

MICROBIAL DYNAMICS OF A HYPERSALINE CREEK: COMMUNITY RESPONSE TO
DISTURBANCE AND CONNECTIVITY TO WILDLIFE

A Dissertation

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ABSTRACT

Estuarine ecosystems can experience extended periods of salinity and temperature stress. In the South Texas region of the northwest Gulf of Mexico (GoM), climate models and current trends support increased temperatures, prolonged droughts, and increased storm severity. It is therefore imperative to assess how climate stress will impact South Texas estuarine ecosystems. Coastal lagoons are particularly vulnerable to disturbance, and the hypersaline Baffin Bay and Upper Laguna Madre Complex is a ‘hotspot’ of environmental change. In this dissertation, factors contributing to hypersaline microbial community dynamics in a hypersaline creek were assessed in three stand-alone research projects: 1) a short-term 2-month study of microbial community dynamics following a flood event, 2) a long-term 18-month study of microbial community dynamics that included flood and freeze events, and 3) a targeted study of wildlife connectivity (Mexican free-tailed bats) to microbial community dynamics and eutrophication. The short-term study revealed that flood events are disturbance events that cause pronounced shifts in microbial community structure. The long-term study revealed the hypersaline community was resilient to flood and freeze events. Additionally, whole genome sequencing of halophilic bacteria uncovered mechanisms of osmoregulation and heavy metal resistance. The targeted study revealed that bat guano is a source of dissolved organic carbon and potentially pathogenic bacteria. Severe heat coupled with severe flooding is anticipated to alter salinity regimes, increase osmotic stress, adversely impact ecosystem stability, and potentially restructure natural communities between drought and flood events. Climate stress will also affect the quality of riparian buffers and the wildlife inhabiting those buffers. A better understanding of microbial

drought and flood resilience is critical to predicting how hypersaline coastal ecosystems will adapt and evolve under future climate scenarios.

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CHAPTER I

INTRODUCTION

Climate Stress

Over the past century, the global ocean has experienced significant climate stress. According to the Intergovernmental Panel on Climate Change (IPCC 2022), global sea-surface temperature (SST) has risen by an average of 0.13 °C per decade since 1901 (IPCC 2022). Moreover, since 1925, the frequency and duration of marine heat waves (MHWs) have increased by 34 and 17%, respectively (Oliver et al. 2018). A record-setting MHW occurred in the North Pacific Ocean between 2013 and 2015 (aka The Blob) when SST increased more than 6 °C (Frölicher and Laufkötter, 2018). This sustained SST increase was correlated with decreased primary productivity and increased mortality of sea lions, whales, and sea birds (Cavole et al. 2016). Similarly, reoccurring MHWs in the West Pacific Ocean (1998, 2002, and 2016) correlate with dramatic coral bleaching in the Great Barrier Reef (Fordyce et al. 2019).

In the Atlantic Ocean, including the Gulf of Mexico (GoM) and the Caribbean Sea, MHWs play a significant role in ecosystem degradation. Beginning in June 2023, an unprecedented MHW has increased North Atlantic SST by as much as 6 °C (Stillman 2023). Concurrently, a severe MHW has increased SST 5 °C above normal off the coast of Southern Florida (Cornwall 2023; Staletovich 2023). These unprecedented STTs are normally not experienced until August or September. Analysis of three ocean temperature databases revealed that Caribbean coral reefs experience five MHWs annually (Bove et al. 2022). A 40-year study of satellite-derived SST along the South Florida coast revealed eight MHW events annually, between 2015 and 2021, with each lasting 70 to 110 days (Androulidakis and Kourafalou 2022). Climate model simulations predict a further 2 to 3 °C SST increase over the 21st century will

exceed coral bleaching thresholds (Lawman et al. 2022). In fact, recent events show warming may be occurring faster than our current models, as global SST hit a record high for the fourth consecutive month with July 2023 being the hottest month in recorded history (NOAA 2023).

Against this backdrop of global ocean warming, climate models reveal increased hurricane risk (Balaguru et al. 2023). Models also project enhanced hurricane frequency for the GoM, where upper-level atmospheric circulation in the Atlantic Ocean will become more favorable to storm development. Further, increases in intensification rates, maximum intensity, and precipitation rate will increase the risk of widespread damage and flooding (Balaguru et al. 2023). In the last 20 years, 53 category 4 or 5 hurricanes formed in the Atlantic Ocean. Additionally, in the last six years, 10 hurricanes made landfall in the United States: Harvey (Rockport, Texas 2017), Maria (Hatteras and Ocracoke, North Carolina 2017), Irma (Cudjoe Key, Florida Keys 2017), Florence (Wrightsville Beach, North Carolina 2018), Michael (Mexico Beach, Tyndall Air Force Base 2018), Dorian (Deer Field Beach, Florida 2019), Delta (Creole, Louisiana 2020), Laura (Cameron, Louisiana 2020), Ida (Port Fourchon, Louisiana 2021), and Ian (Punta Gorda, Florida 2022) (NOAA). Hurricane Harvey caused the most significant rainfall in United States history (60.58 inches near Nederland, Texas), and trends show a 5-7% increase in hurricane-related precipitation over the last century (Balaguru et al. 2023).

Current trends in the 2023 hurricane season show agreement with hurricane risk predictions, with some of the earliest storm events occurring on record (i.e. tropical unnamed January storm, Tropical Storm Arlene [June 1], Tropical Storm Bret [June 19]). However, trends are difficult to interpret in light of unseasonably warm SST in an El Niño year. Warmer SSTs can speed storm development and intensification while upper-atmosphere wind shear associated with El Niño tends to inhibit development or limit intensification. For 2023, the National

Oceanic and Atmospheric Administration (NOAA) Climate Prediction Center forecasted a near-normal season with 12 to 17 named storms, 5 to 9 hurricanes, and 1 to 4 major (category 3, 4, or 5) hurricanes (NOAA 2023). By contrast, the United Kingdom Meteorological Office forecasted a far more active season with 20 named storms, 11 hurricanes, and five major hurricanes (UKMet 2023).

In addition, climate change is making non-hurricane-related heavy precipitation and flooding increasingly more probable. NOAA's NWS rainfall estimates, based on Atlas 14 (1973) and Atlas 2 (2018) data, are standard references, but they do not reflect current trends or keep pace with the rapidly changing climate. A new report, the 8th National Risk Assessment: The Precipitation Problem, analyzed data from 795 NOAA Automated Surface Observing Stations (ASOS) to correct rainfall estimates and flood risks using a peer-reviewed model (Kim et al. 2022; FSF 2023). Findings revealed the increased probability of heavy precipitation and flooding in many regions across the United States. This new ASOS Regional Frequency Analysis shows that 100-year rainfall and flooding events will occur every 5 to 10 years in some regions. A dramatic increase in heavy precipitation and flooding was predicted for some GoM regions including coastal Texas and Louisiana (Kim et al. 2022).

The compounding impacts of decadal trends and plural extreme weather events will test the resiliency of coastal systems. In October 2018, a MHW in the Northern GoM contributed to the intensification of Hurricane Michael, which made landfall in the Florida Panhandle as a category 5 storm (Dzwonkowsky et al. 2020). Presently, the collision of a sustained heat dome and a MHW in Southern Florida is fueling unprecedented SST warming (Cornwall 2023; Staletovich 2023). These extreme events are coupled ocean-atmosphere phenomena. Increases in

SST temperatures are strongly correlated with increases in precipitable water vapor and flood severity (Wang et al. 2018).

Climate change induced droughts will potentially influence coastal hypersalinity in the coming decades (Tweedley et al. 2019). Ocean connectivity, alterations in rainfall and sea level is also expected affect salinity regimes (Tweedley et al 2019). In fact, in the Baffin Bay – Upper Laguna Madre Complex, hypersalinity has been linked to extreme heat caused evaporation, low precipitation and flow, and limited ocean connectivity (Beecraft and Wetz 2022). The ULMC presence within a semi-arid climate zone will also likely be affected by and compound this condition, as these zones are predicted to expand in the future. As these conditions increase in frequency and intensity, hypersalinity will also continue to pose issues to coastal communities and ecosystems.

Vulnerability of Microorganisms

The effects of climate stress (e.g., heatwaves, droughts, and floods) include the loss of economically important fisheries (e.g., salmon and Dungeness crab in the North Pacific) and charismatic megafauna (e.g., whales in the North Atlantic and corals in the Caribbean and West Pacific). Rarely is the loss of microbial biodiversity considered. Yet, most life is microbial. Prokaryotes (i.e., bacteria and archaea) comprise two of life's three domains, and most eukaryotes are microbial, e.g., fungi, algae, and protozoa (Hug et al. 2016). Prokaryotes are the 'unseen majority' (Whitman et al. 1998). Their abundance ($4-6 \times 10^{30}$ cells) is difficult to imagine. By comparison, their abundance far exceeds the estimated number of stars in the universe (10^{24}) (Sogin et al. 2012). They are responsible for nearly half of all primary productivity and carry out vital transformations in biogeochemical cycles (Reinold et al. 2019). For example, prokaryotes alone transform atmospheric nitrogen into biologically usable forms

such as ammonia (LaRoche and Breitbarth 2005). Given their abundance, distribution, and ecological significance, they could provide useful insight into the effects of ecosystem stress.

Prokaryotes also offer enormous potential as model organisms for assessing and quantifying the effects of climate stress. Their small size, fast generation time, high mutation rate, stress tolerance, ability to enter a dormant state, and genome plasticity make prokaryotes immediately responsive to environmental change or perturbation i.e., prokaryotes are nature's first responders. These characteristics help prokaryotes resist and adapt. The 'first responder' concept was convincingly demonstrated by rapid changes in microbial community structure following the Deepwater Horizon and Prestige oil spills (Looper et al. 2013; Acosta-Gonzalez et al. 2015). A comprehensive review of the microbial disturbance literature (n = 247 studies) revealed that 82% reported sensitivity to disturbance (composition [26%], function [21%], both [35%]) (Shade et al. 2012). Of course, this precept also applies to extreme climate events. Flooding associated with Hurricane Floyd in 1999 altered cyanobacteria mat community composition in the Bahamas (Paerl et al. 2003), and flooding associated with Hurricane Harvey in 2017 shifted coastal marine microbial communities toward that of terrestrial soils (Steichen et al. 2020).

The following paragraph defines terms used in this dissertation. A disturbance can be defined as an event that results in a discrete change in the physical or chemical environment with anticipated effects on a community (Glasby and Underwood 1996). Generally, short-term and long-term disturbance events are defined as pulse and press (Bender et al. 1984). Flooding associated with an extreme weather event would qualify as a pulse disturbance while decadal trends like increasing atmospheric CO₂ would qualify as a press disturbance. Resistance is the degree to which a community withstands change; Resilience is the rate at which a community

returns to its original composition; Stability includes elements of resistance and resilience and is defined as the tendency of a community to return to its previous state (Shade et al. 2012).

The microbial response to disturbance can be compositional and functional. Community-level compositional change can be assessed by measuring how disturbance affects diversity. Compositional changes can be evaluated by measuring alpha diversity and beta diversity. Generally, alpha diversity is the measure of diversity in a particular ecosystem or population while beta diversity is the dissimilarity of diversity between ecosystems or populations. The contribution of specific taxa can be determined through linear discriminant analysis (Segata et al. 2011). Favorable experimental designs include the Before-After and Before-After-Control-Impact methods (Smokorowski and Randall 2017). The latter is the preferred method and consists of an experimental treatment plus a control that did not experience the disturbance, but it can be challenging to find an equivalent control.

Both alpha and beta diversity should be considered coarse measurements with inherent error. They are susceptible to sample size bias. Additionally, with respect to 16S rRNA metagenomic sequencing, differences in DNA extraction efficiency and sequencing library sizes can bias downstream analyses (Lande 1996). For example, experiments that yield different numbers of sequence reads result in different community compositions and different levels of diversity. Hence, experiments have an explicit measurement error, but diversity metrics do not account for this error (Willis 2019).

Vulnerability of Hypersaline Lagoons

A recent meta-analysis identified 60 hypersaline lagoons, estuaries, and embayments worldwide (Tweedly et al. 2019). These systems are commonly located in arid and semi-arid tropical and sub-tropical climate zones. Long residence times and minimal ocean connectivity

make these systems acutely vulnerable to pollution, eutrophication, harmful algal blooms, and hypoxia (Kennish and Paerl 2010; Warwick et al. 2018). Climate change is predicted to impact these systems through the alteration of flood and drought (Tweedly et al. 2019). Concurrently, sea level rise and storm events will influence ocean connectivity.

The Laguna Madre in the Northeastern GoM is one of these hypersaline lagoons that were identified. This lagoon has experienced prolonged droughts with reduced freshwater inflows. In 2013, *Syringodium filiforme* seagrass meadows disappeared in response to sustained hypersalinity (50-70) during a prolonged drought (Wilson and Dunton 2017). A long-term analysis of water quality data from the Texas Commission on Environmental Quality (TCEQ) Surface Water Quality Monitoring (SWQM) program characterized sections of the Laguna Madre (Baffin Bay and the Upper Laguna Madre Complex) as ‘hotspots’ of environmental change (Bugica et al. 2020). Upstream freshwater removal by a growing coastal population is predicted to further reduce freshwater inflows (Montagna et al. 2013), and fewer soaking rains are predicted to increase flood severity (Easterling et al. 2017).

The IPCC predicts climate change will increase drought severity in the GoM (Pachauri et al. 2014). Likewise, the Fourth National Climate Assessment predicted the Southern Great Plains (i.e., Kansas, Oklahoma, and Texas) will experience cycles of extreme drought interrupted by severe flooding (Reidmiller et al. 2018). Terrestrial heatwaves and heat domes coupled with increasing SST stand to compound drought to flood conditions. For example, a severe record-setting heat dome settled over the Southern Great Plains and Lower Mississippi Valley in June 2023, prompting the NWS to issue ‘excessive’ heat warnings. The Southeast Regional Climate Center recorded more than 30 records for daily high temperatures across Texas (Baddour 2023). These current trends align with a recent report by the Office of the Texas State Climatologist.

This report presented a 136-year (1900 to 2036) extreme weather assessment and projected the number of 100-degree days will nearly double in the next decade (Nielsen-Gammon et al. 2021).

Harmful ‘brown tide’ algal blooms caused by *Aureoumbra lagunensis* are a defining occurrence in the Baffin Bay and the Upper Laguna Madre Complex. Increased temperatures, prolonged droughts, and reduced freshwater inflows have been associated with these environmentally disruptive but non-toxic brown tides (Cira et al. 2021). In the northwest GoM, brown tides occur nearly annually in the Baffin Bay and the Upper Laguna Madre Complex, with the longest lasting eight years (Buskey et al. 2001). They impair ecosystems by disrupting ecosystem processes (Gobler and Sunda 2012). *A. lagunensis* blooms attenuate sunlight necessary for the productivity of submerged vegetative habitats such as seagrass beds (Onuf, 1996). In the Baffin Bay and the Upper Laguna Madre Complex, brown tide blooms have been implicated in the decreased abundance of seagrasses (Onuf 1996). This loss of seagrass habitat affects higher trophic levels as ecologically and economically important fisheries depend on seagrasses for nursery and foraging habitat (Sheridan and Minello 2003). The loss of seagrass habitat also affects endangered megafauna, such as the green sea turtle (*Chelonia mydas*), that forage in seagrass habitat (Taquet et al. 2006). The disruption of seagrass habitat can also impact larger-scale processes, such as nutrient cycling through decreased dissolved oxygen, sediment stability, and carbon sequestration (Orth et al. 2006; Bricker et al. 2008; Finlayson et al. 2018).

Temperature, salinity, and nutrient concentrations can facilitate the initiation and maintenance of brown tide blooms (Gobler and Sunda 2012). The average surface water temperatures in the Baffin Bay and the Upper Laguna Madre Complex range from 15 to 30 °C between May and October (Buskey et al. 1998; Rhudy et al. 1999), and culture-based studies have determined that *A. lagunensis* growth is optimal at 20 to 25°C (Buskey et al. 1998).

Salinities in the Laguna Madre can exceed 70 (Buskey et al. 1998), and *A. lagunensis* has been observed to proliferate in hypersaline conditions, although hypersalinity is not a strict growth requirement (Buskey et al. 1998; Cira and Wetz 2019). In addition, high levels of organic nutrients have been linked to bloom formation, as blooms have been seen to form in highly eutrophic systems (Wetz et al. 2017). As a result, the physical factors that select for *A. lagunensis* growth, such as high concentrations of organic nutrients, temperatures, and hypersalinity, establish the Baffin Bay and the Upper Laguna Madre Complex as an ideal system for brown tide blooms (Buskey et al. 1998; Rhudy et al. 1999).

Depressed zooplankton grazing activity has been implicated as a contributor to brown tide maintenance (Buskey and Hyatt 1995; Buskey et al. 1997). Grazing may decrease due to a bloom-associated deterioration of environmental parameters (e.g., depleted food sources, increased salinity, predation) (Buskey et al. 1998). Additionally, it was proposed that the physiology of *A. lagunensis* may be partially responsible (Buskey and Hyatt 1995; Buskey et al. 1997). Enveloping the algal cell is a protective sheath of extracellular polymeric substances (EPS) that decreases algal grazing (Liu and Buskey 2000). The EPS layer was subsequently shown to protect cells during zooplankton digestion (Bersano et al. 2002). This depressed grazing activity is thought to be yet another reason for long-term bloom maintenance (Porter 1976; Gobler and Sunda 2012).

The disruption of ecosystem processes following a brown tide bloom can devastate coastal economies reliant on tourism and coastal natural resources (Hoagland et al. 2002; Waycott et al. 2009). In Texas, in 2014, the shrimp, oyster, blue crab, snapper, and drum landings were valued at \$278.4 million (Kildow et al. 2016). Two of Texas' largest recreational fisheries, red drum and spotted seatrout, were estimated to be worth \$350 million and \$220

million, respectively (Vega et al. 2009). A bloom-related decrease in seagrass habitat and water quality would inevitably decrease landings and increase seafood costs (Hoagland et al. 2002). Along the Atlantic seaboard, from 1985 to 1987, a brown tide formed by *Aureococcus spp.* was responsible for mass mortality and recruitment failure in populations of bay scallops, causing the collapse of a multi-million-dollar industry (Kraeuter et al. 2008). Similarly, in the late 1980s through early 1990s, brown tide blooms were responsible for the near collapse of *Mercinaria mercinaria* clam landings (Kraeuter et al. 2008).

The limited flushing of the lagoon is a compounding factor that contributes to bloom initiation and maintenance (Buskey et al. 1998). Residence times for the Laguna Madre have been estimated to range from 300 days to several years (Buskey et al. 1998; Buskey et al. 2001). High residence times allow for large amounts of algal biomass accumulation, further exacerbating low nutrient availability (Gobler and Sunda 2012). High residence times, coupled with decreased freshwater inflows, also contribute to hypersalinity, further selecting for the growth of halotolerant algal species (Gobler and Sunda 2012). The average annual sunshine and subtropical temperatures enhance hypersalinity as evaporation rates typically exceed freshwater inflows (Largier et al. 1997; Mudge et al. 2008). As a result, the lagoon is regarded as a negative or inverse estuary, and the persistence of this inverse condition is thought to have favored the maintenance of the aforementioned eight-year bloom event (Buskey et al. 1998). Conversely, high-magnitude freshwater pulses, produced by tropical storms and hurricanes, can flush the system and terminate persistent blooms. However, the impact of perturbations in temperature, salinity, and nutrients on microbial dynamics in this system is still unknown.

Microbial Dynamics

The role of marine bacteria as decomposers of dissolved organic matter (DOM) was first observed in the 1930s (Waksman and Carey 1935; Waksman and Butler 1937). Phytoplankton production of nutrient-rich extracellular products and sloppy feeding and egestion by zooplankton stimulates bacterial growth (Bell and Mitchell 1972). Not surprisingly, bacterial abundance has been correlated with phytoplankton abundance (Needham and Fuhrman 2016). Thus, the growth and dynamics of bacteria and phytoplankton are closely linked. Further, the diversity of bacterial communities can reflect the diversity of phytoplankton communities (Turner et al. 2009; Andersson et al. 2010; Needham and Fuhrman 2016).

Temperature and salinity are strong drivers of microbial community composition. Both are critical limiting factors for microbial abundance and distribution in marine environments (Pomeroy et al. 2001). For example, mesophilic *Vibrio* species are normally temperature-limited, but increased temperature coupled with substrate abundance can promote bacterial blooms (Turner et al. 2009; Frischkorn et al. 2013; Westrich et al. 2018). Further, although *Vibrio* are halophiles, hypersalinity tends to select against survival (Froelich et al. 2012). Microbes adapt to fluctuations in temperature through cold- and heat-shock responses (Lim and Gross 2010). Adaptation to salinity fluctuations is mediated by the production of osmolytes, e.g., ectoine, glycine betaine, and choline, that help maintain homeostasis under high osmotic pressure (Robert 2005; Setati 2010).

The dynamics of microbial communities are dependent on the partition of microbes between free-living and particle-associated (DeLong et al. 1993; Crump et al. 1999; Hollibaugh et al. 2000). A particle-associated lifestyle provides microorganisms with increased nutritional resources and environmental stability (Azam et al. 1994), and particle-associated microbial communities are compositionally different from free-living communities (e.g., DeLong et al.

1993; Crump et al. 1999; Hollibaugh et al. 2000). When microorganisms attach to phytoplankton, that microhabitat is called the phycosphere. The phycosphere is the aquatic equivalent of the rhizosphere in terrestrial systems (Amin et al. 2012). The rhizosphere is the zone surrounding the roots of terrestrial plants where plant exudates and nutrients stimulate a diversity of microorganisms that play a critical role in terrestrial nutrient cycling (Mendes et al. 2013). Like the rhizosphere, the phycosphere is a zone of complex microbial interactions that play a critical role in marine nutrient cycling (Amin et al. 2012). Further, both zones exhibit significantly different chemistry (e.g., concentrations of organic compounds, oxygen levels, pH) in comparison to the surrounding water or soil, both produce exudates that stimulate microbial chemotaxis and colonization, both support the growth of similar genera such as *Rhizobium* and *Sphingomonas*, and both are dependent on the exchange of similar chemical currencies (e.g., sugars, amino acids, dimethylsulfoniopropionate (DMSP), and signaling molecules) (Seymour et al. 2017).

Microbial dynamics within the phycosphere are partly mediated by intra- and inter-kingdom communication (Amin et al. 2012; Cooper and Smith 2015; Kouzuma and Watanabe 2015). This communication is thought to be essential to the evolution of highly specific and mutualistic interactions between phytoplankton and their phycosphere inhabitants (Zhou et al. 2016). Pheromones are a wide-ranging group of chemicals suspected to aid in this communication (Decho et al. 2011; Zhou et al. 2016). Typically, these pheromones are lipid-based molecules that pass easily across cell membranes without needing energy-intensive facilitated membrane transport (Amin et al. 2012). Phytoplankton communication has been demonstrated in several laboratory studies by the attraction of gametes using carbon-based chain molecules (Pohnert and Boland 1996; Sato et al. 2011). In 2008, it was shown that lumichrome, a

derivative of vitamin B₂, produced by *Chlamydomonas* sp., is capable of stimulating the LasR quorum sensing (QS) receptor in *Pseudomonas aeruginosa* (Rajamani et al. 2008). Similarly, wildtype *Vibrio anguillarum* NB10, a producer of N-acyl-homoserine lactones (AHLs), was shown to regulate algal cell production through QS disruption using this class of communication molecules (Tait et al. 2005).

