

Microbial community response to environmental change during the Anthropocene

By

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Abstract

Microbial community composition and functional potential can be affected by human-derived environmental changes during the Anthropocene with important consequences for elemental cycling and whole ecosystem processes. This study tested the hypothesis that environmental changes impact microbial communities across different spatial and temporal scales. The main objectives of this study were to determine 1) biocorrosion-causing organism colonization and abundance on man-made steel structures, 2) the identity and function of a core microbiome across steel microbial communities, and 3) the response of coastal microbial communities to extreme hurricane disturbance events. Steel microbiomes represent microbial responses to environmental disturbance (i.e., introduction of a novel substrate and surface environment) on small spatial scales but long temporal scales. Conversely, microbial responses to extreme storm events provide insight into disturbances affecting large spatial scales but short temporal scales. Stainless steel (304 and 316) deployments along salinity gradients in two North Carolina estuarine river systems resulted in increased colonization of iron-oxidizing bacteria on

more-corrosion-resistant stainless steel (316) and at higher salinities. A novel iron-oxidizer species *Mariprofundus ferrooxydans* was isolated and sequenced, revealing genes for molybdenum utilization and reactive oxygen species protection, which may represent adaptations towards advanced steel types. Comparisons of microbial communities across stainless steel and historic ferrous-hulled shipwreck steel in the Pamlico Sound, NC revealed a “core steel microbiome” of heterotrophic generalists that likely play important roles in biofilm protection and functional stability for biocorrosion communities. Shifting scales, extreme hurricane events were correlated with changes in total (DNA) and active (RNA) coastal bacterial but not archaeal communities, and in the water column but not in sediments. Offshore marine sites exhibited decreased community diversity and evenness and increased abundance of copiotrophs. Hurricanes were also correlated with increased putative function of pathogenic taxa (i.e., *Prevotella* and *Legionella*) and lignin-degrading taxa, likely causing decreased water quality and increased bacterial production. These environmental disturbances across different spatial and temporal scales show that microbial communities are constantly responding and adapting to survive. Microbial communities have shown to be extremely resilient to Anthropocene conditions, and the microbial responses in this study can be applied to better understand future global change scenarios.

Microbial community response to environmental change during the Anthropocene

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LIST OF ABBREVIATIONS

DNA = deoxyribonucleic acid

RNA = ribonucleic acid

CO₂ = carbon dioxide

FeOB = iron-oxidizing bacteria

SS = stainless steel

16S rRNA = 16S ribosomal ribonucleic acid

ppt = parts per thousand

°C = degrees Celsius

mL = milliliters

DO = dissolved oxygen

mg = milligrams

L = liters

MPN = most probable number

qPCR = quantitative polymerase chain reactive

µm = micrometers

O₂ = oxygen

nM = nanomolar

nm = nanometers

g = grams

kV = kilovolts

PCR = polymerase chain reaction

OTU = operational taxonomic unit

GC content = guanine-cytosine content

ANI = average nucleotide identities

AAI = average amino acid identities

SD = standard deviation

MIC = microbially influenced corrosion

PVC = polyvinyl chloride

bp = base pairs

MAG = metagenome assembled genome

C = carbon

N = nitrogen

P = phosphorous

DOC = dissolved organic carbon

cDNA = complementary DNA

I. INTRODUCTION

Microbial survival in a diverse abiotic world

The vast array of physical and chemical conditions on Earth present complex challenges for survival of biological organisms. Single-cell microorganisms (i.e., bacteria and archaea) have orders of magnitude higher phylogenetic diversity compared to eukaryotes, as seen in the most recent iterations of the tree of life (Hug *et al.* 2016). This phylogenetic diversity of microbes stems from a multitude of energy metabolism pathways enabling survival across a wide variety of environmental conditions (Pace 1997). Single-cell microorganisms also have an increased ability for rapidly adapting to abiotic environmental disturbances and fluctuations compared to larger eukaryotes (Poole *et al.* 2003). This is due to faster cell replication rates, shorter life cycles and larger population sizes in microbes, lending to high mutation rates within a population (Allison & Martiny 2008; Lynch 2010; Comte & del Giorgio 2011). Adaptation of these mutations within a microbial population is strongly impacted by high incidence of horizontal gene transfer and unique cell signaling pathways for recognizing environmental cues such as biofilm formation, sporulation, and dormancy (Marijuán *et al.* 2010; Vos *et al.* 2015). While it may be difficult to directly measure mutations and adaptations within microbes present in the natural environment, comparative analyses of microbial community composition and functional potential across different abiotic conditions can be used to infer environmental adaptability of many microbial taxa under global change scenarios (Logue *et al.* 2015).

In addition to the complexity of physical and chemical conditions that can exist on Earth at any one point in time, the global environment is constantly forcing microbes to adapt to new conditions over time in order to survive (Nguyen *et al.* 2020_a). The global environment is expected to continue changing significantly in the future, and it is important to understand the factors and consequences of environmental change on microbial communities in order to predict future impacts on global ecosystems. In general, single microbial lineages must exhibit, or switch between, physiological traits that permit survival under environmental fluctuations (e.g., facultative anaerobes enduring fluctuations between oxic and anoxic conditions). Otherwise, widespread cell deaths can lead to major shifts in microbial community composition and functional potential (Csonka 1989; Potts 1994; Imlay 2013; Finn *et al.* 2017). Adaptability of microbes to changing environmental conditions is likely crucial to preventing extinction of taxonomic lineages in the distant future. True steady-state conditions arguably do not exist in the natural environment, as environmental change and fluctuation are inherent characteristics of ecosystems across the globe (Nguyen *et al.* 2020_a). Lab-based studies and advanced models have shown that fluctuating environmental conditions promote physiological adaptations that were not found in comparable steady-state environments (Nguyen *et al.* 2020_b), and that historical contingency of previous exposure to environmental fluctuations significantly influences functional resilience (Hawkes & Keitt 2015). This suggests that microbes adapt to their surrounding environment continually over time to ensure survival, and have likely already adapted to characteristics of the Anthropocene such as higher global temperatures and newly

introduced anthropogenic debris (e.g. steel, plastic, etc.) as substrate types for potential colonization.

The degree of environmental change and fluctuations that may be exhibited over time and space within any given environment is largely determined by its physical characteristics. For example, aquatic environments such as oceans and estuaries may experience greater abiotic fluctuations compared to soil, sediment, or living host environments. This can be due to generally faster diffusion and advection of nutrients, oxygen, and salts across spatial scales within liquids. Further, variations in grain size of soils and sediments lead to variable pore sizes within which water and fluctuating abiotic conditions may not distribute evenly, leading to less overall exposure to environmental fluctuations in these environments (Nguyen *et al.* 2020_a). Oceans and estuaries on the other hand have additional exposure to fluctuations from advection currents, tidal cycles, and riverine input following rainfall events. One could argue that tidal cycles make estuaries one of the most fluctuating environments on Earth, as resident organisms must be capable of surviving daily changes in salt concentrations. With salinity among the strongest driving forces toward phylogenetic divergences, some estuarine microbes have distinct adaptations to overcome osmotic stress associated with daily salinity fluctuations such as osmoprotectant genes (Yaakop *et al.* 2016) and duplicate paralogs of important functional genes, each optimized for different salinity concentrations (Sanchez-Perez *et al.* 2008). There may be many different routes of adaptation for salinity fluctuation tolerance, but in the end, only taxa that are prepared for environmental change will survive to make meaningful impacts on biogeochemical

cycles and other ecosystems services. Furthermore, this type of convergent evolution describing multiple routes of adaptation towards withstanding environmental change will likely be even more evident in the future as climate change scenarios predict drastically different environmental conditions.

Of the environmental sensing pathways unique to microbes and beneficial during environmental change, biofilm formation plays a large role in nutrient acquisition for many taxa (O'Toole *et al.* 2000). For example, microbes can colonize surface environments and form biofilms for energy acquisition from the surrounding waters or from the surface environment itself (O'Toole *et al.* 2000; Watnick & Colter 2000; Stanley & Lazazzera 2004; Flemming *et al.* 2016). Cells can either attach to the surface directly as early pioneers of biofilm formation or colonize within the extracellular polymeric substance matrix (i.e., the three-dimensional space of a mature biofilm) without actually attaching to the surface. Once established, cells can fulfill energy requirements from scavenging nutrients within the extracellular matrix or by utilizing vital elements within the surface material itself such as iron from steel surfaces. This iron can be used as a source of electrons for growth of microorganisms such as iron-oxidizing bacteria during biocorrosion processes (Little *et al.* 2006; Emerson *et al.* 2010; Emerson 2019). Many different bacterial taxa have proven capable of surface attachment after otherwise living a planktonic lifestyle in aquatic systems (O'Toole *et al.* 2000). For example, *Caulobacter* sp. exhibits a unique process of cell-cycle-controlled replication of both flagellar planktonic cells and stalked cells for a sessile attached lifestyle (Merker & Smit 1988; Yun *et al.* 1994; Bodenmiller *et al.* 2004). Once formed, these aquatic biofilm

communities can be highly dynamic, providing much more than nutrients alone. Biofilms can supply habitat diversity via localized gradients of physical or chemical conditions (e.g., oxygen) that could enable niche establishment beyond what was available in the surrounding environment (Flemming *et al.* 2016). Biofilms can also promote mutualistic relationships among taxa and sharing of genetic material between neighboring taxa via horizontal gene transfer (Watnick & Colter 2000). These newly acquired genes can ultimately become the source of new adaptations and even novel microbial taxa equipped for survival in future environmental change scenarios.

Another unique response of microbial cells to certain environmental cues is dormancy. Microbes may enter this state of inactivity, nearly resembling a dead cell, whenever environmental conditions are deemed unfavorable for cell survival (Lennon & Jones 2011). Portions of microbial populations have shown dormancy initiation even prior to the onset of unfavorable conditions, possibly due to some form of bet-hedging strategy to prepare for potential future changes within the environment (Jones & Lennon 2010). In aquatic systems, dormant cells have the added advantage of translocation via advection currents, enabling placement in potentially more favorable environmental conditions (Mestre & Höfer 2020). This remarkable ability to avoid cellular demise while also benefitting from relocation essentially enables widespread taxonomic dispersion and ubiquity around the globe, and further advances the potential for adaptation towards different environmental conditions. Additionally, some observed dispersion paths for dormant microbial cells have displayed cyclical patterns, eventually returning to geographic points of origin over time (Mestre & Höfer 2020). This suggests even

further adaptations surrounding dormancy and environmental change that have yet to be fully explored or understood. As an additional consequence of dormancy, comparative microbial community analyses that utilize DNA sequenced from the environment could potentially depict dormant cells as a major portion of the community while these cells are likely not contributing to biogeochemical cycles and ecosystem services (Freedman *et al.* 2015; Cardoso *et al.* 2017). Comparisons of DNA-based surveys (i.e., the total community) to RNA-based surveys (i.e., the active community) are often useful in determining the prevalence of dormancy in an environment and a more accurate depiction of the putative ecosystem functions performed by the community (Campbell *et al.* 2009; Locey *et al.* 2020). The diverse environmental sensing abilities that microbes exhibit are indicative of the diversity of potential adaptations yet to be discovered or yet to be formed, and are important considerations to make when measuring microbial response to environmental change.

Ecological niche strategies of microbes

The role that certain microbial taxa play in ecosystem services can be better understood when classified according to their ecological niche (Carbonero *et al.* 2014). Niche breadth is a calculation used by classical ecologists to determine the degree of a taxon's environmental specialization, or how often they are found across different environment types (Levins 1968). Generalist taxa have a broad spectrum of habitat types, often due to an ability for utilizing multiple sources of nutrients for energy and growth. In contrast, specialist taxa are typically only found within a narrow range of habitat types and have very specific energetic growth requirements. Both of these

lifestyles could be considered adaptations to environmental conditions by fulfilling an empty niche within the ecosystem that may facilitate survival. Most specialist microbes tend to have a slight advantage over generalists on a local scale leading to greater relative abundances in comparison (Mariadassou *et al.* 2015). Specialist microbes also tend to exhibit broader *spatial* distributions compared to generalist microbes, while the opposite is true for larger eukaryotic organisms (Carbonero *et al.* 2014). This could be due to greater intraspecific microbial competition for generalists compared to specialists and is an important distinction to consider when applying general ecological theory to predict response of microbial populations to environmental change.

Even though intra- and inter-specific competition plays an important role in ecological niche fulfillment, environmental filtering (i.e., local habitat conditions) still seems to be the most powerful force shaping microbial community composition and functional potential (Leibold *et al.* 2004; Van der Gucht *et al.* 2007; Logue *et al.* 2015; Lindh *et al.* 2016). This concept has held true across biological domains for nearly a century after Baas Becking first proclaimed “Everything is everywhere, but the environment selects” (Baas Becking 1934). Upon closer examination at the microbial scale in aquatic environments, studies have shown that generalists may be more strongly regulated by environmental conditions and may have greater contributions to the microbial community variation or beta diversity across environment types compared to specialists (Székely & Langenheder 2014; Lindh *et al.* 2016). As such, generalist microbes are clearly core determinants of microbial community ecosystem function. Generalist microbes have also been shown to have speciation rates nearly 20x higher

than specialist taxa lending to an increased ability of avoiding localized extinction (Sriswasdi *et al.* 2017). Coupling this with evidence suggesting more frequent generalist-to-specialist transitions than that of the reverse (Sriswasdi *et al.* 2017), generalist microbes may play more important roles in global microbial dispersion and the evolution and adaptation of traits to withstand environmental change in a rapidly changing world.

Another avenue for ecological niche development among aquatic microbes can be created by differential nutrient concentrations within the environment. Copiotrophic organisms thrive under high nutrient conditions, whereas oligotrophic organisms conversely excel under low nutrient conditions (Poindexter 1981; Koch 2001). Most importantly, these environmental preferences are founded upon clear genomic adaptations such as genomic streamlining in oligotrophs (i.e., minimal metabolic machinery for optimal energy efficiency) or larger cell size, faster replication rates, and greater transcriptional control in copiotrophs (Lauro *et al.* 2009; Swan *et al.* 2013; Cottrell & Kirchman 2016). Microbial genome streamlining in the ocean was likely a reductive evolution process that occurred as a result of metabolic machinery inactivity leading to obsolescence and functional loss (Giovannoni *et al.* 2014). In the end, this reductive process has proven to be a successful ecological strategy within low nutrient environments such as the open ocean.

Regardless of the beneficial aspects of genomic streamlining, oligotrophs arguably would not remain successful without the coexistence of copiotrophs and the

ecosystem services they provide (Li *et al.* 2020). Marine copiotrophs can produce extracellular enzymes that enable breakdown of larger organic polymers which are subsequently utilized by oligotrophs. This cheater-helper paradigm, often referred to as the “Black Queen hypothesis” (Morris *et al.* 2012), has been debated over which ecological role may be more coveted: the cheater or the helper (Fullmer *et al.* 2015; Morris 2015; Ndhlovu *et al.* 2020). Ultimately however, it is likely a near even mutualistic relationship with neither functional group able to thrive without the other (Mas *et al.* 2016). In terms of enduring environmental change, copiotrophs have the clear advantage of prevalent genomic machinery enabling better sensing and integration of environmental stimuli (Lauro *et al.* 2009; Dash *et al.* 2013) and are likely more resilient to change compared to oligotrophs. The long-term success of any taxonomic group of microbes will eventually come down to the physical and chemical conditions that the environment dictates.

Microbial life in the Anthropocene

The Anthropocene is a term often used to describe the present geological time period that is heavily influenced by human activity, often defined by the overarching dominance and outcompeting nature that humans have over natural Earth processes (Crutzen 2006; Ruddiman 2013). The start of the Anthropocene coincides with global industrialization and population growth in the mid-20th century (Zalasiewicz *et al.* 2017). Atomic bomb tests in the early 1950s spread radionuclides worldwide, and these signatures in the rock record provide a more precise reference for the start of the Anthropocene time period (Zalasiewicz *et al.* 2017). Humans have created many

avenues of change to the abiotic world, ranging from the production of chemical compounds not produced by nature to deforestation and excessive burning of fossil fuels (Crutzen 2006). These unnatural chemicals and environmental conditions are all novel factors that had not previously been encountered by ancestral microbial taxa. As such, it is important to understand the mechanisms of modern ecological niche development for microbes under these novel conditions in order to predict the health and stability of future global ecosystems. The impact that fossil fuel has had on atmospheric CO₂ levels is arguably one of the most prominent features of the Anthropocene and is widely accepted in the scientific community as the main culprit behind global warming and its many associated environmental changes (Steffen *et al.* 2011; Ruddiman 2013; Lewis & Maslin 2015). The exhaustive list of factors associated with global warming and climate change is continually being expanded, and investigating the effects of these indirect factors on microbial populations will be equally as important for protecting ecosystem function.

Microorganisms are most importantly major drivers of global biogeochemical cycles including the carbon cycle and have been impacted by the Anthropocene in many ways (Falkowski 2008). The farming and agriculture industry has led to high concentrations of bacteria associated with crop cultivation and livestock waste, producing unusually high concentrations of methane emissions leading to imbalances in the atmospheric carbon cycle (Gillings & Paulsen 2014, Pepper *et al.* 2015). High concentrations of nitrogen-based fertilizers are transported via run-off into surrounding aquatic systems, causing unusually high bacterial production and sometimes anoxic

conditions leading to major shifts in the microbial community composition towards certain taxa (Le *et al.* 2016; Beattie *et al.* 2020). Human activities have also facilitated unnatural dispersion of microbial taxa including pathogenic species via farming and agriculture as well as human travel through ships' cargo and ballast water (Joachimsthal *et al.* 2003; Dobbs & Rogerson 2005; Gillings & Paulsen 2014). Anthropogenic changes have largely caused microbes to either conform to new conditions, migrate to more favorable locations, or succumb to reduced taxonomic fitness and potential localized extinction (Webster *et al.* 2018).

Environmental changes stemming from anthropogenic activities are highly unique from natural environmental forces. For example, humans have created synthetic materials such as metals, plastics, and fabrics that become novel, unnatural substrates for microorganisms to colonize. Humans also create higher extremes in fluctuations of abiotic conditions (e.g., high nutrient concentrations from agricultural run-off) than those that would occur naturally. Microbial response to environmental disturbances during the Anthropocene must be investigated across different spatial and temporal scales in order to better understand how these unnatural conditions affect ecosystem processes. In particular, environmental disturbances within small spatial scales may only affect nutrient cycling and ecosystem function within a small geographic area compared to disturbances across larger spatial scales (O'Brien *et al.* 2016; Langenheder & Lindström 2019). Temporal scales on the other hand are important for understanding the length of time that ecosystem processes are affected. It is crucial to improve our understanding of

how microbes respond and adapt to these changes across different spatial and temporal scales in order to better predict future global ecosystem function.

When considering how to predict changes to microbial communities during the Anthropocene, much less is known about archaea compared to bacteria. It is well known that bacteria play important roles in biogeochemical cycles and the prevalence of human disease (Graevenitz 1977; Cho & Azam 1988). On the other hand, the phylogenetic diversity, global ubiquity, and functional roles of archaea are just starting to be uncovered through genomic sequencing surveys (Baker *et al.* 2020). Interesting to note however, is that some preliminary evidence has shown potential higher resiliency to environmental change in archaea compared to bacteria (Nguyen & Landfald 2015), although much more evidence is needed to confirm. If the trend holds true across multiple studies and environment types, then it will be important to consider the implications this may have on ecosystem function in current and future Anthropocene conditions.

One of the often overlooked effects of climate change is the positive correlation between warmer global temperatures and higher intensity and more frequent extreme storm events (Emanuel 2005; Groisman *et al.* 2005; Knutson *et al.* 2010; Kunkel *et al.* 2010; Holland & Bruyère 2014). Interestingly, not much is known about the effect that higher intensity and more frequent extreme storm events may have on microbial populations and ecosystem function. Many side effects accompany extreme storm events in coastal environments such as inland flooding potentially leading to increased

agriculture and human waste run-off into marine systems. Studies have shown microbial community disruptions from run-off without extreme storms, so it stands to reason that the exacerbated effects from extreme storms will have equally if not more significant consequences to microbial community composition and functional potential.

Furthermore, extreme storm events represent disturbances to microbial communities across large spatial scales (i.e., the entire path that a hurricane follows) and short temporal scales (i.e., the length of time during which extreme precipitation and wind occur). Investigations into these extreme storm events unique to the Anthropocene could potentially reveal important clues about microbial adaptations to this unique geologic time period.

In contrast to conditions observed within the Anthropocene, global oceans in early Earth geologic time periods such as the Paleoproterozoic (2.4 billion years ago) were characterized by anoxia or near anoxic conditions and high soluble iron concentrations (Rouxel *et al.* 2005; Sessions *et al.* 2009; Gumsley *et al.* 2017). Meanwhile, the modern world exhibits highly limited iron concentrations in marine systems even though it is often an essential micronutrient (Archer & Johnson 2000; Moore *et al.* 2001). Humans have alleviated this limitation inadvertently to some extent, as iron sources can be supplied to microorganisms via ship hulls, bridges, piers, and other steel-based infrastructures. It is important to remember that these types of infrastructures are not naturally occurring and could greatly impact microbial function within an ecosystem. Microbial taxa have nevertheless adapted to these man-made structures, utilizing them as sources of iron as well as general surface environments for

biofilm communities (Beech & Sunner 2004; Kip & Van Veen 2015; Emerson 2019).

Steel surface introductions into the environment can also be viewed as Anthropocene disturbances with small spatial scales (i.e., confined to the geographic area pertaining to the man-made infrastructure) but across long temporal scales (i.e., often residing within the environment for decades to centuries).

The goal of this dissertation is to investigate the microbial response to environmental disturbances during the Anthropocene across different spatial and temporal scales. The overall research questions include: 1) what does the colonization potential of corrosion-causing microbes on novel steel substrates tell us about how certain microbes have adapted to man-made sources of iron? 2) What role do non-corrosion-causing microbes have within these steel biofilm communities and how does that impact biofilm function? 3) How do extreme storm events affect coastal microbial communities and what consequence does that have for biogeochemical processes across the ecosystem? These results will ultimately depict unique microbial responses to environmental disturbances from novel substrate introductions and extreme weather events, both of which occur across different spatial and temporal scales. This will help reveal the scales under which Anthropocene disturbances make the most ecological impact with the greatest consequences for ecosystem stability. Investigating these different scenarios and scales will further discourage narrow-sighted views of only small aspects of the Anthropocene, but rather provide a wholistic view of the many challenges that microbes may face during this geologic time period.

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II. ENVIRONMENTAL AND GENOMIC SURVEY OF CORROSION-CAUSING MICROORGANISM COLONIZATION OF STAINLESS STEEL IN AQUATIC ENVIRONMENTS

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Abstract:

Iron-oxidizing bacteria (FeOB) are one of the initial colonizing organisms during microbially influenced corrosion of steel infrastructure. To better understand the abiotic conditions under which FeOB colonize steel, an environmental study was conducted to determine the effects of salinity, temperature, dissolved oxygen, and steel type on FeOB colonization. Stainless steel (304 and 316) was used to determine the potential susceptibility of these specialized corrosion resistant steels. Steel coupon deployments along salinity gradients of two river systems revealed attachment by FeOB at all sites,

with higher abundance of FeOB at higher salinities and on 316 stainless steel compared to 304 stainless steel. This may be due to the presence of molybdenum in 316 stainless steel potentially providing a selective advantage for FeOB colonization. A novel species of *Zetaproteobacteria*, *Mariprofundus erugo*, was isolated from these stainless steel samples. Genes encoding molybdenum utilization and uptake and reactive oxygen species protection were found within its genome, further supporting the evidence from our FeOB abundance data, and may be adaptations of FeOB to colonize surfaces of anthropogenic iron sources such as stainless steel. These results reveal environmental conditions under which FeOB colonize steel surfaces most abundantly, and provide the framework needed to develop biocorrosion prevention strategies for stainless steel infrastructure in coastal estuarine areas.

Importance:

Colonization of iron-oxidizing bacteria (FeOB) on corrosion resistant stainless steel types (304 and 316) has been quantified from environmental deployments along salinity gradients in estuarine environments. Higher FeOB abundance at higher salinities and on the more corrosion resistant 316 stainless steel suggests there may be a higher risk of biocorrosion at higher salinities, and there may be a selective advantage from certain stainless steel alloy metals such as molybdenum towards FeOB colonization. A novel species of FeOB described herein was isolated from our stainless steel coupon deployments, and its genome sequence supports our environmental data, as genes involved in the potential selectiveness towards surface colonization of stainless steel may lead to higher rates of biocorrosion of man-made aquatic

infrastructure. These combined results provide environmental constraints for FeOB colonization on anthropogenic iron sources and build upon previous frameworks towards biocorrosion prevention strategies.

Introduction:

Neutrophilic iron-oxidizing bacteria (FeOB) play important roles in Earth's natural iron cycle and in the corrosion of man-made iron structures (Emerson *et al.* 2010). Corrosion by FeOB has been well documented for freshwater environments often characterized by clogged and degrading pipelines in residential and industrial systems (Emerson *et al.* 2010; Rao *et al.* 2000), and more recently documented on mild steel in estuarine and marine environments (McBeth *et al.* 2011; Dang *et al.* 2011; Mumford *et al.* 2016). Total damages due to corrosion in the United States have been estimated at \$276 billion, with 50% reported to be microbiologically influenced (Little & Lee 2007). Sulfate-reducing bacteria are known to play a major role in biocorrosion, as studies have shown positive correlations with sulfate-reducing bacteria abundance and corrosion severity (Marty *et al.* 2014; Enning *et al.* 2012). Recent evidence has shown that FeOB may be the initial colonizers in the succession of microbial biocorrosion communities (McBeth & Emerson 2016), and thus play equally important roles in biocorrosion. The steel industry has responded to corrosion with more consistent use of corrosion resistant steels, such as the commonly used 304 stainless steel, or the more specialized 316 stainless steel which is better rated for resistance to corrosion in salty, marine environments (Little & Lee 2007).

During corrosion of ferrous metals, oxidation proceeds via oxygen as an electron acceptor and iron (II) as an electron donor. Stainless steel (SS) is designed to combat abiotic oxidation of iron within the steel via the inclusion of alloying metals such as chromium, nickel, and molybdenum that react with oxygen to form a surface oxide film (Hamilton 2003). The formation of an oxide film by the alloying metals creates a barrier that limits accessibility to the electron donating iron and thus greatly reduces the potential for electron transfer and subsequent oxidation and corrosion. This design, however, does not address the impacts that microorganisms have in altering surface film characteristics via colonization as some microorganisms are known to be resistant to metals such as chromium (Cervantes *et al.* 2001), which is present within the stainless steel alloy. Most FeOB are microaerophilic and thrive in low oxygen environments where they have a kinetic advantage over abiotic oxidation of iron (Emerson *et al.* 2010). If FeOB are able to disrupt the oxide film on stainless steel, they should be able to form their typical colonization biofilm that is depleted in oxygen (Mumford *et al.* 2016; Emerson 2019) and subsequently utilize the underlying iron as a source of electrons without competition from abiotic oxidation. In doing so, the formation of this new microbial film by colonizing FeOB cells could allow other fouling material and microorganisms such as sulfate-reducing bacteria to colonize and contribute to this microbial corrosion community (Emerson 2019). This would likely result in a positive feedback loop in which the integrity of the steel surface is compromised by the formation of the biofilm, as a result creating more biologically available iron to fuel the corrosion community. Such a scenario would further support the role of FeOB as primary colonizers on steel surfaces. This would be consistent with previous work by

McBeth and Emerson which showed FeOB as primary colonizers in microbial attachment succession on mild steel surfaces (McBeth & Emerson 2016). Investigating the extent to which FeOB attach and colonize stainless steel will help determine the overall role and importance of FeOB on biocorrosion of steel alloys.

