, 2010). Rarefied bacterial OTU tables were used to calculate alpha diversity metrics and to conduct beta diversity analyses using weighted UniFrac distance matrices. Taxonomy was assigned using the RDP v. 2.12 reference database (Wang *et al.*, 2007). Bacterial Families were also categorized by oxygen requirement using descriptions available in Bergey's Manual of

Bacteriology (Whitman, 2015). Bacterial Families with Genera that have multiple oxygen requirements were classified as "Various". The raw sequence data files were submitted to the NCBI Sequence Read Archive under accession number SRP248232. Additional details for sequence processing and analysis are provided in Supplemental Methods.

Quantitative PCR for human pathogens associated with Hurricane Harvey-derived floodwaters

Species-specific functional genes were chosen as biomarkers to detect and quantify fecal indicator bacteria (*Escherichia coli* and *Enterococcus spp.*), putative pathogenic bacteria in the Family Enterobacteriaceae (*Klebsiella pneumoniae*, *Serratia marcescens*, and *Salmonella enterica*), and other putative pathogenic bacteria (*Pseudomonas aeruginosa* and *Staphylococcus aureus*, Supplemental Table S6). These bacterial species were targeted because OTUs of the same Families were identified in high-throughput sequencing of terrestrial floodwater and sediment samples collected immediately following Hurricane Harvey (Yu *et al.*, 2018). Target gene amplicons were used to establish the standard curve between the threshold cycle (Ct) value and log10 (gene copies) for each pathogenic bacterium individually. To assess gene copy number in a given sample, C_T values of samples were compared to a prepared standard curve that was included in triplicate with each qPCR run (further details are provided in Supplemental Methods). Calculated copy number was normalized to g of wet sponge tissue. The limit of quantification ranged from 20 - 100 gene copies per g tissue.

Statistics

PRIMER-E was used to calculate alpha diversity metrics (Observed OTUs, Shannon Diversity). The weighted UniFrac distance matrix was used to calculate beta-diversity to assess dispersion in bacterial communities among samples at each site within each sponge species and to construct Principle Coordinates Analysis (PCoA) plots to visualize differences in bacterial community structure between sites. PCoA was conducted for all samples (both sponge species), as well as for samples of each species individually. Pairwise Analysis of Similarities (ANOSIM) was used to test for significant differences in bacterial

516 communities between sites using PRIMER-E v7 (Primer-E Ltd). One-way ANOVA was used to compare
517 results such as alpha diversity metrics, beta-diversity metrics, and quantified bacterial abundances among
518 collection dates (within each sponge species). Due to unequal variances in other data sets, (even after
519 transformation), the non-parametric Kruskal-Wallis (with Mann-Whitney post-hoc comparisons and
520 bonferroni correction) was used to compare relative abundances of major microbial taxa among collection
521 dates (within each sponge species). All data are represented as mean (± SEM), unless otherwise stated.
522
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TABLEs AND FIGURES