The interactions in the phycosphere range from mutualistic to communalistic to competitive to predatory (Amin et al. 2012). Ferrier et al. 2002 reported that the addition of native bacterial assemblages significantly enhanced the growth of *Alexandrium fundyense* (Ferrier et al. 2002), and Amin et al. 2012 commented that diatoms appear to “cultivate” the mutualistic bacteria in their phycosphere (Amin et al. 2012). Thompson et al. 2012 identified a relationship between cyanobacteria and unicellular algae where the algae provided fixed carbon in exchange for nitrogen fixed by bacteria (Thompson et al. 2012), with other studies also corroborating this finding (Foster et al. 2011). A similar association was identified when researchers found that cyanobacteria, Alphaproteobacteria, and archaeal groups can supply Vitamin B₁₂, necessary for B₁₂-dependent methionine synthase, to auxotrophic algae in exchange for fixed carbon (Croft et al. 2005). *A. lagunensis*, an auxotroph of B₁, B₇, and B₁₂, could require a similar relationship (Tang et al. 2010). In addition, mutualistic relationships were identified between algae and soil bacteria from the genera *Mesorhizobium*, *Azospirillum*, and *Rhizobium* (Kim et al. 2014). It has been speculated that this exchange of nutrients and vitamins is significant to global nutrient cycling (Cooper and Smith 2015).

Algae and bacteria will compete for available nutrients. In 1985, it was discovered that bacterial and algal communities competed for phosphorous (Bratbak and Thingstad 1985). Also, when in batch culture with bacteria, diatom cell densities decreased, and their growth was only

stimulated during the addition of phosphorous, suggesting that bacteria could scavenge phosphorous better than their algal competitors (Guerrini et al. 1998). This competition and subsequent growth limitation also increased exopolysaccharide production (Guerrini et al. 1998). Besides phosphorous, competition between algae and microbes can also be facilitated by nitrogen limitation. During a mesocosm study, it was shown that benthic microalgae can limit the growth of ammonia-oxidizing bacteria by depleting ammonia (Risgaard-Petersen et al. 2004). Conversely, a separate study showed that ammonia-oxidizing archaea can outcompete diatoms using exoenzymes with a greater affinity for ammonia (Martens-Habbena et al. 2009).

Algicidal bacteria negatively affect algal growth and are thought to influence bloom dynamics, including termination (Rashidan and Bird 2001). Indeed, the phycosphere, besides attracting mutually beneficial bacteria, can attract and harbor predatory microbes capable of lysing algal cells (Amin et al. 2012). This lytic interaction is facilitated through the production of exoenzymes such as glucosidases, chitinases, and cellulases (Mühlenbruch et al. 2018). In 2008, researchers isolated several enriched bacteria strains belonging to the Proteobacteria, Actinobacteria, and Bacteroidetes that were associated with the decline of dinoflagellate cultures (Kim et al. 2008). A similar study was conducted where researchers observed colonization of the phycosphere and lysis of a red tide forming dinoflagellate by *Alteromonas* species (Gammaproteobacteria) and *Thalassobius* species (Alphaproteobacteria) (Wang et al. 2010).

Summary

Over the past century, the global ocean has experienced significant climate stress (IPCC 2022). Increased SST, MHWs, and extreme weather events are increasing in frequency and intensity (Androulidakis and Kourafalou 2022; Lawman et al. 2022; Balaguru et al. 2023). These and related events greatly affect coastal communities and their animal inhabitants (Cavole et al.

2016), causing new and exacerbating existing issues. To add further to these issues, compounding impacts of decadal and multi-decadal trends, with more extreme weather events, such as what happened with Hurricane Michael, when a MHW caused it to intensify to a category 5 storm (Dzwonkowski et al. 2020). Specific to the LM, climate change is also expected to induce drought which are predicted to worsen coastal hypersalinity. In the face of this change, it is imperative to understand how organisms will be affected by this future change. As the ‘workhorse’ of ecosystem processes/services, prokaryotes offer enormous potential as model organisms for assessing and quantifying the effects of climate stress. Here, we wanted to investigate how microbes would respond to disturbance events within a low-flow, hypersaline estuary, as hypersaline systems are acutely vulnerable to disturbances, anthropogenic or environmental. We conducted 1) a short-term 2-month study of microbial community dynamics following a flood event, 2) a long-term 18-month study of microbial community dynamics that included flood and freeze events, and 3) a targeted study of wildlife connectivity (Mexican free-tailed bats) to microbial community dynamics and eutrophication. We hypothesized that disturbance events, including floods and a freeze, would cause a sustained shift in the microbial community structure and that wildlife, specifically Mexican free-tailed bats, could act as a nutrient source contributing to organic eutrophication within the estuary.

CHAPTER II

MICROBIAL COMMUNITY RESPONSE TO FLOOD DISTURBANCE IN A HYPERSALINE LAGOON

Abstract

Coasts experience freshwater inflows of varying magnitude. Flood events can be considered pulse disturbances. In the Northwest Gulf of Mexico (GoM), climate models predict increased salinity variability, storm severity, and coastal flooding. The microbial community response to flood disturbance is critical to understanding the future resilience and stability of coastal ecosystems. We monitored water quality and microbial community composition in a tertiary hypersaline bay following a flood. Changes were monitored weekly at two study sites over 11 weeks: the tidal segment of a low-flow tributary that experienced complete flushing and a distant non-flood site that experienced minimal effects. Salinity variance was accompanied by variance in dissolved oxygen, pH, water temperature, chlorophyll *a*, DOC, DON, DIN ammonia, silicate, and orthophosphate. Salinity and dissolved organic nutrients were dominant drivers of temporal microbial community variance. The flood stimulated a unique microbial consortium, and several bacterial taxa were significantly more abundant in the flood site: Alphaproteobacteria (Rhodobacterales; 6.02%), Gammaproteobacteria (Chromatiaceae; 1.54% and Alcanivoracaceae; 1.10%), and Cyanobacteria (Nostocales; 4.50%). An increase in chlorophyll *a* and silicate concentrations was indicative of an unspecified diatom bloom. Alphaproteobacteria and Gammaproteobacteria have the potential to mediate B-vitamin acquisition and supply diatoms with reduced sulfur compounds, whereas cyanobacteria have the potential to supply reduced carbon and nitrogen compounds. Additionally, some lower abundance flood site taxa [Phycisphaerae (1.48%), Planctomycetacia (2.93%), Kiritimatiellae (2.50%), Mollicutes

(2.91%), and Nitriliruptoria (3.05%)] could potentially perform site specific functions. Together, these results better our understanding of flood-induced shifts in microbial community composition and show that heavy precipitation temporarily (8 weeks) restructured the community until hypersaline conditions (> 40) returned.

Introduction

Disturbances profoundly impact the stability of microbial communities and their ecosystems (Shade et al. 2012). Disturbances can be characterized as pulse (discrete) or press (continuous), although these terms were originally coined to describe perturbation experiments (Bender et al. 1984). Floods are generally regarded as pulse disturbances that alter community composition until the affected system relaxes to its previous state. In this context, stability can be explored by investigating a system's resistance (insensitivity) and resilience (rate of recovery) to flood disturbance.

Current trends and future climate models portend a global increase in hurricane risk (Balaguru et al. 2023). Further, robust satellite data recently revealed that extreme droughts and floods will be the 'most conspicuous consequence' of climate change (Rodell and Li 2023). Regional predictions are less precise, but the Fourth National Climate Assessment predicts the United States Southern Great Plains (i.e., Kansas, Oklahoma, and Texas) will experience increased heavy precipitation events (Easterling et al. 2017). Hurricane Harvey caused the most significant rainfall in United States history (60.58 inches near Nederland, Texas), and trends show a 5-7% increase in hurricane-related heavy precipitation over the last century. The rainfall introduced a significant shift in the microbial community toward bacteria more commonly associated with terrestrial soils and freshwater habitats, but the shift reversed toward estuarine and marine bacteria within one month (Steichen et al. 2020).

Hypersaline lagoons are particularly vulnerable to flood disturbance. The Laguna Madre, in the Northwest Gulf of Mexico (GoM), has experienced prolonged droughts interrupted by heavy precipitation events (Wilson and Dunton 2018). Upstream freshwater removal by a growing coastal population is predicted to further reduce freshwater inflows (Montagna et al. 2013), and fewer soaking rains are predicted to increase flood severity (Easterling et al. 2017). Reduced freshwater inflow coupled with severe flooding will increase osmotic stress among lagoon inhabitants and potentially restructure microbial communities between flood and drought events. A better understanding of flood resilience and recovery is critical to predicting how microbial communities will adapt and evolve to future climate scenarios.

A recent meta-analysis identified 60 hypersaline lagoons, estuaries, and embayments worldwide (Tweedly et al. 2019). The Laguna Madre is a large hypersaline lagoon system found in the northern Gulf of Mexico on the Texas coast. These systems are commonly located in arid and semi-arid tropical and sub-tropical climate zones. Climate change will impact these systems through the alteration of flood and drought. Concurrently, sea level rise will influence ocean connectivity. This study assessed how microbial communities respond to flood disturbance in a tertiary bay (Laguna Salada) inside the Laguna Madre complex. We hypothesized that flooding would cause a significant and sustained shift in microbial community structure. Findings establish a knowledge baseline for understanding the microbial flood response in this system, but the results can be extrapolated to better understand flood disturbance in distant hypersaline systems.

Materials and Methods

Study Sites

The flood study site was located in Los Olmos Creek (27°16'23.62" N, 97°48'08.01" W), a tributary that flows into the Laguna Salada segment of Baffin Bay, Texas, United States (Figure 1). The creek is a shallow, low-flow tributary, and the tidal segment is hypersaline (Tunnell and Judd 2002); however, heavy rainfall can flush the system (Cira et al. 2021). The Laguna Salada is a well-documented reservoir for persistent brown tide blooms that may aid the formation of more expansive blooms throughout Baffin Bay and the Laguna Madre (Cira and Wetz 2019). The non-flood site was located at Riviera Beach (27°17'00.09" N, 97°39'52.80" W) at the confluence of the Laguna Salada and Baffin Bay, 13.7 km from the Los Olmos Creek flood site (Fig. 1).



Fig. 1 Map of the flood (red) and non-flood (blue) study sites in the western Laguna Salada segment of Baffin Bay, Texas, United States. Figure rendered via GIS. Map credit Md

Mahabubur Rahman

Sample Collection

Surface water samples for microbial analysis (1 L) were collected in duplicate in sterile 1 L polypropylene bottles from the flood (Los Olmos Creek) and non-flood (Riviera Beach) study sites. Samples for water quality (1 L) were collected were also collected from surface water. Samples were collected weekly over 11 weeks: June 27 through September 7, 2018. The first sampling event (June 27, 2018) followed a high-intensity storm (invest 91L on June 19, 2018) responsible for significant flooding in Los Olmos Creek. Sampling concluded 81 days later (September 7, 2018) after heavy rainfalls again flooded the system. All samples were transported on ice and processed within four hours of collection.

Water Quality Parameters

Water temperature (°C), pH, dissolved oxygen (%), and salinity were recorded with a YSI 556 Multi Probe System (YSI Incorporated, Yellow Springs, OH, United States). Wind speed (mph) and air temperature (°C) were measured using a Kestrel 3000 Wind Meter (Kestrel Meters, Boothwyn, PA, United States). To quantify dissolved organic carbon (DOC) and total dissolved nitrogen (TDN), aliquots were pressure filtered through combusted Ahlstrom disc filters (Ahlstrom -MUNKSJÖ, Helsinki, Finland) into 60 mL bottles using a hand syringe and filter attachments and stored at -20°C [Wetz et al. 2017]. Both DOC and TDN were analyzed simultaneously with the Shimadzu TOC-Vs Analyzer (Kyoto, Japan) using Standard Methods 5310 High-Temperature Combustion [Wetz et al. 2017]. Dissolved organic nitrogen (DON) was determined by subtracting dissolved inorganic nitrogen (ammonium, nitrate + nitrite (NOX) from TDN [Wetz *et al.*, 2017]. Inorganic nutrient (e.g. orthophosphate, silicate, nitrites, nitrates, and ammonium) concentrations were determined from 25 mL aliquots of flood and non-flood samples. As with DOC or TDN, aliquots were pressure filtered through combusted Ahlstrom disc filters (Ahlstrom -MUNKSJÖ, Helsinki, Finland) into 30 mL bottles using a similar syringe and attachment apparatus and stored at -20°C. Filtrate was analyzed with a QuAatro inorganic nutrients autoanalyzer (Seal Analytical, United Kingdom) using Standard Methods 4500-P F for orthophosphate, 4500-NO₃ F for nitrates and nitrites, 4500 SiO₂ C for silicate, and 4500-NH₃ G for ammonium [Wetz et al. 2017]. To determine chlorophyll *a* concentrations, 25 mL aliquots were vacuum filtered onto 25 mm Whatman GF/F filters (Whatman plc, Maidstone, United Kingdom) (stored at -20°C), washed in an acetone solution, and then the filtrate was analyzed using a Turner Designs Fluorometer (Turner Designs, San Jose, California) Standard Methods 10200 H.3 [Wetz et al. 2011]. Briefly, nutrient samples were... Water transparency was measured

using a transparency tube fixed with a Secchi disk (cm) (Carolina Biological Supply Company, Burlington, NC, United States).

Statistical Analysis

ANOVA, Kendall's tau correlation, and principal component analysis (PCA) were used to characterize site conditions and identify differences in their physiochemical parameters.

DNA Isolation

To concentrate seston, 100 mL subsamples were filtered through low-protein binding 0.22 µm polyethersulfone (PES) filters (MilliporeSigma, Burlington, MA, United States). Total genomic DNA was isolated from the filters using a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA quantity (ng/µl) and quality (A260/A280 and A260/A230 absorbance ratios) were measured using a biospectrophotometer (Bio-Rad, Hercules, CA, United States). The DNA was stored at -80 °C.

16S rRNA Gene Amplicon Sequencing

The 515F (5' – GTG YCA GCM GCC GCG GTA A – 3') and 806R (5' – GGA CTA CNV GGG TWT CTA AT – 3') primers were used to amplify the V4 region of the 16S rRNA gene sequence (Walters et al. 2015) using a HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, United States). The following cycling conditions were used for amplification: 94 °C for 3 minutes, 30 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds, and 72 °C for one minute, followed by a 5-minute hold at 72 °C. The 16S rRNA gene was sequenced on an Illumina MiSeq platform using paired-end chemistry (2 x 300 bp) at Molecular Research L.P. (Shallowater, TX, United States).

Taxonomic Analyses

Raw sequence reads were processed using QIIME version 1.9 (Caporaso et al. 2010, 2012) and QIIME2 version 2018.11 (Bolyen et al. 2019). Barcodes were extracted from the paired-end reads using the ‘extract_barcodes.py’ tool in QIIME. The following steps were performed within QIIME2. Reads were demultiplexed and denoised with DADA2 (Callahan et al. 2016), resulting in amplicon sequence variants (ASVs). Trim lengths of 242 bp were used on both the forward and reverse reads. In addition to denoising the data, DADA2 filtered sequences for quality, removed chimeric sequences, and merged paired-end reads. A phylogenetic tree was then generated using the ‘q2-phylogeny’ pipeline with default settings, which was used to calculate phylogeny-based diversity metrics. Taxonomy was assigned using a Naïve Bayes classifier trained on the SILVA v. 132 99% OTUs database (Quast et al. 2013), including only the 250 bases from the V4 region bound by the 515F/806R primer pair. Reads mapped to chloroplast and mitochondrial sequences were filtered out from the sequence variants table using the ‘filter_taxa’ function. Faith’s phylogenetic distance (PD) and the Shannon-Weiner (SW) diversity index were calculated for all samples using the ‘q2-diversity’ plugin.

Data were then imported into phyloseq (McMurdie and Holmes 2013) using the ‘import_biom’ and ‘import_qiime_sample_data’ functions and merged into a phyloseq object. All samples maintained high read counts and did not require proportional transformation to a normalized read count. Beta diversity was analyzed using a distance matrix based on weighted UniFrac (Lozupone et al. 2011) distances calculated in phyloseq using the ‘q2-diversity’ plugin. As stated in Lozupone et al. 2011, briefly, UniFrac measures the difference between two collections of sequences (e.g. 16S rRNA molecules sequenced from different microbial samples) as the amount of evolutionary history unique to either, which is measured as the fraction of branch length in a phylogenetic tree that leads to descendants of one sample or the other but not

both. A principal coordinate analysis (PCoA) was calculated and plotted from these distances. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences between communities using the ‘vegan’ (Oksanen et al. 2019) and ‘pairwiseAdonis’ (Arbizu 2017) packages in R version 3.5.2 (R Core Team, 2017). To test if variance was due to dispersion of variability among groups, permutational analyses of multivariate dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes with the ‘vegan’ package in R. Microbial community differences were further confirmed via linear discriminant analysis (LDA) effect size (LEfSe) using the LEfSe tool on the Galaxy server (<https://huttenhower.sph.harvard.edu/galaxy/>). Genera that comprised more than 1.00% of the communities’ relative abundance were analyzed using default settings with the significance threshold set to a p-value of < 0.01.

Results

Site Conditions

Approximately one week before the first sampling event on June 27, 2018, tropical invest 91L (June 19, 2018) caused widespread flooding in the Baffin Bay watershed. The flooding raised the creek stage from 0.64 to 3.75 m, flushing the system. Following the flooding event, negligible precipitation (<2 cm) was experienced until the conclusion of the study, 81 days later (September 7, 2018), when heavy rainfalls again flooded the system. Throughout the study, we observed the expansion joints in the Highway 77 overpass were home to a large Mexican free-tailed *Tadarida brasiliensis* bat colony (Fig. 2). We also observed abundant garbage dumping (e.g., discarded furniture, Styrofoam cups, plastic bags, cigarette butts, broken glass, tires, plastic and rubber car parts) and signs of road runoff (e.g., erosion around the highway and bridge supports, large ditches, and the absence of vegetation).



Fig. 2 Mexican free-tailed bat (*T. brasiliensis*) colony. In a clockwise direction from the upper left, panels show guano concentrated along the wrack line, guano and urine discoloration along bridge support, a zoomed-out view of the expansion joints, and a zoomed-in view of the same expansion joints showing roosting bats

Water Quality Parameters

Appendix A summarizes the water quality parameters. Salinity in the flood site increased from 0.94 to 64.81. By contrast, in the non-flood site, salinity increased from 21.39 to 41.69. The flood and non-flood site recovered hypersaline conditions (>40) after 8 weeks. Dissolved oxygen (DO) in the flood site increased from 40.00% to supersaturated after 7 weeks, while DO at the non-flood site increased from 78.10% to supersaturated after 4 weeks. pH ranged from 7.15 to 9.23 and 7.96 to 8.48 in the flood and non-flood sites, respectively. Chlorophyll *a* was significantly different between sites (ANOVA; p -value < 0.05), ranging from 23.19 to 471.85 $\mu\text{g/L}$ at the flood site and 1.86 to 12.83 $\mu\text{g/L}$ at the non-flood site. Water temperature was more consistent, ranging from 30.00 to 32.70 $^{\circ}\text{C}$ at the flood site and 28.54 to 31.70 $^{\circ}\text{C}$ at the non-

flood site. Certain nutrient concentrations (i.e., DO, DOC, DON, DIN, ammonia, nitrate, nitrite, orthophosphate, silicate) were significantly different between sites (ANOVA; p -value < 0.05), but other concentrations (ammonia, nitrate, nitrite, orthophosphate, dissolved inorganic nitrogen, and silicate concentrations showed minimal variance within sites. Organic nutrient concentrations in the flood site were highly elevated compared to the non-flood site. Flood site DOC and DON averaged 2,603.60 and 210.75 $\mu\text{mol/L}$, in stark contrast to 770.80 and 80.87 $\mu\text{mol/L}$ at the non-flood site. The PCA demonstrated that the flood site was impacted by variance in salinity as well as dissolved oxygen, pH, water temperature, chlorophyll a , DOC, DON, DIN ammonia, silicate, and orthophosphate (Fig. 3).

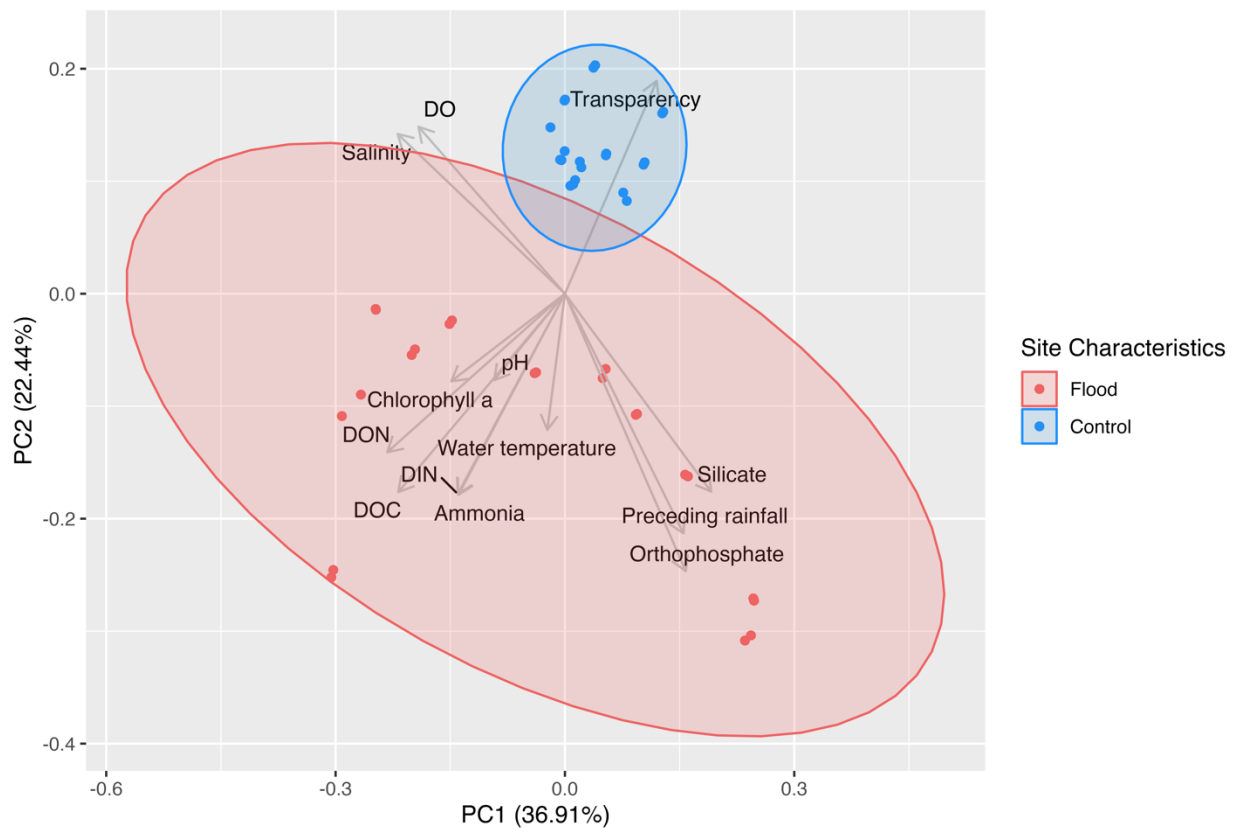


Fig. 3 Principal component analysis (PCA) of water quality parameters of the flood (Los Olmos Creek) and non-flood (Riviera Beach) sites

Microbial Community Diversity and Relative Abundance

Alpha diversity values (Fig. 4) were not significantly different between sites. To further determine community differences, microbial community composition was visualized using PCoA (Fig. 5) with a clear distinction between flood and non-flood communities (PERMANOVA; adj. p-value < 0.05), and salinity was a driver of community variability. Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia were the three most commonly observed classes at either site (Fig. 6 and 7). Bacteroidia and Gammaproteobacteria each composed 17.89 and 12.60%, respectively, at the flood site and 15.99 and 14.86% at the non-flood site. While comprising only 10.22% of the flood site microbial community, Alphaproteobacteria comprised nearly one-third (29.56%) of the non-flood site community. Although identified at both sites, “open water” Alphaproteobacteria groups, such as SAR-116 clades and the AEGEAN-169 marine group, were only detected at the non-flood site, comprising 16.83% over half of the Alphaproteobacteria community. Oxyphotobacteria (8.51%) and an unidentified phylum (6.55%) were observed to be the fourth and fifth most abundant taxa, respectively, composing 15.06% of the flood site community in comparison to 4.96% of the non-flood community. Alternatively, the Campylobacteria (7.33%) and Acidimicrobiia (4.25%) were the fourth and fifth most abundant classes observed in the non-flood community, considerably more than the flood site community (0.63% and 0.64%). One class, Actinobacteria, was similarly abundant at the flood and non-flood sites (4.14 and 4.08%, respectively).