The two most common types of stainless steel, 304 and 316, are rated differently based on their environmental application. Stainless steel 304 is composed of 18% chromium, 8% nickel, 2% manganese, <1% carbon, phosphorous, sulfur, silicon, and nitrogen, with the remaining composition balanced by iron. The composition of stainless steel 316 differs with 16.6% chromium, 10% nickel, 2% molybdenum, 2% manganese, <1% carbon, phosphorous, sulfur, silicon, and nitrogen, and balanced by iron (Metal Samples Company 2017). Two big differences to note between the stainless steel types include the addition of molybdenum in 316SS, and the lower overall percentage of iron in 316SS. Molybdenum is considered more resistant to corrosive environments and therefore makes 316SS better rated for salty marine environments (Little & Lee 2007). It is commonly known that rust occurs more rapidly in saltwater, which is in part due to the role of sulfate-reducing bacteria as described above, but also largely due to the higher conductivity of saltwater and increased concentration of chloride ions. These ions provide a plentiful supply of electrons that enable more redox reactions and hence more iron oxidation. These estuarine environments can have potentially high salinities, and thus are more vulnerable to chemical iron oxidation. For this reason, estuarine infrastructure is often built using stainless steel in hopes to resist corrosion, but the

biological effect on stainless steel corrosion is often ignored due to a lack of research and understanding.

Previous studies have investigated the role of FeOB and sulfate-reducing bacteria on corrosion of 316SS within industrial freshwater systems (Xu *et al.* 2007). Sulfate-reducing bacteria exhibited a higher corrosion rate than FeOB, yet a combination of FeOB and sulfate-reducing bacteria yielded an even higher corrosion rate than sulfate-reducing bacteria alone. Stainless steel in the marine environment has been shown to be colonized by phylogenetically diverse groups of microorganisms (Vandecastelaere *et al.* 2010), with evidence that other types of bacteria, such as manganese-oxidizing bacteria, also affect the occurrence of biocorrosion (Baker *et al.* 2003). These studies demonstrate that microbial community interactions on stainless steel are important, and further investigation of FeOB colonization as it varies in the environment will help determine the conditions under which stainless steel is most vulnerable to biocorrosion.

Salinity also plays an important role in the distribution of microbial species and community structures. Previous studies have described dispersal of FeOB lineages along an estuarine river in Maine, with salinity as the determining factor for FeOB community composition according to 16S rRNA tagged pyrosequencing results (McBeth *et al.* 2013). Marine FeOB (*Zetaproteobacteria*) were limited to salinities greater than 5 ppt, whereas freshwater FeOB (*Betaproteobacteria*) were mostly limited to less than 5 ppt. This study builds upon those results by presenting a long-term environmental study

using generalized FeOB enrichment cultures (Emerson & Floyd 2005) to describe variation in relative abundance and colonization of FeOB in the environment across steel types, salinity gradients, and fluctuations in temperature and dissolved oxygen.

The goal of this study was to assess the influence of abiotic factors on FeOB colonization and potential biocorrosion in estuarine environments. The differences in composition between two stainless steel types, 304SS (18% chromium, 8% nickel) and 316SS (16.6% chromium, 10% nickel, 2% molybdenum), were hypothesized to cause differences in FeOB colonization due to differences in oxide film characteristics. The 304SS was hypothesized to have higher FeOB colonization due to its higher percentage of iron available for FeOB utilization, and its lower rating for corrosion resistance in salty environments. The influence of salinity on FeOB colonization was unclear prior to this study because FeOB lineages and their impact on biocorrosion span across both freshwater and marine environments (Emerson *et al.* 2010; Rao *et al.* 2000; McBeth *et al.* 2011). Due to a wider salinity tolerance range (Chiu *et al.* 2017), marine FeOB were expected to colonize at a higher number of sites. Both psychrophilic and thermophilic lineages of FeOB have been previously reported (Edwards *et al.* 2003; Brierly 1978), indicating a wide range of potential colonization temperatures. Growth rates of other closely related neutrophilic FeOB are highest at 20 - 30 °C (Chiu *et al.* 2017), and so higher colonization was expected in this range of *in situ* temperatures. Dissolved oxygen concentrations were expected to be lower at sites with higher FeOB abundance as they are generally thought to be microaerophilic organisms. These results will not only shed light on the environmental growth constraints of FeOB, but it will also provide

a framework for which industrial and residential property owners can use to minimize biocorrosion of structural property.

Materials and Methods:

Field Deployments:

Sampling sites were chosen at regularly spaced intervals along two estuarine rivers in order to maximize diversity of environmental treatment factors (Table II.1, Fig. II.1). Stainless steel (SS) coupons (1/2" x 3" x 1/16") (Metal Samples Company 2017) were secured via cable ties inside modified 50 mL conical centrifuge tubes. The bottom conical part of the tube was cut off, and the cap left off, leaving an open-ended cylinder with one coupon inside for water to freely flow through while the coupons were protected from attachment of larger organisms and debris. One coupon tube of each SS type (304 and 316) was secured vertically to a small plastic crate and staked into the ground at five sites along the Neuse River and six sites along the Pamlico River. Coupons were kept above the sediment layer, and within the water column. There were continual coupon deployments and retrievals every 6 - 8 weeks for 1.5 years in order to ensure adequate time for colonization of FeOB and full seasonal cycle variability, but not long enough for succession of other microorganisms to take over the community (McBeth & Emerson 2016). Salinity, water temperature, and bulk water dissolved oxygen concentrations at each site were recorded upon retrieval of the coupon using an electronic YSI model 30 (Xylem Inc.) for temperature and salinity and YSI model ProODO (Xylem Inc.) for dissolved oxygen concentration. One long-term salinity sensor (Onset® HOBO®) was deployed at one site (Cedar Island) to determine degree of

salinity fluctuation during coupon deployment (Fig. II.S1). Salinity fluctuated throughout the deployment period, but the larger fluctuations stabilized quickly, and the majority of the deployment time remained near the salinity average. While still submerged, the plastic crate was gently turned on its side and cable ties securing the centrifuge tubes with coupons were cut in preparation for sample collection in an effort not to disturb the biological material attached to the coupons. Any coupons that were buried in sediment due to heavy storms or exposed due to low water level were not used for analysis. Coupons were collected with sterilized forceps and placed in a new centrifuge tube containing 50 mL *in situ* water from the site of collection and placed on ice for transport back to the lab for processing.

Table II.1: Sampling site descriptions including retrieval salinity, water temperature, and dissolved oxygen measurements. See Figure II.1 for specific site locations.

Site	Latitude	Longitude	River System	Salinity (ppt) [Range] (Average)	Temp (°C) [Range] (Average)	D.O. (mg/L) [Range] (Average)
1. Estuarium (Washington, NC)	35.5399	-77.0537	Pamlico	[0 - 0.5] (0.2)	[6.6 - 28.8] (18.1)	[3.9 - 10.1] (6.5)
2. Mallard Creek (Washington, NC)	35.4754	-76.9261	Pamlico	[2.6 - 11] (5.9)	[4.6 - 31.2] (17.2)	[3.5 - 11] (6.7)
3. Goose Creek (Washington, NC)	35.4668	-76.9249	Pamlico	[1.2 - 8.0] (5.1)	[5.9 - 32.7] (22.1)	[4.2 - 12] (7.6)
4. North Creek (Bath, NC)	35.4286	-76.7088	Pamlico	[7.3 - 16.6] (10.8)	[8.4 - 32.6] (20.5)	[3.1 - 10.8] (6.9)
5. Wright's Creek (Belhaven, NC)	35.4115	-76.5891	Pamlico	[9.1 - 17.9] (11.4)	[8 - 32] (20.6)	[3 - 11.6] (7.7)

6. Swan Quarter (Swan Quarter, NC)	35.3765	-76.4845	Pamlico	[10.5 - 19] (15.3)	[10 - 24.5] (19.1)	[5.4 - 8.6] (6.9)
7. Fisher's Landing (New Bern, NC)	34.9988	-76.9808	Neuse	[1.8 - 5.4] (3.5)	[15.7 - 31.8] (24.9)	[6.5 - 13.5] (10.4)
8. Cahooque Creek (Havelock, NC)	34.9176	-76.8527	Neuse	[3.6 - 9.6] (5.9)	[8.6 - 33.8] (23.6)	[4.3 - 12] (8.4)
9. Pin Oak Ct (Merrimon, NC)	34.9313	-76.8023	Neuse	[12.4 - 19.2] (15.9)	[23.7 - 32.4] (26.9)	[5.5 - 8.4] (7.2)
10. Matthew's Point (Havelock, NC)	34.9096	-76.7624	Neuse	[7.9 - 15.4] (12.5)	[8.7 - 33.4] (21.6)	[4.4 - 13.2] (8.9)
11. Cedar Island (Cedar Island, NC)	35.0049	-76.3054	Neuse	[14 - 21.2] (17.3)	[10.9 - 30] (22.4)	[2.8 - 10.2] (6.4)

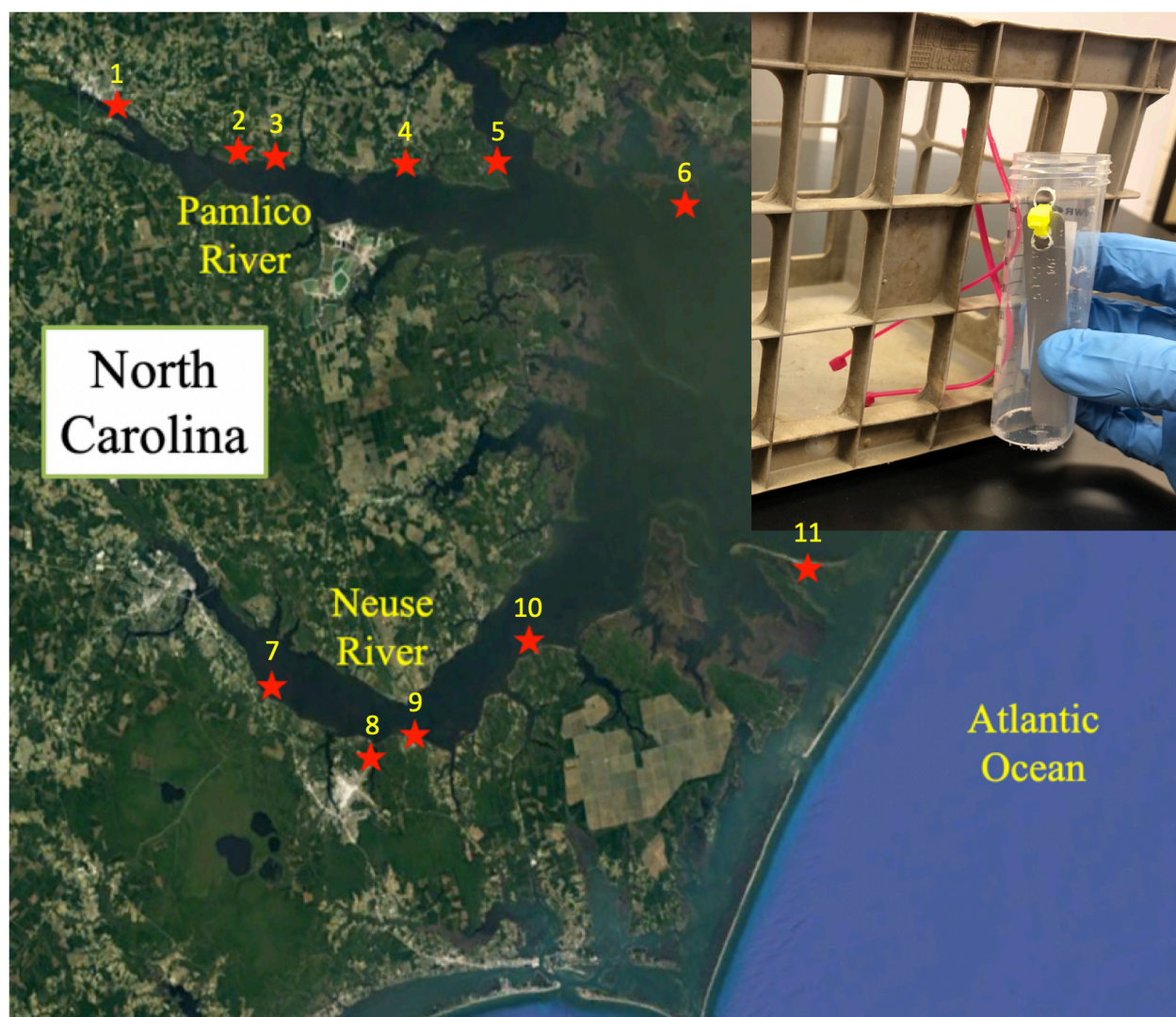


Fig. II.1: Map of coupon deployment sites. Red stars represent coupon deployment sites on Neuse River and Pamlico River, North Carolina (Numbered 1-11; see Table II.1). Coupon attachment setup shown in upper right corner.

Sample Processing:

Using enrichment media that is reported to grow nearly all types of neutrophilic, microaerophilic FeOB allowed for a cost effective and minimally biased method of processing hundreds of samples from each stainless steel type and from different salinities. After retrieval, samples from seven out of the eleven sites were randomly

selected for FeOB enrichment culturing and abundance estimates in order to account for sample loss (physical loss or burial/exposure), incubator space, and equipment limitations in the lab. Standardized FeOB enrichment media (Emerson & Floyd 2005) was prepared and autoclaved. Vitamins and trace minerals (ATCC® MD-VS, MD-TMS) were added after autoclaving according to the media recipes. For sites less than 5 ppt at time of collection, freshwater media (~0.4 ppt) was used for enrichment cultures, and for sites greater than 5 ppt, estuary media (~18 ppt) (Field *et al.* 2016) was used. This threshold of 5 ppt was deemed appropriate due to reports showing freshwater FeOB tolerance up to 5 ppt, and marine FeOB tolerance down to 5 ppt (McBeth *et al.* 2013; Chiu *et al.* 2017).

A most probable number (MPN) method utilizing a series of replicates and dilutions was used to estimate relative abundance of FeOB colonization on stainless steel. The most vulnerable salinities and stainless steel types to FeOB attachment and subsequent biocorrosion were determined by recording differences in relative abundance of FeOB for each sample without the bias of measuring only certain species or classes of FeOB (i.e. *Zetaproteobacteria*). Stainless steel microbial communities are less dense and thus less ideal for 16S rRNA gene sequencing to identify FeOB, which are already considered to be low-abundance rare species (Emerson *et al.* 2010). Similarly, quantifying colonization using other molecular methods such as qPCR, which requires selecting for a specific lineage of bacteria prior to analysis, is also troublesome when many different possible lineages of FeOB may be present on any given sample and at potentially very low abundances. MPN methods used in this study thus allowed

us to quantify FeOB colonization with the least amount of potential bias. Each coupon was aseptically scraped, vortexed, and 1.5 mL of the 50 mL of sample inoculated into petri dishes containing 13.5 mL of media for a total plate volume of 15 mL. Each sample was plated to 3 replicates, with 4 dilutions of each replicate (12 plates total for each steel type sample, 24 plates total for each site retrieval) for MPN estimates. For each dilution transfer, the previous plate was mixed via a combination of circular and zig zag motions to ensure random mixing, followed by pipetting in a random fluid motion as to not pipette entirely from the same spot on the plate. Five mg of zero-valent iron powder (-200 mesh; <74 μm , Alfa Aesar by Thermo Fisher Scientific) was then added to each plate as a source of iron for any FeOB that may be present in the sample.

After inoculation, MPN enrichment cultures were stored in sealed containers containing BD GasPak EZ Campy Container Systems to obtain microaerobic conditions (~1% O_2 headspace). Containers were incubated at 20 °C for 21 days to ensure growth by any FeOB cells that may be present (Emerson & Floyd 2005). An incubation temperature of 20 °C was chosen as the treatment for all sample cultures in order to avoid slow growth rates that may affect the results after 21 days, and to align with previously reported optimum growth temperatures (Chiu *et al.* 2017). Positive growth for each plate was determined first visually, then if necessary, confirmed with both light microscopy for distinctive biological iron oxide morphologies and fluorescence microscopy for distinctive bean-shaped cells attached to the iron oxides, and additional confirmation via subsequent enrichment plate transfer. Positive growth by FeOB appears as very distinctive suspended flocculant orange clumps, which indicate iron

oxides of biological origin, and can be described as intertwined stalks, tubular sheaths, short dreads, or even amorphous (Chan *et al.* 2009; Chan *et al.* 2011; Kato *et al.* 2015; Mori *et al.* 2017).

Data Analysis:

An MPN calculator (Curiale 2012) was used to estimate abundance based on which of the 12 plates for each sample exhibited growth after the incubation period. Raw MPN abundance data was visualized using ggplot (Wickham & Wickham 2007) in R (R Core Team 2013) and then using a general linear model with salinity as a predictor variable and steel type as a fixed effect used to predict FeOB relative abundance (Marschner *et al.* 2018). Residuals of the raw count data were not normally distributed along the predicted trend of the general linear model and were skewed across several orders of magnitude. A number of outlier data points additionally had high influence on the predicted linear trend, and therefore the data were logarithmically transformed in order to interpret statistical trends across salinity and steel type. After log transformation, residuals of the data exhibited a more appropriate normal distribution along the predicted trend without the strong influence of outlier data points, resulting in a better overall fit for general linear model analysis. Data analysis R code can be found at https://github.com/cgarr017/ECU_dissertation_code/blob/main/chapter1.

Species Isolation and Sequencing:

Serial dilution isolation methods were used to isolate FeOB species from Mallard Creek (Feb. 2017, 4 ppt, 316SS) and North Creek (Feb. 2017, 7.3 ppt, 316SS). The

16S rRNA gene was sequenced from each isolate to identify its lineage, followed by whole genome sequencing of the novel FeOB isolate, *M. erugo*, to investigate potential stainless steel alloy related genes. Several high FeOB abundance samples from each steel type, from high and low salinity sites, and from each river were chosen to attempt isolation of pure cultures of FeOB species present in each sample. Two rounds of dilutions-to-extinction led to the isolates described above. A total of 5 dilutions were used, from 10^{-1} to 10^{-5} , in 10-fold dilution increments. Individual orange clumps of biological iron oxides were selectively pipetted and transferred to 1.5 mL centrifuge tubes containing 1 mL of the same enrichment media used in the MPN plates. All centrifuge dilution tubes were then vortexed and 100 μ L was pipetted from each dilution into separate petri plates containing 15 mL of enrichment media and 5 mg of zero-valent iron mesh. Once growth was visible in the plates (typically after 3 - 5 days of incubation in microaerophilic containers as described above), material from the most diluted plate exhibiting growth was selectively transferred and the serial dilution was repeated. Pure cultures were confirmed using epifluorescence microscopy to identify the presence of a single cell morphology. Cell samples were dyed with 50nM SYTO® 13 green fluorescent nucleic acid stain (Invitrogen™) and viewed using an Eclipse Ni-E upright microscope (Nikon Instruments Inc.) under 1000x magnification with FITC excitation and emission spectrum (~500 nm peak wavelength). Microscope images were visualized using NIS-Elements BR 4.60.00 imaging software and Y-T TV microscope camera (Nikon Instruments Inc.). Pure cultures were additionally confirmed by inoculation onto nutrient agar (9 g/L nutrient broth (Remel™) and 15 g/L bacteriological agar (VWR International, LLC)) to confirm the absence of heterotrophic contaminant

microbes. Samples for scanning electron microscopy imaging were mounted on a 0.2- μ m-pore-size polycarbonate filter, air-dried, and coated with platinum. Samples were imaged on a Thermo Scientific™ Apreo scanning electron microscope at 4000x magnification, with an accelerating voltage of 2kV.

Individual isolated FeOB strains were extracted for DNA using a MoBio DNeasy PowerSoil kit (Qiagen, Inc.). The 16S rRNA gene was PCR amplified using universal 16S primers 8F and 1492R (Lane 1991; Turner *et al.* 1999) The PCR products were purified using QIAquick PCR purification kit (Qiagen, Inc.). The 16S rRNA gene was sequenced by GeneWiz® (South Plainfield, NJ) in order to identify the isolates. The sequence data were then aligned and cleaned using Sequencher® (Gene Codes Corporation, Ann Arbor, MI). The aligned 16S rRNA gene sequences were then imported into BLAST (Altschul *et al.* 1990) to identify the closest neighboring species. The aligned 16S rRNA gene sequences were also imported into the ZetaHunter program (McAllister *et al.* 2018) to identify which *Zetaproteobacteria* OTU the isolates were most similar to.

The genomes of both isolates were sequenced by CGEB-IMR© (Dalhousie University, Halifax, NS, Canada) using Illumina MiSeq. The paired-end reads were trimmed using Trimmomatic (Bolger *et al.* 2014) and quality filtered using FastQC (Andrews 2010). The reads were then assembled using SPAdes (Bankevich *et al.* 2012) and annotated using RASTtk (Aziz *et al.* 2008; Overbeek *et al.* 2013; Brettin *et al.* 2015). The draft genome sequence of P3 consists of 59 scaffolds. It has a genome size of 2,898,355 bp, GC content of 54.7%, and 2,831 protein coding sequences. The draft

genome sequence of P7 consists of 115 scaffolds, 2,986,037 bp, 54.7% GC content, and 3,019 protein coding sequences. Average nucleotide identities (ANI) were calculated using OrthoANI (Yoon *et al.* 2017), and average amino acid identities (AAI) were calculated using a web-based calculator (Newman Lab 2013). Maximum likelihood phylogenetic trees for 16S rRNA and RubisCO gene comparisons with other similar bacteria lineages were constructed using MEGAX (Kumar *et al.* 2018) using the Tamura-Nei nucleotide substitution model for 16S rRNA, and the Jones-Taylor-Thornton amino acid substitution model for RubisCO, each with 1000 bootstrap iterations.

Corrosion coupon material from February 2017 and May 2017 retrievals was extracted for DNA using a MoBio DNeasy PowerSoil kit. DNA samples were sent to CGEB-IMR© for 16S rRNA gene sequencing using improved internal transcribed spacer marker gene primers (Walters *et al.* 2015) on an Illumina MiSeq machine. Microbial community data were processed and analyzed using Mothur v1.41.3 (Schloss *et al.* 2009) and the associated curation pipeline (Kozich *et al.* 2013).

Data Availability:

The GenBank accession numbers for the 16S rRNA gene sequences of *Mariprofundus erugo* strain P3 and *Mariprofundus erugo* strain P7 are MK554583 and MK554584, respectively. The NCBI accession numbers for *M. erugo* P3 and *M. erugo* P7 whole-genome sequences are SAMN11634928 and SAMN11634956, respectively.

Results:

A total of 140 stainless steel coupon samples were collected from six sites on the Pamlico River and five sites on the Neuse River in North Carolina from February 2017 to July 2018 with a range of temperatures, salinities, and dissolved oxygen (DO) concentrations (Table II.1). Coupon deployments lasted for a minimum of 6 weeks, or a maximum of 8 weeks depending on weather conditions and personnel availability. Samples were retrieved from water temperatures as low as 4.6 °C and as high as 33.8 °C, while salinities ranged from 0 ppt at the most inland site, to 21.2 ppt at the site closest to the ocean. DO ranged from 2.84 - 13.5 mg/L. FeOB colonization occurred at all sites and salinities that were sampled, across a wide range of temperature and DO concentrations, and on both stainless steel types showing that their attachment to stainless steel in estuarine environments is widespread, though some salinities exhibited colonization more frequently and more abundantly than others (Fig. II.2; Fig. II.S2). Some variation in salinity at individual sites was observed, as it is a tidally influenced estuarine environment, though site-specific salinity was consistent for the majority of deployment time (Cedar Island March 2018 range: 5 - 22 ppt; average: 14.8 ppt) (Fig. II.S1). Salinity was a significant environmental factor as FeOB cell abundance increased with increasing salinity (Linear regression test: $df = 139$, $z = 4.29$, $p = 0.0001$). Dissolved oxygen concentrations did not significantly correlate with FeOB cell abundances (Linear regression test: $df = 139$, $z = -0.92$, $p = 0.36$). Temperature also did not significantly correlate with FeOB cell abundances (Linear regression test: $df = 139$, $z = 0.58$, $p = 0.58$) (Fig. II.S3); however, site specific seasonal trends in FeOB colonization reveal more FeOB colonization in Spring and Fall months (Fig. II.3)

suggesting it may be a contributing factor. The average FeOB cell abundance at lower salinities (<10 ppt) (306 cells/coupon (SD = 701)) was significantly lower than FeOB cell abundance at higher salinities (>10 ppt) (925 cells/coupon (SD = 1534)) (T-test: df = 74, $t = -2.87$, $p = 0.0054$). Chiu *et al.* (2017) reported salinity growth ranges for the estuarine FeOB *M. aestuarium* and *M. ferrinatatus* as 7 – 31.5 ppt with an optimum range of 14 – 17.5 ppt, therefore a threshold of above and below 10 ppt was deemed appropriate in this study for summarizing results. The highest FeOB abundance of 8,000 cells/coupon was recorded at a salinity of 19.7 ppt on 316SS. Abundance was significantly correlated with salinity whether deployment or retrieval salinity measurements were used in the model (data not shown). Continuous salinity data was collected for a select few deployments in order to identify short-term fluctuations (one deployment shown in Fig II.S1; similar trends for remaining deployment data). Continuous salinity data was limited to a minimal number of deployments due to a lack of equipment availability.