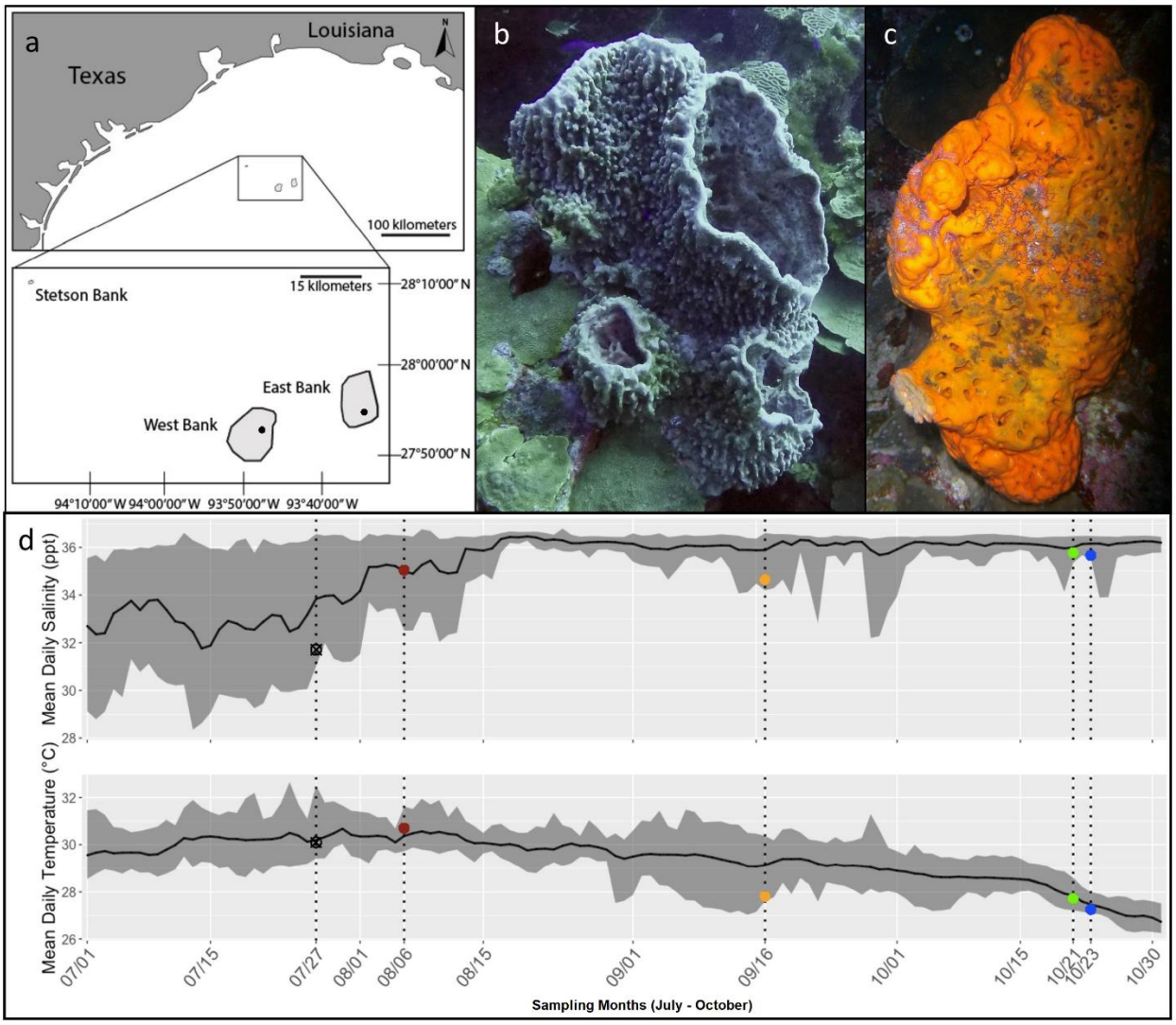
Table 1. Summary of sample collections from two reef sponge species at the East Bank (EB) and West Bank (WB) of the Flower Garden Banks National Marine Sanctuary (northwest Gulf of Mexico) and amplicon sequencing results of the V4 region of the 16S rRNA gene from sponge-associated bacterial communities. Richness (Observed OTUs) and diversity (Shannon H') were calculated from rarefied OTU tables. Data are presented as mean ± (sem).