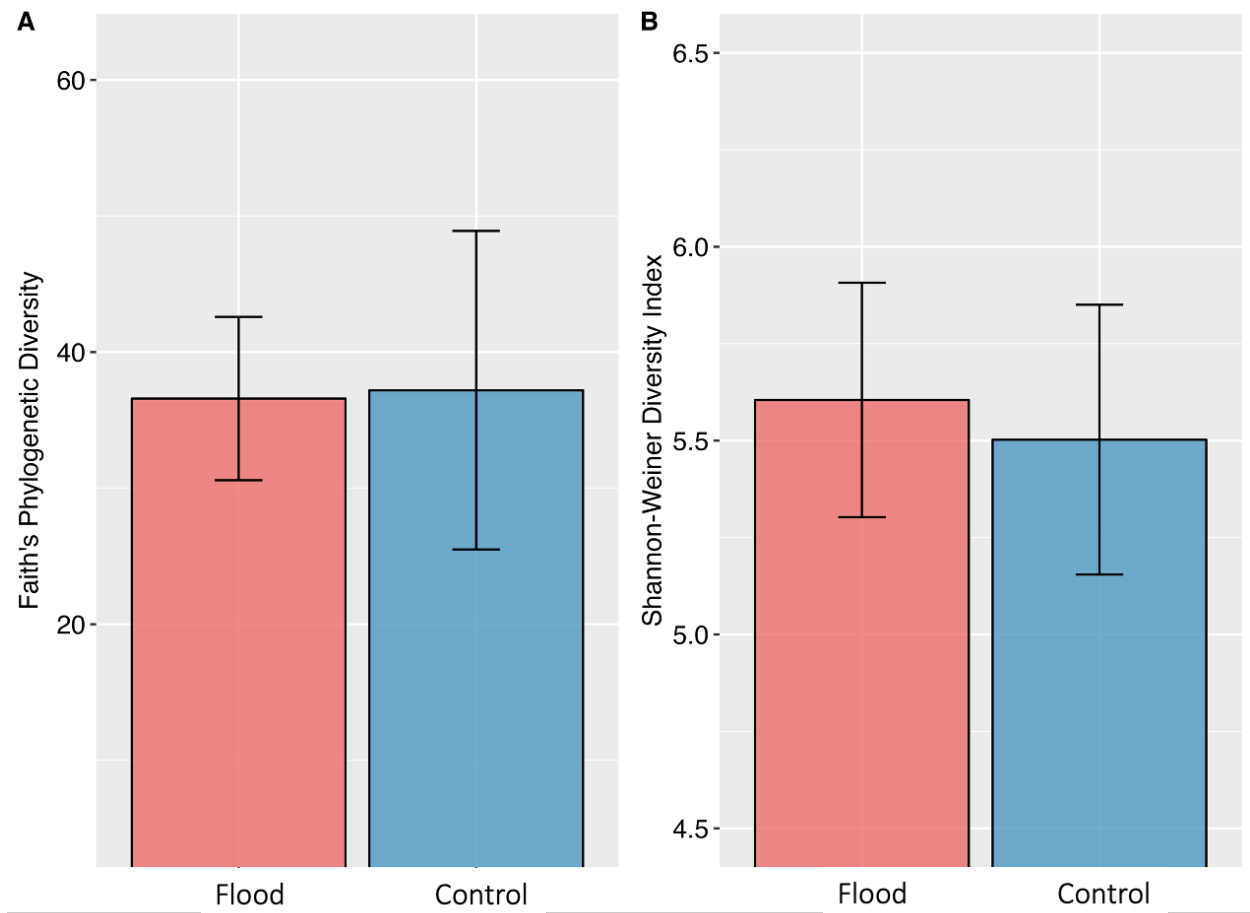


Fig. 4 Subset bar plots of Faith's phylogenetic diversity (A) and Shannon-Weiner diversity (B) values for Los Olmos Creek (red) and Riviera Beach (blue) sites. Site-specific communities were not significantly different according to either measure ($p > 0.05$)

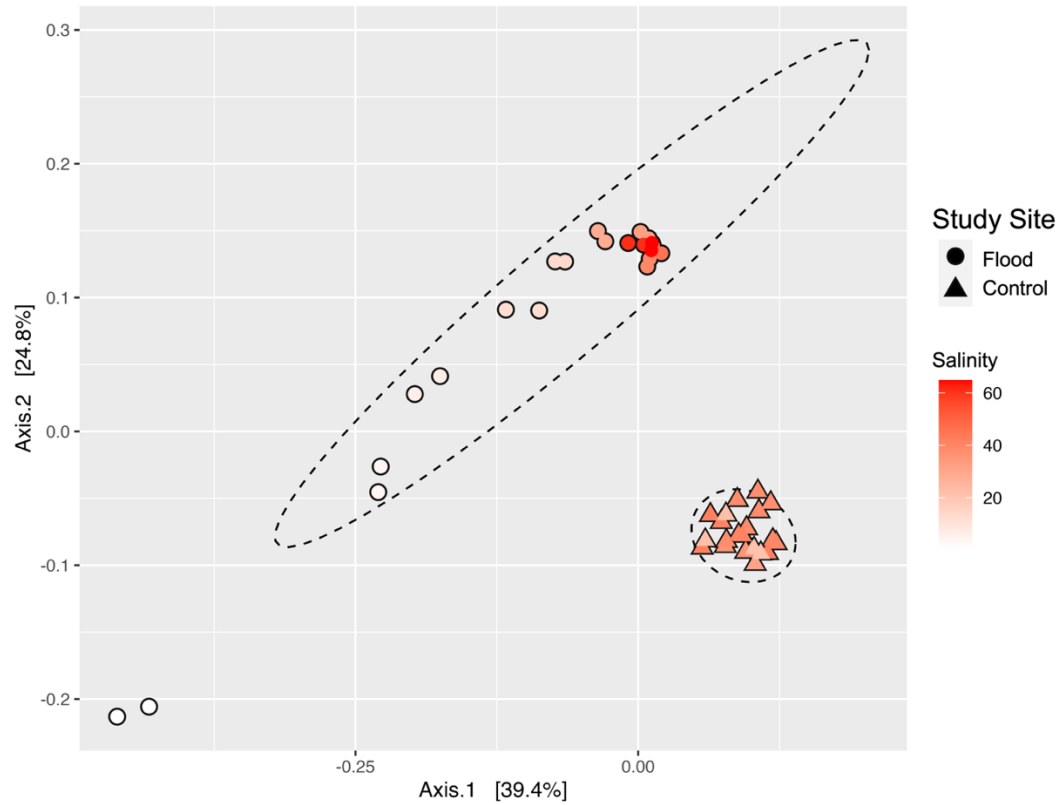


Fig. 5 Principal coordinate analysis (PCoA) of beta diversity values from the flood (Los Olmos Creek) and non-flood (Riviera Beach) sites using weighted UniFrac distance values. Flood and non-flood communities were compositionally distinct (p-value < 0.05)

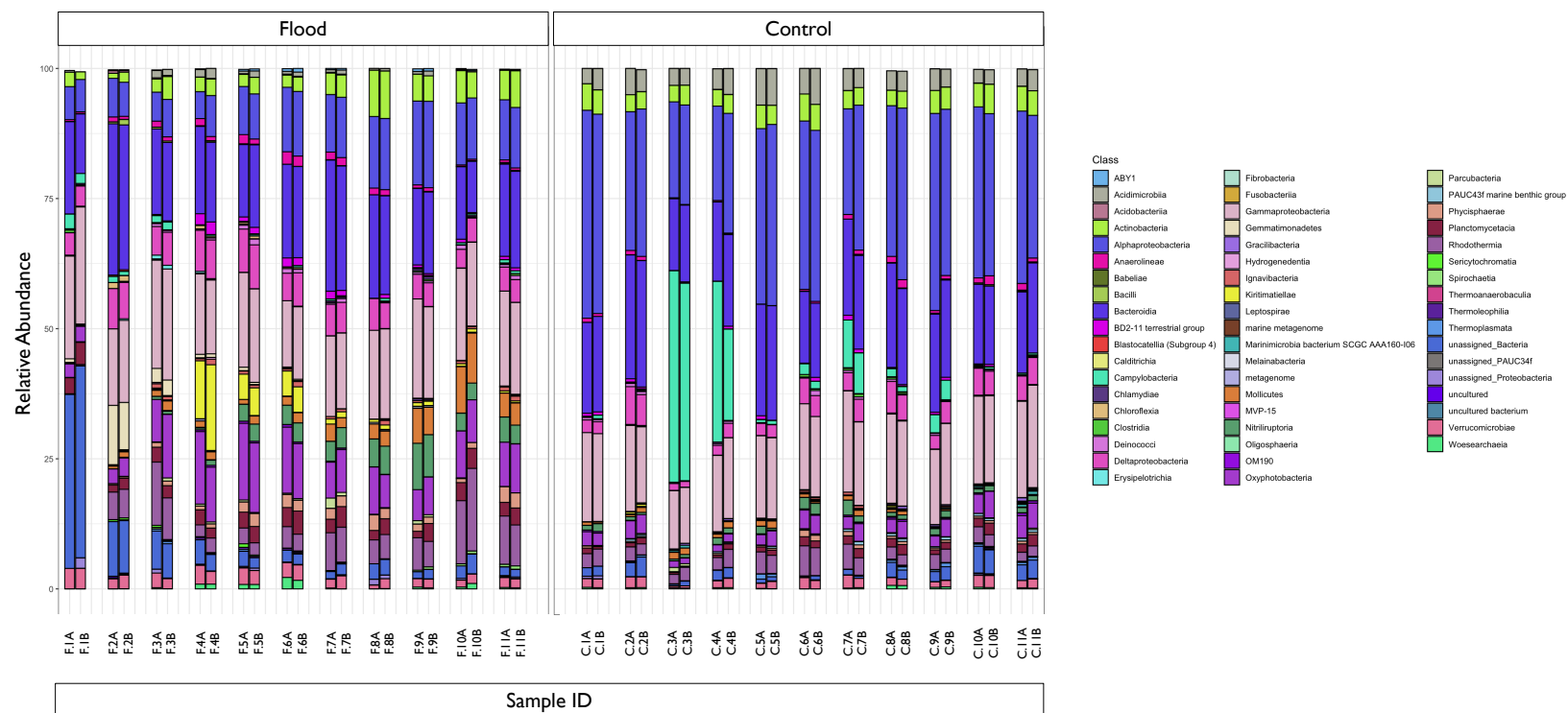


Fig. 6 Relative abundance bar plots of observed microbial communities within the flood and non-flood sites (1.00% abundance cutoff)

Enriched Flood Site Taxa

Linear discriminant analysis (LDA) effect size (LEfSe) was implemented to further investigate and confirm differences in the flood and non-flood communities. Several groups were overrepresented in the flood community compared to the non-flood community. Members of Gemmatimonadetes (1.38%), Phycisphaerae (1.48%), Verrucomicrobiae (2.37%), Kiritimatiellae (2.50%), Mollicutes (2.91%), Planctomycetacia (2.93%), Nitrospirae (3.05%), Betaproteobacteria (4.59%), and Balneolia (5.85%) composed over a quarter of the flood community (27.60%) and were all enriched compared to the non-flood (p -value < 0.05). These taxa were all significantly less abundant in the non-flood community (Fig. 7). By contrast, fewer bacterial taxa were enriched in the non-flood site e.g., Campylobacteria (7.33%), Acidimicrobiia (4.25%), and Flavobacteriales (Bacteroidia; 8.55%). However, neither Campylobacteria nor Acidimicrobiia composed over 1% (0.3 and 0.5%) of the flood site community, whereas Flavobacteriales composed 5.21%.

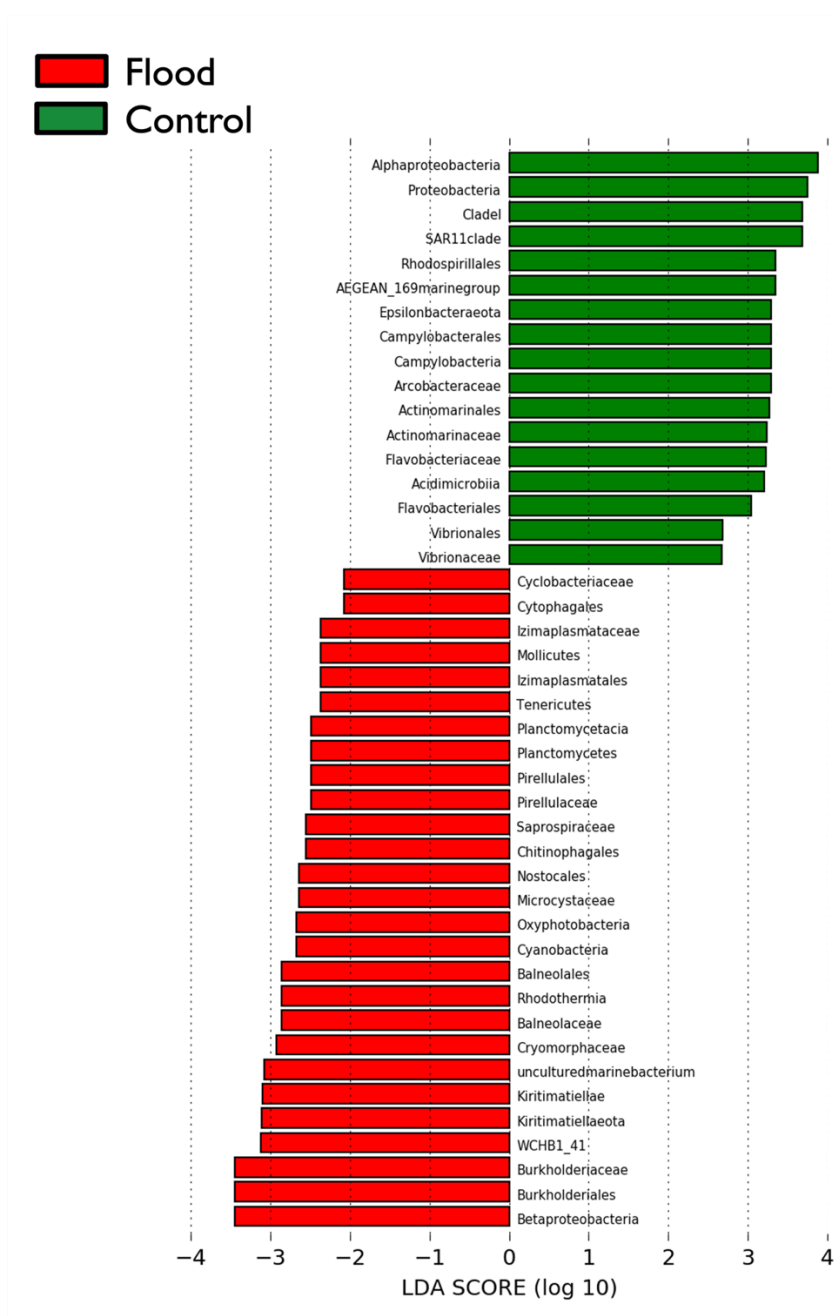


Fig. 7 Linear discriminant analysis (LDA) effect size (LEfSe) histogram depicting enriched taxa of the flood (Los Olmos Creek; red) and non-flood (Riviera Beach; green) site samples (p-value < 0.05)

Discussion

Microorganisms offer enormous potential as model organisms for assessing the effects of disturbance. Their small size, fast generation time, high mutation rate, and genome plasticity make microorganisms immediately responsive to environmental change or perturbation. A comprehensive review of the microbial disturbance literature (n = 247 studies) revealed that 82% reported sensitivity to disturbance (Shade et al. 2012). Analysis of water quality data from the Texas Commission on Environmental Quality (TCEQ) Surface Water Quality Monitoring (SWQM) program characterized sections of the Laguna Madre (Baffin Bay and the Upper Laguna Madre Complex) as ‘hotspots’ of environmental change (Bugica et al. 2020). This study is the first to assess how microbial communities respond to flood disturbance in low-flow and inverse estuaries.

Los Olmos Creek shows signs of eutrophication. Dissolved organic nitrogen was highly elevated compared to the non-flood, and increasing chlorophyll *a* and silicate suggested the occurrence of an unspecified diatom bloom. Low inflow, high residence times, hypersalinity, and elevated chlorophyll *a* and organic nitrogen concentrations in Baffin Bay are historically associated with persistent brown tide algal blooms (*Aureoumbra lagunensis*) (Wetz et al. 2017; Cira and Wetz 2019; Bugica et al. 2020; Cira et al. 2021; Beecraft and Wetz 2022; Chin et al. 2022). The maintenance of the most persistent bloom recorded, lasting eight years, is thought to be the sum of these factors resulting in prolonged hypersalinity, light attenuation, and the disruption of grazer populations (Buskey et al. 1995; 1998). Interestingly, guano from a large Mexican free-tailed bat colony (*T. brasiliensis*, identified in this study) may provide additional nutrients to support bloom formation and maintenance. The Texas Clean Rivers Program monitors the eutrophication of the creek, and it may soon be listed as impaired under Section 303(d) of the United States Clean Water Act due to a history of elevated fecal bacteria

concentrations (Lucas Gregory, Associate Director of Research, Texas Water Resources Institute, personal communication). Additional research is needed to assess the role guano-derived nutrients play in the eutrophication of this system.

A previous study reported that salinity was the best predictor of benthic macrofauna diversity in Baffin Bay (Breux et al. 2019). Here, beta diversity (weighted UniFrac) revealed that the flood and non-flood microbial communities differed significantly. However, alpha diversity was not significant. Non-significant (ANOVA; $p\text{-value} > 0.05$) alpha diversity values (Faith's PD) can be explained by the presence of fewer taxa and the presence of more phylogenetically similar taxa. In contrast, the non-flood community maintained more phylogenetically dissimilar taxa. Periodic flooding events may act as pulse disturbances that diminish species diversity. Alternately, the return to hypersalinity may select for the growth of more phylogenetically similar taxa. Previous studies in Baffin Bay have shown that pulsed inflows temporarily increase phytoplankton diversity (Beecraft and Wetz 2022; Chin et al. 2022). Disturbance typically increases diversity, and the intermediate disturbance hypothesis suggests periodic disturbance will maintain the highest level of diversity (Petraitis et al. 1989). However, we anticipated that extreme flood disturbance that completely flushes the system would temporarily decrease diversity. Lower diversity suggests decreased stability and may reflect the ephemeral nature of an ecosystem that swings between drought and flood.

Based on the relative abundance of specific bacterial taxa, Los Olmos Creek supported a distinct microbial consortium. Enriched bacteria included Rhodobacterales (Alphaproteobacteria) (6.02%) and Verrucomicrobiae (2.37%). Rhodobacterales and Verrucomicrobiae may support algal growth and algal blooms through the provision of B vitamins (Croft et al. 2005; Gómez-Consarnau et al. 2018). Oxyphotobacteria (Cyanobacteria) were also enriched in the flood site.

For example, the Oxyphotobacteria composed 8.51% of the flood consortium. Additionally, the order Nostocales, especially members of the family Microcystaceae, were enriched, comprising 4.50% of the flood community. Cyanobacteria could affect algal bloom dynamics by providing reduced forms of nitrogen and contributing to the organic carbon pool via carbon fixation. The overabundance of cyanobacteria may also reflect the taxa's proclivity for dissolved organic nitrogen in a eutrophic system.

The majority of Gammaproteobacteria identified within the flood community co-occurred in the non-flood. However, some Gammaproteobacteria, such as the Chromatiaceae (1.54%) and Alcanivoracaceae (1.10%), were enriched in the flood community. The Chromatiaceae are a group of phototrophic purple sulfur bacteria (PSB) capable of utilizing organic and inorganic sulfur compounds (Frigaard and Dahl 2008). Runoff from the Highway 77 overpass may explain the presence of Alcanivoracaceae, which have been associated with oil contamination (Yakimov et al. 2019). Nonpoint source runoff from roadways pollutes aquatic environments with a range of pollutants e.g., fertilizers, pesticides, various organic nutrients, and sewage (Huber et al. 2000; Wada et al. 2015, 2020; Ma et al. 2018; Zhao et al. 2022). Future research could be warranted to investigate the potential role of road runoff and oil pollution in this system.

Betaproteobacteria, specifically members of Burkholderiaceae, were also enriched within the flood community (3.19%). Aside from the Gammaproteobacteria mentioned above, members of the Burkholderiaceae have also been known to utilize sulfur compounds as energy sources (Frigaard and Dahl 2008). Additionally, the Burkholderiaceae includes known animal and plant pathogens and symbionts commonly found in polluted aquatic environments (Coenye 2014). Sewage pollution and nutrient pollution in Los Olmos Creek may select for the growth and maintenance of this family.

Cyclobacteriaceae and Saprospiraceae were the only members of the Bacteroidetes enriched in the flood community, making up 8.46% of the 17.84% total detected. Bacteroidetes are commonly associated with various algae (Amin et al. 2012; Buchan et al. 2014; Qu et al. 2020; Vidal-Melgosa et al. 2021). Bacteroidetes elaborate enzymes capable of degrading laminarin, a storage form of glucose produced by brown macroalgae (class Phaeophyceae) (Huang et al. 2021; Unfried et al. 2018; Vidal-Melgosa et al. 2021), pointing to a possible ecological function. Saprospiraceae are important degraders of complex carbon sources and exhibit algicidal behavior (Furusawa et al. 2003; Mayali 2004). Additionally, both families have been associated with wastewater treatment systems globally (Kondrotaitė et al. 2022), suggesting that sewage pollution may select for their abundance. The outfall of the Riviera Water Control and Improvement District WWTP empties into Los Olmos Creek immediately upstream of the sampling site (Nueces River Authority, personal communication). Nonpoint source agricultural runoff may also select for Cyclobacteriaceae and Saprospiraceae, but previous studies have only investigated its effects on brown tide phytoplankton blooms (Buskey et al. 1999; Felix and Campbell 2019).