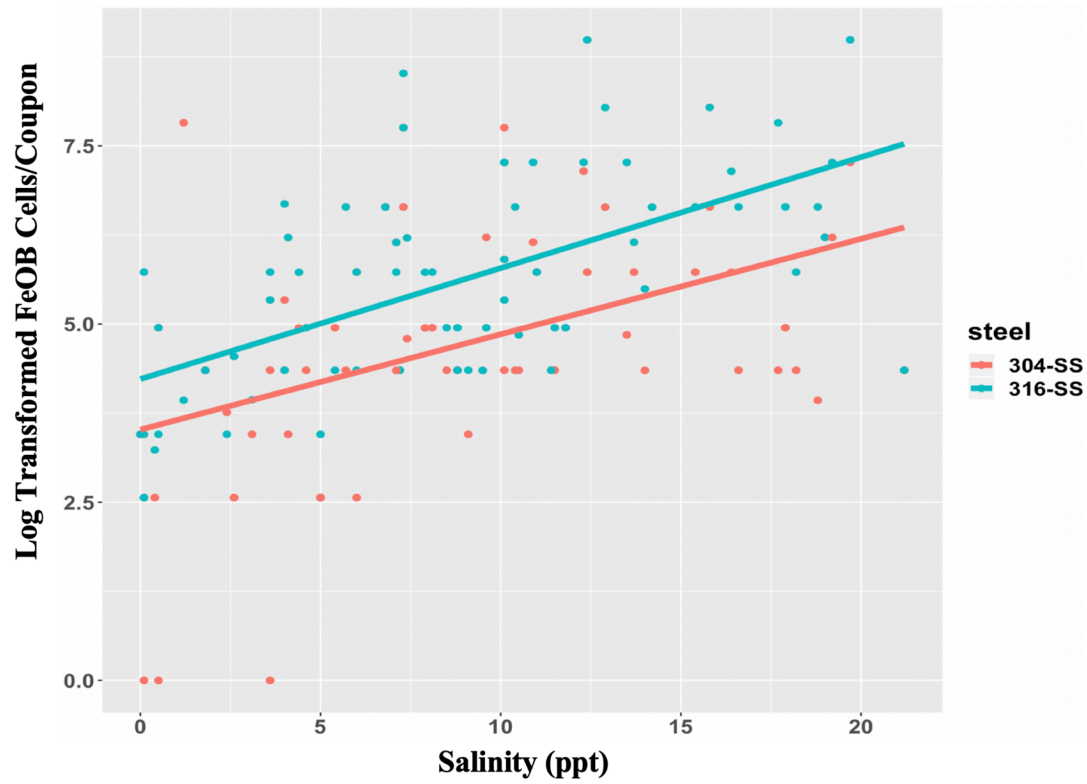


Fig. II.2: Log transformed general linear model shows higher iron-oxidizing bacteria abundance on 316 stainless steel compared to 304 stainless steel ($df=139$, $z=2.35$, $p=0.018$) and at higher salinities ($df=139$, $z=4.29$, $p=1.8e-5$).

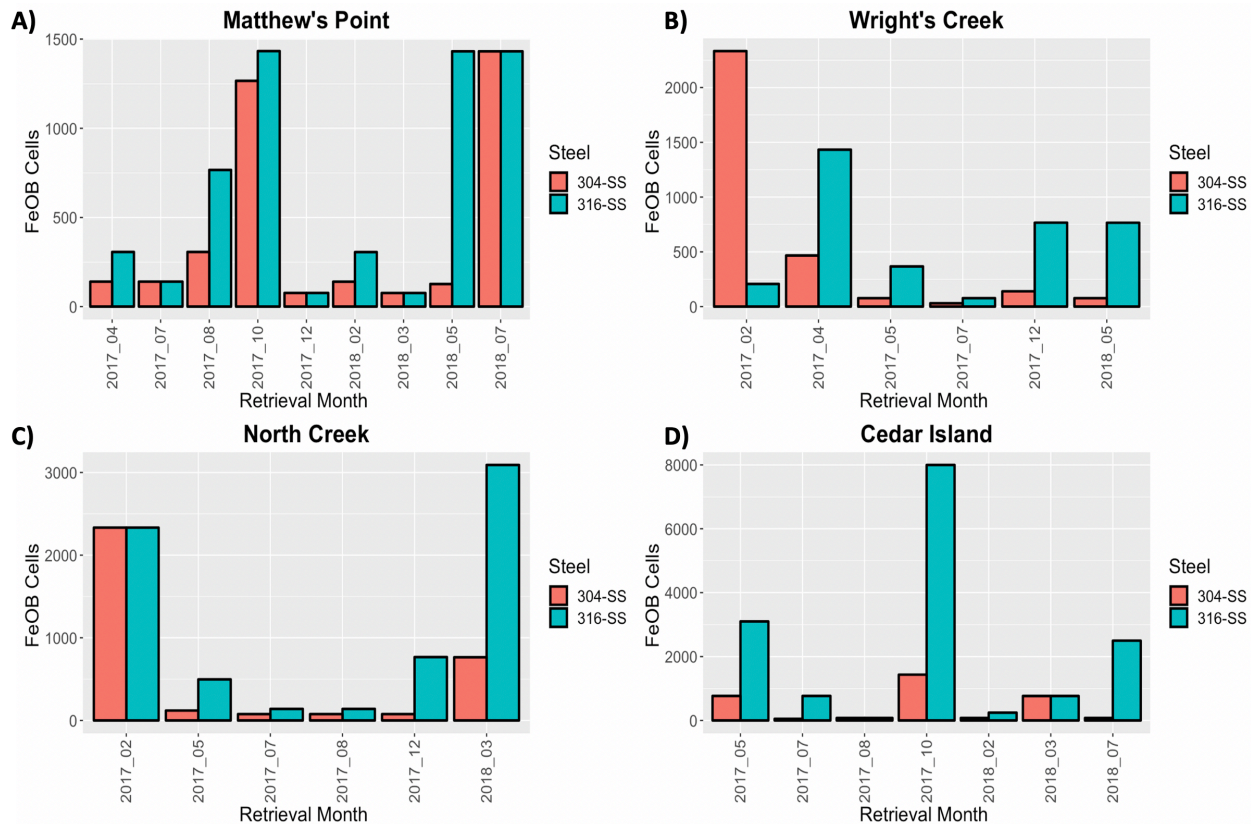


Fig. II.3: Iron-oxidizing bacteria abundance plots for several high salinity sites by retrieval month to show seasonal trends. Cell abundance is equal or higher on 316 stainless steel for all retrievals except for February 2017 at Wright's Creek. A) Matthew's Point exhibited highest cell abundance in May, July, August, and October. B) Wright's Creek exhibited highest cell abundance in February and April. C) North Creek exhibited highest cell abundance in February and March. D) Cedar Island exhibited highest cell abundance in May, July, and October.

FeOB cell abundance was significantly higher on 316SS and lower on 304SS (General linear model: $df = 139$, $z = 2.35$, $p = 0.018$; Fig. II.2) (T-test: $df = 86$, $t = -2.46$, $p = 0.016$). Dark orange rust coloration was visually evident on four different 316SS samples, but never on 304SS (Fig. II.S4). This coloration is indicative of a compromised surface oxide film and exposure of the underlying iron. The 304SS sample (Fig. II.S4) exhibited high algal and fish egg colonization, but no evidence of corrosion. A total of 64.3% of sample retrievals exhibited a higher abundance of FeOB cells on 316SS, 8.6%

of sample retrievals exhibited a higher FeOB cell abundance on 304SS, and 27.1% of sample retrievals exhibited FeOB cell abundances that were estimated to be equal on both stainless steel types (Fig. II.3). This trend for steel type preference appears to be even stronger at higher salinities and during temperate months. Multiple iron oxide morphologies (twisted stalks and tubular sheaths (Fig. II.S5)) were observed via epifluorescent microscopy in our enrichment cultures suggesting that multiple species of FeOB can colonize stainless steel. This supports the use of culture-based MPN methods as it can aid in cultivating multiple types of FeOB as quantitative PCR of specific FeOB taxa may miss some of these organisms.

A novel *Zetaproteobacteria* OTU (ZOTU (McAllister *et al.* 2018)) has been successfully cultivated and identified from positive MPN enrichment cultures. Two different strains were isolated from Mallard Creek (Feb. 2017, 4 ppt, 316SS) and North Creek (Feb. 2017, 7.3 ppt, 316SS). The two isolate strains represent a novel species of *Zetaproteobacteria*, referenced hereafter as *Mariprofundus erugo*, strain P3 (isolated from Mallard Creek) and strain P7 (isolated from North Creek). They are members of the same OTU, as they are 99.6% identical in 16S rRNA gene sequence, and they exhibit 98.96% average nucleotide identity (ANI) and 98.3% average amino acid identity (AAI) across the whole genome. While members of a new ZOTU, they are most similar to other isolates including *Mariprofundus ferrooxydans* PV-1 (Singer *et al.* 2011) (16S rRNA gene sequence: 96.5% identity PV-1 to P3, 95.8% identity PV-1 to P7; whole genome sequence: **ANI**: 74.46% PV-1 to P3, 74.41% PV-1 to P7; **AAI**: 73.68% PV-1 to P3, 73.4% PV-1 to P7), (Fig. II.4). The P3 and P7 genome are both 99% complete,

0.84% redundant, and 0% strain heterogeneity (Parks *et al.* 2015). The RubisCO genes utilized for carbon fixation pathways in the *M. erugo* draft genomes are Type II, indicating an affinity for low oxygen and/or higher CO₂ conditions. The RubisCO amino acid sequence is phylogenetically most similar to the RubisCO sequence found in a freshwater FeOB, *Ferriphaselus* sp. R-1 (Krepiski *et al.* 2012) (Fig. II.S6). It has been suggested that *cyc2* is primarily responsible for energy acquisition in marine FeOB via electron transport (Chiu *et al.* 2017; Barco *et al.* 2015; McAllister *et al.* 2018). A homolog to the *Cyc2* protein sequence from *M. ferrooxydans* PV-1 was found in the *M. erugo* draft genomes (75% AAI). Another FeOB isolate, *Mariprofundus* sp. DIS-1 (Mumford *et al.* 2016) was isolated from mild steel coupons deployed in seawater off the coast of Maine. *M. erugo* has a higher GC content (54.7%) compared to Strain DIS-1 (48.6%), and Strain DIS-1 has both Type I and Type II RubisCO genes, whereas the genome of *M. erugo* only shows Type II. Thus Strain DIS-1 may be better adapted for both high and low oxygen affinities, and also has genes to protect against reactive oxygen species such as catalase and catalase-peroxidase genes. Interestingly, *M. erugo* also contains coding sequences for protection against reactive oxygen species and oxidative stress, specifically Cytochrome c551 peroxidase and superoxidase dismutase. These proteins may provide an advantage in colonizing steel in oxygenated waters, though the presence of only Type II RubisCO genes suggests they still prefer low oxygen conditions while metabolically active on surface environments.

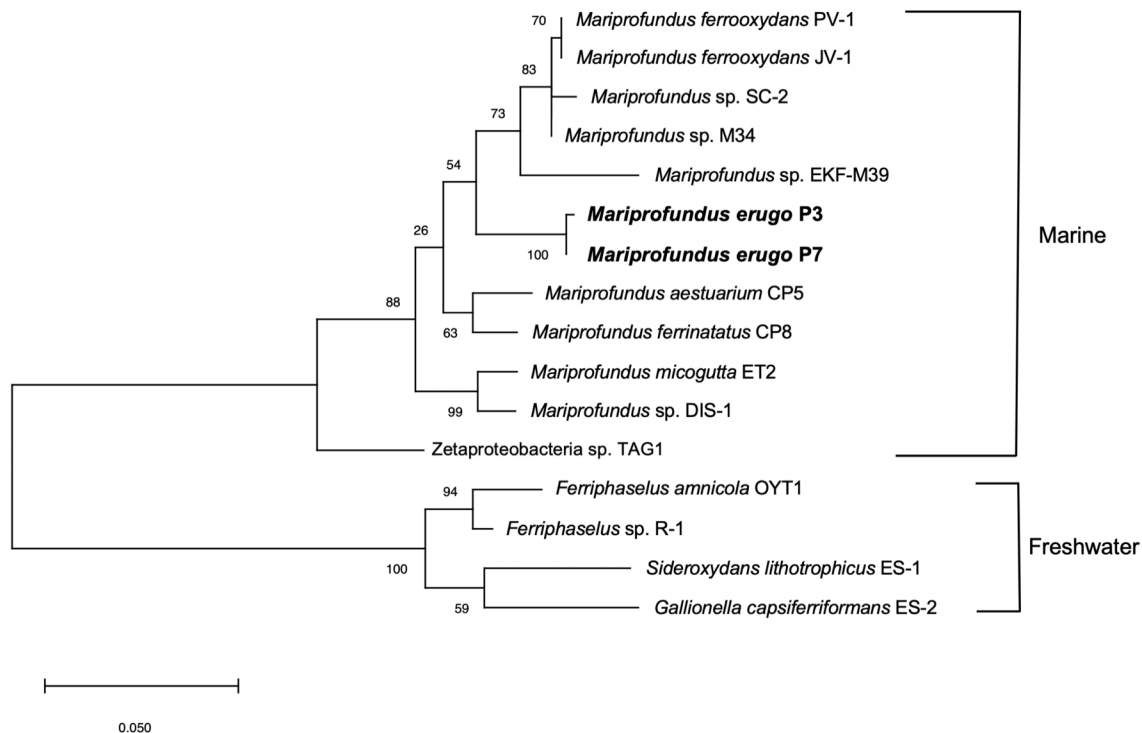


Fig. II.4: Maximum likelihood phylogenetic tree showing relatedness of 16S rRNA gene for *Mariprofundus erugo* P3 and P7 (bold) compared to other known iron-oxidizing bacteria. Scale bar represents amount of evolutionary change in DNA, or average number of nucleotide substitutions per site. Bootstrap values represent number of times out of 100 that the same branch was observed when reconstructing the tree.

Mariprofundus erugo strain P3 and P7 iron oxide morphologies were imaged with scanning electron microscopy (Fig. II.5). Both strains exhibit twisted stalk morphologies, characterized as helical spirals. Additional species of FeOB also colonized the stainless steel coupons in this study, as evident by the multiple iron oxide morphologies observed from enrichment culture microscopy (Fig. II.S5). FeOB from marine environments that exhibit sheath morphologies, like those found in the enrichment cultures, have been observed previously (Fleming *et al.* 2013; Barco *et al.* 2017), but not yet taxonomically identified, thus isolation and identification is an ongoing project.

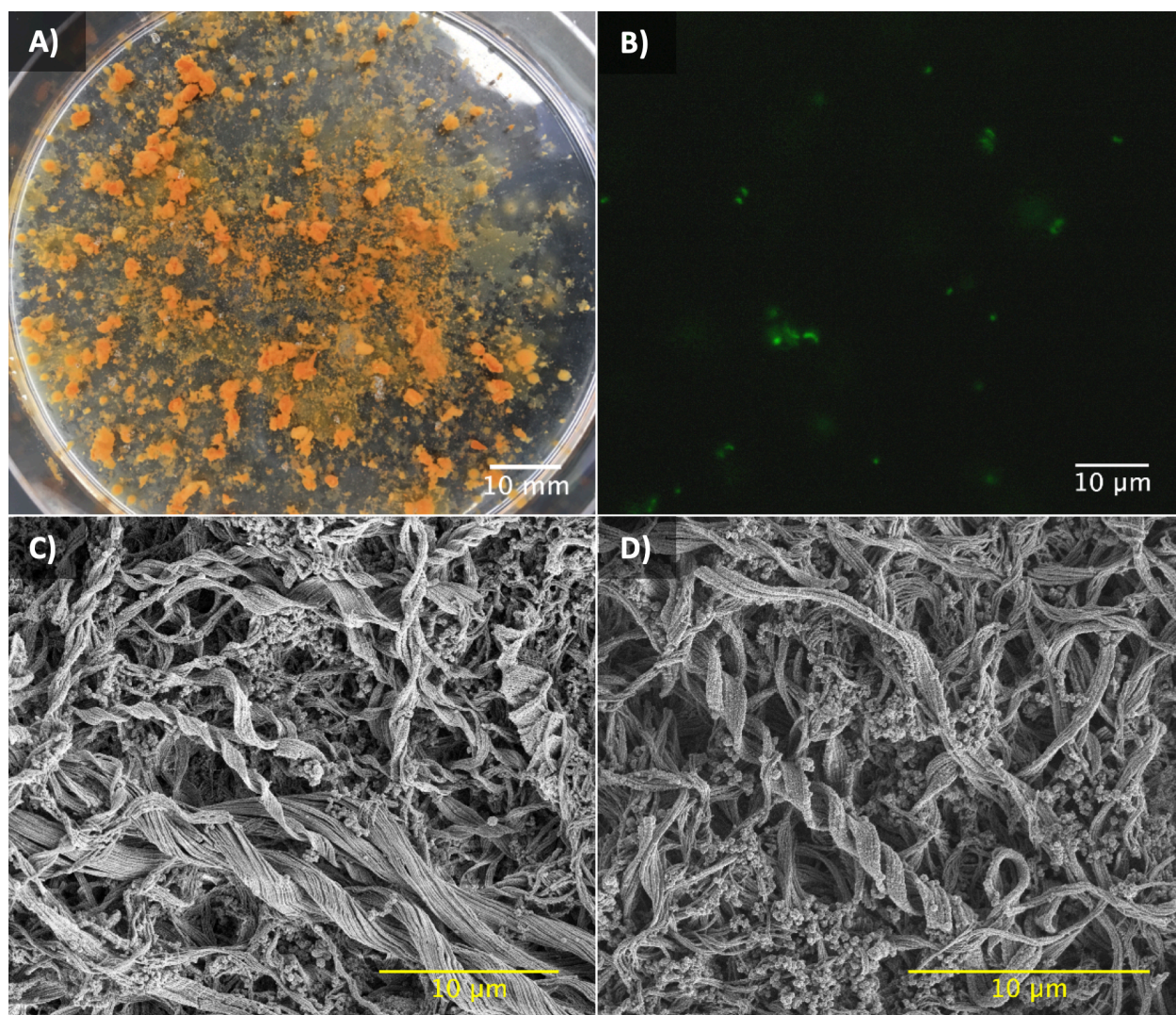


Fig. II.5: Representative images of *Mariprofundus ferrooxydans*. A) Pure culture of strain P7 forming orange flocculant clumps suspended in liquid Estuary media with zero-valent iron under microaerophilic conditions, B) Strain P7 cells under fluorescence after staining with SYTO13, C) Scanning electron microscopy image of strain P3, D) Scanning electron microscopy image of strain P7 representing diversity within the iron oxide morphologies produced by both strains of *M. ferrooxydans*.

Discussion:

The extensive long-term study presented here describes an increase in FeOB colonization and abundance on stainless steel at higher salinities and more so on

316SS than on 304SS. This study reports that FeOB colonizing stainless steel in the natural environment, thus showing that two commonly used stainless steel types exhibited FeOB colonization at all salinities (0 - 21.2 ppt), all temperatures (4.6 °C - 33.8 °C), and all DO concentrations (2.84 - 13.5 mg/L) that were sampled. Moreover, these results show that FeOB are not only capable of colonizing across a wide range of conditions, but also largely resilient to short-term fluctuations in environmental conditions. The variations in abundance of FeOB across salinity gradients reveal not only the differences in biocorrosion risks, but also suggest optimal environmental conditions for FeOB growth, which could lead to higher proportions of FeOB within the overall microbial community. McBeth *et al.* (2013) reported the presence or absence of FeOB lineages along a salinity gradient, and Xu *et al.* (2007) revealed the importance of FeOB during corrosion of stainless steel in freshwater industrial systems. This study builds upon that to quantify variation of FeOB colonization and potential biocorrosion at different salinities. The stainless steel microbial communities in this study were also analyzed via 16S rRNA gene sequencing, but FeOB lineages were rarely found within the sequence data, and always in low proportion (<0.1%). This supports their role as primary colonizers during early community succession and likely rare community members after initial colonization. Sulfate-reducing bacteria were found within the 16S rRNA gene sequence data, but also in low community proportions (1-2%) and with no significant difference in proportion between 304SS and 316SS (T-test: df = 24, t = -0.177, p = 0.86). Previous reports of FeOB cell succession during biocorrosion reveal complex community relationships. McBeth and Emerson (2016) found that *Zetaproteobacteria* comprised 12% of the microbial community on mild steel coupons

after 9 days of deployment in coastal seawater and only 0.9% after 22 days, whereas Barco *et al.* (2017) found no detection of *Zetaproteobacteria* after two weeks on mild steel in coastal seawater and 0-8% after one month. FeOB cell abundance data from this study present a reliable dataset that reinforces the fact that FeOB are recurring members of biocorrosion communities, and can further be used to describe FeOB preferences towards different conditions.

Our results showing higher abundances of FeOB at higher salinities for 140 total samples indicate that stainless steel may be more vulnerable to biocorrosion in higher salinities. This trend towards higher salinities further suggests that the marine class of FeOB, *Zetaproteobacteria*, may be more likely to colonize stainless steel than the freshwater class of FeOB, *Betaproteobacteria*. This is further supported by the 16S rRNA gene sequence data, within which no iron-oxidizing *Betaproteobacteria* taxa were found. This trend could also be influenced to some extent by the fact that marine systems are generally more iron limited than terrestrial freshwater systems. *Zetaproteobacteria* are known to tolerate salinities down to 3.5 ppt (Chiu *et al.* 2017), but they may have a higher optimal growth salinity. Results from this study show multiple iron oxide morphologies confirmed via microscopy which suggests that multiple species of FeOB beyond the *Zetaproteobacteria* class are capable of colonizing stainless steel. Occasional large fluctuations in salinity, such as at Cedar Island the highest salinity site, were likely due to heavy rainfall events in combination with tidal cycles. The attached microbial community would be affected by these fluctuations and their presence at the time of retrieval suggests that stainless steel colonizing FeOB

seem to withstand salinity variations within tidally influenced estuarine environments. The environmental variability likely contributes to the variability in the FeOB abundance data at higher salinities.

Temperature and dissolved oxygen were not statistically significant variables affecting trends in FeOB cell abundance, but these variables did seem to have a qualitative seasonal effect and possible correlation with salinity while still less important of a factor overall. Samples taken at temperatures less than 10 °C exhibited extremely low abundances, while moderate to high temperature samples (22 - 33 °C) had higher abundance (>3000 FeOB cells/coupon) and typically aligned with high salinity measurements. This suggests that FeOB may have optimal growth and colonization conditions in the Spring through Fall months in regions similar to North Carolina, but only when salinity is also higher and thus more favorable. Temperature did not seem to be a determining factor for FeOB growth at lower salinity sites (<10 ppt). During winter months, as with many other types of organisms, general metabolism of FeOB may be overall slower and result in lower abundance and lower risk for subsequent biocorrosion. It is possible that FeOB remain dormant in the sediment as “seeds” until environmental conditions are more favorable, which may be a common strategy even more so during winter months. Dissolved oxygen exhibited lower concentrations at sites with higher FeOB cell abundance, which supports the microaerophilic lifestyle of FeOB, but the bulk DO measurements likely do not accurately reflect the microenvironment formed by colonizing microorganisms on the surface of the stainless steel.

Stainless steel has proved to be more vulnerable to colonization by FeOB and potentially to subsequent biocorrosion than previously considered, and vulnerability is highest at salinities greater than 10 ppt. While 316SS may be more resistant to chemical corrosion, the results in this study suggest that 316SS may be a more favorable substrate for FeOB colonization, and thus more research should be done to determine if 316SS exhibits a higher rate of biocorrosion in marine environments. Note that some coupon deployments resulted in higher FeOB colonization on 304SS, with the highest relative abundance on 304SS recorded at Wright's Creek in February 2017. Many different environmental sources of variation (e.g., weather events and associated sediment disruption affecting surface access of one coupon more than the other) could have led to FeOB colonization contrasting the overall trend favoring 316SS, and the Wright's Creek anomaly is not believed to be site-specific. The direct effect of FeOB colonization towards biocorrosion was also not specifically addressed in this study but could be an important topic of future research. The preference of FeOB towards 316SS was surprising not only because 316SS is rated more corrosion resistant, but also because 316SS has less iron by weight overall that is available for FeOB to utilize. Stainless steel 316 is known in the steel industry as being more corrosion resistant in marine environments in part due to the added component of molybdenum, which hardens and strengthens the steel, thus decreasing the potential damage from the chloride ions in saltwater (Little & Lee 2007). However, Hayes *et al.* proposed that molybdenum in stainless steel may provide more of a stabilization effect for the oxide film whereas chromium provides more of the corrosion resistance effect (Hayes *et al.* 2006). From a biological perspective, it is possible that molybdenum may be a

metabolic benefit for FeOB and thus leads to higher colonization and abundance of FeOB cells creating a low oxygen microenvironment that promotes the colonization of other corrosive microorganisms such as sulfate-reducing bacteria. From a mechanistic perspective, it is possible that FeOB may be removing molybdenum from the stainless steel as a micronutrient for growth (Friedrich *et al.* 1986), thus disturbing the stabilization effect from molybdenum on the oxide film and exposing an inherently lower amount of chromium available for corrosion resistance. Ongoing studies will help to characterize the response of *M. erugo* to these individual stainless steel alloy metals such as molybdenum at various concentrations.

Molybdenum is often incorporated into enzymes for proper metabolic function in bacteria (Friedrich *et al.* 1986) and could have other undiscovered metabolic benefits for FeOB as well. While typically not considered a limited micronutrient in marine systems, molybdenum is extremely limited in freshwater systems (Glass *et al.* 2012). Thus molybdenum should theoretically be limited in estuarine environments where these two opposing systems meet. Some marine FeOB are known to have genes for molybdenum cofactor biosynthesis and for transport of molybdenum (Singer *et al.* 2011; Field *et al.* 2015), suggesting that molybdenum may be a necessary enzyme cofactor similar to other organisms. Interestingly, Strain P3's draft genome has three different protein-coding sequences for molybdenum transport (ModA, ModB, ModC) and one protein coding sequence for molybdenum binding (ModE) (Fig. II.S7), none of which are present in *M. ferrooxydans* PV-1. These sequences are found however in *Mariprofundus* sp. EKF-M39 (Field *et al.* 2015). These sequences were not found in the

P7 strain genome, but it may be due to genome incompleteness. These proteins could be utilized during colonization of stainless steels that contain molybdenum alloys. These four molybdenum-based proteins are all located on the same operon in the genome sequence, indicating that they are more likely to be conserved sequences than if they were distant from each other within the genome. This sequence cluster is also neighboring sequences for cobalt-zinc-cadmium resistance protein CzcA, efflux RND periplasmic transporter protein, AcrR transcriptional regulator protein, and two hypothetical proteins that are also found in multiple marine and freshwater FeOB. These molybdenum related proteins may allow the molybdenum alloy in 316SS to be a metabolic benefit to the FeOB cells, and thus favored over 304SS. As FeOB are believed to “prime” the surface of steel creating more favorable colonization conditions for other corrosive microorganisms such as sulfate-reducing bacteria (McBeth & Emerson 2016), further investigation of sulfate-reducing bacteria colonization on different stainless steel types would be useful. If FeOB are beneficial to corrosion communities and its members as suspected, then quantitative MPN experiments of sulfate-reducing bacteria on stainless steel could be hypothesized to follow similar correlation trends to those found in this study.

Out of thirteen relatively abundant *Zetaproteobacteria* OTUs that are globally distributed, only two have previously been linked to corrosion (Emerson 2019). With the discovery of a third corrosion associated OTU, *M. erugo* increases support for the theory that there may be selective pressure on these ancient organisms as a result of colonizing new anthropogenic iron sources. As of now, *M. erugo* is the only reported

FeOB isolated from stainless steel. Genomic evidence such as molybdenum related genes and protection against reactive oxygen species and oxidative stress suggest that it may be adapted specifically for oxygenated surface environments made out of advanced steel types, though its lifestyle may not be strictly limited to such. The high sequence similarity of strain P3 and P7's Cyc2 protein sequence to that of *M. ferrooxydans* PV-1 (75% AAI) suggests that the general iron oxidation mechanisms of *M. erugo* may be conserved among *Zetaproteobacteria*. The high sequence similarity of strain P3 and P7's RubisCO amino acid sequence to that of a freshwater FeOB suggest common evolutionary history and potential adaptation for estuarine environments. The given genomic evidence suggests either horizontal gene transfer from (or to) freshwater FeOB due to the close proximity of estuarine environments to freshwater species, or a transition of marine descendants followed by adaptation and niche expansion. The MPN results show higher FeOB cell abundance at higher salinities, but also a seemingly strong resilience to wide salinity fluctuations which means that FeOB are important organisms to consider when planning for biocorrosion prevention strategies in nearly all types of estuarine environments. All of these results combined can be further utilized to improve our understanding of the *Zetaproteobacteria* class of microorganisms, which is more diverse than previously identified.