					Sequence Analysis Summary		
Species	Date	Site	Health Status	N	High Quality Sequences[#]	Observed OTUs[^]	Shannon H'
Various species	Jul. 2016	EB	Dead*	8	48,706 (5,970)	470 (46)	3.286 (0.234)
Agelas clathrodes	Aug. 2016	EB	Diseased	4	108,475 (8,413)	1,338 (79)	3.600 (0.613)
	Oct. 2017	EB	Healthy	8	98,916 (4,999)	840 (46)	3.670 (0.087)
		WB	Healthy	5	73,449 (9,982)	801 (60)	3.355 (0.277)
		EB	Healthy	9	29,487 (2,797)	500 (52)	2.822 (0.258)
		WB	Healthy	8	28,893 (1,194)	541 (31)	3.345 (0.288)
	Oct. 2018	EB	Healthy	10	29,728 (1,111)	910 (20)	4.072 (0.056)
		WB	Healthy	10	29,721 (1,039)	805 (16)	3.901 (0.063)
Xestospongia muta	Aug. 2016	EB	Diseased	5	74,783 (18,104)	987 (77)	4.073 (0.111)
	Sept. 2017	EB	Healthy	2	103,021 (2,718)	1,189 (105)	4.527 (0.332)
		WB	Healthy	3	107,784 (6,555)	999 (62)	3.806 (0.530)
		EB	Healthy	9	36,437 (4,643)	790 (68)	4.386 (0.195)
	Oct. 2017	EB	Healthy	2	28,125 (1,076)	1,219 (58)	4.977 (0.044)
		WB	Healthy	3	30,113 (1,305)	916 (170)	4.136 (0.431)
	Oct. 2018	EB	Healthy	10	31, 029 (3,026)	1,142 (36)	4.552 (0.092)
		WB	Healthy	10	30,808 (1,944)	1,113 (70)	4.449 (0.117)

*samples collection from a variety of dead invertebrate tissue

[#]quality filtering included removal of low quality, short, Mitochondrial, Chloroplast, and Unassigned reads

[^]Operational Taxonomic Units (OTUs) after rarefying to equal depth (20,000 reads)



760

761 **Figure 1**. **Summary of study site, host taxa and local abiotic conditions associated with this study.** A)
762 Map of Flower Garden Banks National Marine Sanctuary (northwest Gulf of Mexico) with sites of sponge
763 collection indicated as black dots; B) Representative *Xestospongia muta* sponge; C) Representative *Agelas*
764 *clathrodes* sponge; D) Surface Salinity (ppt, top) and temperature (°C, bottom) at buoy V (3 km from
765 sampling sites) spanning the months July through October in which sampling occurred for this study. Black
766 lines represent daily means from 2013–2018 and grey shaded areas encompass minimum and maximum
767 values from 2013–2018. Dotted lines and symbols represent mean daily values on the date of each sample
768 collection: black circle = 27 July 2016, dark red circle = 6 August 2016, orange circle = 16 September 2017,
769 green circle = 21 October 2017, and blue circle = 23 October 2018.