Balneolaceae was one of the most abundant taxa and heavily enriched in the flood site, composing 7.06% of the community. This family is part of a newly proposed phylum (Balneolaeota) with an affinity for highly alkaline and saline environments (Munoz et al. 2016; Vavourakis et al. 2018). Balneolaceae are common constituents of tannery waste-contaminated soils, characterized by high metal and salt concentrations (Lukhele et al. 2022). Balneolaceae were also shown to be abundant in salt lakes on the Tibetan Plateau of China (He et al. 2022). The hypersaline conditions at Los Olmos Creek may select for this family's abundance. Additionally, heavy metals (e.g., arsenic, lead, chromium, and nickel) present in herbicides and

pesticides and introduced through agricultural runoff may select for their abundance (Defarge et al. 2018; Alengebawy et al. 2021).

In contrast to the more abundant taxa (such as Balneolaceae), several groups were enriched in much lower concentrations. Collectively, these rare taxa [Phycisphaerae (2.02%), Planctomycetacia (3.12%), Kiritimatiellae (1.41%), Mollicutes (4.50%), Nitriliruptoria (4.84%), and Deltaproteobacteria (Bdellovibrionaceae, 1.58%)] composed 17.47% of the flood community. Rare taxa can play a disproportionate role in ecosystem functioning (Shade et al. 2014), and their enrichment suggests they serve a function in the flood community. For example, Bdellovibrionaceae are well-documented algal predators (Caiola and Pellegrini 1984; Lee et al. 2018; Bauer and Forchhammer 2021), and Phycisphaerae can metabolize complex organic compounds produced by a suite of algal species (Fukunaga et al. 2009; Yoon et al. 2014; Rios-Del Toro et al. 2018). Additionally, Planctomycetacia, Kiritimatiellae, Mollicutes, and Nitriliruptoria have been associated with algal blooms (Kallscheuer et al. 2021), hypersaline cyanobacterial mats (Spring et al. 2015), plant and animal pathogens (Miles 1992), and complex nitrogen degradation (Sorokin et al. 2009).

This study characterized the microbial response to flood disturbance in a hypersaline lagoon. The flood site exhibited higher variability in salinity, dissolved oxygen, pH, water temperature, chlorophyll *a*, DOC, DON, DIN ammonia, silicate, and orthophosphate. The flood site's distinct microbial consortium included taxa that may perform site-specific functions. Future flood disturbance, brought on by prolonged droughts, reduced freshwater inflow, and increased storm severity, will test the system's resilience. This study demonstrated that flooding and salinity variation caused a dramatic shift in microbial community composition, highlighting the need for a better baseline understanding of flood effects on microbial communities. In light

of these results, additional long-term research is needed to characterize the microbial community across multiple flood disturbances.

Acknowledgments

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CHAPTER III

RESILIENCE OF HALOPHILE COMMUNITIES FOLLOWING FLOOD AND FREEZE DISTURBANCES IN A HYPERSALINE CREEK

Abstract

Inland hypersaline lakes dominate our understanding of hypersaline systems and halophilic microorganisms. In contrast to their inland analogs, the microbial diversity of coastal hypersaline systems is underexplored. Here, we monitored the water quality and microbial community composition of a hypersaline creek in the northwest Gulf of Mexico. Changes were monitored for 18 months at two study sites: a hypersaline creek and a connected hypersaline bay (12 sampling trips, duplicate sampling, N = 48). Drought, flood, and freeze events were observed. The halophile community was remarkably resistant to flood and freeze disturbances. In particular, a flood decreasing salinity from 78.10 to 19.18 and a freeze decreasing temperature from 27.50 to 12.10 °C did not significantly alter the community's beta-diversity. Regardless, salinity was the dominant environmental driver of community change. The five most abundant taxa (i.e., Synechococcales, Actinobacteria, Balneolaceae, Gammaproteobacteria Incertae Sedis (ord.), and Rhodobacteraceae) were enriched at the more hypersaline creek. These taxa may fill specific functional roles, including commensalism with co-occurring algae or remediation of urban and agricultural pollutants. Less abundant *Salinivibrio siamesis* strains were isolated from the creek, and whole-genome sequencing revealed genes beneficial to osmoregulation and copper resistance, and laboratory testing indicated that salt and copper tolerance were cross-protective. Together, these results better our understanding of halophile community resilience and shed light on mechanisms of osmoregulation and copper tolerance.

Introduction

Hypersaline environments support a diversity of microorganisms across all three domains of life: Bacteria, Archaea, and Eukarya (Oren 2016). High salt concentrations were thought to limit microbial community complexity, but modern culture-independent studies have revealed highly diverse hypersaline communities (Saccò et al. 2021). Osmotic adaption can be achieved through varied mechanisms like high-affinity pumps that couple the import of K^+ with the export of Na^+ (Chen et al. 2019). Additionally, the production or accumulation of osmolytes such as ectoine and glycine betaine enhances salt tolerance and prevents Na^+ accumulation (Robert et al. 2000). For example, the halophilic bacterium *Natranaerobius thermophilus* imports/accumulates K^+ and glycine betaine while simultaneously exporting Na^+ through a large repertoire of $Na^+(K^+)H^+$ antiporters and Na^+ extruding ATPases (Mesbah et al. 2009).

Halophiles survive and thrive at the physico-chemical boundary of life. They tolerate high salt concentrations and often show resilience to high temperatures and UV radiation, conditions common to arid tropical and subtropical ecosystems that experience high evaporation rates. On Mars, halophiles may persist in saline brine pockets in the permafrost, formed by the retreat of water after the planet lost its oceans and atmosphere (Mancinelli et al. 2004). Alternately, life in ancient hypersaline systems on Mars could be investigated by studying analogous systems here on Earth (Perl and Baxter 2020). Hence, halophiles are highly regarded as models for astrobiology (DasSarma et al. 2006).

The biotechnological applications of halophiles remain limited compared to thermophilic and alkaliphilic extremophiles (Oren 2010). Exceptions include the halophilic microalgae *Dunaleilla*, a ‘nutraceutical’ beta-carotene source for humans and agricultural animals (Marino et al. 2020). The halophilic archaea *Halobacterium* is a source of bacteriorhodopsin, a light-activated protein pump with bioelectronic applications like artificial retinas (Singh et al. 2021).

Meanwhile, ectoine, produced by various halophilic bacteria, including *Halomonas*, can protect enzymes against heating, freezing, and drying (Pastor et al. 2010). For instance, ectoine is used as a protectant in molecular biology techniques such as PCR (Ma et al. 2010). Halophiles are gaining attention as a promising source of bioplastics, including polyhydroxyalkanoates (Obruča et al. 2022).

Little is known about the prokaryotic halophiles inhabiting the Laguna Madre. Although hypersalinity is a defining feature of the lagoon, salinity variability between drought and flood can be severe. Hence, the prokaryotes that persist in this system are thought to be resistant to fluctuating osmotic stress. This study explored the occurrence of prokaryotic halophiles at the level of communities (i.e., 16S rRNA gene sequencing) and individuals (whole-genome sequencing). We hypothesized that 1) the halophile community would be resistant to flood and freeze disturbances and 2) halophilic individuals would possess varied genetic strategies/mechanisms for osmoadaptation.

Materials and Methods

Sampling Site

The Laguna Madre is a negative estuary in the northwest Gulf of Mexico. Padre Island, the longest barrier island worldwide, limits connectivity and flushing with the Gulf of Mexico. Residence times for the Laguna Madre range from 300 days to several years (Buskey et al. 1998). High residence times, rapid evaporation rates, prolonged droughts, and minimal freshwater inflows contribute to hypersalinity. The lagoon has an average freshwater inflow balance of $-451,882,931 \text{ m}^3 \text{ y}^{-1}$, indicating that evaporation exceeds precipitation and freshwater inflow (Tweedley et al. 2019). Periods of hypersalinity select for the growth of halotolerant species. For example, the lagoon experiences near annual ‘brown tide’ algal blooms caused by

the halotolerant microalgae *Aureoumbra lagunensis* (e.g., Wetz et al. 2017; Cira and Wetz 2019). The most persistent and expansive bloom persisted for eight years (Buskey et al. 1995).

Sample Collection

Water samples (1 L) were collected in duplicate from Los Olmos Creek (LOC) (27°16'23.62" N, 97°48'08.01" W) and Riviera Beach (RB) (27°17'00.09" N, 97°39'52.80" W) in the Upper Laguna Madre Baffin Bay Complex (Fig. 1). Twelve sampling events occurred 01/15/2020, 02/24/2020, 05/18/2020, 06/15/2020, 09/13/2020, 10/16/2020, 12/18/2020, 01/22/2021, 02/26/2021, 03/22/2021, 05/25/2022, and 06/18/2021. Los Olmos Creek is a shallow, typically hypersaline tributary that flows into the Laguna Salada (Tunnell and Judd, 2002). Riviera Beach is a public recreational area at the confluence of the Laguna Salada and Baffin Bay, 13.7 km from Los Olmos Creek.



Fig. 8 Map depicting the Los Olmos Creek and Riviera Beach study sites in the Laguna Salada segment of Baffin Bay, Texas, United States. Figure rendered via GIS. Map credit Md

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Water Quality Parameters

Water temperature (°C), pH, dissolved oxygen (%), and salinity were recorded with a YSI 556 Multi Probe System (YSI Incorporated, Yellow Springs, OH, United States). Wind speed (mph) and air temperature (°C) were measured using a Kestrel 3000 Wind Meter (Kestrel Meters, Boothwyn, PA, United States). Concentrations of NO_3^- , NO_2^- , and NH_4^+ were measured using a SEAL AQ300 Discrete Analyzer (AQ300 methods EPA-148-D, EPA-115-D, and EPA-126-D, respectively). Water transparency (cm) was measured using a transparency tube fixed with a Secchi disk (Carolina Biological Supply Company, Burlington, NC, United States).

DNA Isolation

Water samples (100 mL) were filtered through low-protein binding 0.22 μm polyethersulfone (PES) filters (MilliporeSigma, Burlington, MA, United States). Total genomic DNA was isolated from the filters using a DNeasy PowerSoil kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA quantity (ng/ μl) and quality (A260/A280 and A260/A230 absorbance ratios) were measured using a biospectrophotometer (Bio-Rad, Hercules, CA, United States). The DNA was stored at -20°C.

16S rRNA Gene Sequencing

The 515f (5' – GTG YCA GCM GCC GCG GTA A – 3') and 806r (5' – GGA CTA CNV GGG TWT CTA AT – 3') primers were used to amplify the V4 region of the 16S rRNA gene (Walters et al. 2015). Amplification was achieved using a HotStar Taq Plus Master Mix Kit (Qiagen, Valencia, CA, United States) using the following cycling conditions: 94°C for 3 minutes,

30 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for one minute, followed by a 5-minute hold at 72°C. Amplicons were visualized in a 2% agarose gel, pooled and purified with calibrated Ampure XP beads (Beckman Coulter, Indianapolis, IN, United States), and sequencing was performed on an Illumina MiSeq platform using paired-end chemistry (2 x 300 bp) at Molecular Research L.P. (Shallowater, TX, United States).

Microbial Community Analysis

Sequence reads were processed using QIIME version 1.9 (Caporaso et al. 2010) and QIIME2 version 2018.11 (Bolyen et al. 2019). Barcodes were removed from the paired-end reads using the ‘extract_barcodes.py’ tool in QIIME. The following steps were performed within QIIME2. Reads were demultiplexed and denoised with DADA2 (Callahan et al. 2016), resulting in amplicon sequence variants (ASVs). Trim lengths of 242 bp were used on both the forward and reverse reads. In addition to denoising the data, DADA2 filtered sequences for quality, removed chimeric sequences, and merged paired-end reads. A phylogenetic tree was then generated using the ‘q2-phylogeny’ pipeline with default settings, which was used to calculate phylogeny-based diversity metrics. Taxonomy was assigned using a Naïve Bayes classifier trained on the SILVA v. 132 99% OTUs database (Quast et al. 2013), including only the 250 bases from the V4 region bound by the 515F/806R primer pair. Reads mapped to chloroplast and mitochondrial sequences were filtered out from the sequence variants table using the ‘filter_taxa’ function. Faith’s phylogenetic distance (PD) and the Shannon-Weiner diversity index were calculated for all samples using the ‘q2-diversity’ plugin. Data were then imported into phyloseq (McMurdie and Holmes, 2013) using the ‘import_biom’ and ‘import_qiime_sample_data’ functions and merged into a phyloseq object. All samples maintained high read counts and did not require proportional transformation to a normalized read count. Beta diversity was analyzed using a distance matrix

based on weighted UniFrac (Lozupone et al. 2011) distances calculated in phyloseq using the ‘q2-diversity’ plugin. As stated in Lozupone et al. 2011, briefly, UniFrac measures the difference between two collections of sequences (e.g. 16S rRNA molecules sequenced from different microbial samples) as the amount of evolutionary history unique to either, which is measured as the fraction of branch length in a phylogenetic tree that leads to descendants of one sample or the other but not both. A principal coordinate analysis (PCoA) was calculated and plotted from these distances. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences between communities using the ‘vegan’ (Oksanen et al. 2019) and ‘pairwiseAdonis’ (Arbizu, 2017) packages in R version 3.5.2 (R Core Team, 2017). To test if variance was due to dispersion of variability among groups, permutational analyses of dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes with the ‘vegan’ package in R. Community differences were further confirmed via linear discriminant analysis (LDA) effect size (LEfSe) using the LEfSe tool on the Galaxy server (<https://huttenhower.sph.harvard.edu/galaxy/>). Genera that comprised more than 0.10% of the communities’ relative abundance were analyzed using default settings with the significance threshold set to a p-value of < 0.01 .

Halophile Isolation

Water samples (1 L) were collected from LOC on May 21 and June 13, 2018. Samples were enriched in alkaline peptone water (APW; Oxoid, Basingstoke, England) prepared with filter-sterilized water (0.22 μm) from the creek and incubated overnight (18 hours) at 30 °C with shaking (80 rpm). Halophiles were isolated via direct plating on thiosulfate-citrate-bile-salts-sucrose agar (TCBS; BD, Franklin Lakes, NJ, USA) incubated overnight at 30 °C. Agar was supplemented with Instant Ocean (Spectrum Brands, Blacksburg, VA, USA) to match the

hypersalinity of the creek, measured with a refractometer (Sper Scientific, Scottsdale, AZ, USA). Isolated colonies were cryopreserved (25% glycerol, v/v, -80 °C) for further characterization.

Whole-Genome Sequencing

Selected isolated colonies (N = 5) were grown in Difco marine broth (MB; BD, Franklin Lakes, NJ, USA) overnight (18 hours) at 30 °C with shaking (80 rpm). Cultures (1.8 mL) were pelleted by two rounds of centrifugation (10,000 x g, 30 seconds), and DNA was isolated from the pelleted cells using a DNeasy Ultraclean Microbial Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. The DNA was quantified and assayed for quality (A_{260}/A_{280}) using BioPhotometer D30 (Eppendorf, Hamburg, Germany) and stored at -20 °C. Genomic DNA was sequenced with an Illumina HiSeq instrument at Molecular Research LP (Shallowater, TX, USA) using paired-end chemistry (2 x 250 bp). Sequence reads were pre-processed by trimming adapter sequences and low-quality bases with Trim Galore! (version 0.4.0), which is a Python wrapper for CutAdapt (Martin 2011) and FastQC (version 0.11.7) (Andrews 2010). The draft genomes were assembled *de novo* with the SPAdes assembler (version 3.9.0) (Nurk et al. 2013) and filtered for length to remove contigs shorter than 500 bp. QUAST (Gurevich et al. 2013) was used to assess assembly quality. The draft genomes were initially annotated with RASTtk (Aziz et al. 2008), but the final annotation was completed with the National Center for Biotechnology Information's (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016).

Halophile Identification and Characterization

Preliminary species assignment to the *Salinivibrio* genus was determined with the “view closest neighbors” tool in RASTtk. The assignment of these strains to the *Salinivibrio* genus was confirmed by average nucleotide identity (ANI) comparison with publicly available *Salinivibrio*

genomes using FastANI (Jain et al. 2018). The 96% identity threshold was used as the species boundary. Genes responsible for osmoregulation and heavy metal resistance were detected using RASTtk by searching for subsystem features related to osmotic stress. Salt and copper tolerance was investigated by growing the strains under a range of copper concentrations (0, 50, 125, and 250 ppm) under normal (30) and hypersaline (120) conditions. Strains were grown on salt agar broth/plates (5% peptone, 1% yeast extract, w/o 1.5% bacto agar) prepared with artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA). Cultures were grown in broth overnight (18 hrs) at 30 °C with shaking (120 rpm). Strains and positive controls (*Vibrio parahaemolyticus* RIMD2210633 and ATCC 17802) were spotted (10 uL) in duplicate and grown overnight (20 hours) at 30 °C

Statistical Analyses

ANOVA was performed to determine differences in the physical parameters between sites. Kendall's tau correlation and principal component analysis (PCA) were used to characterize site conditions.

Results

Site Conditions

Drought, flood, and freeze events were observed during the study. From May 15 to May 30, 2020, heavy rainfalls supplied the watershed with 4.85 inches of precipitation. Over the New Year holiday (2020–2021), a winter storm delivered heavy rainfall, flushing the system and significantly reducing the salinity. Soon after, another winter storm swept the area from February 13 to February 20, 2021, causing a significant and unprecedented freeze event for South Texas and much of the central United States. The immediate effects of the freeze were evident, as the shores of both sites were covered with dead fish and crabs. Dead bats were observed at Los

Olmos Creek. The last recorded rain event flooded the system with 8.6 inches of rain one week before the May 25, 2021, sampling trip. During this flood, the creek stage increased to a max of 3.4 ft with a maximum discharge rate of 50 cubic feet per second (CFS).

Water Quality Parameters

Appendix B summarizes the water quality parameters during the sampling period. Salinity at Los Olmos Creek ranged from 2.57 to 82.19. By contrast, salinity at Riviera Beach ranged from 16.05 to 49.78. Both sites maintained hypersalinity (> 35) throughout the first ten sampling trips. Dissolved oxygen (DO) at LOC ranged from 11–105.5%, while DO at RB ranged from 65–130.7%. pH ranged from 7.57–8.66 and 7.66–8.37 at the creek and beach sites. Water temperature was more consistent, ranging from 12.10–32.52 °C at the creek and 14.60–28.93 °C at RB. Nutrient concentrations (i.e., ammonia, nitrate, and nitrite) were significantly different between sites (ANOVA; p -value < 0.05). The PCA demonstrated that variations in salinity, temperature, and DO significantly impacted the creek site (Fig. 2).

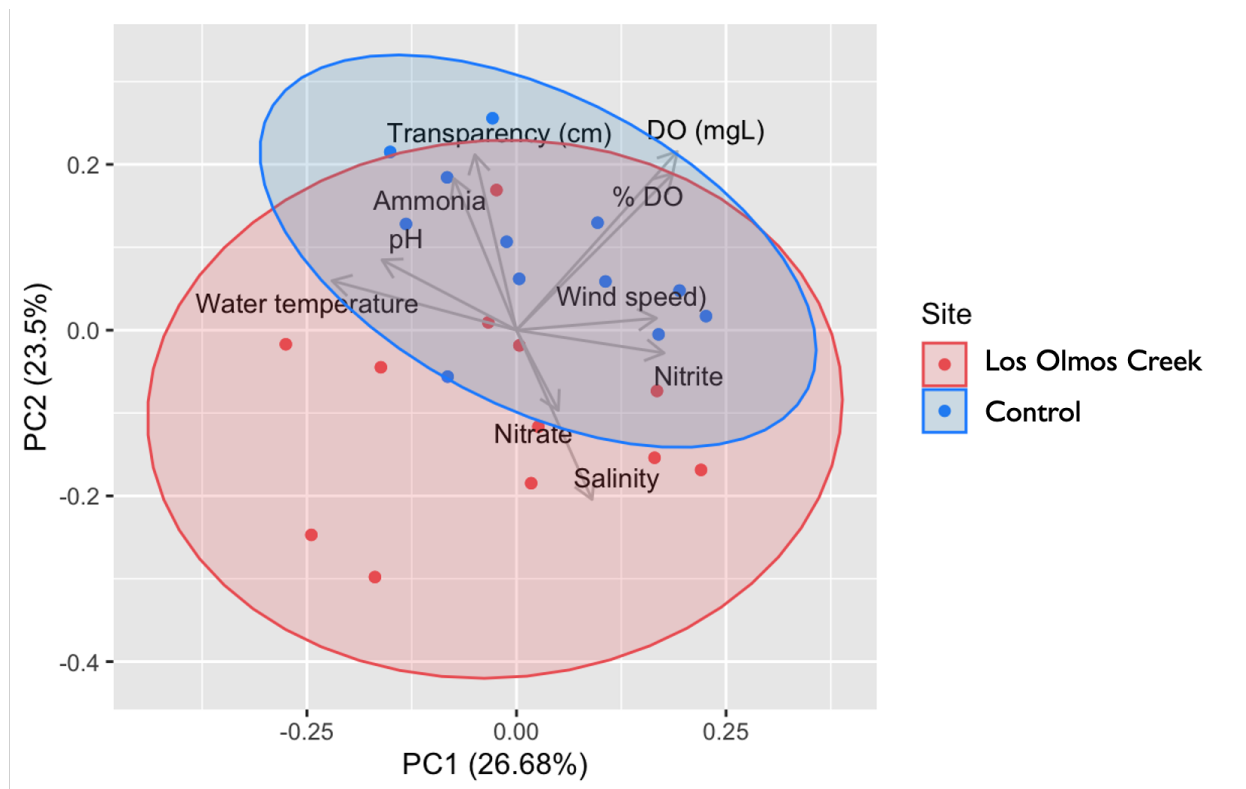


Fig. 9 Principal component analysis (PCA) of water quality parameters showing clustering of similar samples. The size and direction of the vectors show which variables were most responsible for the dissimilarity between Los Olmos Creek and Riviera Beach sites

Microbial Community Diversity and Relative Abundance

Los Olmos Creek and Riviera Beach microbial communities maintained relatively high alpha diversities. Figure 3 shows bar plots of Faith's PD (A) and SW diversity values (B) observed throughout the study. Faith's PD indicated that the RB site maintained significantly higher alpha diversity values ($p\text{-value} < 0.05$), although the differences were not significant when SW values were compared.