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Supplemental Materials:

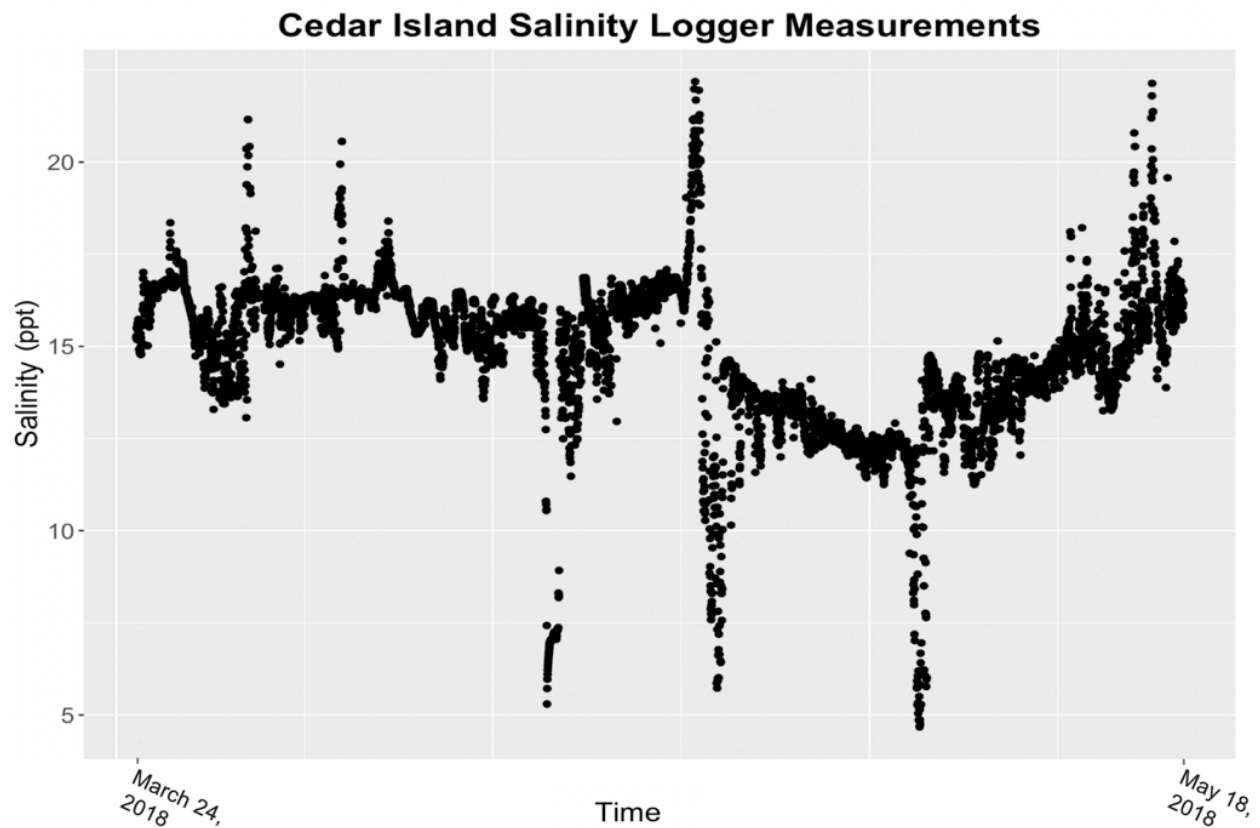


Fig. II.S1: Salinity time series from one coupon deployment in March 2018 for eight weeks at Cedar Island, NC.

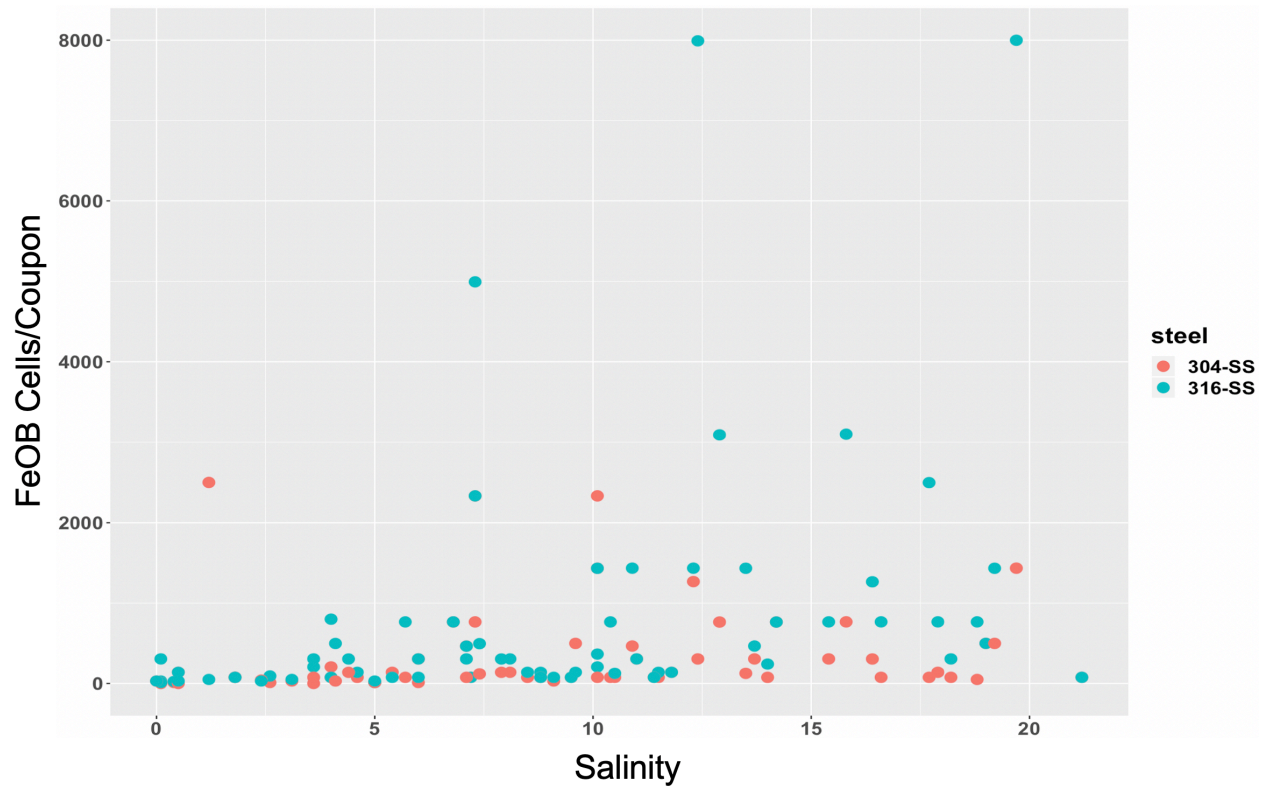


Fig. II.S2: Iron-oxidizing bacteria raw abundance data from all sample retrievals. Cells were generally more abundant on 316 stainless steel than on 304 stainless steel and at higher salinities.

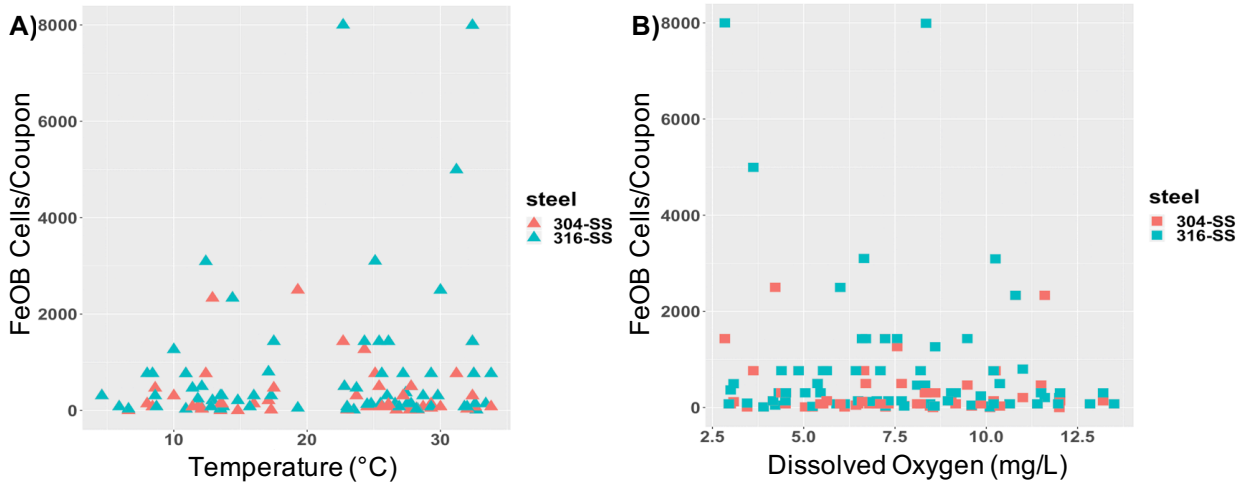


Fig. II.S3: A) Iron-oxidizing bacteria abundance with temperature from all coupon samples. Highest cell abundance samples exhibited higher temperatures. B) Iron-oxidizing bacteria abundance with dissolved oxygen from all coupon samples. Highest cell abundance samples exhibited lower dissolved oxygen concentrations.

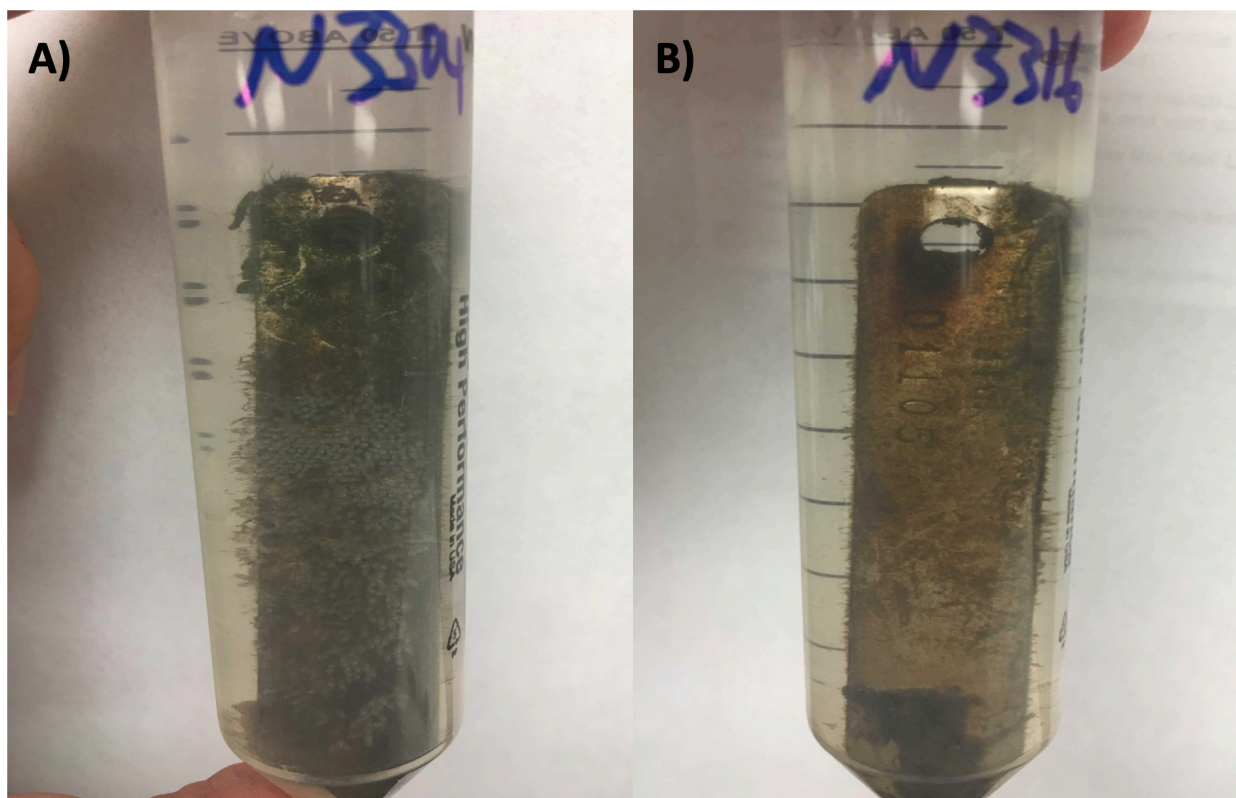


Fig. II.S4: 1/2" x 3" x 1/16" coupons deployed for six weeks. A) 304 stainless steel covered in algae and fish eggs with no visible rust coloration. B) 316 stainless steel with dark orange rust coloration.

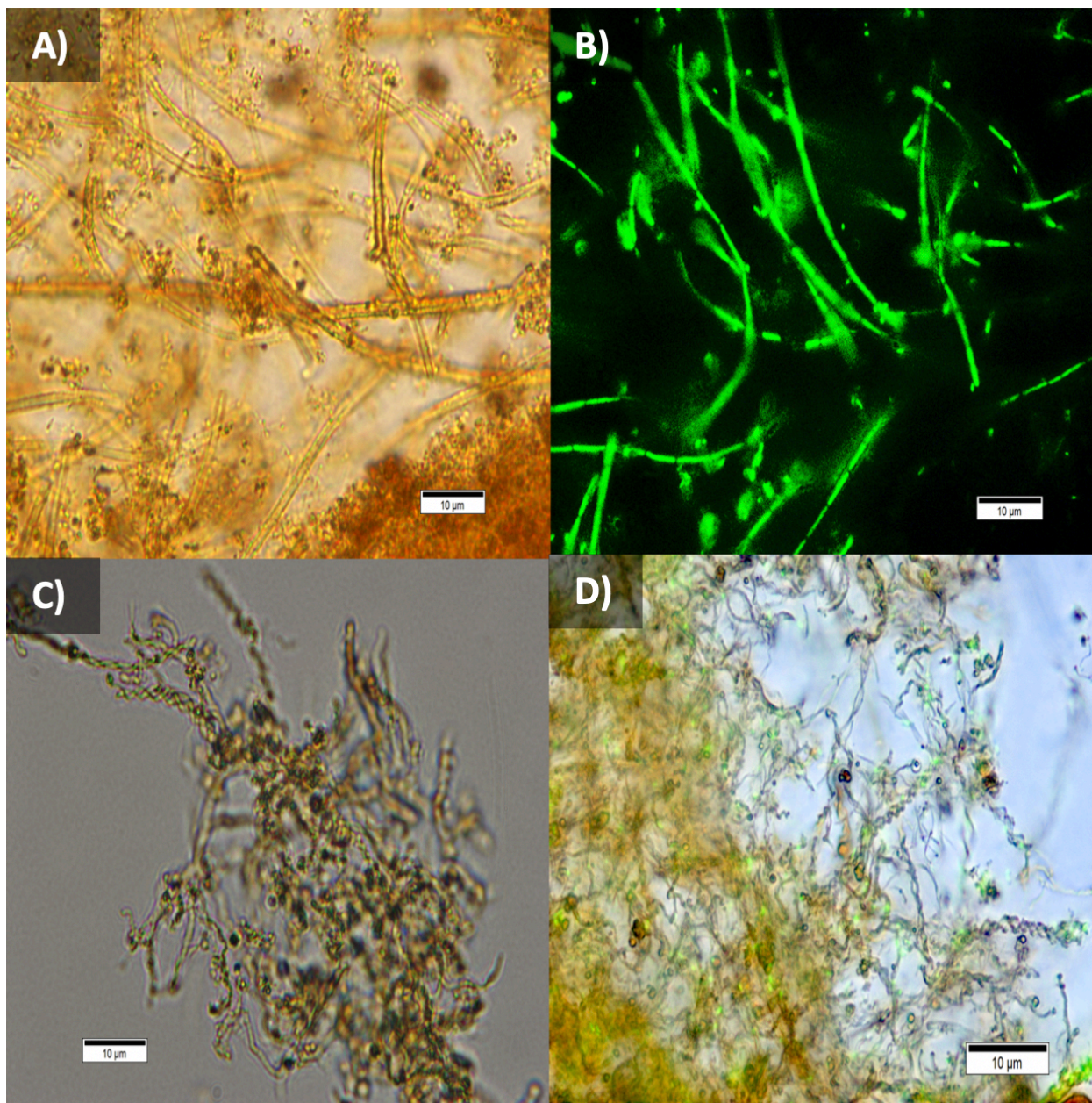


Fig. II.S5: Representative images of enrichment cultures from stainless steel coupon inocula. A) Light microscopy of tubular iron oxide sheaths. B) Fluorescent microscopy showing iron-oxidizing bacteria cells inside hollow tubular sheaths. C) Light microscopy of twisted iron oxide stalks. D) Fluorescent image overlaid on brightfield image with iron-oxidizing bacteria cells at ends of twisted stalks

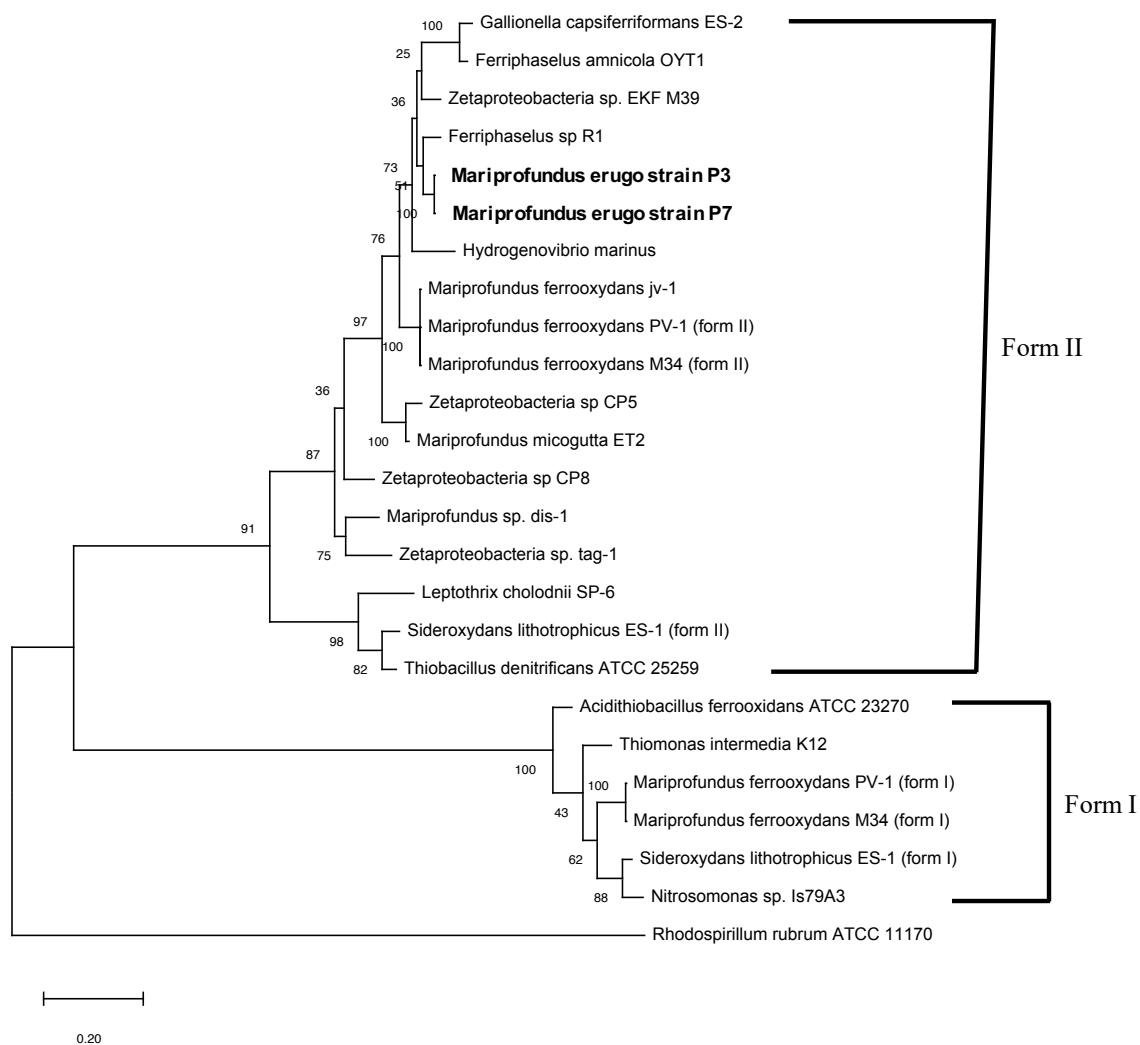


Fig. II.S6: Maximum likelihood phylogenetic tree showing relatedness of RubisCO protein sequences for *Mariprofundus erugo* P3 and P7 (bold) compared to other closely related bacteria species. Scale bar represents amount of evolutionary change in DNA, or average number of nucleotide substitutions per site. Bootstrap values represent number of times out of 100 that the same branch was observed when reconstructing the tree.

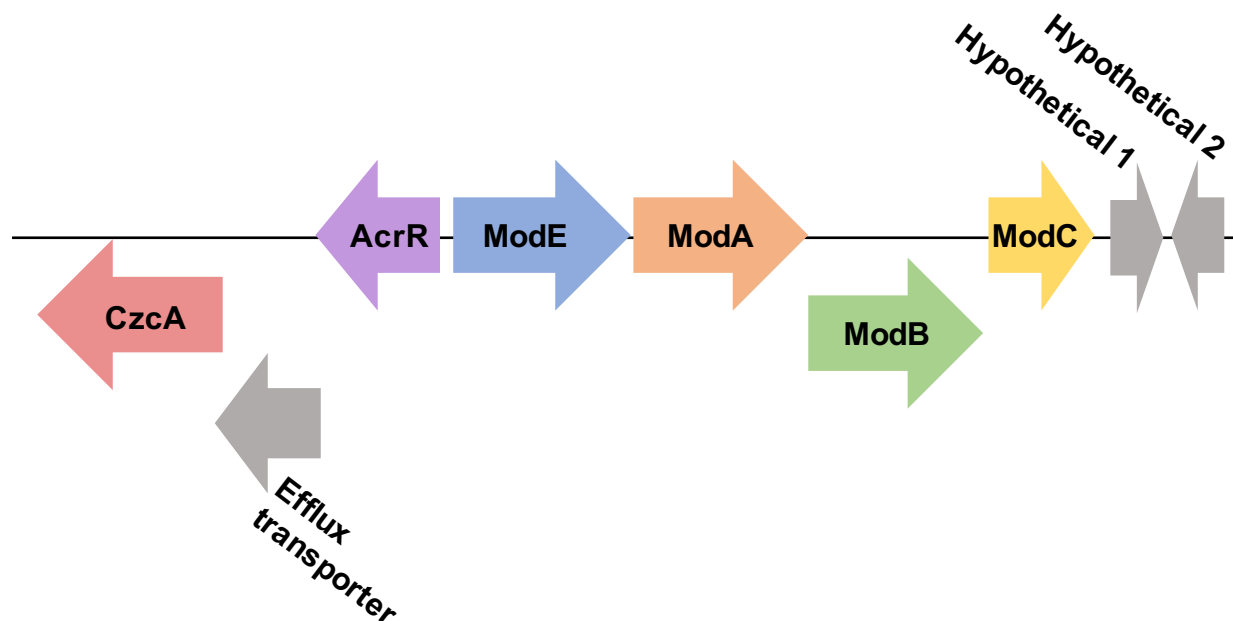


Fig. II.S7: Gene operon of molybdenum-related proteins (ModA, ModB, ModC, ModE) from *Mariprofundus erugo* P3 draft genome. Other neighboring sequences include cobalt-zinc-cadmium resistance protein CzcA, Efflux transporter protein, AcrR transcriptional regulator protein, and two hypothetical proteins that are also found in multiple freshwater and marine iron-oxidizing bacteria.

III. IDENTITY AND FUNCTION OF NON-CORROSION-CAUSING MICROORGANISMS COMPRISING A “CORE STEEL MICROBIOME” ACROSS STAINLESS STEEL AND SHIPWRECK STEEL IN AQUATIC ENVIRONMENTS

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Abstract:

Microorganisms attached to aquatic steel structures play key roles in nutrient cycling and structural degradation processes. Corrosion-causing microbes are often the focus of studies involving microbially influenced corrosion, yet the roles of remaining community members remain unclear. This study characterizes the composition and functional potential of a “core steel microbiome” across stainless steel types (304 and 316) and historic shipwreck steel along salinity gradients in North Carolina estuaries. We found higher phylogenetic evenness and diversity on steel surfaces compared to sediment, and at lower salinities. The core steel microbiome was composed of heterotrophic generalist taxa, and community composition was most strongly influenced by salinity. Substrate type was a secondary factor becoming more influential at higher

salinities. The core steel microbiome included members of *Sphingobacteriia*, *Cytophagia*, *Anaerolineaceae*, *Verrucomicrobiaceae*, *Chitinophagaceae*, and *Rheinheimera*. While salinity led to phylogenetic separations across the whole microbial community, functional genes were conserved across salinity and steel type. Generalist taxa on steel surfaces likely provide functional stability and biofilm protection for the community with limited functional trade-offs compared to surrounding environments. Further, characterization of a core steel microbiome increases the understanding of these complex steel surface microbial communities and their similarities to core microbiomes in other environments.

Introduction:

Microbially influenced corrosion (MIC) has been well described for its corrosion-causing community members and significant global costs of infrastructure destruction (Emerson *et al.* 2010; Beech & Sunner 2004; Little & Lee 2007; Dang & Lovell 2016; Emerson 2018). Much less is known about the core microbial community members “behind the scenes” which promote biofilm formation and functional stability among these MIC sites on steel surfaces. Recent evidence suggests that the abundance of corrosion-causing community members varies based on environmental factors such as substrate, salinity, and successional timing (McBeth *et al.* 2011; McBeth *et al.* 2013; McBeth & Emerson 2016; Garrison *et al.* 2019; Price *et al.* 2020). If the core microbial members are also shifting in community composition, then the potential for biocorrosion and nutrient-cycling ecosystem services (i.e., carbon, nitrogen, phosphorous) are likely also impacted. Characterization of the entire microbial

community is important, because less dominant and rare microbial community members maintain important roles in shaping community function (Hol *et al.* 2010; Shade *et al.* 2014; Aanderud *et al.* 2015; Kleindienst *et al.* 2016; Jousset *et al.* 2017). The characterization of a “core steel microbiome” across variations in environmental conditions could greatly improve the understanding of steel surface community functional potential. The distribution and abundance of functional genes within these steel microbial communities are also largely unknown and could provide further supporting evidence of the core steel microbiome functional role across different environmental conditions, as well as the overall functional potential of these microbial communities colonizing steel surfaces.