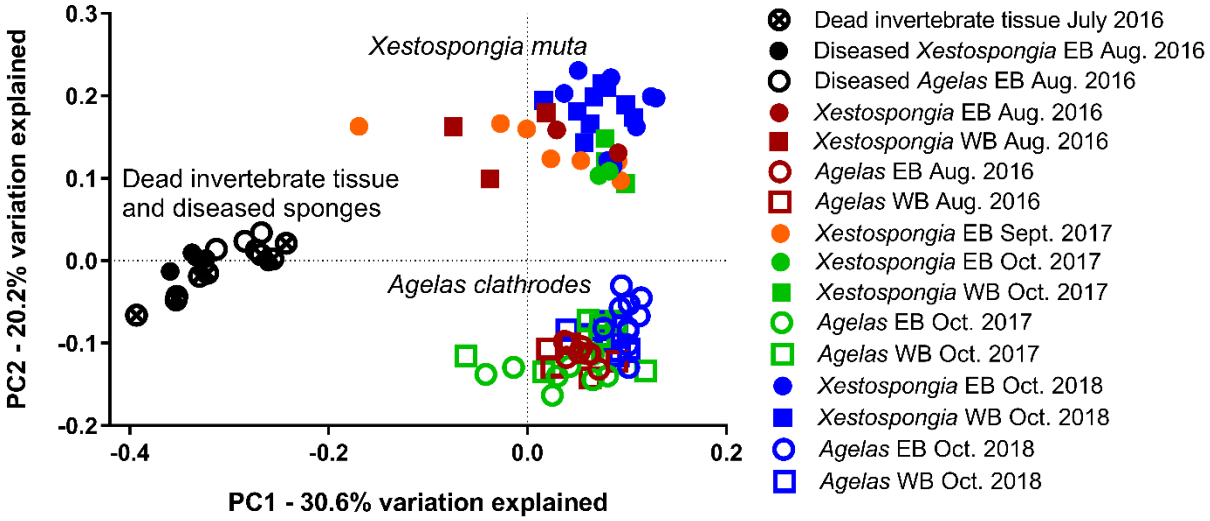
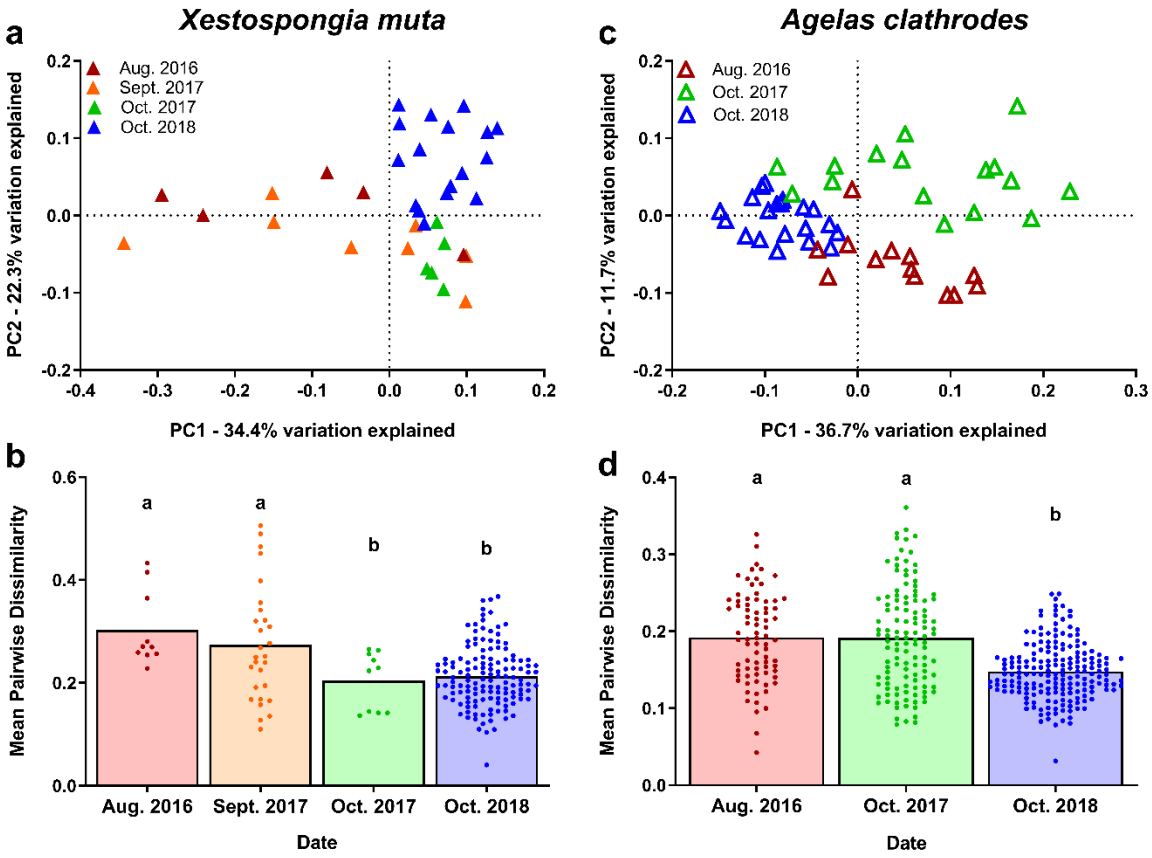