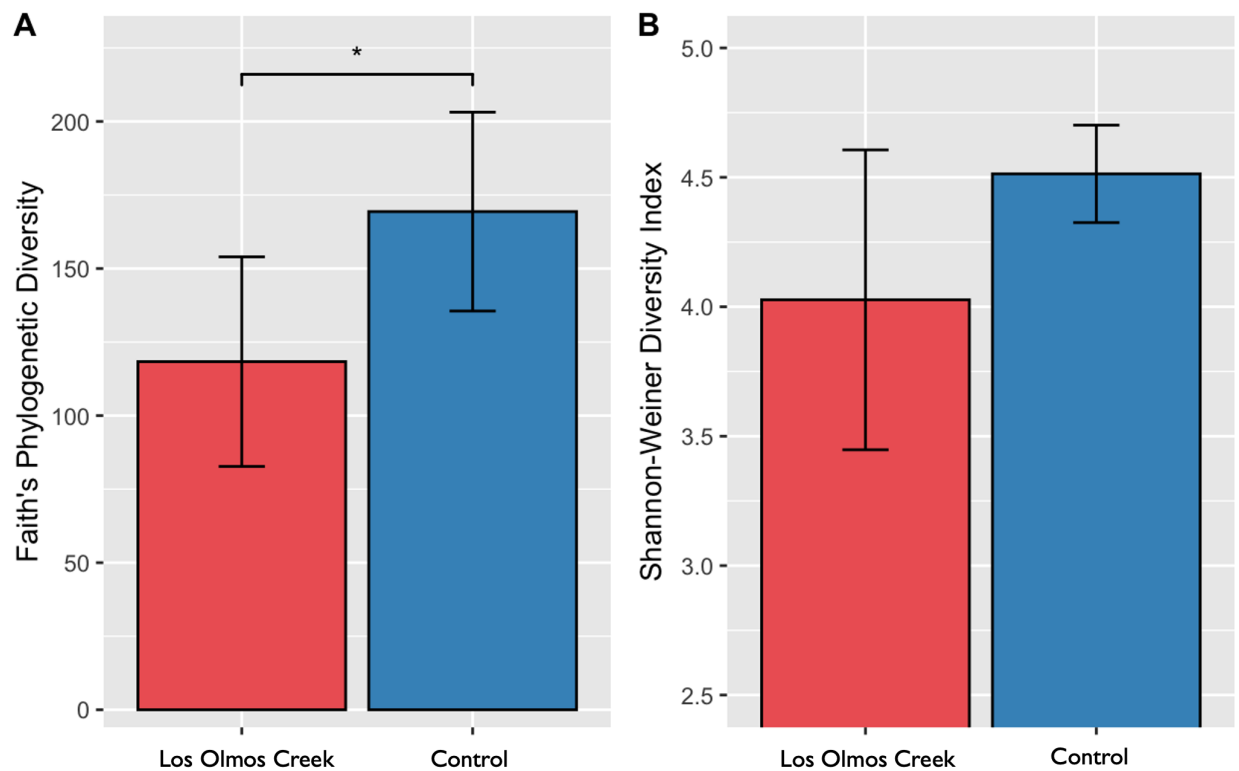


Fig. 10 Subset bar plots of Faith's phylogenetic diversity (A) and Shannon-Weiner diversity (B) values for Los Olmos Creek (red) and Riviera Beach (blue) sites. The asterisk indicated that site-specific communities were significantly different according to Faith's phylogenetic diversity ($p < 0.05$)

Microbial community compositions were visualized using PCoA, as shown in Figure 4, with a clear distinction between site communities (PERMANOVA; adj. p-value < 0.05). Cyanobacteria were the most observed taxa at both sites. Actinobacteria and Rhodothermia were the second and third most abundant taxa, respectively, at LOC, whereas Alphaproteobacteria and Bacteroidia were the second and third most abundant taxa observed at RB, respectively (Fig. 5-6). Cyanobacteria, specifically those belonging to Synechococcales, composed nearly 1/5 of each site's communities, at 18.75% for LOC and 14.98% for RB. Nitriliruptorales Actinobacteria and SAR-11 Alphaproteobacteria were roughly equal proportions, representing 12.22% and 12.24%

of LOC and RB communities. The third most abundant groups observed, Balneolales of Rhodothermia and Chitinophagales of Bacteroidia, composed 10.76% of LOC's community and 8.50%, respectively. An unknown family of Gammaproteobacteria (7.73%) and Rhodobacteraceae (Alphaproteobacteria; 6.32%) were observed to be the fourth and fifth most abundant groups of LOC's community. Alternatively, members of Actinomarinaceae (Acidimicrobiia; 6.58%) and Balneolaceae (Rhodothermia; 6.52%) made up the fourth and fifth most abundant groups of RB. Several other groups were shared in similar, low average concentrations among the respective study (LOC, RB) sites, such as Cryomorphaceae (0.83 and 1.22%) and Phycisphaeraceae (1.10 and 0.86%).

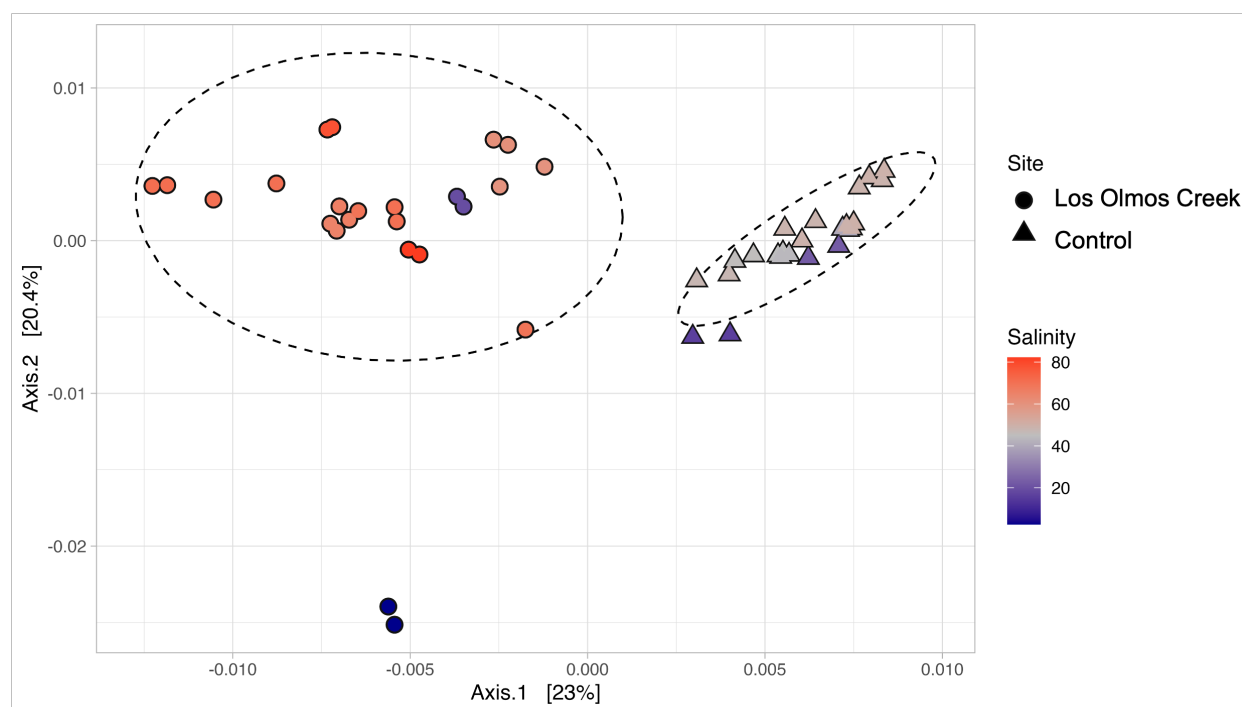


Fig. 11 Principal coordinate analysis (PCoA) of beta diversity values showing the dissimilarity of samples from Los Olmos Creek and Riviera Beach using weighted UniFrac distance values. Ellipses represent 95% confidence intervals. Coloring represents a salinity gradient that increases from fresh (blue) to hypersaline (red)

Enriched Taxa

Linear discriminant analysis (LDA) effect size (LEfSe) was implemented to further investigate and confirm community differences. Besides the most abundant groups observed, several were overrepresented at LOC compared to RB's community. Puniceicoccaceae (1.10%), Terasakiellaceae (1.22%), Izemoplasmataceae (1.65%), Crocinitomicaceae (4.00%), Comamonadaceae (1.07%), PeM15 Actinobacteria (2.47%), and an unassigned Gammaproteobacteria (1.08%) collectively composed over 1/10th of LOC's community (12.59%). These taxa were significantly less abundant in RB's community (Fig. 6). While the RB site maintained relatively the same number of enriched taxa, these groups are more typical of marine systems such as marine Alphaproteobacteria (e.g., SAR-11 [13.35%], AEGEAN-169 marine group [3.24%]).

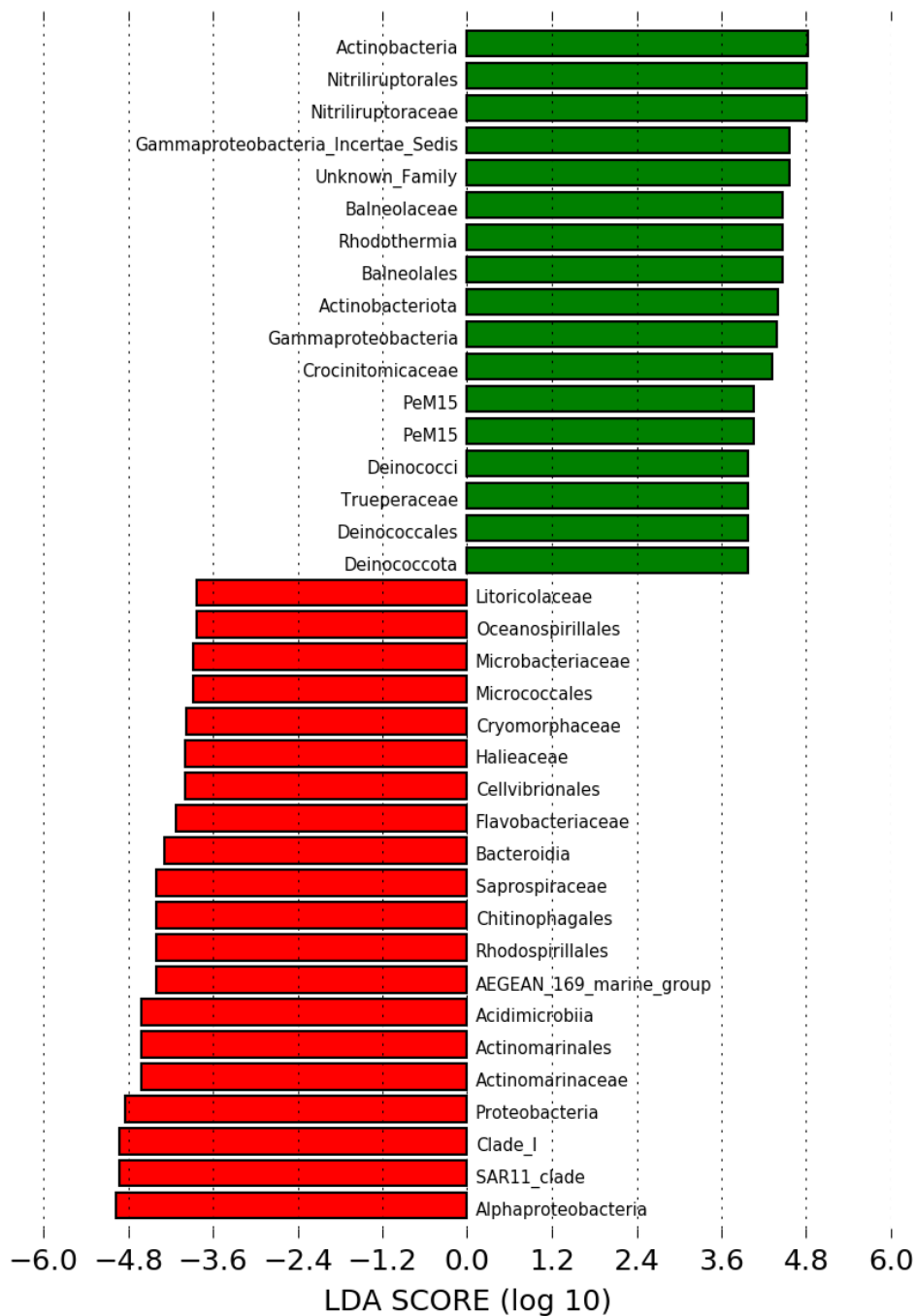


Fig. 12 Linear discriminant analysis (LDA) effect size (LEfSe) histogram depicting taxa enriched in Los Olmos Creek (green) and Riviera Beach (red) (p-value < 0.05). Only taxa passing a 1% relative abundance cutoff were included.

Culturable Halophile Identification and Characterization

The draft *Salinivibrio* genomes (VGrn1, VYel1, VYel5, VYel6, and VYel7) were deposited in GenBank under BioProject PRJNA494150. Table 1 shows the genome assembly metrics. ANI comparison revealed that our five genomes shared >97% ANI with the NCBI reference genome for *S. siamensis* JCM14472. According to the RASTtk subsystem feature counts, all genomes possessed genes responsible for choline and betaine uptake and betaine biosynthesis. The genomes also harbored various genes responsible for heavy metal resistance: chromium, mercury, cobalt, zinc, cadmium, and especially copper. Multidrug efflux pumps and mutations associated with fluoroquinolone resistance were also detected. At normal salinity (30), the *Salinivibrio* strains were less tolerant of copper compared to the controls. However, at hypersaline conditions (120), the *Salinivibrio* strains could grow at 50 and 125 ppm copper concentrations, whereas the control strains showed no growth (Table 2, Fig. 7).

Table 1 Summary of the genome assembly metrics for the five *S. saimesis* strains sequenced in this study

Strain	Size (bp)	N50	%GC	Contigs	Coding sequences	RNAs
VGrn1	3,304,387	385,503	50.6	27	3,118	87
VYel1	3,354,949	192,436	50.6	39	3,175	95
VYel5	3,301,751	312,886	50.6	28	3,098	94
VYel6	3,629,081	312,480	50.2	33	3,624	91
VYel7	3,300,891	313,704	50.6	31	3,109	88

Table 2 Growth of *S. siamensis* strains and controls at a range of copper concentrations (0, 50, 125, and 250 ppm) under normal (30) and hypersaline (120) conditions. Observations of growth were defined as high (+++), medium (++), slight (+), and none (-)

<i>Salinity</i>	30	30	30	30	120	120	120	120
<i>Copper ppm</i>	0	50	125	250	0	50	125	250
<i>ATCC 17802</i>	+++	+++	+	-	-	-	-	-
<i>RIMD2210633</i>	+++	+++	-	-	-	-	-	-
<i>VGrn1</i>	+++	+	-	-	+++	++	+	-
<i>VYel1</i>	+++	+	-	-	+++	++	+	-
<i>VYel5</i>	+++	+	-	-	+++	++	+	-
<i>VYel6</i>	+++	+	-	-	+++	++	+	-
<i>VYel7</i>	+++	+	-	-	+++	++	+	-

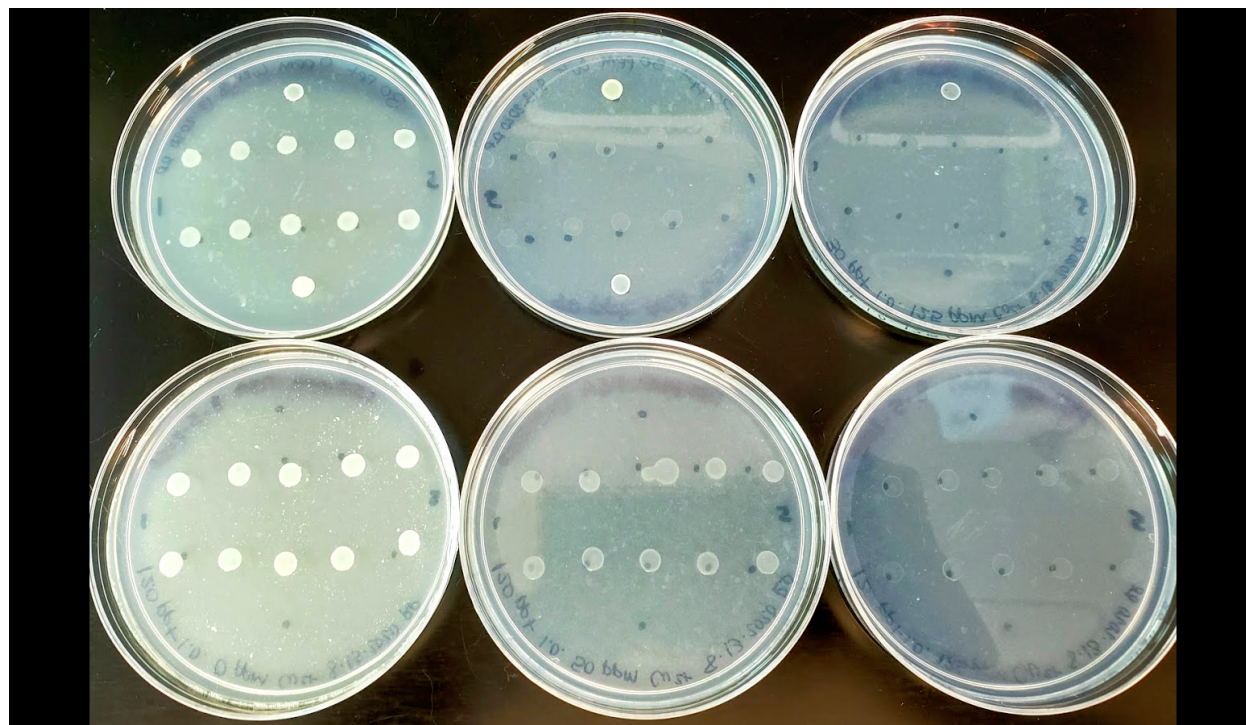


Fig. 13 Growth of *S. siamensis* and control strains under 0, 50, and 125 ppm copper (columns 1, 2, and 3, respectively) at normal (30) and hypersaline (120) conditions (rows 1 and 2, respectively). Control strains were spotted at the top and bottom of the plates (RIMD2210633 and ATCC 17802, respectively), and the *S. siamensis* were spotted in duplicate from left to right (VGrn1, VYel1, VYel5, VYel6, and VYel 7)

Discussion

Inland hypersaline lakes dominate our understanding of hypersaline systems and halophilic microorganisms. These inland systems are surprisingly abundant, accounting for nearly half (44%) of all inland water volume (Messenger et al. 2016). They support complex halophilic microbial communities and are natural laboratories for studying salinity resilience and adaptation (Sacco et al. 2021). The most recognized hypersaline systems include the Great Salt Lake (United States), the Dead Sea (Israel), the Crimean Salt Lake (Crimea), Lake Dangxiong (Tibet), Laguna Puilar (Chile), Gaet'ale Pond (Ethiopia), Kati Thanda-Lake Eyre (Australia), the Coorong Lagoon (Australia), and Deep Lake (Antarctica). Less is known about coastal hypersaline systems and their halophilic inhabitants. Tweedly et al. (2019) identified 60 coastal lagoons, 34 estuaries, and 7 marine embayments with hypersaline conditions. In contrast to their inland analogs, the microbial diversity of these coastal hypersaline systems is underexplored.

The salinity of the Laguna Madre varies significantly with flood and drought. Normal hypersaline conditions (40-80) give way to fresh or brackish (0-35) during flood events and brine (>80) during prolonged drought (Tweedly et al. 2019). In this study, Los Olmos Creek experienced a profound salinity range: 2.57 to 82.19. In comparison to the RB site, salinity was the dominant variable defining the creek's environmental conditions. Hypersalinity can select against diversity by limiting the growth of non-halotolerant species. This contrast in diversity

was confirmed by trends in Faith's and Shannon-Weiner diversity, with the halophilic creek community exhibiting less diversity. This observation suggests the presence of fewer taxa or more phylogenetically similar taxa in Los Olmos Creek. Beta diversity further confirmed the two communities were clearly and significantly distinct. Halophiles probably contribute to diversity at the RB study site as conditionally rare members of the microbial community.

Significant weather events may act as pulse disturbances that alter species composition (Girvan et al. 2005). Los Olmos Creek experienced two flood events and a freeze event during the study period. The final flood event before the study's conclusion reduced salinity from 78.10 to 19.18 and further reduced it to 2.57, while the winter freeze event reduced water temperatures from 27.50 to 12.10 °C, keeping the system suppressed below 20 °C until May. According to the PCoA plot, the first flood event did little to alter the microbial community structure despite a 58.92 salinity decrease. In contrast, the second flood event resulted in beta diversity values far outside the confidence interval. This result suggests the halophile community is resistant to significant flood events, but within limits. Surprisingly, the unprecedented freeze event had no obvious effect on beta diversity. We postulate the halophilic community was insulated against the freeze by the hypersalinity of the water (70.46 to 82.19). In terrestrial systems, soil salinity can increase the freeze tolerance of plants and animals (Syvertsen and Yelenosky 1988; Silva et al. 2013). Similarly, saltier brine solutions improve freeze tolerance in the halophilic bacterium *Planococcus halocryophilus* (Heinz et al. 2019).

Although Los Olmos Creek and Riviera Beach supported distinct microbial communities, they shared notable taxonomic similarities. For instance, the five most abundant taxa in Los Olmos Creek were also found at Riviera Beach. However, all five were enriched at the creek, and their enrichment/abundance was significantly correlated with salinity. The two study sites

were located 13.7 km apart in the Laguna Salada. Thus, overlap in bacterial community composition was anticipated despite significant differences in environmental conditions. Cyanobacteria, specifically Synechococcales, was notably the most abundant group observed at either study site. Additional abundant taxa, notable for enrichment in Los Olmos Creek, were Actinobacteria, Balneolaceae, Gammaproteobacteria Incertae Sedis (ord.), and Rhodobacteraceae.

In 2018, Hall et al. reported that *Synechococcus* was abundant during an *A. lagunensis* brown tide bloom in Guantanamo Bay, Cuba (Hall et al. 2018). The abundance of *Synechococcus* could be explained by the regular occurrence of brown tide blooms in Baffin Bay and the Laguna Salada (Cira and Wetz 2019). Laguna Salada, where Los Olmos Creek is located, is highly eutrophic and has been postulated to act as a reservoir for brown tide-forming *A. lagunensis* (Rhudy et al. 1998; Cira and Wetz 2019). A combination of elevated dissolved organic nutrients and hypersalinity could co-select for *A. lagunensis* and *Synechococcus* in this system (Rosales et al. 2005). Although the function of *Synechococcus* in this system was not experimentally verified, many Cyanobacteria, including *Synechococcus*, can transform atmospheric nitrogen into biologically useful forms through nitrogen fixation. However, cyanobacterial nitrogen fixation may be inhibited by exceptionally high dissolved organic nitrogen concentrations in this eutrophic system (Wetz and Cira 2017).

Actinobacteria, specifically those belonging to the Nitriliruptoraceae, were highly enriched at Los Olmos Creek. This family is known for its halotolerance, ability to utilize aliphatic nitriles, nitrite oxidation, and its association with harmful algal bloom organisms (Sorokin et al. 2009; Lukwambe et al. 2019). Nitriliruptoraceae has been implicated as a major constituent in salt-stressed soils (Wang et al. 2021), and the family may be continually

introduced to the creek through stormwater runoff and periodic flooding. Alternately, they may persist in association with halophilic plants as members of the *Salicornia* rhizosphere microbiome (Begmatov et al. 2019). Interestingly, Nitrospiraceae and Balneolaceae (below) are enriched by the amendment of agricultural soils with biochar, which is a beneficial amendment in green waste and manure composting and shows promise in the amelioration of coastal saline and alkaline soils for enhanced agricultural production (Qiu et al. 2019; Zhang et al. 2022).

Balneolaceae (formerly Rhodothermia) have been previously observed at Los Olmos Creek (Munoz et al. 2016; unpublished data). Composing 10.76% of the creek's community, Balneolaceae was one of the most abundant individual bacterial groups. The family has an affinity for hypersaline and hyperalkaline environments (Munoz et al. 2016; Vavourakis et al. 2018; Xia et al. 2016, 2017). A previous study revealed that Balneolaceae was dominant in tannery waste dumpsite soils contaminated by heavy metals and organophosphate insecticides (Lukhele et al. 2022). Conkle and Hormoz-Estrabad revealed high concentrations of the organophosphate insecticides atrazine, malathion, and chlorpyrifos in Baffin Bay (Conkle and Hormoz-Estrabad 2018). Balneolaceae's abundance at Los Olmos Creek may reflect its role as a natural remediator in a creek impaired by agricultural pollution. Additionally, reports of Balneolaceae enrichment by oil contamination (Zhang et al. 2022) suggest that runoff from the Highway 77 overpass may also shape the creek's microbial community.