Microbial communities on both mild steel and stainless steel are highly diverse (Vandecastelaere *et al.* 2010; McBeth *et al.* 2011), and thus the presence of many non-corroding organisms must also help provide favorable conditions for biocorrosion, nutrient cycling, and overall community function. Aquatic sediment microbial communities often have higher phylogenetic diversity and higher Shannon diversity than those found in the water column (Lozupone & Knight 2007; Feng *et al.* 2009), but data is limited for diversity indices of steel surfaces in aquatic environments. Phylogenetic species evenness has been shown to have a positive correlation with bacterial productivity and overall functional stability (Wittebolle *et al.* 2009; Haig *et al.* 2015; Schmidt *et al.* 2020), and can also be used as an indicator for core microbiomes (Helmus *et al.* 2007; Shade & Handelsman 2012). Comparisons of community diversity indices between steel surfaces and other substrates (i.e., sediment and surface

controls) could broaden the overall understanding of steel surface microbial function and a potential core steel microbiome.

The characterization of a core steel microbiome could be an important step in determining the conditions under which steel is most vulnerable to microbially influenced corrosion, or even conversely, more likely to exhibit corrosion inhibition. This is further strengthened by the fact that biocorrosion is a complex process that has yet to be definitively connected to a single biochemical reaction or a single group of microbes, and with many different protein secretions and metabolites likely playing important secondary roles in biocorrosion (Kip & van Veen 2015). The importance of characterizing a core microbiome has recently expanded beyond host associated environments in order to more accurately define the health of microbial communities and their potential response to ecological disturbances (Shade & Handelsman 2012). If there are common microbial members across multiple assemblages within the same habitat after sampling across space and time, then those members may comprise a core microbiome (Berg *et al.* 2020). These core members may be governed by either functional or phylogenetic redundancy, and determining the degree of each could indicate how well these communities are adapted to and/or prepared for global change (Shade & Handelsman 2012). The dynamic environmental conditions within estuaries and the wide variety of steel types available in these environments for colonization could be analogous to global change. With the use of omics techniques to identify both phylogeny and function, this study aims to characterize a core steel microbiome based on the similarities of steel microbial communities across variables including salinity,

steel type, and timescale, while also highlighting the differences in functional potential between communities on steel and control substrates.

The influence of salinity on overall microbial communities is well known for aquatic systems and is the most significant environmental factor regulating phylogenetic divergence and community composition (Bouvier & del Giorgio 2002; Lozupone & Knight 2007; Herlemann *et al.* 2011). However, the degree to which salinity controls bacterial diversity is not always consistent. Higher salinity environments have been shown to have higher phylogenetic diversity, i.e., decreased taxonomic relatedness (Lozupone & Knight 2007), while other studies have shown decreasing bacterial richness, evenness, and diversity with increasing salinity (Oren 2002; Wang *et al.* 2012; Nawar 2016). Others have even shown no clear trend between salinity and bacterial Shannon diversity (Hu *et al.* 2016). Uncovering the bacterial diversity relationship for steel surface environments will indicate whether phylogenetic divergence occurs in core steel microbiomes across salinity, as well as provide insight into potential differences in bacterial productivity and functional stability.

Timescale is also important to consider, as corrosion-causing organism abundance can shift from iron-oxidizer dominated to sulfate-reducer dominated in a matter of days (McBeth & Emerson 2016). If the core steel community is largely the same or similar across time, then any changes in corrosion-causing organism niche establishment could be more appropriately inferred as a direct cause of chemical or abiotic factors rather than shifts in the core steel microbiome. The chemical composition

of steel substrates often changes over long timescales due to oxidation and reduction microbial processes, which create metabolic byproducts such as hydrogen sulfides, iron oxides, and carbon-based organics (Emerson 2018) which could presumably also influence core microbial member assembly. While it may be difficult to say how these communities shift over timescales of years without extremely long-term experiments, historical ferrous-hulled shipwrecks provide a mechanism for studying in situ long-term effects. A study involving the same shipwreck used in this study showed that relative iron-oxidizer abundance varied significantly based on sampling location on the ship (Price *et al.* 2020). Further analysis of the whole microbial community from these samples could help determine whether corrosion-causing organism abundance is more heavily controlled by abiotic or biotic factors. Combining data from these shipwreck samples with short-term stainless steel coupon deployment samples (Garrison *et al.* 2019) allows a core steel microbiome to be characterized across salinity gradients, three different steel types, and two different timescales. The increased combinations of environmental conditions found in this study provide a more encompassing view of the microbial communities associated with steel and the role of their environment.

An analysis of microbial community composition and function was conducted using 16S rRNA gene sequence data and metagenomic sequencing data from three different types of steel surfaces in North Carolina estuarine environments: 304 stainless steel (304SS) (18% chromium and 8% nickel), 316 stainless steel (316SS) (16.6% chromium, 10% nickel, and 2% molybdenum), and historical ferrous-hulled shipwreck steel (exact composition unknown). These previous studies found that the abundance of

corrosion-causing microbes colonizing the steel samples varied with environmental factors (Price *et al.* 2020; Garrison *et al.* 2019), suggesting that community functional genes and the presence of a core microbiome may also vary with these factors and have important impacts on biocorrosion potential. This study enables a wholistic view of aquatic steel microbial communities that builds upon these previous foundational studies. Microbial community phylogeny and functional genes were also analyzed within nearby sediment samples and surface control samples (PVC biofoul plates and empty oyster shells attached to the shipwreck) to further illustrate the uniqueness of steel surfaces as a novel ecological niche. These comparisons further allow us to determine how steel surfaces may have selected for specific microbial assemblages and how core metabolic functions have adapted to these unique environments. In contrast to previous MIC community studies which often lack metagenomic sequencing, this study provides some context for functional genes found on steel surfaces in addition to characterizing a core steel microbiome.

Methods:

Field Deployments and Sample Processing:

Stainless steel coupons were collected in February and May 2017 after being deployed for 57 and 40 days respectively, from five sites on the Pamlico River and five sites on the Neuse River, North Carolina, with salinities ranging from 0.1 ppt to 15.8 ppt (Garrison *et al.* 2019). Coupons were deployed along two estuarine river systems in NC and processed according to the methods outlined in Garrison *et al.* (2019). Substrate types for analysis from these stainless steel sites included 304SS, 316SS, nearby

sediment, and biofouling plates made out of polyvinyl chloride (PVC) plastic as a non-metal control. Plastics such as PVC are also susceptible to fouling and deterioration but do not undergo corrosion in terms of iron oxidation. Stainless steel and PVC have no difference in aquatic bacterial colonization potential (Pedersen 1990), which makes them ideal surface controls to compare to steel biocorrosion communities (Garrison *et al.* 2019; Price *et al.* 2020). Shipwreck samples were collected in September 2017 from a ferrous-hulled World War II gunboat located in the Pamlico Sound, North Carolina, at a salinity of 17.9 ppt (Price *et al.* 2020). Shipwreck sample types for analysis included metal pieces of the ship that appeared visibly corroded (either orange, black, or both orange and black), or not visibly corroded. All non-visibly corroded ship pieces consisted of a mixture of empty oyster shells attached to ship substrate (referred to herein as oyster surface controls). Nearby sediment was also analyzed for community comparisons to steel surfaces, according to the methods outlined in Price *et al.* (2020). Sampling site details are listed in Table III.S1.

16S rRNA Sequencing and Analysis:

This study analyzed 16S rRNA gene amplicon sequencing data to determine microbial community composition from samples related to stainless steel sites (Garrison *et al.* 2019) and shallow water shipwreck sites (Price *et al.* 2020) (Table III.S1). Extracted DNA were sent to CGEB Integrated Microbiome Resource (Dalhousie University, Halifax, Canada) for sequencing. 16S sequences were processed using a Phusion polymerase PCR amplification method (Comeau *et al.* 2017), targeting the V4-V5 region of the 16S rRNA gene, and sequenced using the Illumina MiSeq platform.

The V4-V5 region is the most reliable hypervariable region for representing the full length of the 16S rRNA gene and the largest majority of bacteria phylogeny (Yang *et al.* 2016). The V6-V8 region is able to capture a larger diversity of marine bacterial taxa (Willis *et al.* 2019); however, the sampling sites in this study are strongly influenced by terrestrial and freshwater environments which is why the v4-v5 region was used. Amplicon sequence data was processed and annotated using the mothur v1.41.3 pipeline (Schloss *et al.* 2009). Output from mothur based on 97% OTU classifications was used to create CCA and NMDS ordination plots using a Bray-Curtis similarity matrix method in R with vegan and ggplot2 packages and the metaMDS function (R Core Team 2013). Significant differences between groups in CCA and NMDS plots were tested using an analysis of similarities (ANOSIM (Clarke 1993; Warton *et al.* 2012)) in R. Significance and degree of correlation of environmental variables within the CCA community composition plots was then tested using permutational multivariate analysis of variance (PERMANOVA (Anderson 2005); Adonis function in R). The most influential taxa towards differences seen in microbial community compositions between sample types was calculated using a similarity percentage analysis (SIMPER (Clarke 1993; Warton *et al.* 2012)) in R. Community diversity indices (richness, evenness, and Shannon diversity (Shannon 1948)) were calculated using the diversity function within vegan. Boxplots depict 5 key summary statistics including the median, 25% and 75% percentiles, and the feasible range of the data ($\pm 1.5 \times$ the interquartile range) represented by the two whiskers. Linear regression of Shannon diversity was tested using a general linear model with salinity as the predictor variable. Significant differences between diversity index values were tested using a Mann-Whitney U test

(McKnight 2010) (wilcox.test function in R). An indicator species analysis was performed using the indicpecies package in R to determine OTUs within the dataset that were deemed indicators for certain sample types and/or environmental conditions such as steel surface associated samples (De Cáceres & Legendre 2009). The indicpecies function determined probability percentages based on exclusivity (whether or not the OTU occurred only in that sample type) and fidelity (whether or not the OTU occurred at all sites within that sample type). Levins' niche breadth analysis was further used to determine whether OTUs within the dataset could be categorized as generalists or specialists, and thus whether or not certain sample types contained higher proportions of either category. Niche breadth values were calculated using the formula $B = 1/\sum(p_i^2)$, where B is niche breadth and p is proportion of OTU within a sample i (Levins 1968). Three-dimensional surface plots were created using Matlab v.R2017b to visualize distributions of specialists and generalists across sample types. Data analysis R code: https://github.com/cgarr017/ECU_dissertation_code/blob/main/chapter2.

Shotgun Metagenomic Sequencing and Analysis:

Shotgun metagenomic sequencing was used to determine the functional genes present on eight samples from two of the stainless steel sites with salinities of 3.6 ppt and 6 ppt (Site N3 and N11; Table III.S1). Samples included 304SS, 316SS, sediment, and PVC biofoul surface control for each site. Both 316SS samples from these sites were orange and visibly corroded. Eight samples from the shipwreck site with salinity of 17.9 ppt were also sequenced for shotgun metagenomics. Samples included three visibly corroded ship pieces, one sediment sample, one oyster-ship non-corroded

surface control, one surrounding seawater sample, and two drilled shipcore samples extracted from the interior of the ship's hull. One 304SS sample was not included in the metagenomic results due to a low number of raw reads resulting in few genes recovered compared to the other samples. Metagenomic samples were prepared using an Illumina Nextera Flex kit at CGEB Integrated Microbiome Resource center (Comeau *et al.* 2017). Samples were enzymatically sheared and tagged with adaptors, PCR amplified using barcodes, then purified, normalized, and pooled for loading. Samples were sequenced using the Illumina NextSeq platform, with 2X sequencing depth for ~8 million paired-end 150+150 bp reads (16 million single reads) resulting in ~2.4 Gb per sample. Metagenomic raw reads were trimmed and confirmed using TrimGalore v.0.4.5 (Krueger 2012) and FastQC v.0.11.8 (Andrews 2010), respectively. Trimmed reads were concatenated, then assembled using SPAdes v.3.13.0 (Bankevich *et al.* 2012). Assembled contigs were functionally annotated using MG-RAST v.4.0.3 (Meyer *et al.* 2008) to search for functional genes of interest. Significant differences between metagenomic gene abundance were tested using a Mann-Whitney U test (McKnight 2010) (wilcox.test function in R). FeGenie (Garber *et al.* 2020), LithoGenie (<https://github.com/Arkadiy-Garber/LithoGenie>), Prodigal v2.6.3 (Hyatt *et al.* 2010), and HMMer v.3.3 (<https://hmmer.org/>) were used for gene annotations and estimating MIC-related protein abundance.

Assembled metagenomic contigs were further binned into metagenome assembled genomes (MAGs) using MaxBin v.2.2.7 (Wu *et al.* 2016), Concoct v.1.1.0 (Alneberg *et al.* 2014), and MetaBAT v.2.12.1 (Kang *et al.* 2019). Additional program

dependencies utilized by these programs included bowtie v.2.3.5.1 (Langmead & Salzberg 2012) and samtools v.1.9 (Li *et al.* 2009). Results from all three binning methods were analyzed using DAS Tool v.1.1.2 (Sieber *et al.* 2018) to determine the MAGs that had the highest completeness (>50%) and lowest contamination (<10%). Quality and bin statistics were further confirmed using CheckM v.1.0.18 (Parks *et al.* 2015), which also relies on pplacer v.1.1.alpha19 (Matsen *et al.* 2010), prodigal v.2.6.3 (Hyatt *et al.* 2010), and HMMer v.3.3 (<http://hmmer.org/>). High quality MAGs were annotated using RASTtk v.2.0 (Aziz *et al.* 2008; Overbeek *et al.* 2014; Brettin *et al.* 2015) in order to identify 16S rRNA genes and functional genes of interest. For MAGs that did not have 16S rRNA genes identified by RASTtk, barrnap v.0.9 (<https://github.com/tseemann/barrnap>) was used to look for short 16S rRNA gene fragments that may have been cut off during contig assembly. Any short 16S rRNA gene fragments identified in the MAG were used to search for the longer accompanying sequence in the raw reads of that sample. Any longer 16S rRNA gene sequence was used in a search query in BLAST (Altschul *et al.* 1990) to identify the bacterial taxonomy with the highest percent identity. Any MAGs that did not recover reliable 16S rRNA were classified using MetaSanity v.1.2.0 (Neely *et al.* 2020).

Data Availability:

Sequence data for this project are stored in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. 16S rRNA amplicon sequence data for the shipwreck samples can be found under the accession numbers SRR12148233-SRR12148251. Amplicon data for the stainless steel samples

and metagenomic data for both can be found under the BioProject accession number PRJNA674937.

Results:

Higher evenness and diversity on steel surfaces compared to sediment

Microbial community richness was lower on surface environments (steel, biofoul PVC, and oyster shell) compared to sediment for stainless steel sites with salinity < 10 ppt (Mann-Whitney U test: $W = 271.5$, $p = 0.0153$), but there was no difference in richness between surface environments and sediment for sites > 10 ppt ($p > 0.05$) (Fig. III.1). Microbial community evenness was higher on surface environments (stainless steel, ship steel, biofoul PVC, and oyster-ship substrate) compared to sediment for stainless steel sites < 10 ppt (Mann-Whitney U test: $W = 56$, $p = 0.0004$) and for the shipwreck site (17.9 ppt) ($W = 0$, $p = 0.0003$), but there was no difference in evenness at stainless steel sites > 10 ppt ($p > 0.05$). Overall, Shannon diversity decreased with increasing salinity, regardless of sample type (Linear regression test: $df = 70$, $t = -12.37$, $p < 0.0001$). More specifically, Shannon diversity was higher on steel surfaces compared to sediment and surface controls for the shipwreck site (Mann-Whitney U test: $W = 1$, $p = 0.003$), but there was no difference in diversity between surface environments and sediment for stainless steel sites at all salinities.

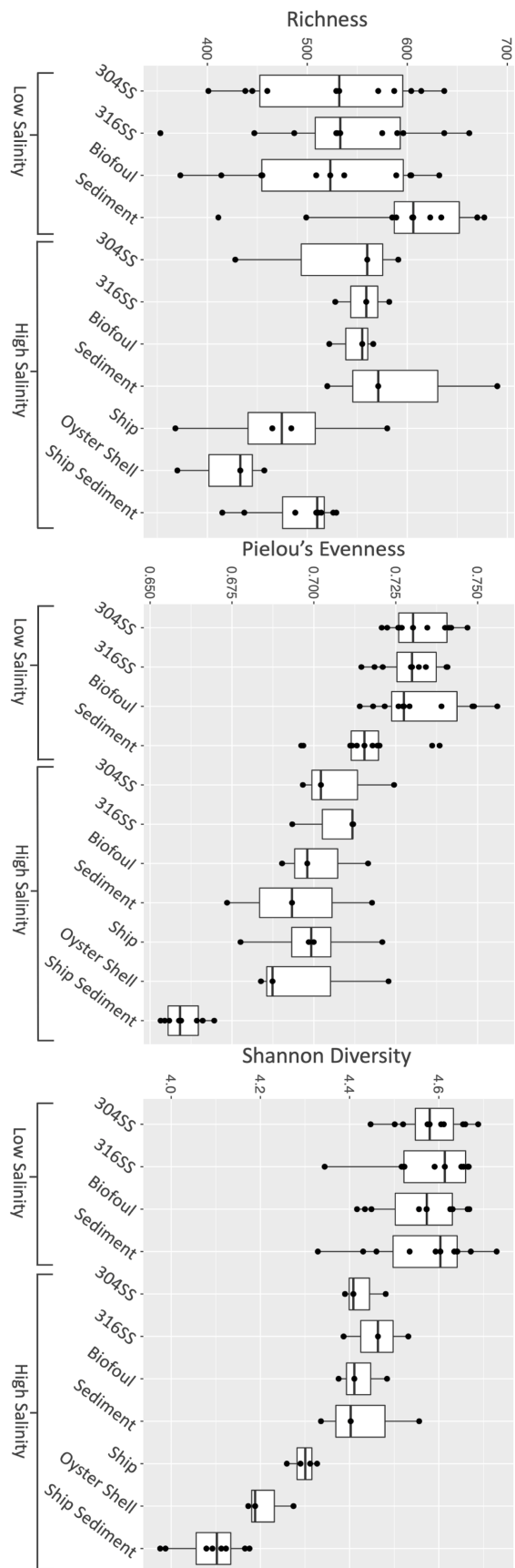


Fig III.1. Species richness (top), evenness (middle), and Shannon diversity (bottom) across sample types based on 97% similarity classifications of OTUs from 16S rRNA gene sequencing data. Samples were separated into two groups for analysis: low salinity (< 10 ppt) and high salinity (> 10 ppt) for more accurate fine-scale comparisons between sample types. Microbial community evenness was higher on surface environments (SS, ship, biofoul, and oyster shell) compared to sediment for low salinity sites (Mann-Whitney U test: $W = 56$, $p = 0.0004$) and for the shipwreck site ($W = 0$, $p = 0.0003$). Shannon diversity was higher on steel surfaces than both sediment and surface controls for the shipwreck site only ($W = 1$, $p = 0.003$).

Salinity had greatest influence on community composition, followed by substrate

Salinity exhibited the strongest correlation with microbial community composition (PERMANOVA test: $R^2 = 0.275$, $p = 0.001$), followed by substrate (PERMANOVA test: $R^2 = 0.173$, $p = 0.001$) (Fig. III.2). Timescale was also a significant variable, although it presented the weakest correlation (PERMANOVA test: $R^2 = 0.020$, $p = 0.006$). Microbial communities were not significantly different between 304SS and 316SS, although stainless steel communities as a whole (304SS and 316SS) were significantly different from ship steel communities (ANOSIM test: $R = 0.299$, $p = 0.019$). Differences in microbial community composition between stainless steel and ship steel were most strongly influenced at the class level by *Gammaproteobacteria* (SIMPER analysis: 11.6%), *Alphaproteobacteria* (11.2%), *Betaproteobacteria* (10.9%), *Planctomycetia* (9.0%), unclassified *Bacteroidetes* (8.9%), and *Deltaproteobacteria* (7.5%).

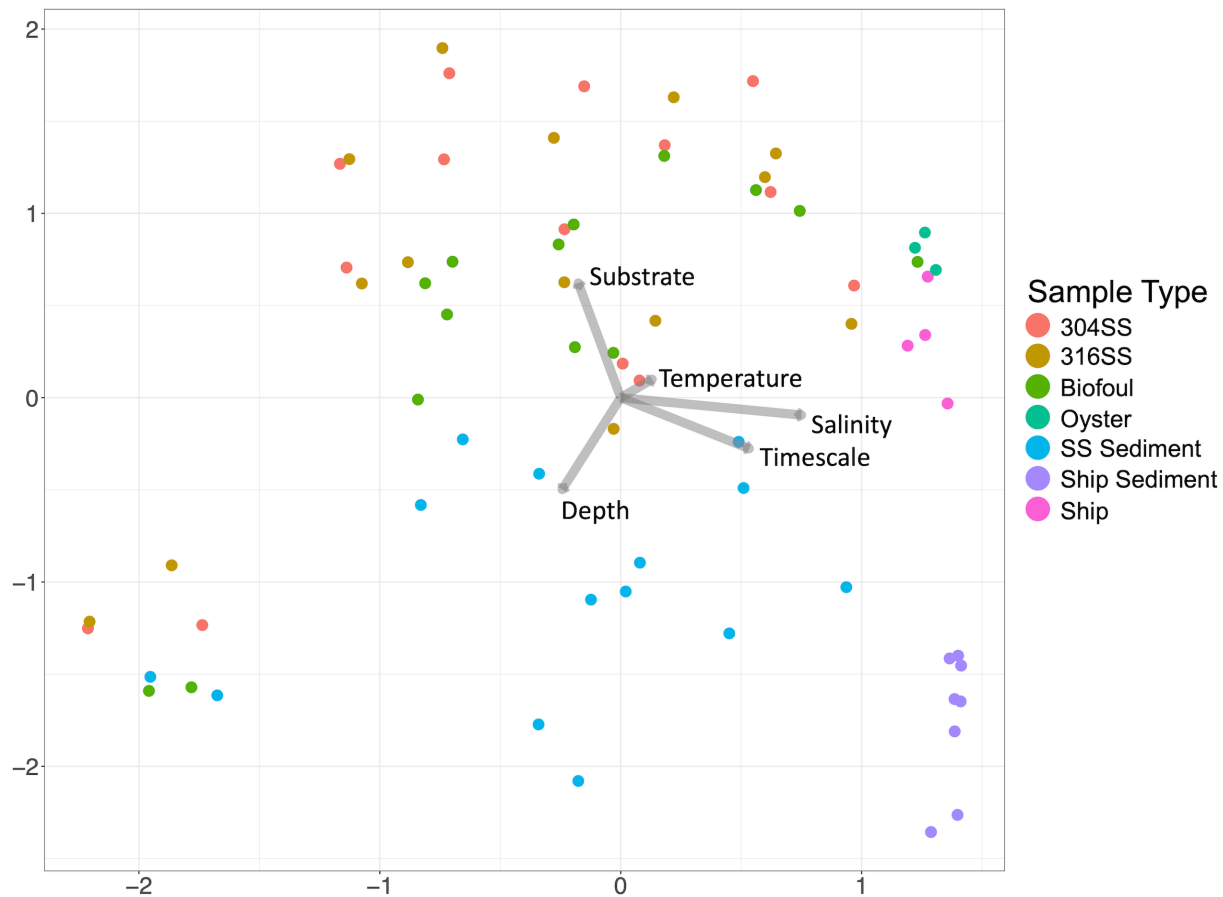


Fig III.2. Canonical correspondence analysis (CCA) plot based on 16S rRNA gene sequencing data. Each datapoint represents a unique microbial community composition from one sample. Distance between points represents the degree of difference between community composition of each point. Salinity exhibited the strongest correlation with microbial community composition (PERMANOVA test: $R^2 = 0.275$, $p = 0.001$).

Interestingly, differences in community composition between sample types were more evident at higher salinities. Stainless steel and biofoul samples were significantly different from sediment at salinities > 10 ppt (ANOSIM test: $R = 0.4872$, $p = 0.0232$), but not at salinities < 10 ppt (Fig. III.S1). This suggests that there may be increased microbial selectiveness or specialization due to environment at higher salinities. Additionally, there were two 316SS coupons from two different sites that had visible

orange corrosion byproducts, while the rest of the SS coupons exhibited varying degrees of fouling and detritus material but never any orange coloration. These two corroded coupons had no difference in community composition compared to the non-corroded coupons from the same deployment time and site (Fig. III.S2). Each pair of 304SS and 316SS samples, regardless of corrosion, grouped together based on site. This suggests that community composition seemed to be the same regardless of the presence or absence of corrosion, while also supporting salinity as the most important factor towards community composition. Clearly, salinity must be taken into account when identifying a core steel microbiome.

Core steel microbiome composed of generalist taxa

Some microbial taxa were expected to vary in abundance across steel types in accordance with results from a previous study showing higher abundance of corrosion-causing organisms on certain stainless steel types (Garrison *et al.* 2019). A core microbiome of consistent steel colonizers was expected to emerge as a result of aquatic steel surfaces enabling novel niche establishment. For example, it was expected that core steel microbial taxa may separate based on salinity due to physiological constraints across phylogenies. Results supported this as *Betaproteobacteria* were more abundant across lower salinity samples (Fig. III.3), regardless of sample type, consistent with their known freshwater habitat preference (Wu *et al.* 2006; Wang *et al.* 2012; Emerson 2018). There were more *Deltaproteobacteria* in the sediment samples compared to the steel samples, but only in higher salinity sites. *Alphaproteobacteria*, *Flavobacteria* and *Sphingobacteriia* were more abundant on surface samples (steel and

surface controls) compared to sediment samples at higher salinity sites. Low salinity sites showed almost no difference in proportion of dominant taxa across sample types, whereas higher salinity sites showed higher phylogenetic differentiation across sample types. Bacterial genera exhibited similar trends across salinity as those seen at the class level (Fig. III.S3). *Flavobacterium*, *Rhodopirellula*, *Erythrobacter*, and *Hyphomonas* were all found at higher proportion on surface environments (steel and surface controls) compared to the sediment at high salinity sites.