Figure 2. Principle Coordinate Analysis of the weighted UniFrac distance matrix for bacterial communities analyzed in this study. Empty symbols = *Agelas clathrodes* samples; filled symbols = *Xestospongia muta* samples. Black symbols = samples from dead invertebrate tissue (DIT) and diseased sponges during (July 2016) and immediately after (August 2016) the Localized Mortality Event (LME); Red symbols = visually healthy sponge samples collected immediately after the 2016 LME. Orange symbols = samples collected immediately following Hurricane Harvey (Sept. 2017) from visually healthy sponges; Green symbols = samples collected one month following Hurricane Harvey (Oct. 2017) from visually healthy sponges. Blue symbols = samples collected in a no-flood (baseline) year from visually healthy sponges (Oct. 2018). Circles = samples collected from East Bank (EB); squares = samples collected from West Bank (WB) of the Flower Garden Banks National Marine Sanctuary (northern Gulf of Mexico).



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789 **Figure 3.** Sponge-associated bacterial communities differed in composition and variability following
790 extreme storm-derived floods. Principle Coordinate Analysis of the weighted UniFrac distance matrices for
791 visually healthy a) *Xestospongia muta* and c) *Agelas clathrodes* bacterial communities. Mean (with
792 individual value dots) pairwise dissimilarity values for b) *X. muta* and d) *A. clathrodes*. Red = August 2016.
793 Orange = September 2017; Green = October 2017. Blue = October 2018 associated with no flooding stress.
794 Bars represent mean (± sem). Within a species, bars that do not share a letter are significantly different
795 based on one-way ANOVA with Tukey's comparisons ($p < 0.05$).

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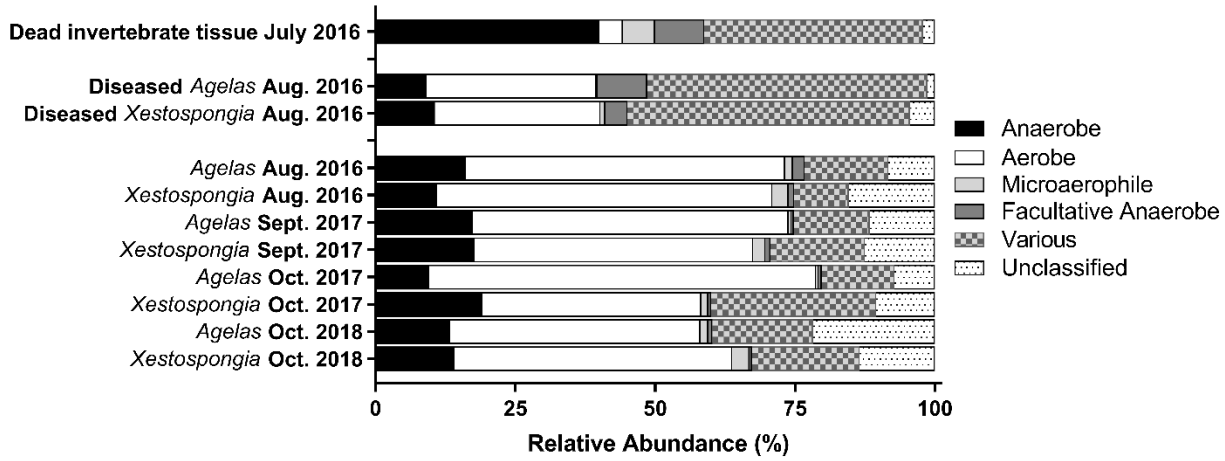

Figure 4. Percent abundance of bacterial sequences classified by oxygen requirement, for dead invertebrate tissue (DIT) samples (July 2016), diseased sponges (August 2016), and visually healthy sponges (2016, 2017, 2018). Flood events occurred in the region in 2016 and 2017, but not in 2018. Bacterial taxonomy was assigned using the RDP v2.12 database, and oxygen requirements of each Family were classified as using Bergey's Manual of Systematics of Archaea and Bacteria (Whitman et al., 2015). Families with more than one type of oxygen requirement were categorized as 'Various'.