Little is known about uncultured Gammaproteobacteria Incertae Sedis. Understanding of this enigmatic group and its placement in the Gammaproteobacteria remains unclear.

Wenzhouxiangella is a close relative isolated from an alkaline soda lake (Sorokin et al. 2020). A recent study used network analysis to demonstrate that Gammaproteobacteria Incertae Sedis was

a keystone taxa in microflora associated with *Spartina alterniflora* salt marsh (Gao et al. 2021), and a separate study reported Gammaproteobacteria Incertae Sedis was a dominate member of the microbial community in a hyperalkaline serpentinizing spring (Trutschel et al. 2023). This taxon was involved in the degradation of the PAH phenanthrene in mangrove sediments (Tiralerdpanich et al. 2021), and it potentially plays a role in the degradation of petroleum compounds in shrimp ponds and urban mangroves impacted by oil pollution (Marcial Gomes et al. 2008; Lin et al. 2017). Phenanthrene is used to manufacture pesticides, and background levels can be found in agricultural soils and products (Roszko et al. 2020). Although the occurrence of PAH and oil pollution in Los Olmos Creek has not been experimentally verified, the creek receives drainage from an agricultural watershed and a major highway.

Rhodobacteraceae (Alphaproteobacteria) was the fifth most abundant group, composing 6.32% of the Los Olmos Creek community. Rhodobacteraceae was previously identified as a member of the creek community in a previous study (Bachand et al. 2023, unpublished). The Baffin Bay Upper Laguna Madre Complex experiences near annual *A. lagunensis* brown tide blooms (Rhudy et al. 1998; Cira and Wetz 2019). Many algal species, including *A. lagunensis*, are B vitamin auxotrophs (Croft et al. 2005; Gómez-Consarnau et al. 2018). Rhodobacterales may provide vitamin B₁ (Croft et al. 2005; Gómez-Consarnau et al. 2018). Similarly, the less abundant but enriched Verrucomicrobiae (1.24%) may supply vitamin B₆ (Gómez-Consarnau et al. 2018).

Salinivibrio is a genus of halophilic Gram-negative bacteria belonging to the family Vibrionaceae and class Gammaproteobacteria that are ubiquitous across aquatic and terrestrial hypersaline environments (Fernández et al. 2014). Although this genus was not ranked among the most abundant taxa at Los Olmos Creek taxa, it was a culturable member of the microbial

community. Like other Vibrionaceae, thresholds between species are difficult to discern without the benefit of whole-genomes. The genus underwent extensive reclassification when a group from Spain published the first genome-scale taxonomic analysis (de la Haba et al. 2019). According to a genome-scale ANI analysis, the strains isolated from Los Olmos Creek were identified as *S. siamensis*. Attributes common to the genus include resistance from heavy metals, UV light, and hypersalinity (Gorriti et al. 2014).

Mechanisms of osmoregulation and heavy metal resistance may be critical to surviving a hypersaline creek impacted by agricultural and urban pollution. The *S. siamensis* strains isolated in this study harbored a variety of genes that likely aid survival in Los Olmos Creek. To facilitate growth in hypersaline conditions, these strains possess genes responsible for choline and betaine uptake and betaine biosynthesis. The production or import of osmolytes helps maintain homeostasis under high osmotic pressure (Gorriti et al. 2014), and osmolyte production can increase or decrease in response to salinity fluctuations (Somero and Yancey 2010). Genes encoding resistance to copper and other heavy metals may protect against agricultural or highway pollution, while genes encoding resistance to fluoroquinolones may protect against residual antibiotics. Copper is commonly used in pesticides (Willis and Bishop 2016), and Los Olmos Creek has a history of fecal indicator bacterial pollution (personal communication, Sam Sugarek, Nueces River Authority). Interestingly, mechanisms for osmoregulation and copper resistance appear to be cross-protective, seeing that copper resistance was highest when strains were grown under hypersaline (120) conditions; however, the explanation for cross-protection will require further research.

In summary, Los Olmos Creek is a hypersaline system that experiences profound salinity shifts and receives drainage from a highway overpass and an agricultural watershed. The creek's

halophilic community appears resistant to flood and freeze disturbance. The halophiles thriving in the creek include Cyanobacteria, Actinobacteria, Balneolaceae, Gammaproteobacteria Incertae Sedis, and Rhodobacteraceae. The enrichment of Cyanobacteria and Rhodobacteraceae may result from eutrophication, and both may provide co-occurring algal species with nutrients or vitamins. The enrichment of Actinobacteria, Balneolaceae, and Gammaproteobacteria Incertae Sedis may be a consequence of urban or agricultural contaminants, and they may act as natural remediators. Preliminary analysis of *Salinivibrio* genomes revealed mechanisms of osmoregulation, heavy metal resistance, and antibiotic resistance. These findings represent critical steps to understanding the microbial community dynamics in hypersaline systems.

Acknowledgments

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CHAPTER IV

CONNECTIVITY OF WILDLIFE AND EUTROPHICATION IN A COASTAL HYPERSALINE CREEK

Abstract

Hypersaline lagoons are particularly vulnerable to eutrophication. In the Laguna Madre in the northwest Gulf of Mexico, exceptionally high concentrations of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) are drivers of recurrent *Aureoumbra lagunensis* brown tide algal blooms. This study explored the possibility that wildlife contributes to DOC and DON in this system. We characterized the carbon and nitrogen isotopic composition of bat guano (Mexican free-tailed *Tadarida brasiliensis*) and seston from a hypersaline creek. Potential linkage between the guano and seston microbial communities was also investigated through 16S rRNA gene sequencing. Guano and seston carbon composition showed significant overlap, while guano nitrogen composition resembled published values for livestock waste. Rhodobacteraceae (Alphaproteobacteria) was a dominant member of the guano and seston microbial communities. Interestingly, the guano community was enriched in potentially pathogenic bacteria e.g., Staphylococcales, Bacillales, Enterobacteriales, Clostridia, and Campylobacteriales, which may pose a health threat to recreational users. Findings suggest that guano contributes to DOC pools in this system and could act as a pathogen reservoir. Additionally, findings highlight the importance of considering alternative nutrient sources when investigating eutrophication and the need for wildlife management considerations during the planning of infrastructure projects.

Introduction

A recent meta-analysis identified 60 hypersaline lagoons, estuaries, and embayments worldwide (Tweedley et al. 2019). These systems are commonly located in arid and semi-arid

tropical and sub-tropical climate zones. Long residence times and minimal ocean connectivity make these systems acutely vulnerable to pollution, eutrophication, harmful algal blooms, and hypoxia (Kennish and Paerl 2010; Warwick et al. 2018). The Laguna Madre, in the northwest Gulf of Mexico (GoM), is a large hypersaline lagoon and some segments show signs of eutrophication. In particular, the northern segment of the lagoon, the Baffin Bay and Upper Laguna Madre Complex, experiences elevated dissolved organic carbon (DOC), dissolved organic nitrogen (DON), and chlorophyll concentrations in comparison to other Texas estuaries (Wetz et al. 2017).

Harmful ‘brown tide’ algal blooms caused by the microalgae *Aureoumbra lagunensis* are common in the Baffin Bay and Upper Laguna Madre Complex. These blooms impair ecosystems by disrupting benthic productivity (Gobler and Sunda 2012). Namely, *A. lagunensis* blooms attenuate sunlight necessary for the productivity of submerged seagrass, and decades of recurrent blooms have been implicated in seagrass loss throughout the lagoon (Onuf 1996). This loss of seagrass habitat negatively impacts important fish nursery and foraging habitat (Sheridan and Minello 2003). Endangered megafauna, like the green sea turtle (*Chelonia mydas*), also depend on seagrass beds for foraging (Taquet et al. 2006).

The complex’s exceptionally high DOC and DON concentrations likely promote brown tide bloom formation and maintenance (Wetz et al. 2017). The closely related brown tide-forming *Aureococcus anophagefferens* outcompetes other phytoplankton when high DON is paired with low dissolved inorganic nitrogen (DIN) (Gobler et al. 2011). The DON to DIN ratio (~9:1) is exceptionally high in Baffin Bay (Wetz et al. 2017). Similarly, *A. lagunensis* brown tide blooms in the Mosquito and Indian River Lagoons (Florida, USA) were associated with elevated DON and lower DIN (Gobler et al. 2013). The Laguna Salada arm of Baffin Bay, where Los

Olmos Creek drains, maintains elevated DOC and DON concentrations (Wetz et al. 2017; Cira and Wetz 2019; Felix and Campbell 2019). Low fresh water inflows and high residence times are thought complicit in the buildup of organic nutrients. With respect to these findings, it was postulated that Los Olmos Creek acts as a reservoir for *A. lagunensis*, seeding larger bloom events across the complex when conditions are supportive (Cira and Wetz 2019).

Multiple sources likely contributed to the exceptionally high nitrogen concentrations within the ULMC. The isotopic composition of DON indicates sewage is the primary source of reactive nitrogen in Baffin Bay (Felix and Campbell 2019). Other sources include atmospheric deposition, livestock waste, and fertilizer. A subsequent study concluded groundwater contaminated with sewage/septic waste was the dominant DON source in the Laguna Salada (Murgulet et al. 2021). Submarine groundwater discharge can significantly contribute to DON in coastal systems (Santos et al. 2021). In lagoons with limited flushing and high residence times, even several nutrient sources could have disproportionately large roles in nutrient biogeochemistry.

In this study, we explored wildlife as a potential source of nutrient pollution in Los Olmos Creek. Specifically, we hypothesized that a large Mexican free-tailed bat colony (*Tadarida brasiliensis*), roosting in the Highway 77 overpass above the tidal segment of the creek, is a potential nutrient source. For this purpose, we analyzed carbon and nitrogen stable isotope composition. Additionally, we utilized 16S rRNA gene sequencing to identify potential overlap in the guano and water microbial communities. Few studies have provided a comparison of these disparate techniques. Linkage between the bat colony and nutrient pollution could further explain this system's nutrient biogeochemistry and could lead to the development of remediation strategies based on wildlife management.

Methods

Study Site

Los Olmos Creek (LOC) ($27^{\circ}16'23.62''$ N, $97^{\circ}48'08.01''$ W) flows into the Laguna Salada segment of Baffin Bay, Texas, United States (Figure 1). The creek is a shallow, low-flow tributary, and the tidal segment is typically hypersaline (Tunnell and Judd, 2002). The Laguna Salada is a documented reservoir for persistent *A. lagunensis* brown tide blooms that may aid the seeding of more expansive blooms throughout Baffin Bay and the Laguna Madre (Cira and Wetz, 2019) (Figure 1).



Fig. 14 Map of the Los Olmos Creek (red) study site in the Laguna Salada segment of Baffin Bay, Texas, United States. Figure rendered via GIS. Map credit Md Mahabubur Rahman.

Sample Collection

On January 13th, 2022, 30 replicate samples of guano, grab water, and saltgrass/cordgrass (N=30 replicates per sample type, N=90 total) were collected and transported to the laboratory on ice. Fifteen 1 L surface water samples were from a 1 m² portion of the creek after having allowed sediment to settle after being disturbed. Samples were split into lab duplicated and were immediately filtered and prepared for packaging. Bat guano was sampled directly from accessible Highway 77 expansion joints and pillar outfalls where the colony roosts. Guano was collected using sterile spatulas and 50 mL conical tubes. Randomly selected patches of *Distichlis spicata* and *Spartina spartinae* were collected using sterile scissors and 50 mL conical tubes.

Water Quality Parameters

Physical parameters [water temperature (°C), pH, dissolved oxygen (%), and salinity] were recorded with a YSI 556 Multi Probe System (YSI Incorporated, Yellow Springs, OH, United States). Wind speed (mph) and air temperature (°C) were measured using a Kestrel 3000 Wind Meter (Kestrel Meters, Boothwyn, PA, United States). Water transparency was measured using a transparency tube fixed with a Secchi disk (cm) (Carolina Biological Supply Company, Burlington, NC, United States).

Stable Isotope Composition

Guano was dried and powdered, and aliquots of the powdered samples (1.45-1.55 mg) were weighed into tin capsules. Seston was concentrated by vacuum filtrating 100 mL water samples onto combusted 47 mm GF/F filters (0.7 µm pore size) and fumed in a desiccator with HCl to remove carbonates. Filters were then trimmed to 25 mm and packaged in tin capsules. Saltgrass/cordgrass samples were rinsed with sterile deionized (DI) water, washed in 5% HCl and rinsed again using sterile DI water. Cleaned samples were then dried and powdered. Aliquots of the powdered samples (2.5-3 mg) were weighed into tin capsules. All samples were stored in a

desiccator until analyzed. Stable isotope ratios of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in guano, seston, and saltgrass/cordgrass were determined at the University of California Davis Stable Isotope Facility. Briefly, per UC Davis's protocols, plant, filters, and guano samples were analyzed on elemental analyzer isotope ratio mass spectrometer (EA-IRMS) systems. Samples were combusted at 950 °C in a reactor packed with chromium oxide and silvered copper oxide. Oxygen was dosed with sample introduction to ensure complete combustion. Following combustion, residual oxygen and nitrogen oxides were removed by passing the combustion products over reduced copper at 650 °C. Water was trapped by magnesium perchlorate and phosphorous pentoxide. CO_2 and N_2 are separated by a gas chromatography column in the Sercon EAs and by an adsorption trap in the Elementar EAs. After separation, an aliquot of the analyte gases is carried to the IRMS for measurement. Quality control and assurance materials were calibrated against international reference materials, including IAEA-600, USGS40, USGS41, USGS42, USGS43, USGS61, USGS64, and USGS65. All are directly traceable to the primary isotopic reference material for each element (i.e., VPDB for $\delta^{13}\text{C}$ and Air for $\delta^{15}\text{N}$). Calibration procedures for solids were applied identically across reference and sample materials. First, a pure CO_2 or N_2 reference gas was used to calculate provisional isotopic values of the sample peaks. Next, isotopic values were adjusted for changes in linearity and instrumental drift using in-house reference materials, Nylon Powder and Chitin or Alfalfa Flour. Finally, measurements were scale-normalized to the primary reference materials using in-house reference materials, Glutamic Acid and Enriched Alanine or Caffeine. Elemental totals were calculated based on IRMS peak area using linearity references as a calibration curve. Quality control reference materials used post peak calculations were bovine liver, amaranth flour, and keratin. Isotopic values were visualized using ggplot2 (Wickham 2016), reshape (Wickham 2007),

reshape2 (Wickham 2007), plyr (Wickham 2011), and dplyr (Wickham et al. 2023) packages in R version 3.2.5 (R Core Team 2017).

DNA Isolation

DNA was isolated from guano using a DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. To concentrate seston, 100 mL water samples were filtered through low-protein binding 0.22 µm polyethersulfone (PES) filters (MilliporeSigma, Burlington, MA, United States). DNA was isolated from seston using a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA quantity (ng/µl) and quality (A260/A280 and A260/A230 absorbance ratios) were measured using a biospectrophotometer (Bio-Rad, Hercules, CA, United States). The DNA was stored at -80°C.

16S rRNA Gene Amplicon Sequencing

The 515F (5' – GTG YCA GCM GCC GCG GTA A – 3') and 806R (5' – GGA CTA CNV GGG TWT CTA AT – 3') primers were used to amplify the V4 region of the 16S rRNA gene sequence (Walters et al. 2015) using a HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, United States). The following cycling conditions were used for amplification: 94°C for 3 minutes, 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for one minute, followed by a 5-minute hold at 72°C. The 16S rRNA gene was sequenced on an Illumina MiSeq platform using paired-end chemistry (2 x 300 bp) at Molecular Research L.P. (Shallowater, TX, United States).

Microbial Community Analysis

Raw sequence reads were processed using QIIME version 1.9 (Caporaso et al. 2010, 2012) and QIIME2 version 2018.11 (Bolyen et al. 2019). Barcodes were extracted from the

paired-end reads using the ‘extract_barcodes.py’ tool in QIIME. The following steps were performed within QIIME2. Reads were demultiplexed and denoised with DADA2 (Callahan et al. 2016), resulting in amplicon sequence variants (ASVs). Trim lengths of 242 bp were used on both the forward and reverse reads. In addition to denoising the data, DADA2 filtered sequences for quality, removed chimeric sequences, and merged paired-end reads. A phylogenetic tree was then generated using the ‘q2-phylogeny’ pipeline with default settings, which was used to calculate phylogeny-based diversity metrics. Taxonomy was assigned using a Naïve Bayes classifier trained on the SILVA v. 132 99% OTUs database (Quast et al. 2013), including only the 250 bases from the V4 region bound by the 515F/806R primer pair. Reads mapped to chloroplast and mitochondrial sequences were filtered from the sequence variants table using the ‘filter_taxa’ function. Faith’s phylogenetic distance (PD) and the Shannon-Weiner (SW) diversity index were calculated for all samples using the ‘q2-diversity’ plugin. Data were then imported into phyloseq (McMurdie and Holmes 2013) using the ‘import_biom’ and ‘import_qiime_sample_data’ functions and merged into a phyloseq object. All samples maintained high read counts and did not require proportional transformation to a normalized read count. Beta diversity was analyzed using a distance matrix based on weighted UniFrac (Lozupone et al. 2011) distances calculated in phyloseq using the ‘q2-diversity’ plugin. As stated in Lozupone et al. 2011, briefly, UniFrac measures the difference between two collections of sequences (e.g. 16S rRNA molecules sequenced from different microbial samples) as the amount of evolutionary history unique to either, which is measured as the fraction of branch length in a phylogenetic tree that leads to descendants of one sample or the other but not both. A principal coordinate analysis (PCoA) was calculated and plotted from these distances. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences

between communities using the ‘vegan’ (Oksanen et al. 2019) and ‘pairwiseAdonis’ (Arbizu 2017) packages in R version 3.5.2 (R Core Team, 2017). To test if variance was due to dispersion of variability among groups, permutational analyses of multivariate dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes with the ‘vegan’ package in R. Microbial community differences were further confirmed via linear discriminant analysis (LDA) effect size (LEfSe) using the LEfSe tool on the Galaxy server (<https://huttenhower.sph.harvard.edu/galaxy/>). Genera that comprised more than 0.10% of the communities’ relative abundance were analyzed using default settings with the significance threshold set to a p-value of < 0.01.

Results

Water Quality Parameters

The air temperature was 8.9 °C, and the water temperature (16.4 °C) was nearly 10 °C lower than January averages (unpublished data). pH was slightly alkaline (8.25), dissolved oxygen (187.5%) indicated supersaturation, and salinity was below normal (29.66) for this hypersaline creek. The water was turbid, and the transparency was only 9.4 cm.

Stable Isotope Compositions

Appendix C provides a summary of isotopic compositions. Guano carbon isotope compositions ($\delta^{13}\text{C}$) ranged from -21.06 to -18.42‰ with a mean $\delta^{13}\text{C}$ value of -19.87‰, and similar seston carbon isotope compositions ($\delta^{13}\text{C}$) ranged from -21.19 to -16.51‰ with a mean $\delta^{13}\text{C}$ value of -20.32‰ (Figure 2). Markedly higher saltgrass/cordgrass carbon isotope compositions ranged from -14.63 to -14.11‰ with a mean $\delta^{13}\text{C}$ value of -14.33‰. Analysis of variance (ANOVA) showed that guano and seston $\delta^{13}\text{C}$ values were not significantly different (p-value >0.05). Guano nitrogen isotope compositions ($\delta^{15}\text{N}$) ranged from 4.99 to 7.01‰ with a

mean $\delta^{15}\text{N}$ value of 6.36‰, and dissimilar seston nitrogen isotope compositions ranged between 7.25 to 9.60‰ with a mean $\delta^{15}\text{N}$ value of 8.86‰ (Figure 3). Saltgrass/cordgrass nitrogen isotope compositions ranged from 10.57 to 12.18‰ with a mean $\delta^{15}\text{N}$ value of 11.42‰. ANOVA showed that all sample type $\delta^{13}\text{C}$ values differed significantly (p -value < 0.05).

The guano and seston C:N ratios overlapped: guano ranged from 4.45 to 5.04, and seston ranged from 4.40 to 7.54 with means of 4.76 and 5.85, respectively. By contrast, the saltgrass/cordgrass samples showed higher and wider-ranging C:N ratios (21.77 to 26.29) and a larger mean of 23.48.