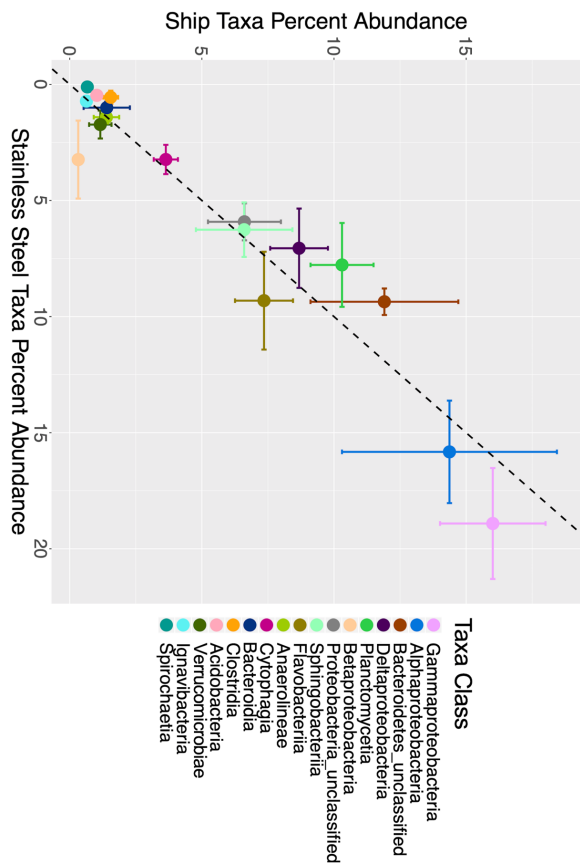
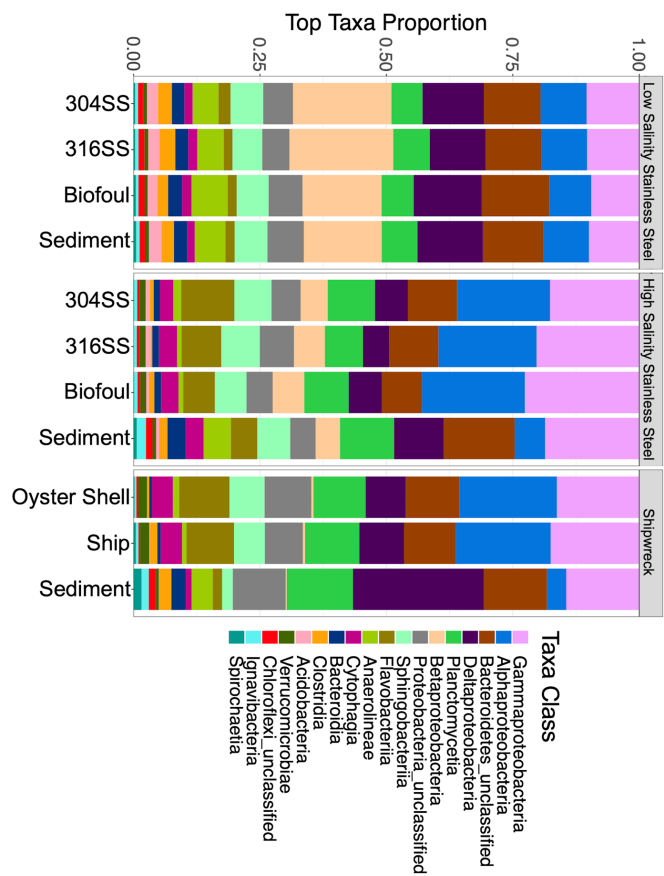


Fig III.3. Class-level taxa plot (top) based on 16S rRNA gene sequence classifications. Higher salinity sites (> 10 ppt) showed greater phylogenetic differentiation across sample types than lower salinity sites (< 10 ppt). *Alphaproteobacteria*, *Flavobacteria* and *Sphingobacteriia* were more abundant on the surface samples (steel and surface controls) compared to the sediment samples, but only at higher salinity sites. Stainless steel taxa abundance plotted against ship taxa abundance at the class level (bottom) indicated that *Alphaproteobacteria*, *Sphingobacteriia*, *Cytophagia*, *Anaerolineae*, and unclassified *Proteobacteria* were closest to the 1:1 ratio line (with error bars overlapping the line) with > 1% abundance, and thus were most likely to be part of a core steel microbiome. Only stainless steel samples from high salinity sites were used to compare to shipwreck steel samples in this plot.

A core steel microbiome can be further interpreted by plotting shipwreck taxa abundance against stainless steel taxa abundance to identify taxa with the most similar abundance across steel types (Fig. III.3). Datapoints that fell on the 1:1 ratio dashed line, or that have error bars overlapping the line, were determined more likely to be part of a core steel microbiome as opposed to datapoints that did not. Datapoints below the dashed line represent microbes with a higher preference towards stainless steel colonization, while datapoints above the line represent microbes that preferred more traditional steel alloys (i.e., carbon-based or mixed steel alloys less resistant to corrosion). Only high salinity sampling sites (> 10 ppt) were used for core microbiome characterization in order to identify the forces beyond salinity that drive core microbiome composition and function. *Alphaproteobacteria*, *Sphingobacteriia*, *Cytophagia*, *Anaerolineae*, and unclassified *Proteobacteria* were the taxonomic classes closest to the 1:1 ratio line with > 1% abundance, and thus most likely to be part of a core steel microbiome (also supported by the taxa plots above showing a higher preference of these taxa on steel surface samples). All of these taxa except *Alphaproteobacteria* had relatively weak influence on the differences in community composition between

stainless steel and ship steel: *Sphingobacteriia* (SIMPER analysis: 3.7% influence towards community differences), *Cytophagia* (2.5%), *Anaerolineae* (2.0%), *Alphaproteobacteria* (11.2%). At the genus level, unclassified *Anaerolineaceae* and unclassified *Verrucomicrobiaceae* were among the closest genera to the core steel dashed line (Fig. III.S4). In examining the major outliers from the core steel dashed line, *Gammaproteobacteria*, *Flavobacteria*, and *Betaproteobacteria* were more prevalent on the stainless steel coupons, while *Deltaproteobacteria*, *Planctomycetia*, and unclassified *Bacteroidetes* were more prevalent on the shipwreck steel. At the genus level, unclassified *Rhodobacteraceae* and unclassified *Flavobacteriaceae* were more prevalent on the stainless steel, while unclassified *Planctomycetaceae* and unclassified *Saprospiraceae* were more prevalent on the shipwreck. These results indicate that the core steel microbiome for the steel types and samples used in this study were likely generalist heterotroph microbes that play important supporting roles for specialists that contribute more directly to biocorrosion.

A Levins' niche breadth analysis (Levins 1968) further determined that sediment samples overall exhibited a greater proportion of specialist microbe species compared to surface environment samples (Fig. III.S5). A lower niche breadth value indicates a specialist lifestyle which is more selective in its functional role in the environment, whereas a higher niche breadth value indicates a generalist lifestyle which is more likely to use more commonly available sources of energy in a given environment (Levins 1968; Cockburn 1991). All samples had large proportions of high niche breadth values and thus generalist taxa; however, sediment samples simultaneously exhibited

significant abundances of low niche breadth values indicative of specialists as well, whereas the steel surface samples did not. This result further supports that generalist microbes likely comprised the majority of the core steel microbiome in this study.

An indicator species analysis (De Cáceres & Legendre 2009) supported the niche breadth analyses, also indicating higher proportions of specialist taxa in sediments and high proportions of generalists on steel surfaces. Several genera exhibited both high exclusivity (present only in one sample type and not in other sample types) and high fidelity (present across all samples within one sample type) in the shipwreck sediment samples (indicspecies test: $p < 0.05$), indicating that these taxa may be unique to this sample type only. These taxa included potential sulfate-reducing genera such as *Desulfospira*, *Desulfatibacillum*, and *Desulfobacterium*, as well as the sulfur-oxidizer *Thiolarillus*. These also included a benzoate-oxidizing sulfate-reducer *Desulfocarbo*, and an aromatic hydrocarbon-degrader *Thalassospira*, which could be a result of crude oil remnants from the ship's fuel tank still present in the surrounding sediment. No generalist taxa were identified for indicator species analysis using samples from all salinity values, however, when only including high salinity sites (> 10 ppt), some potential core steel generalists became more evident. *Leucothrix* was an indicator for 304SS samples only ($p < 0.05$) and is a heterotroph whose filamentous morphology may help form biofilm communities. *Sphingobium* was an indicator for both 304SS and 316SS samples ($p < 0.05$), which supports results from the 1:1 ratio plot above (Fig. III.3) above as potentially important steel community members. *Pelolinea*,

another filamentous heterotroph, was an indicator for combined ship and ship sediment sample groups ($p < 0.05$).

Five high-quality metagenome assembled genomes (MAGs) were recovered from the steel samples ($> 50\%$ complete and $< 10\%$ contamination (CheckM v1.0.18 (Parks *et al.* 2015))) (Table III.S2). Two of these MAGs were classified as *Rheinheimera* sp. and two were classified as *Chitinophagaceae*. The fifth MAG was classified as *Sulfurovum* sp., a sulfur-oxidizer. The steel surface associated MAGs exhibited larger average genome size (mean = 2.63Mbp, standard deviation [SD] = 0.93 Mbp) than surface control MAGs (mean = 1.76Mbp, SD = 0.58Mbp), and lower average GC content (mean = 42.3%, SD = 5.0%) than surface control MAGs (mean = 49.2%, SD = 9.4%). These genome characteristics could be inherently associated with core steel microbiomes, although the small number of MAGs recovered limits interpretation, and additional studies may be warranted for further analysis of individual taxa members. Metagenomic analysis also revealed a higher relative abundance of glycoside hydrolase genes, which are indicators for identifying the presence of generalist taxa such as cellulose-, chitin-, and other polysaccharide-degraders (Edwards *et al.* 2010), on steel samples (mean = 0.22%, SD = 0.03%) compared to surface controls (i.e., oyster shell and biofoul plates) (mean = 0.17%, SD = 0.03%) (Mann-Whitney U test: $W = 3$, $p = 0.066$) (Fig. III.4).

Steel surface functional genes were conserved across salinity while phylogeny diverged

Functional genes were hypothesized to follow similar trends to taxonomic data, with varying degrees of influence by salinity, substrate, and timescale. Metagenomic analysis using Hidden Markov model (HMM)-based categorization of biogeochemically-relevant genes (FeGenie (Garber *et al.* 2020) and LithoGenie (<https://github.com/Arkadiy-Garber/LithoGenie>)) revealed MIC-related genes (i.e., iron oxidation and reduction, sulfur oxidation and reduction) at similar relative abundances across all stainless steel and ship steel types (Fig. III.4, Fig. III.S7 & III.S8). Both stainless steel sites used in metagenomic analysis were low salinity sites (6 ppt and 3.6 ppt) compared to the shipwreck site (17.9 ppt), and thus salinity did not have a significant impact on the relative abundance of MIC-related genes. This is in contrast to the 16S rRNA gene sequence results which showed many phylogenetic separations in steel surface community compositions based on salinity. There was also no significant difference in glycoside hydrolase percent abundance between low salinity stainless steel samples and high salinity ship steel samples, suggesting that functional genes not related to MIC may also be conserved across salinity more so than phylogeny. There was a higher abundance of iron oxidation (Cyc1, Cyc2, FoxABC, FoxEYZ, Sulfocyanin, PioABC, MtoAB), iron reduction (CymA, MtrCAB, OmcF, OmcS, OmcZ, FmnA-dmkA-fmnB-pplA-ndh2-eetAB-dmkB, DFE_0448-0451, DFE_0461-0465, MtrCB, MtrAB, MtoAB-MtrC), sulfur oxidation (DsrABCEFGHK, dioxygenase sdo), sulfate reduction (CysN, aprA, sopT, apsK), and oxygen reduction (CytCoxidase coxAB, ccoNOP, ubiquinol oxidase CyoE, oxidase CydAB) proteins found on steel surfaces compared to surface control sample types (although not statistically significant; Mann-Whitney U test:

W = 160, p = 0.116), consistent with steel surfaces having a higher functional potential for MIC compared to surface controls (Fig. III.S6-III.S8). There were also higher percentages of oxygen reduction related genes in the 304SS, 316SS, and biofoul control samples compared to the nearby sediment samples (Fig. III.S6) (W = 1, p = 0.19), suggesting an increased use of oxygen as an electron acceptor and therefore indicative of a more oxidative environment rather than a reducing environment on surface samples compared to sediment samples.

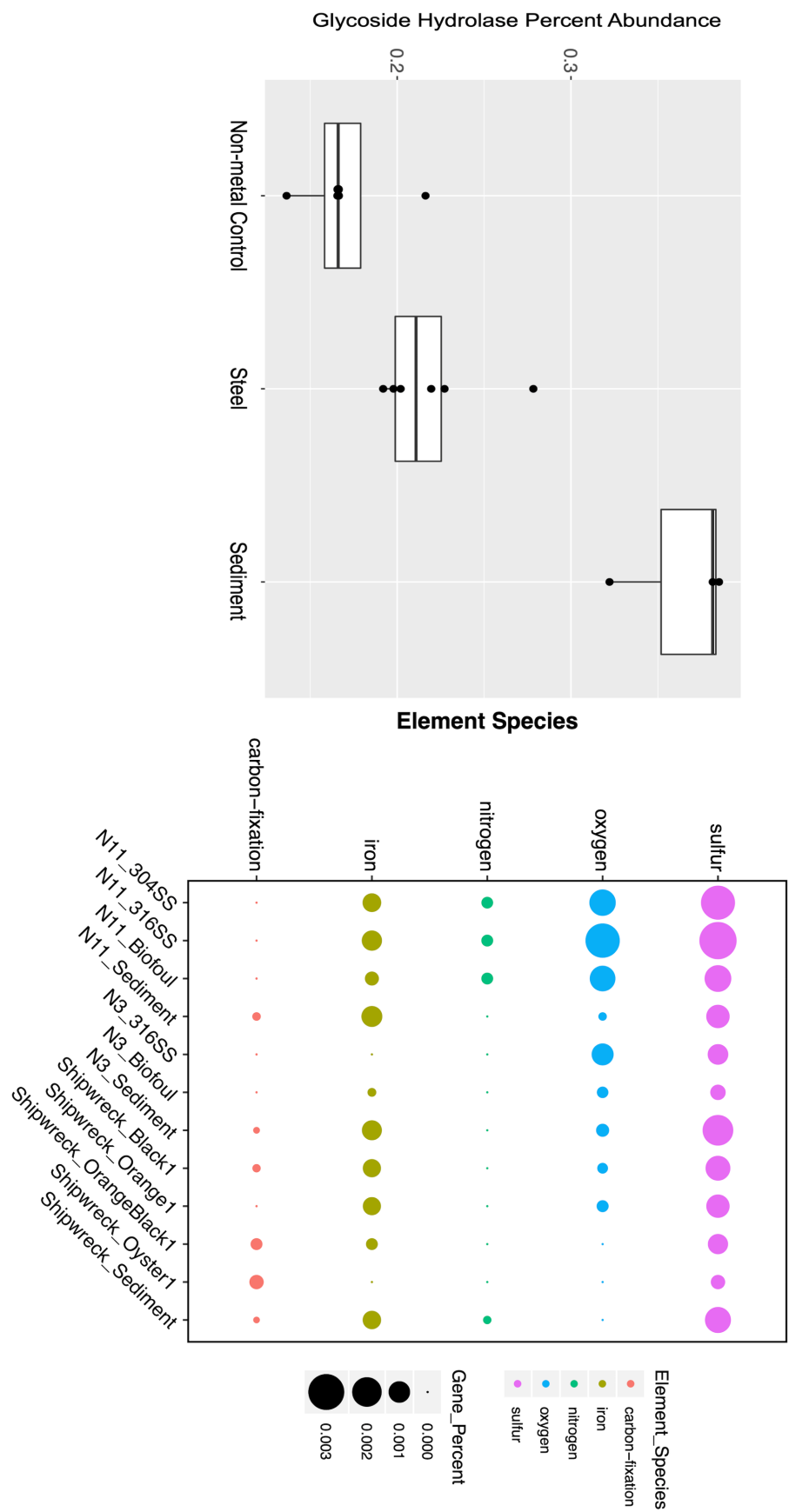


Fig III.4. Glycoside hydrolase boxplot (top) shows a higher relative abundance of glycoside hydrolase genes on steel samples compared to surface controls (oyster shell and biofoul plates) based on metagenomic sequence data (Mann-Whitney U test: $W = 3$, $p = 0.066$). Functional gene bubble plot (bottom) shows that relative abundance of MIC-related genes such as iron oxidation/reduction and sulfur oxidation/reduction were not significantly affected by salinity (site N11 = 6 ppt, site N3 = 3.6 ppt, shipwreck site = 17.9 ppt).

Discussion:

The results from this study provide insight into the community composition and functional potential of a core steel microbiome. Bacterial Shannon diversity on steel surfaces was inversely related to salinity, as Shannon diversity values increased with decreasing salinity. Furthermore, community evenness was more influential than species richness in determining Shannon diversity. Evenness was not only higher at low salinity sites, but also higher on steel surfaces compared to sediment. This suggests that steel surfaces may exhibit higher bacterial productivity and functional stability than that of nearby sediment (Wittebolle *et al.* 2009; Haig *et al.* 2015; Schmidt *et al.* 2020). This is surprising considering the greater environmental fluctuations seen in the water column compared to the sediment, and this indicates that the stability provided by protective biofilms on steel surfaces must be significant. Evenness can also be used as a signal for differences in core microbiomes (Shade & Handelsman 2012), and thus these results argue that steel surfaces have unique core microbiomes from that of sediment, and that core steel microbiomes will vary based on the environment's salinity.

Salinity was the most influential factor for bacterial community composition on steel surface, similar to trends observed in other environment types (Bouvier & del

Giorgio 2002; Lozupone & Knight 2007; Herlemann *et al.* 2011). Interestingly, substrate seemed to become a more significant factor as salinity increased. There was a greater difference in Shannon diversity between substrate types at the shipwreck site, which had the highest salinity (17.9 ppt), compared to stainless steel sites (0.1-15.8 ppt). There was also a greater difference in community composition between substrate types at high salinity stainless steel sites compared to low salinity sites (Fig. III.3, Fig. III.S1, Fig. III.S3). More specifically, *Alphaproteobacteria*, *Flavobacteria* and *Sphingobacteriia* exhibited greater abundances on surface substrates compared to sediment at higher salinities. This suggests a potential preference of these organisms towards surface attachment and an increased bacterial selectiveness of substrate at higher salinities. The core steel microbiome may have more overlapping community members with sediment and surface control environments as salinity decreases.

Heterotrophic generalists consistently emerged as important community members within the core steel microbiome in this study. Higher proportions of generalists were found on steel at high salinities, and several generalist taxa were significant indicators for steel surface substrates. Their taxonomic groups had the most equal proportion of abundance across different steel types (Fig. III.3), and they represented the majority of the MAGs recovered from steel samples. An important biofilm functional gene, glycoside hydrolase, was also found at a higher abundance on all steel surface types compared to surface control groups. These combined results are overwhelming evidence that generalist taxa likely dominate the core steel microbiome. The core steel microbiome taxa at higher salinity sites (>10 ppt) included members of

Sphingobacteriia, *Cytophagia*, *Anaerolineaceae*, *Verrucomicrobiaceae*, *Chitinophagaceae*, and *Rheinheimera*. The similarity in average abundance of *Alphaproteobacteria* across steel types supported their role as potential core steel members, however, their high variability and large influence towards community differences (SIMPER analysis: 11.2%) suggest that their core steel members need to be taxonomically refined beyond the class level. Even so, they are among the most phylogenetically diverse classes (Hug *et al.* 2016), and functional diversity could be beneficial in steel biofilm communities. *Sphingobacteriia* are known to be important members of aquatic biofilms that degrade biopolymers such as cellulose and chitin that tend to accumulate on these surface environments (Sack *et al.* 2014; Battin *et al.* 2016). Less is known about *Cytophagia* functional roles, but they are closely related in phylogeny to *Sphingobacteriia*. *Anaerolineaceae* are anaerobic fermenters of a wide variety of carbon sources (McIlroy *et al.* 2017) and could play an important functional role in the deeper biofilm layers closest to the surface environment. *Verrucomicrobiaceae* are known to be general heterotrophs and are also capable of degrading biopolymers such as cellulose, chitin, and xylan (Cabello-Yeves *et al.* 2017). *Chitinophagaceae* and *Rheinheimera* are both generalist heterotrophs that contribute to, or have genes associated with biofilm production and functional stability (e.g. pilin proteins) (Schuster and Szewzyk 2016; Sack *et al.* 2014; Mai-Prochnow *et al.* 2004).

Recent studies have shown supporting evidence of the importance of generalist taxa on steel biofilm communities. Moura *et al.* (2018) found increased rates of corrosion with the presence of generalists such as *Rhodobacteraceae*,

Flavobacteriaceae, and *Pseudomonadaceae* in marine microcosm experiments. Mugge *et al.* (2019) similarly found increasing abundances of *Alphaproteobacteria*, *Flavobacteriia*, *Saprospiraceae*, and *Rhodobacteraceae* on steel surfaces over time in another marine microcosm corrosion experiment. Steel biofilm communities can often contain carbon-based organic material from many different sources accumulated on the surface environment and thus limit both the attachment ability of other organisms and access to underlying iron sources for growth. Without heterotrophic generalists capable of degrading these potentially complex carbon-based materials, steel biofilms would likely have much lower functional potential. Generalist species have also been shown to be less affected by environmental factors (Pandit *et al.* 2009), suggesting a potential competitive advantage over specialists in environments that are subjected to constant change such as steel surfaces in estuaries. Steel surfaces found in more static environments could presumably exhibit smaller proportions of generalists and greater proportions of specialists resulting in less biofilm protection and stability, and thus may be an interesting direction for future studies. We also acknowledge that the steel surface community compositions were phylogenetically separated across the spectrum of salinity in this study (0 – 17.9 ppt), and there were not enough substrate type comparisons to make a strong case for classification of a low salinity core steel microbiome. Additional studies could potentially resolve this with an increased number of steel types sampled from low salinity sites.

The higher abundance of glycoside hydrolase genes on steel surface samples compared to surface controls suggests that cellulose-, chitin- and polysaccharide-

degraders, which are important members of marine biofilm communities (Christensen *et al.* 1998; Vu *et al.* 2009), are also important to steel surface microbial communities and may even have a syntrophic advantage to colonizing alongside corrosion-causing microbes. The oyster shell and biofoul surface controls likely also have biofilms within which these genes would be beneficial, thus the higher abundance of glycoside hydrolase found on steel samples suggests a potential greater need for the gene on steel compared to the surface controls. All of the high-quality MAGs on the steel surface samples, except for the *Sulfurovum* sp. (sulfur-oxidizer), also contained glycoside hydrolase genes, further supporting these MAGs as being potential core steel microbes, and glycoside hydrolase as an important functional gene for biofilm communities. Other biofilm-related genes were found within the MAGs, such as pilin functional genes, which are critical to surface colonization, biofilm formation, and social interactions with other cells (Tomaras *et al.* 2003; Mandlik *et al.* 2008; Anyan *et al.* 2014). The presence of these genes decreases the possibility that any of these organisms were found on these steel surfaces by chance alone. Potential future studies could clarify further by using a higher number of samples for shotgun metagenomics and/or single cell genomics in order to identify additional genomic trends across steel surface associated MAGs. The steel surface samples also exhibited higher proportions of iron oxidation/reduction and sulfur oxidation/reduction genes compared to surface controls, consistent with these steel surface samples hosting a microbial community that is capable of corrosion metabolisms. The stainless steel samples used for metagenomic analysis were chosen due to the orange rusted appearance of the 316SS coupon, but not on the 304SS coupon, in both cases. Even with the difference in visual appearance, the abundance of

iron, sulfur, and glycoside hydrolase functional genes were comparable across 304SS, 316SS, and ship steel samples. Both of the visually corroded stainless steel coupons were also from low salinity sites (6 ppt and 3.6 ppt), and as a result, salinity did not seem to have a significant impact on functional potential of steel microbial communities regardless of any biogeochemical processes that were occurring on the steel surface.

As seen in the results above, steel surface samples were highly separated based on phylogeny due to both salinity and substrate. However, a large suite of functional genes seemed to be conserved across these salinity and substrate barriers. This is in contrast to previous work showing functional trade-offs and divergence accompanying phylogenetic divergence for microbial communities in the water column across salinity gradients (Dupont *et al.* 2014). It is possible that steel surface communities exhibit less functional divergence across salinity compared to pelagic communities. This could be due to increased concentrations and more consistent supplies of nutrients on steel surfaces compared to the water column (copiotrophic vs. oligotrophic). In addition, the majority of bacterial functional traits have been shown to be largely polyphyletic (Martiny *et al.* 2013), and this characteristic may be featured more strongly on these surface communities. The findings in this study support the notion that bacterial functional roles can be both polyphyletic and conserved on steel surface communities. The increased selectiveness inferred across substrates at higher salinities also suggests that freshwater taxa associated with steel surface communities may have a greater number of functional roles compared to higher salinity taxa, or that a greater number of taxa from higher salinities perform the same functional roles within the surface communities.

We acknowledge that these results are merely a first step in determining the importance and role of core steel microbiomes, and additional studies focusing on their functional potential across a larger number of samples and within other environment types and steel types will be needed to fully understand submerged steel surface microbiomes and how they might compare to other core microbiomes. The results from this study show that functional potential within these communities can be maintained across salinity, substrate, and timescale. Heterotrophic generalist taxa appear to be important members of the core steel microbiome that likely contribute to the overall formation and stability of these biofilm communities. These submerged steel structures seem to be important participants not only for iron and sulfur cycles, but also for carbon and other heterotrophic nutrient cycling processes in the environment. This study also supports core microbiomes as being important for ecosystem health and function in environments other than the often-referenced host-associated systems. The core steel microbiome “behind the scenes” is not always easily observed compared to microbes associated with visible corrosion by-products, but its role in biofilm formation and functional stability is important for the overall steel community health. As our understanding of these core steel microbiomes improves even further, we can ultimately gain a stronger grasp on MIC prevention and the overall fate of submerged steel structures.

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Supplemental Materials:

Table III.S1. Descriptions of sampling sites used for sequence data analysis.

Site	Site ID	Months Sampled	Retrieval Salinity (ppt)	Substrate Samples	Sequence Techniques
Estuarium (Washington, NC)	P1	February 2017, May 2017	0.1, 0.1	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Mallard Creek (Washington, NC)	P3	February 2017, May 2017	4, 2.6	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Goose Creek (Washington, NC)	P5	February 2017	3.6	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
North Creek (Bath, NC)	P7	February 2017, May 2017	7.3, 7.4	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Wright's Creek (Belhaven, NC)	P9	February 2017, May 2017	10.1, 10.1	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Fisher's Landing (New Bern, NC)	N1	May 2017	1.8	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Cahooque Creek (Havelock, NC)	N3	May 2017	3.6	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹ , Metagenomics ²
Pine Cliff (Havelock, NC)	N5	February 2017	7.2	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Cedar Island (Cedar Island, NC)	N9	May 2017	15.8	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹
Matthew's Point (Havelock, NC)	N11	May 2017	6	304SS, 316SS, Biofoul, Sediment	16S Amplicon ¹ , Metagenomics ²
Pappy's Lane Shipwreck (Rodanthe, NC)	Shipwreck	September 2017	17.9	Visibly Corroded Ship (Orange, Black, or Both), Oyster-ship Substrate, Seawater, Sediment, Drilled Shipcore	16S Amplicon ¹ , Metagenomics ²

				Above and Below Sediment Interface	
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¹ = 16S rRNA v4-v5 Illumina PE sequencing; ² = Illumina PE 2x 150bp sequencing

Table III.S2. Descriptions of high-quality metagenome assembled genomes (MAGs) obtained from stainless steel and shipwreck sites.