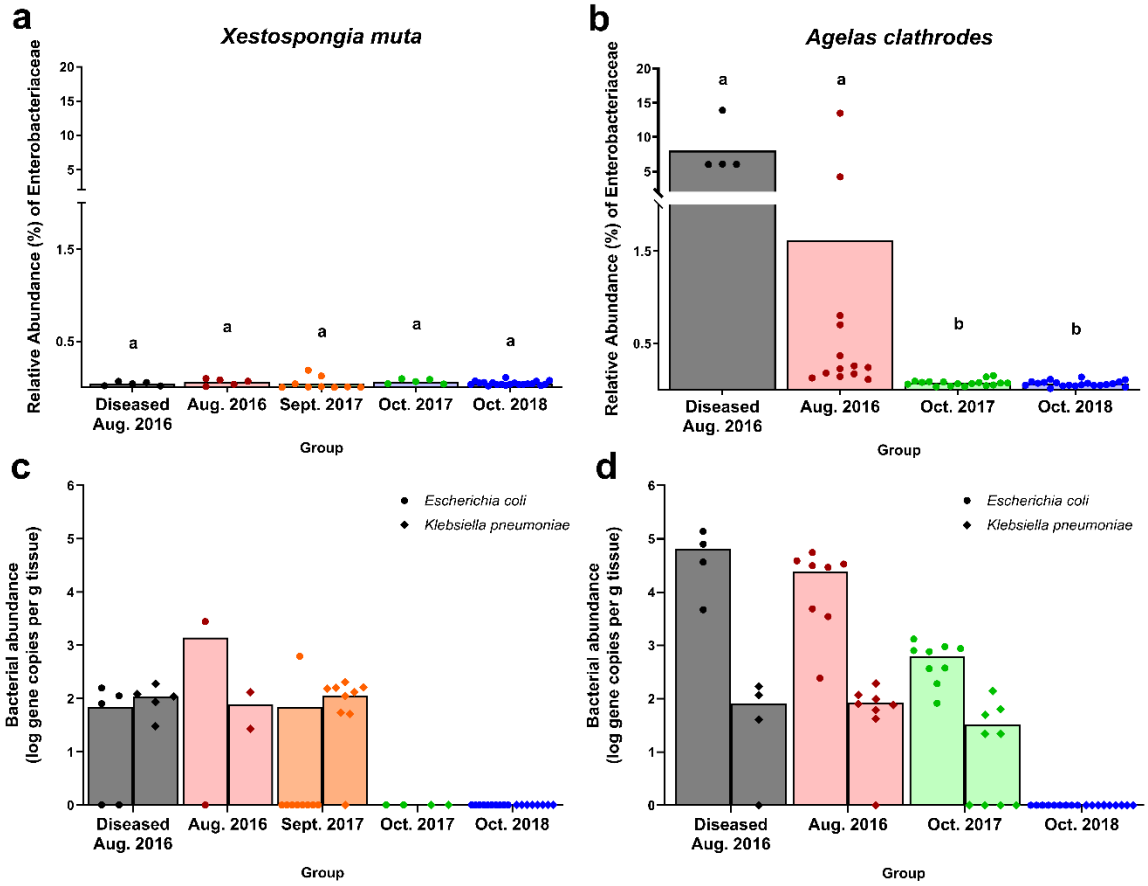

Figure 5. Relative abundance of sequences in the Family Enterobacteriaceae (a, b) and to specific human pathogens (*Escherichia coli;* c, and *Klebsiella pneumoniae)*; d), across sites and years. Data in (a,b) are based on Illumina MiSeq amplicon sequencing of the V4 region of the bacterial 16S rRNA gene, and are presented as mean ± sem. Data in (c,d) are quantitative PCR amplification of the bacterial *ybbW* and *phoE* genes for *E. coli* and *K. pneumoniae*, respectively. Black bars = affected sponges in 2016; red bars = healthy sponges in 2017; green bars = healthy sponges in 2017; blue bars = healthy sponges in 2018 (no flood, baseline year). Groups that share a letter (in a, b) are not significantly different based on Mann-Whitney pairwise comparisons across groups within a species. ND = not detected.