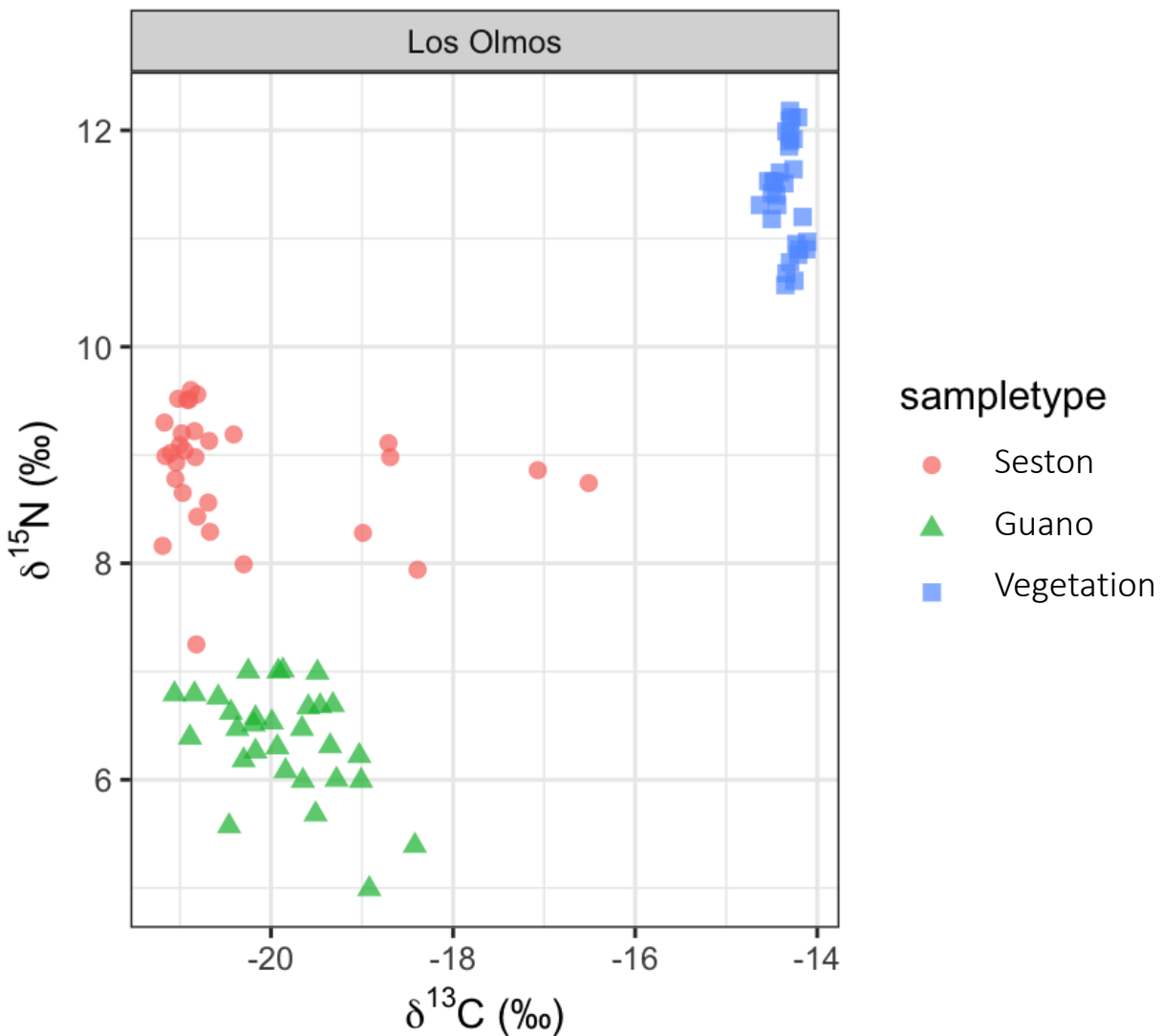


Fig. 15 Scatterplot of nitrogen and carbon isotopic values for guano, seston, and saltgrass/cordgrass

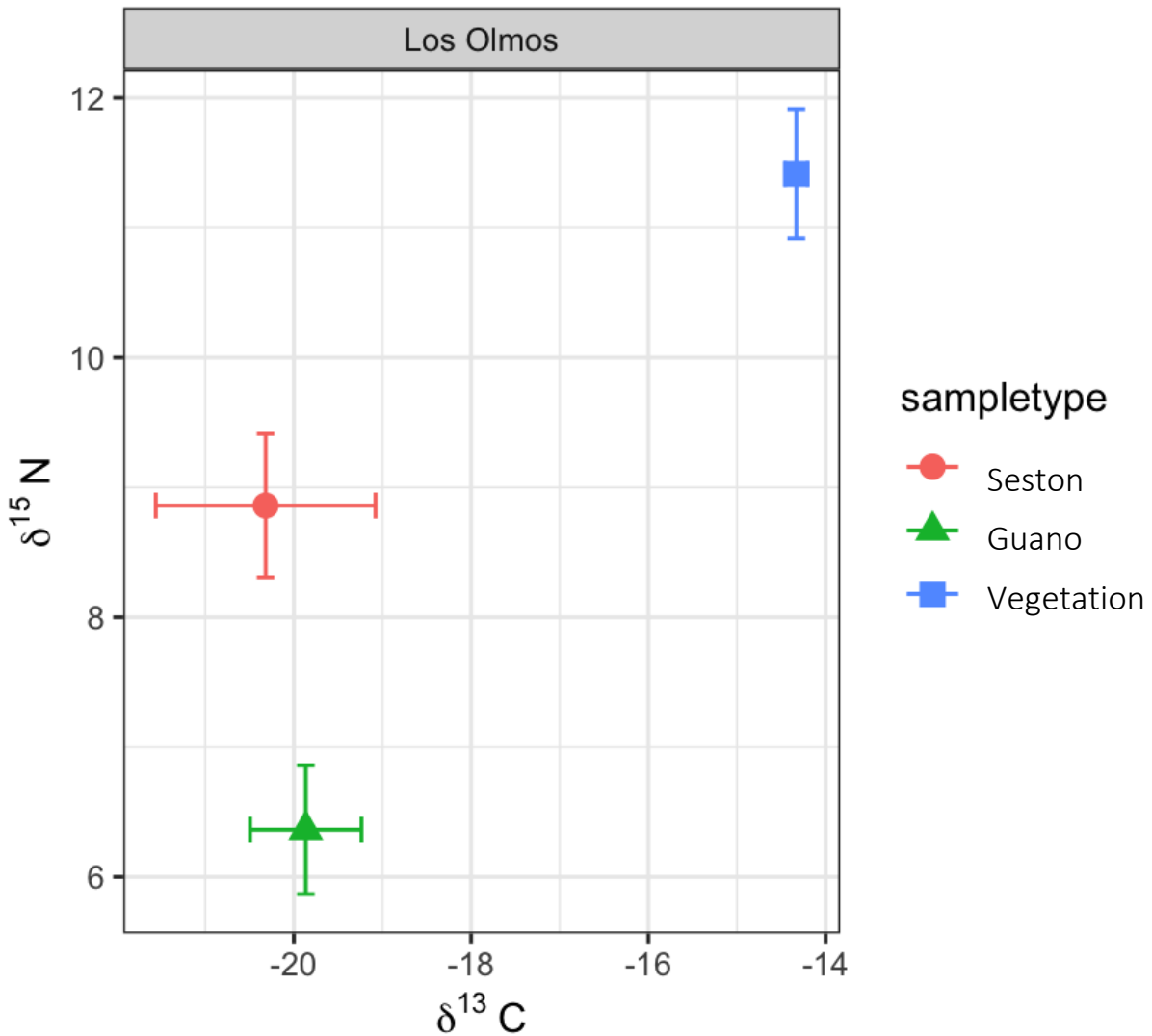


Fig. 16 Scatterplot of nitrogen and carbon isotopic values for guano, seston, and saltgrass/cordgrass with calculated mean and standard deviation

Microbial Community Diversity and Relative Abundance

Guano and seston microbial community alpha diversity is illustrated in Figure 9. Faith's PD was not significantly different between sample types (Figure 9A) ($p\text{-value} > 0.05$). However, SW diversity values were significantly different, with seston maintaining higher diversity (Figure

9B) (p -value < 0.05). PCoA analysis of beta-diversity showed the two communities were significantly different (Figure 10) (PERMANOVA; adj. p -value < 0.05). Linear discriminant analysis (LDA) effect size (LEfSe) analysis (Figure 12) highlights enriched taxa in the guano and seston communities. The guano community was disproportionately dominated the Pseudoalteromonadaceae (Alphaproteobacteria; 30.38%), Vibrionaceae (Gammaproteobacteria; 14.18%), Saccharospirillaceae (Gammaproteobacteria; 10.06%), Alteromonadaceae (Gammaproteobacteria; 8.63%), Oceanospirillaceae (Gammaproteobacteria; 5.79%), Arcobacteraceae (Campylobacteria; 5.40%), Enterococcaceae (Bacilli; 2.38%), unassigned Gammaproteobacteria (8.25%). The seston community was disproportionately dominated by Cyanobiaceae (Cyanobacteria; 13.01%), Saprospiraceae (Bacteroidia; 9.95%), Microcystaceae (Cyanobacteria; 8.76%), Nitriliruptoraceae (Actinobacteria; 8.70%), Balneolaceae (Rhodothermia; 6.84%), Microbacteriaceae (Actinobacteria; 6.38%), Chromatiaceae (Gammaproteobacteria; 4.81%), Pirellulaceae (Planctomycetes; 4.36%), PeM15 Actinobacteria (4.14%), and unknown Gammaproteobacteria (2.31%). Interestingly, one taxon was enriched in both the guano and water communities, Rhodobacteraceae (Alphaproteobacteria), at 4.41% and 2.32%, respectively.

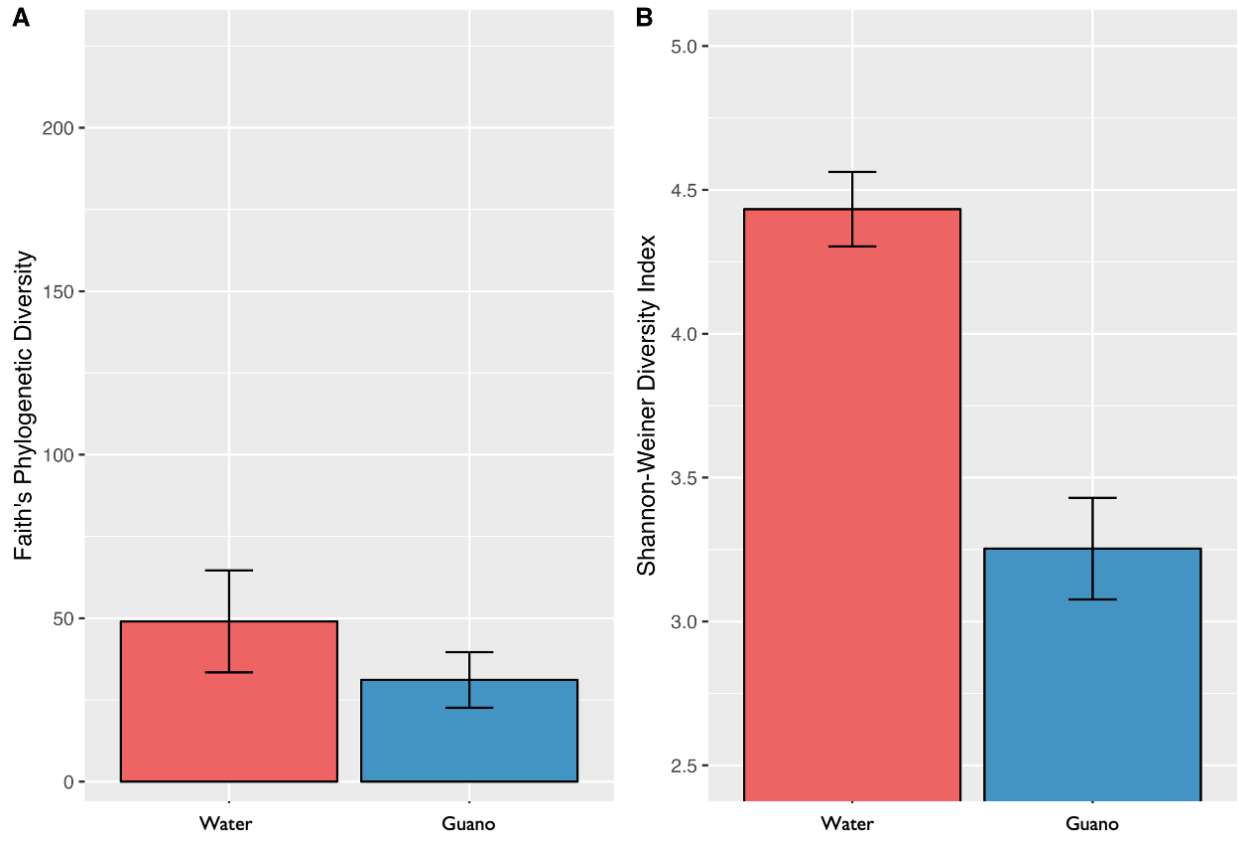


Fig. 17 Subset bar plots of Faith's phylogenetic diversity (A) and Shannon-Weiner diversity (B) values for water/seston (red) and guano (blue)

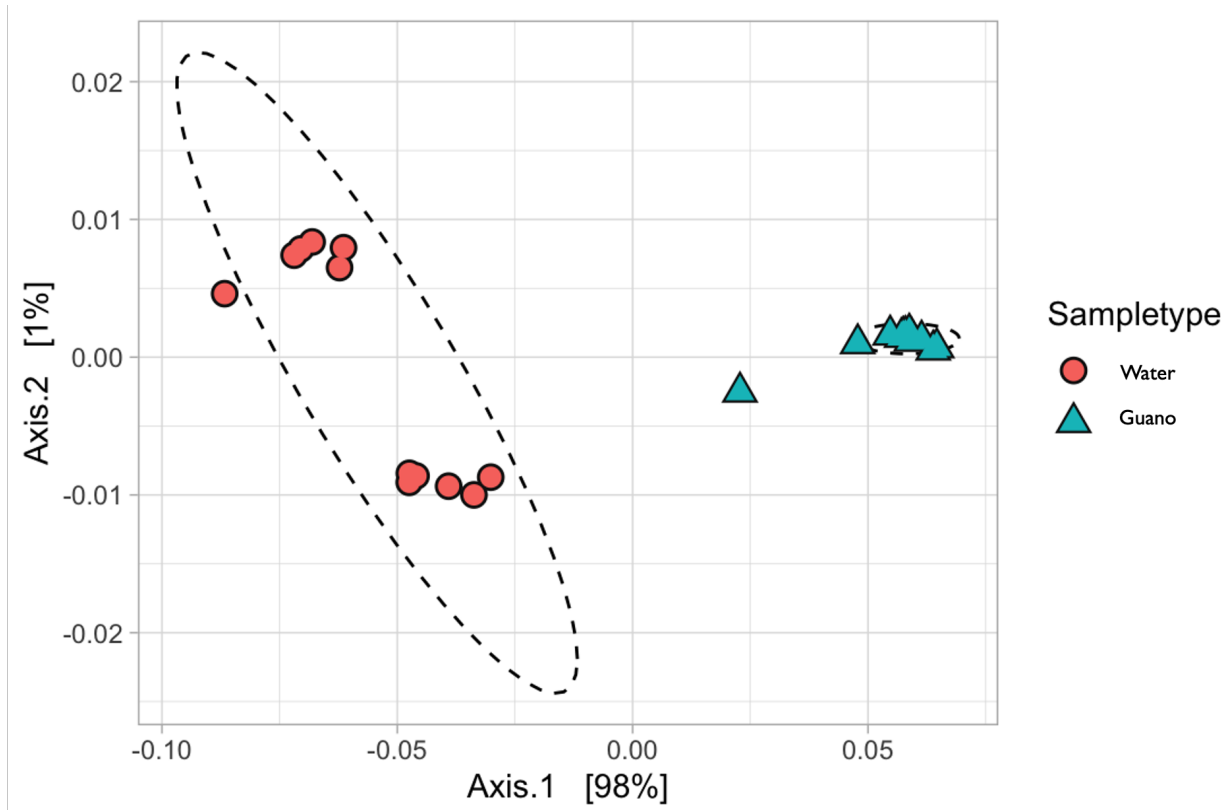


Fig. 18 Principal coordinate analysis (PCoA) of beta diversity values showing the dissimilarity of samples from water/seston and Guano using weighted UniFrac distance values

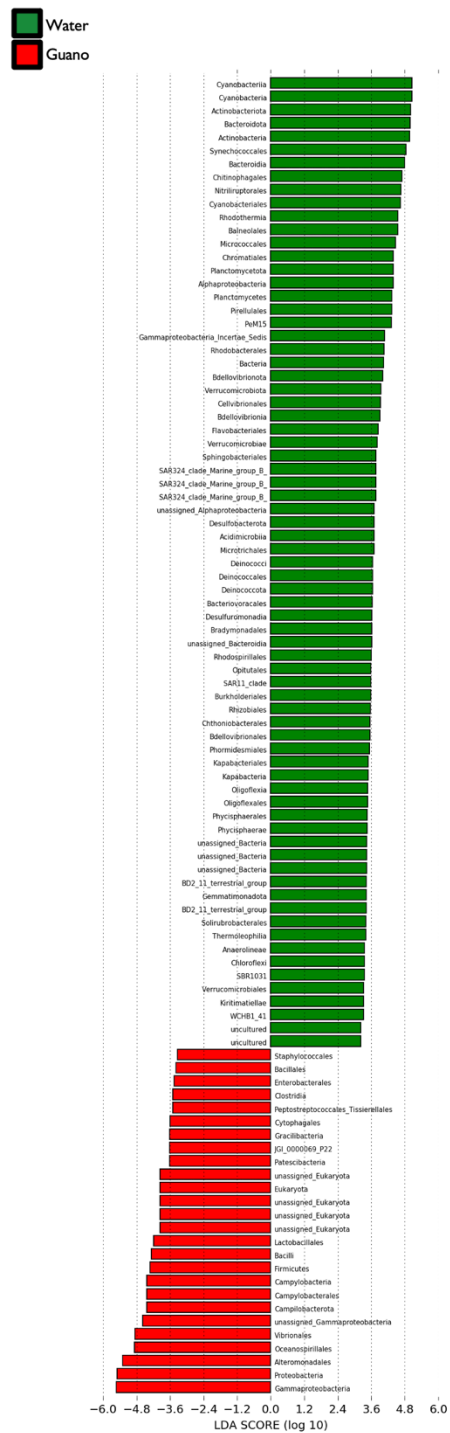


Fig. 19 Linear discriminant analysis (LDA) effect size (LEfSe) histogram depicting enriched taxa enriched in water/seston (green) and guano (red) (p -value < 0.05). Only taxa passing a 1% cutoff were included

Discussion

An exhaustive analysis of surface water quality data collected by the TCEQ SWQM program revealed that Baffin Bay and the Upper Laguna Madre Complex are hotspots of environmental change (Bugica et al. 2020). In addition to episodes of hypoxia and regular brown tide algal blooms, extremely high DOC and DON concentrations have become a defining feature of Baffin Bay (Wetz et al. 2017; Cira and Wetz 2019). Previous studies have implicated septic waste and septic-contaminated groundwater intrusion as primary sources of reactive nitrogen species (Felix and Campbell, 2019; Murgulet et al. 2021). However, the contribution of wildlife to the eutrophication of Baffin Bay is unknown.

Feral swine and bats are potential DOC and DON contributors. Invasive feral swine are endemic across Texas, with an estimated 2.6 million of the United States' 6.9 million animals residing in the state (Kinsey, 2020). Feral swine are especially abundant in rural areas where they cause significant crop and environmental damage through vegetation rooting and subsequent soil erosion. Mexican free-tailed bats are found throughout Texas and the Western Hemisphere. Caves are a common habitat for large colonies, but urban areas can support numerous small roosts, as shown by a survey in Waco, Texas, that identified 54 summer roosts (Li and Wilkens, 2015). Although large Central and North Texas colonies migrate south to Mexico for winter, smaller colonies persist year-round in West and South Texas. Examples of year-round roosts include a bridge colony in Big Bend Ranch State Park (Kasper and Yancy, 2018).

This study is the first report of a large year-round colony roosting in the Highway 77 overpass at Los Olmos Creek. While eutrophication in Baffin Bay is a complex problem involving multiple nutrient sources across the bay's watersheds, we hypothesized that bats are a contributing factor. Stable isotope composition analysis revealed overlap between guano and

seston $\delta^{13}\text{C}$ ratios, suggesting that guano contributes DOC in this system. Per the criteria established by the seminal work of Post 2002, resource similarity and trophic position calculations further support linkage between guano and seston (Post 2002). $\delta^{15}\text{N}$ ratios were higher and significantly different. While $\delta^{15}\text{N}$ values did not support linkage between guano and DON, more substantial anthropogenic sources of reactive nitrogen (e.g., septic waste, fertilizer, livestock waste) may mask a minor linkage. By contrast, cordgrass/saltgrass samples maintained much higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and appeared unaffiliated with guano or seston.

Potential linkage between guano and eutrophication was also explored through microbial community analysis. Alpha and beta diversity analyses revealed clear and significant distinctions between these two communities. However, overlap in the relative abundance of some taxa was observed. In particular, Rhodobacteraceae (Alphaproteobacteria) was a dominant member of the guano and seston microbial communities. This group has been associated with fecal and oil-polluted environments and with the degradation of complex organic materials (Dong et al. 2014; Størdal et al. 2015; Lawhon et al. 2022). The Rhodobacteraceae are notable for the provision of B-vitamins during association with harmful algal blooms caused by B-vitamin auxotrophs like *A. lagunensis* (Croft et al., 2005; Gómez-Consarnau et al., 2018).

All microbial families enriched in the seston (e.g., Nitriliruptoraceae, Microbacteriaceae, PeM15, Saprospiraceae, Balneolaceae, Pirellulaceae, Chromatiaceae, Rhodobacteraceae) were observed in our previous studies (Paxton et al., unpublished). Cyanobacteria, specifically those belonging to Synechococcales and Cyanobacteriales, was the most abundant group in the seston community. Synechococcus abundance has been correlated with elevated nutrient concentrations and fecal pollution (Rhudy et al. 1999; Cira and Wetz 2019). Moreover, Synechococcus abundance may be related to recurrent brown tide blooms in this system, as Synechococcus was

co-dominant with *A. lagunensis* brown tide blooms in Guantanamo Bay, Cuba (Hall et al. 2018). The halotolerant Nitriliruptoraceae were also enriched during this study. This group can utilize a wide range of nitrile compounds, nitrite oxidation, and its association with harmful algal bloom organisms (Sorokin et al. 2009; Lukwambe et al. 2019). Additionally, Nitriliruptoraceae has been implicated as a major constituent in salt-stressed soils (Wang et al. 2021).

The microbial taxa enriched in the guano included marine and enteric bacteria and potential pathogens. The single largest group enriched within the guano community was Pseudoalteromonadaceae (Gammaproteobacteria). This group is widely distributed and known for its wide-ranging metabolic capacities (Ivanova et al. 2014). Pseudoalteromonadaceae have been identified as members of fresh and marine communities, and they play an important role in detrital degradation and carbon cycling (Sheu et al. 2017; Tinta et al. 2023). Yet, to our knowledge, this study is the first report of Pseudoalteromonadaceae being a major constituent of a bat guano microbial community.

The majority of enriched guano bacteria belonged to Gammaproteobacteria. Of this group, two taxa were highly enriched: Vibrionaceae and Saccharospirillaceae. Members of these families are commonly isolated from marine environments (Labrenz et al. 2003; Choi and Cho 2010; Shahinpei et al. 2014). The enrichment of both may be explained by the bat's insectivorous diet coupled with the location of the roost i.e., insects with aquatic larval stages are likely prey. Vibrionaceae contains well-known pathogens, such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Oliver 2005; Drake et al. 2007), as well as *Salinivibrio* species, which are known to inhabit hypersaline environments (Fernández et al. 2014). While *Vibrio* has been isolated from bat guano, little is known about their role in the bat microbiome (Mühldorfer 2012), and less is known about bats as a vector for *Vibrio* biogeography. Additional enriched

taxa that may pose a threat to human health include Staphylococcales, Bacillales, Enterobacteriales, Clostridia, and Campylobacteriales. The loading of potentially pathogenic guano-associated bacteria may be a concern for recreational users in Baffin Bay.

In summary, a strong linkage between bat guano and eutrophication was not observed in this system. However, guano appears to contribute to DOC pools based on the similarity between guano and seston stable isotope composition. Additionally, some bacterial taxa, particularly Rhodobacteraceae, were enriched in both guano and seston microbial communities. These findings warrant further investigation of potential linkages between wildlife and eutrophication in this system. A longer-term study exploring linkage with bats, feral swine, and livestock may shed light on additional unknown nutrient sources.

CHAPTER V

SUMMARY

A recent meta-analysis identified 60 hypersaline lagoons, estuaries, and embayments worldwide (Tweedley et al. 2019). Typically located in arid/semi-arid tropical/sub-tropical climate zones, these hypersaline systems are acutely vulnerable to a wide range of environmental and anthropogenic stressors. Long residence times and limited ocean connectivity can compound and worsen the effects of pollution, eutrophication, algal blooms, and hypoxia (Kennish and Paerl 2010; Warwick et al. 2018). Further, climate change, including sea level rise and the increased frequency or intensity of storm events, is predicted to impact these systems and compound existing issues through alterations in flood and drought cycles as well as ocean connectivity (Tweedley et al. 2019). Yet little is known about marine hypersaline systems compared to their inland analogs.

The Laguna Madre is an expansive hypersaline lagoon in the Northwest GoM. The lagoon shows signs of eutrophication: elevated dissolved organic nutrients, algal blooms, bacterial pollution, and hypoxia. Mitigation is complicated by prolonged droughts, long residence times, reduced freshwater inflows, and limited ocean connectivity. Residence times for the Laguna Madre range from 300 days to several years (Buskey et al. 1998; Buskey et al. 2001). Persistent brown tide *A. lagunensis* algal blooms are especially disruptive, resulting in the loss of seagrass meadows (Gobler and Sunda 2012; Wilson and Dunton 2017). These issues will be compounded by continued freshwater removal by a growing coastal population and climate related changes in flood and drought cycles (Montagna et al. 2013). Hence, the Laguna Madre is a model system for investigating how anthropogenic pressures and climate change will affect hypersaline lagoons.