MAG#	Sample Type	Bin Method	Complete (%)	Contamination (%)	Identification	Identification method	Genome size (Mbp)	GC content (%)
1	304SS	Maxbin	51.65	5.17	<i>Rheinheimera</i> sp.	MetaSanity	3.12	47.9
2	304SS	Concoct	79.91	2.01	<i>Chitinophagaceae</i>	MetaSanity	2.5	38.1
3	316SS	Concoct	87.61	3.97	<i>Rheinheimera</i> sp.	16S rRNA BLAST	3.89	47.6
4	316SS	Concoct	69.03	1.48	<i>Chitinophagaceae</i>	16S rRNA BLAST	2.18	38.2
5	Ship	Concoct	73	3.58	<i>Sulfurovum</i> sp.	16S rRNA barnap	1.44	39.7
6	Ship Sediment	Concoct	68.54	5.07	<i>Sulfurovum</i> sp.	MetaSanity	1.53	40.2
7	Biofoul	Maxbin	55.78	4.25	<i>Chitinophagaceae</i>	16S rRNA barnap	1.76	38.6
8	Seawater	Maxbin	100	1.7	<i>Cryomorphaceae</i>	MetaSanity	2.75	47.8
9	Seawater	Concoct	84.6	7.04	<i>Litoricola</i>	MetaSanity	1.55	50.6
10	Seawater	Concoct	83.32	5.76	<i>Synechococcus</i>	MetaSanity	1.96	64.2
11	Seawater	Concoct	72.95	6.26	<i>Burkholderiaceae</i>	MetaSanity	1.02	53.9

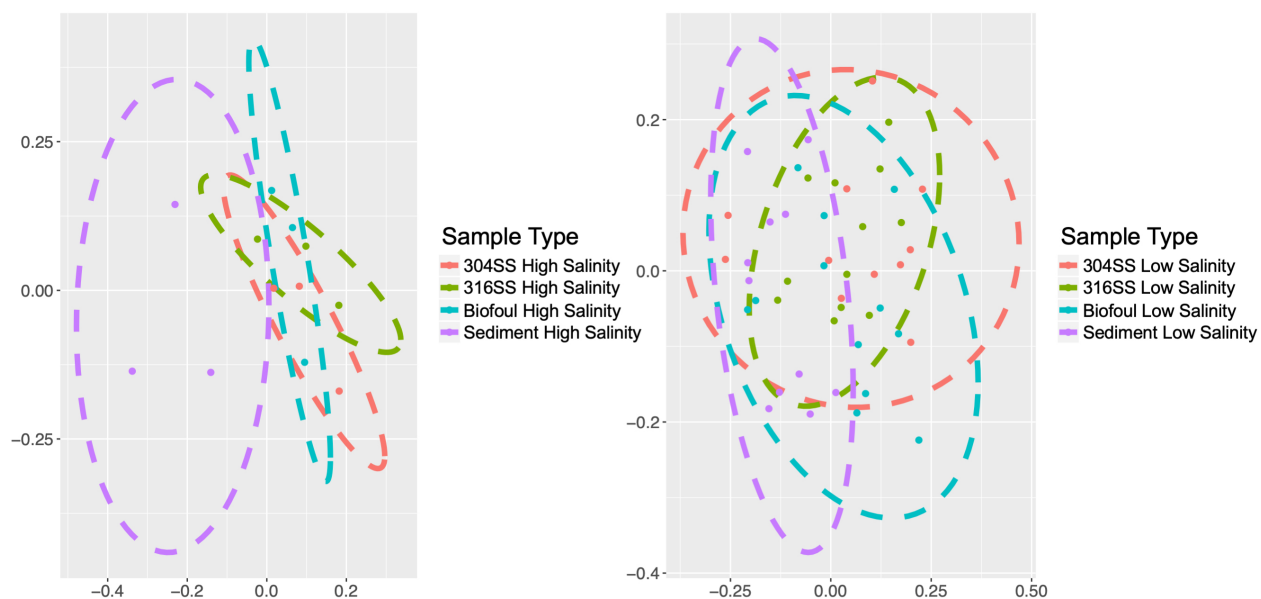


Fig III.S1. Non-metric multidimensional scaling plots of stainless steel site samples based on 16S rRNA gene sequencing data. Each datapoint represents a unique microbial community

composition from one sample, and distance between points represents the degree of difference between community composition. Stainless steel and biofoul samples were significantly different from sediment at salinities > 10 ppt (left) (ANOSIM test: $R = 0.4872$, $p = 0.0232$), but not at salinities < 10 ppt (right).

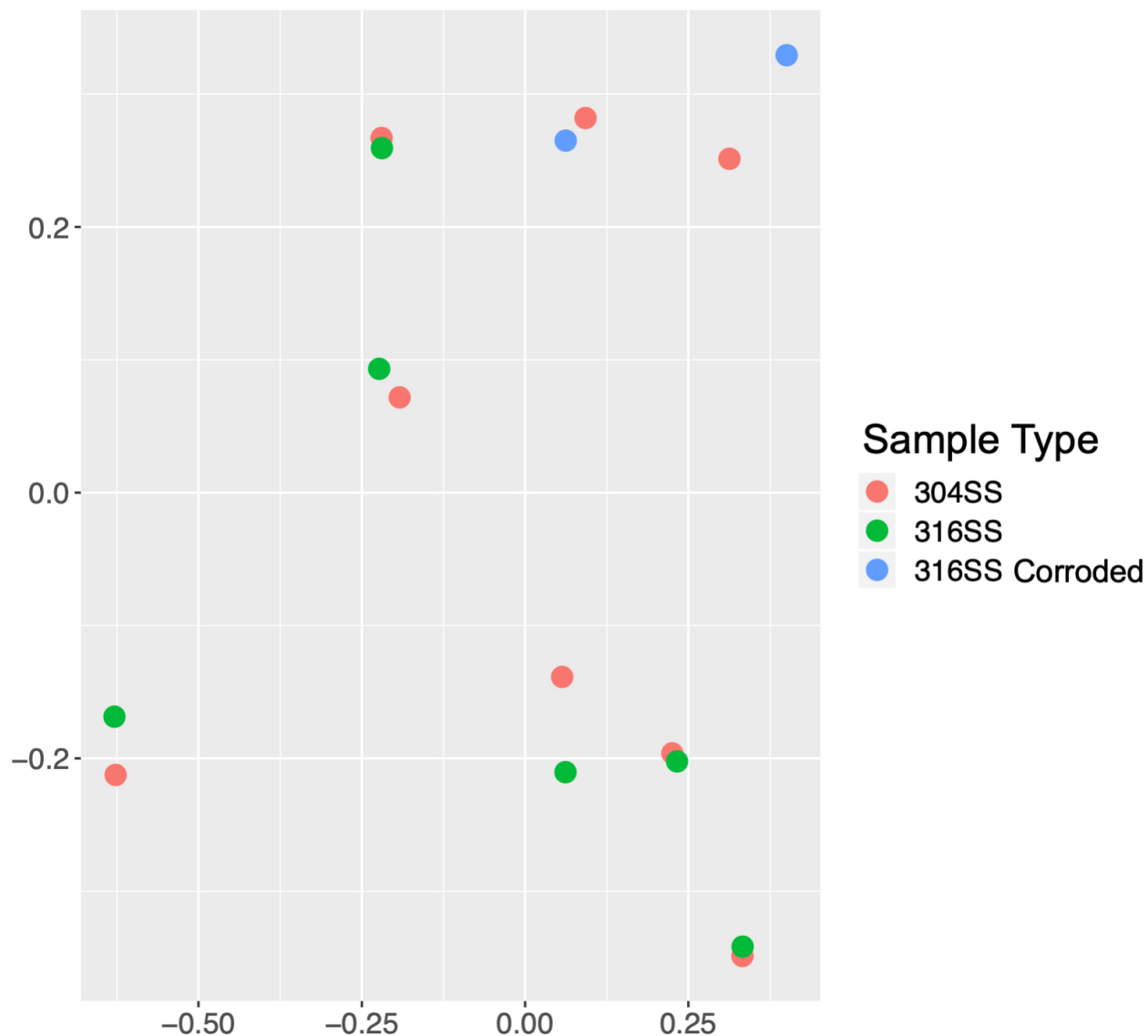


Fig III.S2. Non-metric multidimensional scaling plot of stainless steel samples based on 16S rRNA gene sequencing data. Each datapoint represents a unique microbial community composition from one sample, and distance between points represents the degree of difference between community composition. Each pair of 304SS and 316SS samples group together based on site. Two corroded samples (blue) had no difference in overall microbial community

composition compared to the non-corroded 304SS samples from the same corresponding deployment time and site.

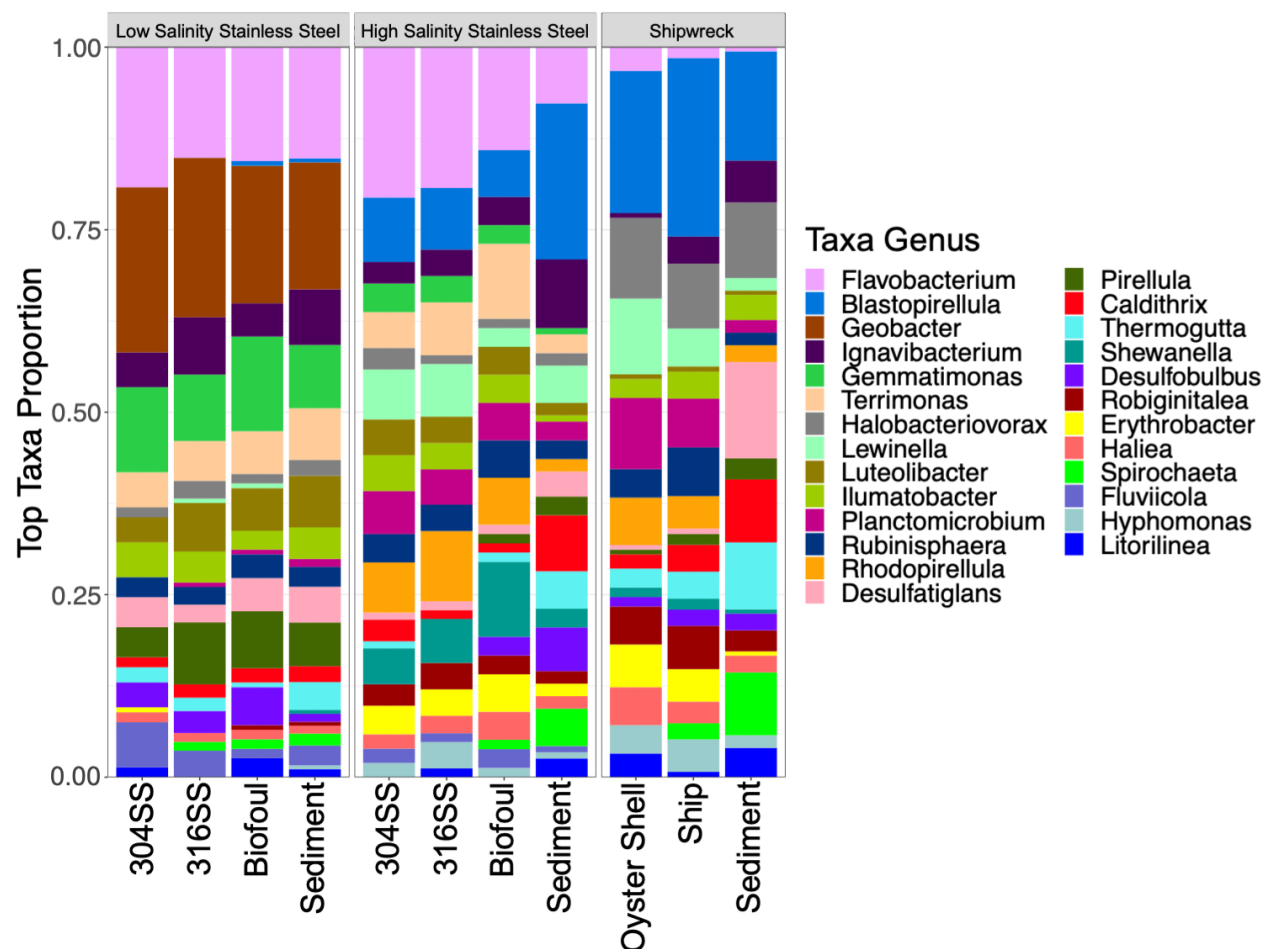


Fig III.S3. Genus-level taxa plot for different sample types based on 16S rRNA gene sequence classification. Higher salinity sites (> 10 ppt) showed greater phylogenetic differentiation across sample types than lower salinity sites (< 10 ppt). *Flavobacterium*, *Rhodopirellula*, *Erythrobacter*, and *Hyphomonas* were all found in higher proportion on the surface environment samples (steel and surface control) compared to the sediment at high salinity sites.

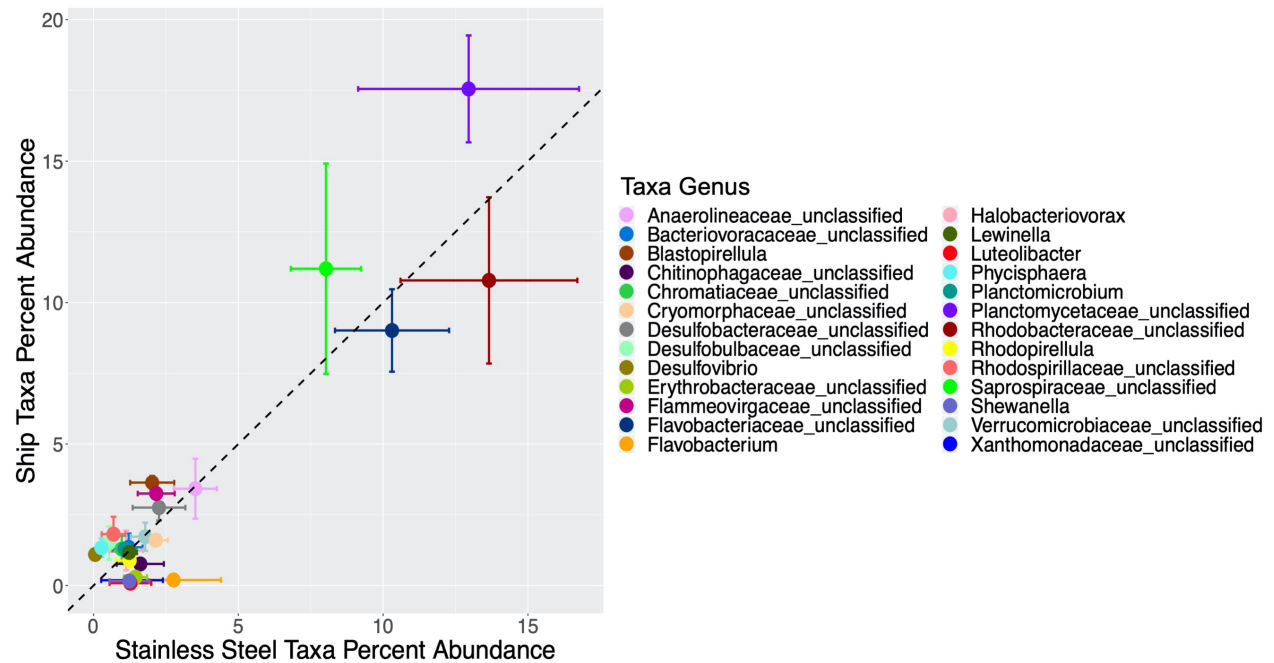


Fig III.S4. Stainless steel taxa abundance plotted against ship taxa abundance based on 16S rRNA classification at the genus level indicated that unclassified *Anaerolineaceae* and unclassified *Verrucomicrobiaceae* were closest to the 1:1 ratio line (with error bars overlapping the line) with > 1% abundance, and thus were most likely to be part of a core steel microbiome. Only high salinity stainless steel sites (> 10 ppt) were used to compare to shipwreck samples in this plot.

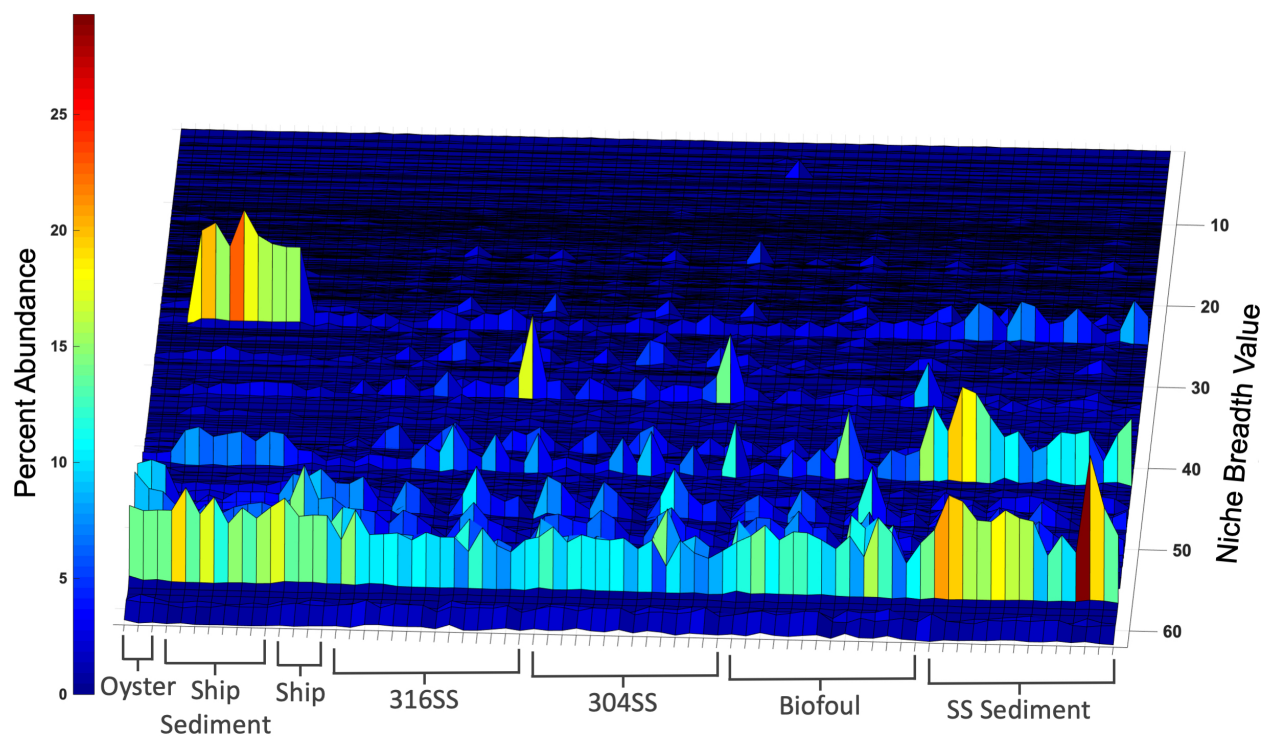


Fig III.S5. Levins' niche breadth analysis of all samples based on 16S rRNA gene sequence data. A lower niche breadth value indicates a specialist lifestyle and a higher niche breadth value indicates a generalist lifestyle. All samples had large proportions of high niche breadth values and thus generalist taxa, while sediment samples overall also had large proportions of low niche breadth values and thus a greater proportion of specialist microbes compared to surface environment samples.

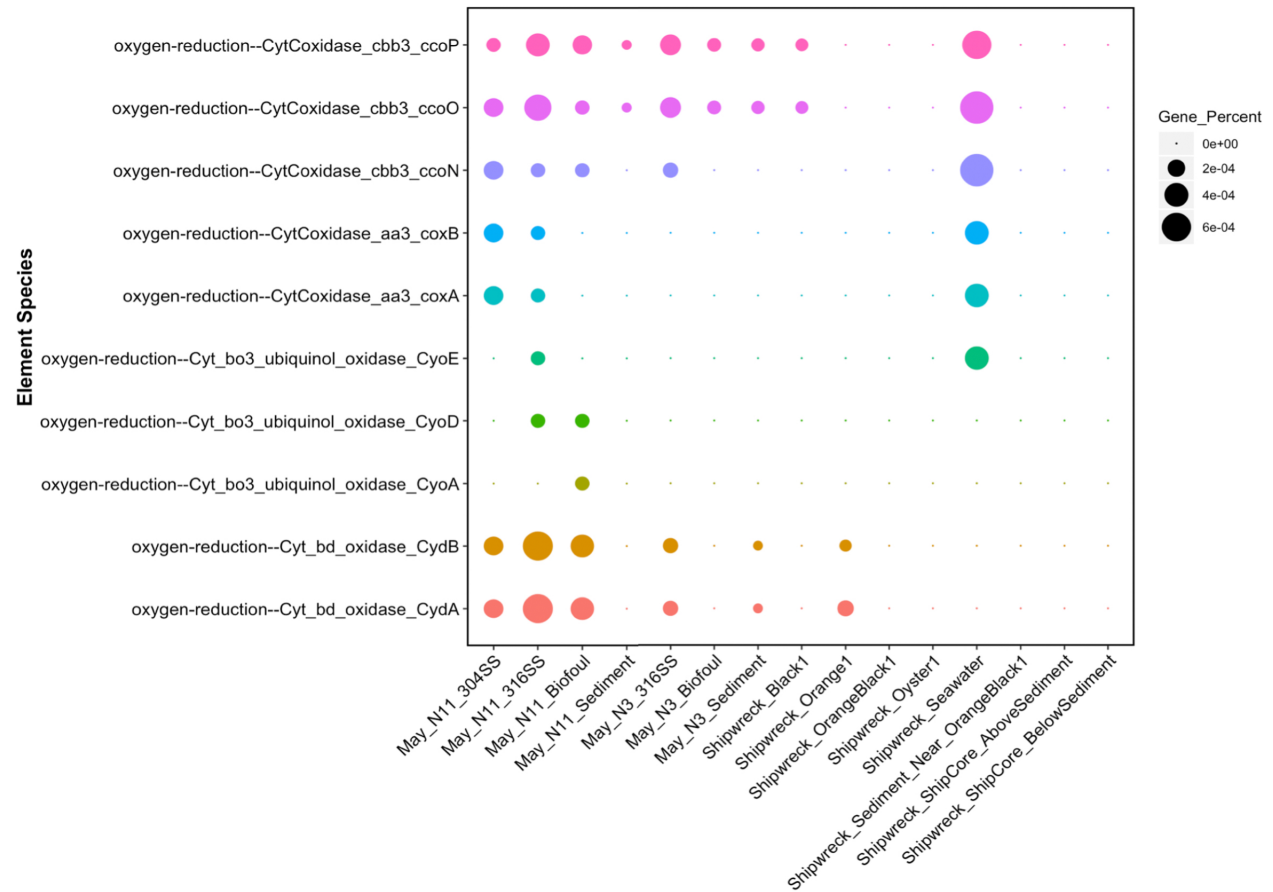


Fig III.S6. Hidden Markov model (HMM) classification of oxygen-related genes based on metagenomic sequencing data.

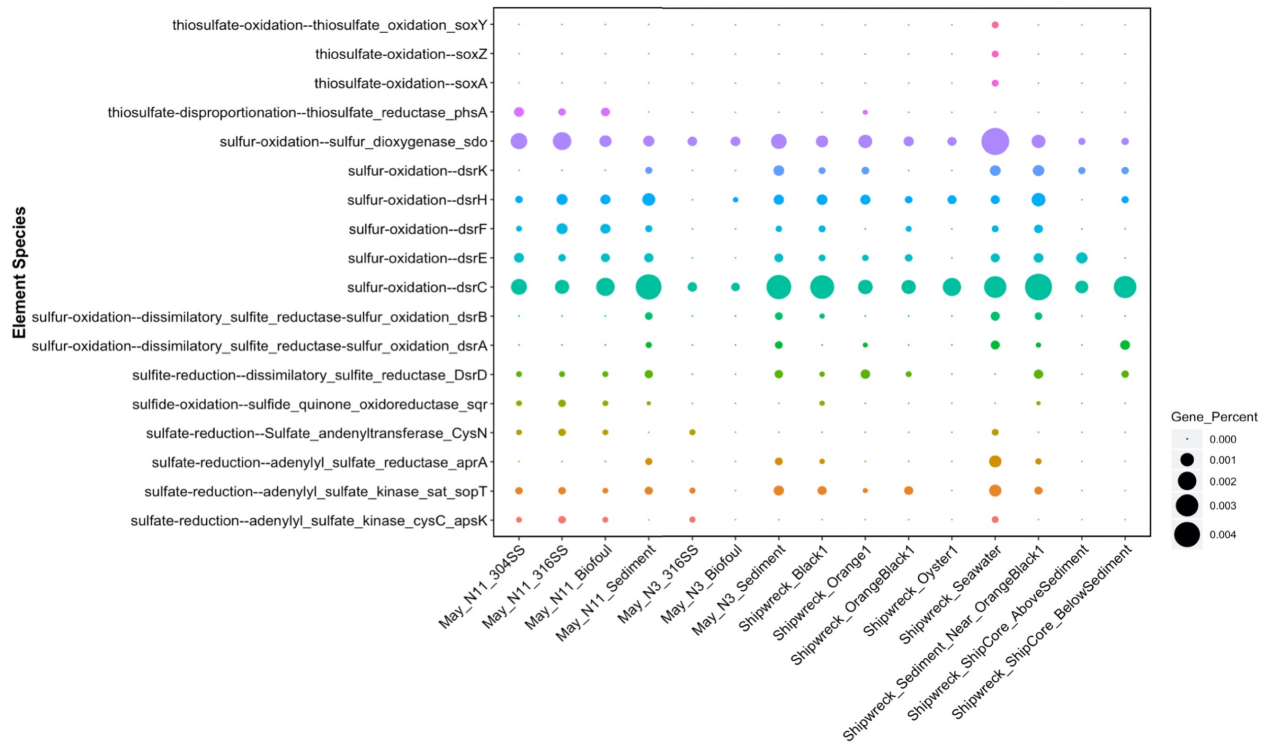


Fig III.S7. Hidden Markov model (HMM) classification of sulfur-related genes based on metagenomic sequencing data.

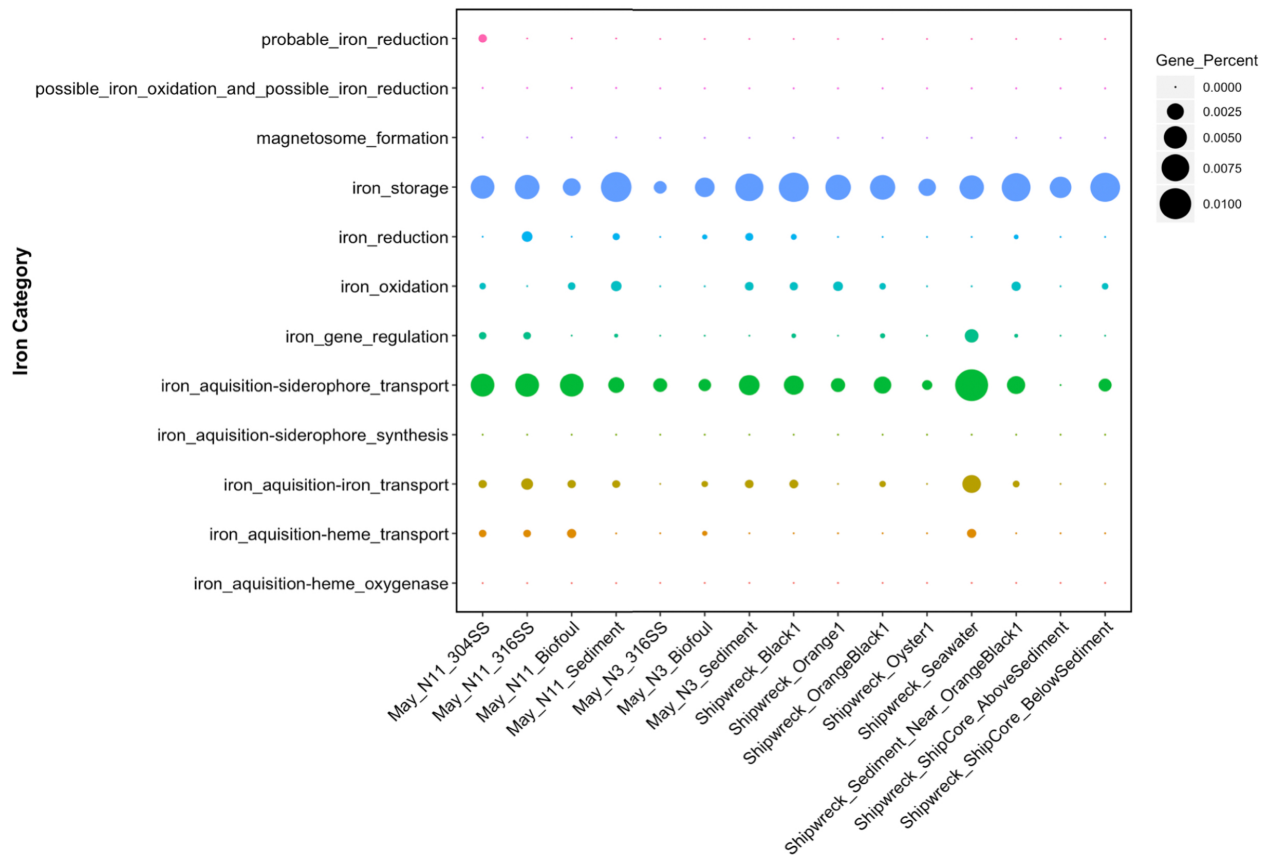


Fig III.S8. Hidden Markov model (HMM) classification of iron-related genes based on metagenomic sequencing data.