The purpose of this dissertation was to investigate the impact of disturbances on the microbial dynamics of a low-flow, inverse, hypersaline system. We also explored connectivity between wildlife and microbial dynamics. Chapter two was a short-term 2-month study of microbial community dynamics and various water quality metrics following a major flood event. The third chapter was a long-term 18-month study of microbial community dynamics and various water quality metrics that included flood and freeze disturbances. The fourth chapter was a targeted study of connectivity between the microbial community and a resident Mexican free-tailed bat colony. Resilience in response to disturbance in a system that experiences profound environmental change was a theme. We hypothesized the microbial community composition would be resilient to disturbance and connectivity with wildlife was assumed.

The short-term 2-month study revealed that flood events are pulse disturbance events that cause pronounced shifts in microbial community structure. The shift occurred across a salinity gradient and included a transition between freshwater to hypersaline taxa. Results also revealed exceptionally high organic nutrient concentrations that were perhaps the highest recorded for the Upper Laguna Madre Baffin Bay Complex. Salinity and dissolved organic nutrients were dominant drivers of temporal microbial community variance. Specifically, the disturbed flood site supported a unique microbial consortium and several bacterial taxa were significantly more abundant: Alphaproteobacteria (Rhodobacterales; 6.02%), Gammaproteobacteria (Chromatiaceae; 1.54% and Alcanivoracaceae; 1.10%), and Cyanobacteria (Nostocales; 4.50%). Additionally, some lower abundance taxa [Phycisphaerae (1.48%), Planctomycetacia (2.93%), Kiritimatiellae (2.50%), Mollicutes (2.91%), and Nitriliruptoria (3.05%)] were enriched at the flood site. Together, these results show that flooding induced a dramatic shift that stabilized only after the return of hypersalinity.

The long-term 18-month study allowed the investigation of multiple disturbance events. Interestingly, the broader temporal perspective revealed that the microbial community composition was resistant to flood and freeze events accompanied by profound decreases in salinity and temperature. For instance, the most significant flooding event decreased salinity from 78.10 to 19.18 but this change was not accompanied by a shift in microbial community composition. Similarly, an unprecedented freeze event decreased temperature from 27.50 to 12.10 °C with no significant change in microbial community structure. The ability to withstand extreme environmental fluctuations could be a hallmark of the system's microbial inhabitants. Whole genome sequencing of halophilic bacteria reinforced this precept with the discovery of mechanisms for osmoregulation such as choline and betaine uptake and betaine biosynthesis. Additionally, laboratory experimentation revealed that mechanisms for surviving hypersalinity appear to be cross-protective against heavy metal (copper) pollution. Together, these results better our understanding of halophile community resistance and shed light on mechanisms of osmoregulation and copper tolerance under extreme conditions.

The targeted study revealed that seston and guano microbial communities shared significant overlap in carbon isotope composition; however, nitrogen isotope composition were significantly different. Rhodobacteraceae (Alphaproteobacteria) was a dominant member of the seston and guano microbial communities. Moreover, the guano community was enriched in potentially pathogenic bacteria e.g., Staphylococcales, Bacillales, Enterobacteriales, Clostridia, and Campylobacteriales, which may pose a health threat to recreational users. Findings suggest that guano contributes to DOC pools and could act as a pathogen reservoir. Additionally, findings highlight the importance of considering alternative nutrient sources when investigating

eutrophication, considering that previous studies have not explored wildlife as a source of nutrient eutrophication.

The dominant taxa common to these three studies may play specific functional roles in the system. Synechococcales can transform atmospheric nitrogen into biologically useful forms through nitrogen fixation, and *Synechococcus* was shown to be co-dominant with an *A. lagunensis* brown tide bloom in Guantanamo Bay, Cuba (Hall et al. 2018). Actinobacteria, specifically those belonging to the Nitriliruptoraceae, are known for halotolerance, the ability to utilize aliphatic nitriles, and previous studies have demonstrated association with harmful algal blooms (Sorokin et al. 2009; Lukwambe et al. 2019). Balneolaceae have an affinity for hypersaline and hyperalkaline environments (Munoz et al. 2016; Vavourakis et al. 2018; Xia et al. 2016, 2017) and they are commonly found in systems contaminated by heavy metals and organophosphate insecticides (Lukhele et al. 2022). Gammaproteobacteria Incertae Sedis can be a dominant taxa in salt marshes (Gao et al. 2021) and hyperalkaline serpentinizing springs (Trutschel et al. 2023), and they may play a role in the degradation of PAHs, pesticides, and oil pollution (Gomes et al. 2008; Lin et al. 2017; Roszko et al. 2020; Tiralerdpanich et al. 2021). Rhodobacterales may provide B vitamins needed by *A. lagunensis*, which is a B vitamin auxotrophs (Croft et al. 2005; Gómez-Consarnau et al. 2018). Of course, partial 16S rRNA gene analysis is not an accurate predictor of function and these roles would require further study and verification.

In summary, we observed that microbial diversity was impacted by disturbance events, but we also observed remarkable resilience. The constraints of resilience will require further investigation to offer predictions about resilience to future climate related disturbance events, but the results of the dissertation establish a baseline understanding of microbial community

dynamics, and this baseline could provide insight into the dynamics of distant coastal hypersaline systems. Interestingly, hypersalinity may insulate against freeze events, mechanisms of osmoregulation may offer cross-protection to heavy metal resistance, and bats may contribute to eutrophication and pathogen loading. Collectively, these results better our understanding of coastal hypersaline microbial dynamics. A better understanding of microbial resilience is critical to predicting how hypersaline coastal ecosystems will adapt and evolve under future climate scenarios.

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APPENDIX A: CHAPTER 1 METADATA

Date	Sample ID	Water Temp. (°C)	Air Temp. (°C)	Wind Speed (mph)	Wind Direction	Days Since Last Precipitation	Last Rainfall (in)	Transparency (cm)	DO (%)	Salinity	pH	DOC (µmol/L)
6.27.18	Flood 1	31.50	29.80	5.00	SSE	6.00	5.57	27.20	40.00	0.94	7.15	1012.95
7.5.18	Flood 2	30.00	27.50	1.90	ENE	14.00	5.57	18.10	43.70	4.20	8.14	1270.05
7.11.18	Flood 3	31.70	29.20	2.30	SSE	3.00	1.22	15.00	56.30	6.75	8.39	1461.95
7.19.18	Flood 4	30.60	30.20	4.10	SSE	11.00	0.58	10.00	54.40	12.00	8.48	1746.65
7.25.18	Flood 5	30.65	0.00	2.80	N/A	18.00	0.58	11.80	67.50	13.12	8.71	1972.15
8.1.18	Flood 6	30.34	33.61	1.30	NE	21.00	1.22	7.20	49.90	28.38	8.92	2693.55
8.8.18	Flood 7	30.30	32.28	1.70	SW	0.00	1.22	8.80	155.00	32.11	9.12	2942.50
8.15.18	Flood 8	30.02	31.50	4.70	SSE	3.00	0.11	8.10	173.40	39.60	9.23	3611.50
8.22.18	Flood 9	30.59	32.40	3.90	S	11.00	0.11	11.60	109.50	45.45	8.65	3872.00
8.29.18	Flood 10	32.70	33.20	8.50	SE	17.00	0.90	12.70	110.70	61.20	8.61	4086.20
9.7.18	Flood 11	31.04	91.00	3.40	SE	2.00	0.15	13.20	124.90	64.81	8.55	3969.75
6.27.18	Non-flood 1	29.70	30.30	6.40	SSE	6.00	1.05	46.10	78.10	21.39	7.96	737.75
7.5.18	Non-flood 2	29.40	29.20	4.10	ESE	9.00	0.20	22.40	70.60	22.36	7.96	655.50
7.11.18	Non-flood 3	31.10	30.30	6.70	SW	3.00	0.58	42.00	71.70	31.20	0.00	747.80
7.19.18	Non-flood 4	20.10	29.80	6.50	SSE	11.00	0.58	31.10	100.30	36.00	8.48	792.50
7.25.18	Non-flood 5	31.13	32.56	3.20	SSE	18.00	0.58	43.80	161.23	39.56	8.25	813.85
8.1.18	Non-flood 6	31.26	30.27	1.70	NNE	25.00	0.58	39.70	89.10	37.59	8.24	797.75
8.8.18	Non-flood 7	29.80	30.17	6.10	S	0.00	0.58	13.40	112.70	38.56	8.30	783.15
8.15.18	Non-flood 8	28.54	30.78	8.40	SSE	4.00	0.25	21.10	100.20	38.19	8.11	793.95
8.22.18	Non-flood 9	30.97	31.30	4.30	WSW	11.00	0.25	22.80	124.40	41.25	8.16	825.45
8.29.18	Non-flood 10	31.70	30.78	13.80	E	18.00	0.25	24.20	124.50	41.69	8.34	791.90
9.7.18	Non-flood 11	29.78	85.60	5.00	SE	2.00	1.15	19.40	114.50	40.88	8.26	739.15
Date	Sample ID	TDN	Ammonia (mM)	NN (mM)	Nitrate (mM)	Nitrite (mM)	orthoP (mM)	Silicate (mM)	DIN (mM)	DON (mM)	DIN:DIP	Chlorophyll <i>a</i> (mg/L)
6.27.18	Flood 1	83.95	12.69	1.17	0.58	0.59	31.41	283.58	13.85	70.10	0.45	23.19
7.5.18	Flood 2	91.45	2.52	0.81	0.37	0.44	25.23	406.89	3.33	88.05	0.10	40.31
7.11.18	Flood 3	105.35	1.07	0.72	0.25	0.47	17.41	426.80	1.79	103.55	0.10	46.80
7.19.18	Flood 4	140.55	1.88	0.69	0.23	0.46	5.34	407.10	2.57	138.00	0.45	40.09
7.25.18	Flood 5	157.45	1.84	0.57	0.14	0.43	0.57	340.52	2.41	155.05	4.25	56.51
8.1.18	Flood 6	216.70	1.59	0.89	0.51	0.38	0.90	109.06	2.49	214.30	2.75	106.02
8.8.18	Flood 7	229.90	1.63	1.56	1.19	0.36	1.17	74.23	3.19	226.65	2.70	294.90
8.15.18	Flood 8	283.20	1.55	0.54	0.23	0.31	1.02	33.66	2.09	281.10	2.05	471.85
8.22.18	Flood 9	360.15	43.77	5.38	0.96	4.42	1.47	56.45	49.15	310.95	33.75	89.43
8.29.18	Flood 10	334.90	6.37	0.68	0.23	0.45	1.22	51.07	7.05	327.85	5.80	57.67
9.7.18	Flood 11	423.90	18.61	2.56	1.69	0.87	1.92	59.43	21.17	402.70	11.00	84.82
6.27.18	Non-flood 1	75.05	1.27	0.45	0.21	0.23	3.37	105.50	1.72	73.35	0.50	10.77
7.5.18	Non-flood 2	61.05	0.83	0.39	0.20	0.19	1.35	141.55	1.22	59.80	0.90	9.18
7.11.18	Non-flood 3	79.95	1.41	1.87	1.68	0.19	0.72	154.08	3.28	76.65	4.60	1.86
7.19.18	Non-flood 4	86.00	1.83	0.51	0.33	0.18	0.52	82.91	2.34	83.65	4.50	3.95
7.25.18	Non-flood 5	93.75	3.44	0.64	0.42	0.22	0.70	114.77	4.08	89.70	5.85	4.31
8.1.18	Non-flood 6	88.35	1.27	0.46	0.24	0.22	0.54	148.52	1.72	86.60	3.30	6.08
8.8.18	Non-flood 7	83.55	0.95	0.41	0.23	0.18	0.65	122.30	1.35	82.20	2.15	8.23
8.15.18	Non-flood 8	83.40	2.34	0.42	0.21	0.21	0.47	134.41	2.76	80.60	6.10	6.59
8.22.18	Non-flood 9	94.25	2.26	0.47	0.23	0.24	0.49	118.47	2.73	91.55	5.55	5.97
8.29.18	Non-flood 10	84.70	0.89	0.39	0.19	0.19	0.51	62.25	1.28	83.40	2.55	10.17
9.7.18	Non-flood 11	84.75	2.01	0.50	0.32	0.17	0.40	116.95	2.51	82.25	6.30	12.83

APPENDIX B: CHAPTER 2 METADATA

Date	Sample ID	Water Temp. (°C)	Air Temp. (°C)	Wind Speed (mph)	Wind Direction	Days Since Last Precipitation	Last Rainfall	Transparency (cm)	DO (%)	Salinity	pH	Ammonia (mM)	Nitrate (mM)	Nitrite (mM)
1.15.20	LOC 1	32.52	26.00	10.90	SSE	1	0.10	10.00	95.70	59.81	8.18	4.39	4.06	0.36
2.24.20	LOC 2	19.75	21.60	2.20	SSE	3	0.17	11.20	105.50	60.87	7.57	9.87	7.30	0.14
5.18.20	LOC 3	28.33	29.30	3.80	SSW	3	2.23	10.30	108.50	64.99	8.30	44.35	2.40	0.00
6.15.20	LOC 4	30.80	28.40	1.70	NNE	16	2.26	10.20	79.40	66.16	8.55	47.77	2.10	0.00
9.13.20	LOC 5	30.20	25.70	1.50	SW	0	0.10	8.00	13.60	69.30	8.12	36.90	1.80	0.00
10.16.20	LOC 6	27.50	19.40	3.40	S	0	0.30	9.06	11.00	70.50	7.90	8.21	3.11	0.08
12.18.20	LOC 7	12.10	19.00	6.00	ESE	17	0.40	6.40	90.50	82.19	7.82	4.12	2.79	0.05
1.22.21	LOC 8	18.80	20.50	1.10	N	12	1.54	19.67	0.00	70.46	8.33	14.09	4.55	0.82
2.26.21	LOC 9	22.20	22.20	4.80	NW	2	1.20	10.18	77.00	71.22	8.38	9.61	9.04	0.05
3.22.21	LOC 10	20.70	20.80	14.00	N	0	0.22	8.33	83.50	78.10	7.65	6.68	10.68	0.05
5.25.21	LOC 11	28.10	28.20	6.50	NW	1	0.58	16.50	81.00	19.18	8.18	233.95	12.17	0.02
6.18.21	LOC 12	29.90	27.60	4.90	N	14	1.29	15.40	33.00	2.57	8.66	8.70	2.19	0.05
Date	Sample ID	Water Temp. (°C)	Air Temp. (°C)	Wind Speed (mph)	Wind Direction	Days Since Last Precipitation	Last Rainfall	Transparency (cm)	DO (%)	Salinity	pH	Ammonia (mM)	Nitrate (mM)	Nitrite (mM)
1.15.20	RB 1	21.47	26.00	10.90		1	0.10	SSE	119.03	40.43	8.33	1.19	1.67	0.95
2.24.20	RB 2	17.77	21.60	2.50		3	0.17	SSE	101.60	44.98	7.66	18.95	8.13	0.17
5.18.20	RB 3	28.24	28.10	3.80		3	2.23	SSW	130.70	49.78	8.32	180.67	2.83	0.01
6.15.20	RB 4	28.93	30.20	0.90		16	2.26	NNE	110.20	47.77	8.17	156.48	2.45	0.06
9.13.20	RB 5	27.10	27.20	2.20		0	0.10	SE	72.30	45.50	7.91	161.38	2.05	0.00
10.16.20	RB 6	26.00	19.90	3.50		0	0.30	S	65.00	43.80	8.06	22.37	2.40	0.27
12.18.20	RB 7	14.60	18.50	6.90		17	0.40	SE	97.00	49.24	8.10	20.95	2.75	0.11
1.22.21	RB 8	17.70	22.20	0.60		12	1.54	SE	85.00	48.80	8.12	21.46	3.90	0.11
2.26.21	RB 9	20.93	22.90	16.50		2	1.20	NW	109.40	49.30	8.17	17.49	7.45	0.19
3.22.21	RB 10	19.20	21.20	11.50		0	0.22	NW	102.70	48.77	8.02	19.16	13.03	0.09
5.25.21	RB 11	27.40	27.50	7.40		1	0.58	NW	95.00	22.69	8.01	43.27	2.78	0.00
6.18.21	RB 12	28.80	31.00	3.10		14	1.29	N	84.50	16.05	8.37	30.39	1.81	0.02

APPENDIX C: ISOTOPIC PARAMETERS OF GUANO, SESTON, AND
SALTGRASS/CORDGASS SAMPLES

Sample ID	Sample Weight (mg)	$\delta^{13}\text{C}_{\text{VPDB}}$ (‰)	Total C (μg)	$\delta^{15}\text{N}_{\text{Air}}$ (‰)	Total N (μg)	C:N
Guano1	1.5311	-19.49	315.98	6.99	69.69	4.53405595
Guano2	1.471	-19.59	352.40	6.67	77.06	4.57285574
Guano3	1.483	-19.46	349.91	6.68	76.86	4.5527196
Guano4	1.5494	-20.37	451.49	6.47	101.50	4.4480103
Guano5	1.4739	-20.25	438.74	7.00	95.70	4.58446707
Guano6	1.4687	-20.58	347.88	6.76	76.11	4.57093057
Guano7	1.5127	-21.06	469.36	6.79	96.52	4.86283065
Guano8	1.5332	-20.89	456.41	6.39	93.58	4.87695994
Guano9	1.5115	-20.84	419.46	6.79	86.48	4.85018969
Guano10	1.452	-20.30	401.05	6.18	81.98	4.89220092
Guano11	1.492	-20.19	346.07	6.51	73.92	4.68156562
Guano12	1.52	-19.92	284.88	7.00	60.77	4.68813393
Guano13	1.5262	-18.92	382.84	4.99	76.79	4.98550069
Guano14	1.5162	-18.42	373.60	5.39	74.06	5.0446419
Guano15	1.4995	-19.66	401.73	6.47	81.16	4.94989041
Guano16	1.5092	-20.46	381.03	5.57	76.24	4.99758955
Guano17	1.5212	-19.35	342.91	6.31	71.26	4.81208833
Guano18	1.509	-19.03	273.74	6.22	56.34	4.85844168

Guano19	1.5208	-19.28	291.47	6.00	61.60	4.73172384
Guano20	1.5482	-19.01	342.91	5.99	72.22	4.74839995
Guano21	1.4787	-19.84	389.36	6.08	80.54	4.83411594
Guano22	1.5299	-19.51	433.36	5.68	89.90	4.82061383
Guano23	1.5594	-20.44	417.45	6.62	88.67	4.70791989
Guano24	1.5431	-19.87	412.51	7.01	87.71	4.70293516
Guano25	1.4643	-20.17	240.23	6.26	50.50	4.75719542
Guano26	1.5247	-19.93	230.64	6.30	48.66	4.73959507
Guano27	1.5164	-19.32	222.69	6.69	46.46	4.7926229
Guano28	1.4672	-19.65	288.52	5.99	60.80	4.74530856
Guano29	1.5245	-20.17	276.24	6.57	58.74	4.70284934
Guano30	1.4961	-19.99	263.27	6.53	55.46	4.74734364
Seston1	N/A	-21.17	559.71	9.30	84.95	6.58836902
Seston2	N/A	-20.88	726.27	9.60	114.21	6.35935792
Seston3	N/A	-21.02	612.23	9.52	96.47	6.34649919
Seston4	N/A	-20.68	731.43	9.13	128.49	5.69240281
Seston5	N/A	-20.97	701.05	8.65	131.65	5.32515082
Seston6	N/A	-21.00	833.40	9.09	145.31	5.7355276
Seston7	N/A	-20.67	704.40	8.29	137.12	5.13716918
Seston8	N/A	-20.90	789.19	9.51	127.08	6.21035242
Seston9	N/A	-20.69	702.22	8.56	132.07	5.31695989
Seston10	N/A	-20.84	817.02	9.22	141.64	5.76833859
Seston11	N/A	-20.83	624.85	8.98	112.90	5.53448193

Seston12	N/A	-20.30	666.67	7.99	137.17	4.86006315
Seston13	N/A	-21.05	825.32	8.78	149.87	5.50682198
Seston14	N/A	-21.19	538.14	8.16	103.48	5.20038961
Seston15	N/A	-21.16	736.72	8.99	130.75	5.63449828
Seston16	N/A	-18.69	1266.22	8.98	183.79	6.88963882
Seston17	N/A	-20.92	807.12	9.51	132.96	6.07034527
Seston18	N/A	-21.04	891.12	8.93	158.63	5.61766468
Seston19	N/A	-21.10	839.41	9.02	142.36	5.89652951
Seston20	N/A	-20.82	719.19	7.25	163.35	4.40269618
Seston21	N/A	-18.39	1135.73	7.94	195.82	5.79983895
Seston22	N/A	-16.51	1366.12	8.74	181.27	7.53633163
Seston23	N/A	-20.98	793.81	9.20	133.28	5.95594141
Seston24	N/A	-20.95	680.14	9.04	122.01	5.5743603
Seston25	N/A	-18.99	1263.97	8.28	214.41	5.89508023
Seston26	N/A	-17.07	1348.37	8.86	186.59	7.22656506
Seston27	N/A	-20.81	725.92	9.56	123.45	5.88006189
Seston28	N/A	-20.41	784.61	9.19	131.72	5.95644588
Seston29	N/A	-18.71	1344.11	9.11	202.47	6.63872512
Seston30	N/A	-20.81	796.49	8.43	157.20	5.0666683
Grass1	2.8244	-14.30	782.50	12.09	35.28	22.1786118
Grass2	2.7168	-14.26	763.04	11.92	34.04	22.416482
Grass3	2.6277	-14.34	753.51	11.99	31.99	23.5537528
Grass4	2.6922	-14.30	742.45	11.90	33.14	22.4048691

Grass5	2.7168	-14.21	768.88	12.12	33.92	22.6699617
Grass6	2.6412	-14.31	752.86	11.85	32.99	22.8176351
Grass7	2.656	-14.28	768.66	12.12	34.98	21.9735846
Grass8	2.7265	-14.30	778.39	11.95	35.08	22.1910845
Grass9	2.6496	-14.30	773.20	12.18	34.51	22.4051272
Grass10	2.6398	-14.26	774.29	11.64	33.71	22.9680623
Grass11	2.7345	-14.41	990.28	11.61	38.57	25.6733985
Grass12	2.6518	-14.44	950.51	11.31	36.57	25.9900177
Grass13	2.6468	-14.45	954.32	11.40	38.71	24.6538645
Grass14	2.7356	-14.47	994.08	11.53	38.04	26.1326605
Grass15	2.7001	-14.50	986.06	11.42	37.51	26.2898008
Grass16	2.6931	-14.36	967.03	11.51	38.60	25.0528639
Grass17	2.6839	-14.54	969.15	11.53	38.16	25.3951512
Grass18	2.6451	-14.50	952.63	11.18	38.46	24.7717064
Grass19	2.6682	-14.48	956.44	11.52	38.16	25.0622291
Grass20	2.6734	-14.63	966.39	11.31	38.07	25.382087
Grass21	2.7057	-14.20	959.19	10.90	43.53	22.0359395
Grass22	2.7252	-14.12	965.34	10.90	42.63	22.64223
Grass23	2.6579	-14.21	938.41	10.85	41.30	22.7238802
Grass24	2.6766	-14.16	956.02	11.20	41.84	22.8517733
Grass25	2.6231	-14.11	918.00	10.97	42.17	21.7690671
Grass26	2.648	-14.35	908.42	10.57	39.06	23.2548214
Grass27	2.7238	-14.34	960.04	10.68	42.25	22.7218125

Grass28	2.6874	-14.23	944.57	10.95	42.80	22.070244
Grass29	2.6532	-14.30	941.17	10.78	41.82	22.5042444
Grass30	2.6655	-14.25	923.96	10.61	42.33	21.8290445