IV. EFFECTS OF SEQUENTIAL EXTREME STORM EVENTS ON AQUATIC MICROBIAL COMMUNITIES IN OUTER BANKS, NORTH CAROLINA

Abstract:

Extreme storm disturbances have the potential to dramatically alter ecosystem functioning if microbial communities are shifted from steady-state conditions. Storm intensities and frequencies are expected to increase under warming climate scenarios, making investigations into microbial community response to these disturbances a priority for predicting future ecosystem impacts. This study explores the microbial community dynamics following sequential hurricanes across a barrier island system, the Outer Banks, North Carolina. We found that extreme storms were correlated with shifts in the bacterial community structure but not within the archaeal community, and within the water column but not within the sediment. RNA-based community compositions differed from DNA-based, suggesting that the total and active portions of the hurricane-affected community differed, although both DNA and RNA-based profiles remained unique from non-hurricane conditions. We found evidence of microbial community shifts up to 77 days after the first storm and 50 days after the second storm, with significant implications for nutrient cycling in nearshore and offshore environments. Offshore sites near the Gulf Stream had lower microbial community evenness and Shannon diversity and increased relative abundance of copiotrophic microbes compared to non-hurricane conditions. Both carbon cycling and nitrogen cycling appeared to be impacted by the storms, with evidence of potential shifts from autotroph dominated to heterotroph dominated at offshore sites for a period of time. These shifts could have potentially

serious impacts on the balance of global carbon sinks and sources in an increasingly fragile global carbon budget. Further, understanding the geographic-dependent responses of coastal microbial communities to extreme storm disturbances is critical for predicting nutrient cycling and whole ecosystem impacts in current and future climate scenarios.

Introduction:

An increasingly warmer global climate has been linked to higher intensity and more frequent extreme storm events (i.e., tropical storms and hurricanes) (Emanuel 2005; Groisman *et al.* 2005; Knutson *et al.* 2010; Kunkel *et al.* 2010; Holland & Bruyère 2014). The extent to which these storm events might reshape local aquatic microbial communities remains unclear. Historical data suggest that the coast of North Carolina is at particularly high risk for tropical cyclones and hurricanes (Kunkel *et al.* 2010). The barrier island system that Outer Banks, North Carolina features is the shortest distance of anywhere on the East Coast of the United States between freshwater terrestrial inputs and the Gulf Stream at the edge of the continental shelf (~60 km). As a result, disruptions to coastal microbial communities in this area may have a greater potential to affect nutrient flow (i.e., C, N, P) within the Gulf Stream and the overall global ocean. Offshore marine sites near the Gulf Stream have important roles as net sinks for atmospheric CO₂ due to their high primary production which helps to balance increasing amounts of net sources linked to climate change (IPCC 2007; Chen & Borges 2009). Extreme storm events have been shown to lead to erosion and run-off of limited nutrients, followed by increased microbial production and respiration (Bianchi *et al.*

2013). In such situations, coastal and offshore aquatic environments may temporarily switch from net autotrophic to net heterotrophic. This could lead to a positive feedback loop with increasing atmospheric CO₂ causing warmer sea surface temperatures and more frequent tropical storms and hurricanes with high winds and precipitation.

Short timescales are often deemed suitable for environmental disturbance studies on microbial communities, largely due to biomass turnover times ranging from less than a day to one week (Fuhrman *et al.* 2015). Some extreme storm studies have supported this notion by showing significant impacts towards microbial communities in coastal environments lasting less than one week (Okinawa, Japan (Ares *et al.* 2020)), although timescales for a return to pre-storm conditions are not consistent for all storm events and geographic locations with some cases of disruption lasting weeks to months (Oahu, Hawaii (De Carlo *et al.* 2007; Yeo *et al.* 2013); New Orleans, Louisiana (Sinigalliano *et al.* 2007); Houston, Texas (Steichen *et al.* 2020)). These previous studies were all characterized by significant microbial community disruptions as a result of the storm events, often with increased relative abundance of terrestrial-based microbial taxa within these marine systems. These previous results further suggest that the magnitude of effect from tropical storms and hurricanes on coastal microbial communities (i.e., timescale for return to pre-storm conditions) is expected to be highly context dependent. For example, storm impacts could vary based on severity of the storm, proximity to urban areas with high levels of pollution, topography of the coastal region, and magnitude and length of tidal cycles for the area. Microbial community recovery timescale is important because it likely correlates with nutrient cycle disruption

timescale and overall health of an ecosystem. Investigating the variations in response of microbial communities to extreme storms under different contexts is crucial for fully understanding the impact of extreme storms on ecosystem processes. This study aims to provide further insight into the geographic- and system-dependent effects of extreme storms and how different contexts might lead to inconsistencies in timescale of microbial community recovery. Understanding the environmental fluctuations that lead to changes in local microbial communities are also important for increasing knowledge of the steady-state conditions, resiliency, and future climate change scenarios of an ecosystem.

One such mechanism that can lead to microbial community disruptions and increased bacterial production is increased input of allochthonous nutrients (Bergström and Jansson 2000; Zhang *et al.* 2019). Heavy precipitation events are often responsible for increased nutrient loads and overall negative impacts on water quality of coastal ecosystems via eutrophication and hypoxia, introduction of herbicides, dangerous chemicals, and pathogenic bacteria from run-off (Solo-Gabriele *et al.* 2000; Anderson and Taylor 2011; Chen *et al.* 2012; Lewis *et al.* 2012; Chen *et al.* 2018; Mistri *et al.* 2019). The coastal North Carolina region in particular has seen doubling of annual nitrogen loads, tripling of annual phosphorous loads, and enough organic carbon input to flip the coastal region from a net sink to a net source of CO₂ during years with at least one major storm ($\sim 10^6$ kg N, $\sim 10^5$ kg P, and $\sim 10^7$ kg DOC input over a 2 week period following Florence in 2018) (Paerl *et al.* 2018; Paerl *et al.* 2020). This is largely a result of the long residence time (~ 1 year) that characterizes the local Albemarle-Pamlico

estuarine system that flows into the coastal marine waters of North Carolina (Paerl *et al.* 2001). Hurricane Matthew in 2016 significantly impacted the local ecosystem, accounting for ~25% of the total annual carbon input to the Neuse-Pamlico Estuary (Osburn *et al.* 2019). Terrigenous organic matter persisted in the estuarine watershed for several months following Matthew, likely impacting the microbial community and overall ecosystem functioning. It is important to determine how entire microbial communities are affected by extreme storms in these areas characterized by long residence times, and to what extent these impacts reach offshore sites near the Gulf Stream. The goal of this study was to characterize the microbial response to extreme storms specific to the coastal North Carolina area, resolve potential timeframes of microbial community recovery, and compare the degree of impact in marine versus estuarine environments. Significant impacts to offshore marine sites could ultimately lead to greater microbiological impacts to nutrient cycling across warming global oceans.

Carbon “priming” is an example of allochthonous nutrient input that can lead to amplified bacterial production and respiration (Bingemann *et al.* 1953; Bianchi 2011). Priming results from a large influx of allochthonous organic material into a coastal marine system, similar to conditions observed for coastal North Carolina following Hurricanes Florence and Michael in 2018. The carbon priming process is driven by low-molecular-weight plant materials that are highly reactive or easily degradable by microbes (e.g., monosaccharides) (Bingemann *et al.* 1953; Kuzyakov *et al.* 2000) but can also be accompanied by less reactive plant materials (e.g., lignin) that persist

longer in the system (Takii 2003; Bianchi 2011). Priming increases microbial biomass and the production of extracellular enzymes that allow breakdown of less reactive local organic matter (Bianchi 2011). Carbon priming leads to overall higher microbial production and respiration than that which would occur from each local carbon pool without storm-induced surging and flushing of river waters into marine systems. This phenomenon could have serious consequences for the coastal North Carolina carbon budget. Investigating the impact of storm events on the relative abundance of specific genes recovered from microbial communities in response to elevated nutrient loading can further aid in understanding and predicting how future environmental conditions might shape bottom-up regulation of ecosystem processes.

The 2018 hurricane season presented a unique opportunity to investigate the impact of sequential extreme storms on microbial communities and subsequent biogeochemical processes in the coastal North Carolina ecosystem. Hurricane Florence landed on the coast of North Carolina on September 14, 2018. Some areas reported record-breaking rainfall over 76 cm and wind gusts over 45 m s^{-1} (National Weather Service 2018). Florence was a slow-moving and persistent storm system that delivered precipitation for 7 days, creating flood levels for inland rivers that have rarely been seen before. Floodwaters in some Outer Banks areas reached 120 cm (National Weather Service 2018). A second storm, Hurricane Michael (2018) also struck North Carolina after being downgraded to a tropical storm prior to impacting its coast on October 11, 2018. However, Hurricane Michael led to wind gusts over 33 m s^{-1} and floodwaters reaching 94 cm (National Weather Service 2018). The combination of two extreme

storm events relatively close together in timeframe provide a unique view of sequential-storm conditions that may be more frequently observed under warmer future conditions. Eastern North Carolina is a largely rural area with high concentrations of agricultural and animal production that likely have serious coastal ecosystem consequences as a result of storm-induced floodwaters and run-off. Reports of increased relative abundance of foodborne zoonotic pathogens due to Hurricane Florence has already been reported for watersheds of North Carolina that flow into the estuaries of the Outer Banks (Niedermeyer *et al.* 2020). Investigations into the impact of Hurricane Florence and Michael on the estuarine and marine microbial communities in this area is critical to assessing the ecosystem-level consequences.

The analysis of both DNA and RNA from microbial communities provides additional insight into the potential of warming global climates to impact microbial community function and nutrient cycling. Microbial RNA surveys can identify “active” members of microbial communities at the time of sampling while “total” microbial community measurements can be obtained from DNA-based community profiles (Campbell *et al.* 2009). RNA sampling can improve overall analyses because total microbial communities identified from DNA profiles may also include extracellular DNA (Dell’Anno *et al.* 1998), as well as dead or dormant microbial cells that are likely not contributing to nutrient cycling or ecosystem functioning (Freedman *et al.* 2015; Cardoso *et al.* 2017). RNA-based microbial profiling has seldom been used in extreme storm studies on microbial communities and could provide a benchmark for correlating DNA surveys to active functional members of hurricane-impacted communities affecting

ecosystem nutrient flow. Nucleic acid analyses of both sediment and water column samples further provide a more comprehensive view of extreme storm impacts across different substrates or environment types. Analyses of functional gene abundance via shotgun metagenomic sequencing can also link putative biological function to microbial phylogeny associated with extreme storm impacts on microbial communities in past, present, and future studies. These combined methods can ultimately be used to accomplish the overall goal of determining extreme weather disturbance impacts on microbial community functional potential and subsequent implications for ecosystem stability.

Methods:

Sample collections and processing:

Fifteen surface water samples and thirteen sediment samples were collected aseptically in sterilized plastic containers on October 25 and 26, and November 30, 2018 from the Coastal Studies Institute Research Vessel “Blackbeard”, with salinities ranging from 4.2 ppt within the estuary to 33.5 ppt closest to the Gulf Stream (Fig. IV.1) (Table IV.S1). These “hurricane affected” samples were then compared to “non-hurricane affected” surface water and sediment samples collected on July 2 and 3, 2019 (11 surface water and 12 sediment; salinities ranging 8.5 – 29.9 ppt) and March 17, 18 and 22, 2016 (2 surface water and 10 sediment; salinities ranging 30.4 – 36.4 ppt). Hurricane affected sites were chosen with the intention of surveying microbial communities exposed to both the northern (i.e., the Albemarle Sound) and the southern regions (i.e., the Pamlico Sound) of the Albemarle-Pamlico estuary, as well as within

Oregon Inlet and along an E-W transect towards the edge of the continental shelf and Gulf Stream. Geochemical measurements including total suspended solids, dissolved organic carbon, total dissolved nitrogen, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ were also conducted (Mitra *et al.* 2020). Surface water from each site was stored on ice immediately following collection and during transport to the lab. Water samples were aseptically filtered onto MilliporeSigma™ polyethersulfone 0.22 μm filters to capture microbiological cells. Filtered surface water volumes ranged from 200 mL to 4 L depending on how quickly the filter became clogged by particulate material; however, all analyses presented in this study utilize relative microbial abundance rather than absolute abundance. As a result, variations in filtered surface water volumes were insignificant to this study. Whole, separate filters were used for individual nucleic acid extraction methods (DNA and RNA). Filtered water samples were extracted for DNA and RNA using MoBio DNeasy and RNeasy Power Water kits (Qiagen, Inc.). Bulk sediment samples were collected using a Van Veen grab sampler wherever feasible based on water depth and strength of current, and subsamples for analysis were collected aseptically from bulk samples. Sediment samples were placed on dry ice immediately following collection and were stored at -80 °C up until nucleic acid extractions. Sediment samples (~0.25 g) were extracted for DNA and RNA using MoBio DNeasy and RNeasy Power Soil kits (Qiagen, Inc.). Both DNA and RNA were extracted to identify the “total” and “active” microbial communities, respectively, within the water column and sediment at the time of sampling. Extracted RNA was converted to cDNA using a High-capacity RNA-to-cDNA reverse transcription kit (Thermo Fisher Applied Biosystems).

Nucleic acid sequencing and analysis:

Extracted DNA and cDNA were both sequenced for 16S rRNA gene amplicon sequencing to identify microbial phylogeny. 16S rRNA gene amplicon sequencing was performed by CGEB Integrated Microbiome Resource (Dalhousie University, Halifax, Canada). Amplicon sequencing utilized a Phusion polymerase PCR amplification method (Comeau *et al.* 2017) on an Illumina MiSeq platform, targeting the V4-V5 region of the 16S rRNA gene in order to capture the largest majority of microbial phylogeny (Yang *et al.* 2016). Amplicon sequence data were processed using mothur v.1.41.3 (Schloss *et al.* 2009) and its associated analysis pipeline (Kozich *et al.* 2013). Phylogeny was determined based on 97% OTU classifications. Canonical Correspondence Analysis plots were created in R using vegan and ggplot2 packages with the metaMDS function and a Bray-Curtis similarity matrix (R Core Team 2013). An analysis of similarities (ANOSIM (Clarke 1993; Warton *et al.* 2012)) was used to determine significant differences between sample groups in R, and a permutational multivariate analysis of variance (PERMANOVA (Anderson 2005); Adonis test in R) was used to determine significance and correlation between environmental variables and microbial community composition. The vegan package in R was further used to determine community diversity indices (richness, evenness, and Shannon diversity (Shannon 1948)) across all samples. Boxplots depict 5 key summary statistics including the median, 25% and 75% percentiles, and the feasible range of the data ($\pm 1.5 \times$ the interquartile range) represented by the two whiskers. Significant differences between sample groups' diversity indices were confirmed using a Mann-Whitney U test (McKnight 2010) (wilcox.test function in R). A similarity percentage analysis (SIMPER

(Clarke 1993; Warton *et al.* 2012) was used to evaluate microbial taxa with the most significant influence towards differences in community composition between sample groups. rRNA (*rrn*) copy numbers were determined using RDP classifier v.2.13 (Wang *et al.* 2007) combined with the *rrn* operon database (Stoddard *et al.* 2015) (Available at <http://rdp.cme.msu.edu/classifier/classifier.jsp>). Data analysis R code can be found at https://github.com/cgarr017/ECU_dissertation_code/blob/main/chapter3.

Ten DNA samples (Hurricane affected: 2018: 1 marine and 2 estuarine surface water samples, 1 sediment sample; Non-hurricane affected: 2016: 2 marine surface water samples; 2019: 2 marine and 1 estuarine surface water samples, 1 sediment sample) were also sequenced using shotgun metagenomics to identify functional genes associated with hurricane-impacted coastal microbial communities. Shotgun metagenomic sequencing was performed by CGEB Integrated Microbiome Resource (Dalhousie University, Halifax, Canada). Samples were sequenced using an Illumina Nextera Flexkit method (Comeau *et al.* 2017) with an Illumina NextSeq platform and 2x sequencing depth in order to obtain roughly 8 million paired-end reads (150+150 bp) (i.e., 16 million single reads) and roughly 2.4 Gb per sample. Resulting raw reads were then trimmed using TrimGalore v.0.4.5 (Krueger 2012) and checked for quality using FastQC v.0.11.8 (Andrews 2010). After trimming, reads were concatenated and assembled using SPAdes v.3.13.0 (Bankevich *et al.* 2012). MG-RAST v.4.0.3 (Meyer *et al.* 2008) was used to annotate assembled contigs, and annotations were manually searched for carbon cycling genes (i.e., lignin-degradation genes) according to those described by Janusz *et al.* (2017). KBase UI v.2.2.1 (Arkin *et al.* 2018) was used to run

DRAM (Shaffer *et al.* 2020) for metabolic profile functional annotations of other common carbon and nitrogen cycling pathways.

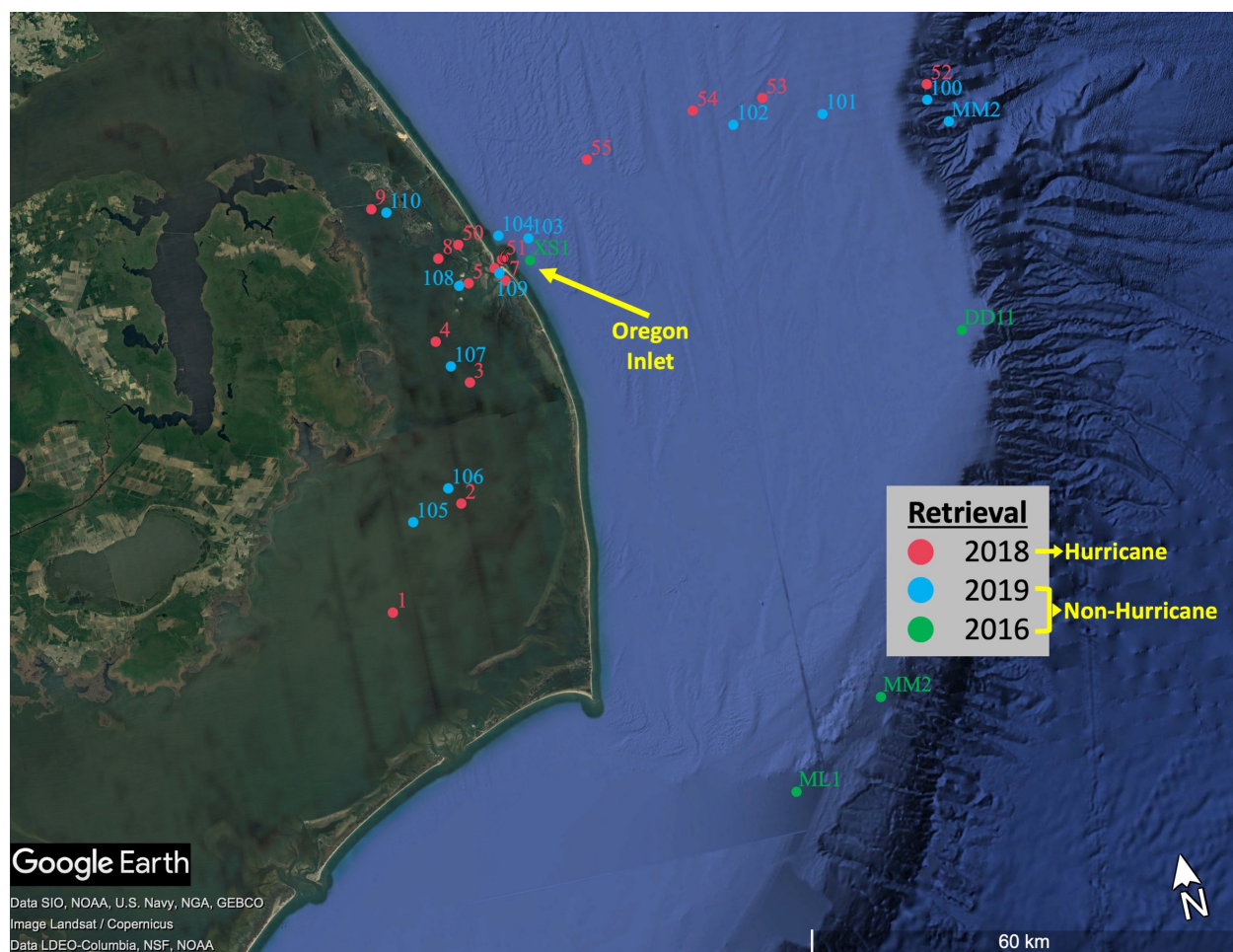


Fig. IV.1. Map of sampling sites from each retrieval. Sites represent both the northern (i.e., the Albemarle Sound) and southern region (i.e., the Pamlico Sound) of the Albemarle-Pamlico estuary, as well as within Oregon Inlet and along an E-W transect towards the edge of the continental shelf and Gulf Stream.

Results and Discussion:

Extreme storms correlated with total (DNA) and active (RNA) bacterial communities but not archaeal communities, and within water column but not in sediment

Bacterial communities isolated from within the water column during the 2018 hurricane season (hurricane effect) were significantly different compared to bacterial community samples taken in 2016 and 2019 (non-hurricane effect) (ANOSIM test: $R = 0.185$, $p < 0.001$) (Fig. IV.2A). Salinity was the most influential variable affecting community composition in the water column, followed by hurricane effect (sampling time point) and then total vs active community (DNA vs RNA), although all three were significant (Fig. IV.2A) (PERMANOVA test: Salinity: $R^2 = 0.357$, $p < 0.05$; Hurricane effect: $R^2 = 0.105$, $p < 0.05$; DNA vs RNA: $R^2 = 0.102$, $p < 0.05$). The persistence of bacterial community shifts within the water-column from the start of the storms for 77 days suggests that extreme storms likely affected biogeochemical processes in this study site for longer time periods than those of similar studies (i.e., Sinigalliano *et al.* 2007, Steichen *et al.* 2020, and Ares *et al.* 2020). These longer disturbance times could be correlated with the long geochemical residence times that define the region (Paerl *et al.* 2001). The extremely low-lying characteristics of the region further compound the effect of inland flooding and nutrient discharge following tropical storms and hurricanes. The high nutrient loads accompanying the inland wetlands and agricultural areas likely fuel bacterial production in the estuarine and marine environments downstream, causing shifts in the bacterial community composition and functional potential.

Sediment bacterial community compositions were not significantly impacted by the hurricanes but did exhibit significant differences between DNA and RNA, and across salinity and water depth (DNA vs RNA: $R^2 = 0.31$, $p < 0.05$; Salinity: $R^2 = 0.059$, $p < 0.05$; Water depth: $R^2 = 0.043$, $p < 0.05$; Hurricane effect: $R^2 = 0.019$, $p = 0.11$) (Fig. IV.2B). The lack of impact on sediment microbial communities suggests that any potential settling of terrestrial microbes from the water column onto the sediment following the storm events likely did not affect sediment microbial community structure or function within the time frames of sampling in this study. Further, these sediment communities may be more resistant or resilient to system-wide disturbances and geochemical fluctuations compared to those within the water column (Bowen *et al.* 2011). This would be consistent with sediment communities exhibiting high rates of cell dormancy, inactivity, and endospore formation (Luna *et al.* 2002; Wörmer *et al.* 2019).

Archaeal community analyses are often not addressed in extreme storm studies on microbial communities, and these results illuminate a clear contrast in response of archaeal communities to extreme storms compared to bacterial communities. Archaea within the water column had no significant influence from the hurricane effect or from DNA vs RNA, while salinity was significant (Salinity: $R^2 = 0.377$, $p < 0.05$; DNA vs RNA: $R^2 = 0.023$, $p = 0.13$; Hurricane effect: $R^2 = 0.011$, $p = 0.41$) (Fig. IV.2C). Archaeal sediment communities also had no significant influence from the hurricane effect, while DNA vs RNA, salinity and water depth were all significant (DNA vs RNA: $R^2 = 0.305$, $p < 0.05$; Salinity: $R^2 = 0.152$, $p < 0.05$; Water depth: $R^2 = 0.060$, $p < 0.05$; Hurricane effect: $R^2 = 0.019$, $p = 0.07$) (Fig. IV.2D). Archaea have been shown to have lower phylogenetic diversity in terrestrial soil environments than any other environment type

(0.72 ± 0.71 Shannon index value in soils compared to 1.70 ± 0.63 in marine sediments) (Auguet *et al.* 2010) which may lead to relatively minimal amounts of archaeal cells getting flushed seaward due to river surging and flooding, although much is still unknown about the diversity of archaea. Once thought to be mainly confined to sediments, marine archaea are now known to be ubiquitous within the water column (DeLong 1992; Santoro *et al.* 2019).

Planktonic marine archaea, similar to bacteria, are important contributors to nitrogen cycling via ammonia-oxidation pathways and to carbon cycling via organic carbon metabolism and inorganic carbon fixation (Santoro *et al.* 2019). Some marine archaeal groups have genomes that appear to be specialized in high-molecular weight compound degradation (Santoro *et al.* 2019) and could play a role in the breakdown of higher complexity terrestrial organic matter introductions into marine systems. It is possible that the contrast in hurricane effect towards bacterial and archaeal communities in this study could be a result of large portions of bacterial taxa outcompeting archaea and increasing abundance in response of newly introduced nutrients. Alternatively, bacterial taxa in this environment may be more adaptable to high nutrient concentrations compared to the resident archaeal taxa. Either scenario could have led to suppressed growth or minimal shifts in archaeal community structure during hurricane conditions. Archaeal communities localized to this study area may also be characterized by metabolic lifestyles unaffected by hurricane conditions (e.g., chemoautotrophy). The vast capabilities of archaeal metabolism such as methane and alkane oxidation and bacterial symbiosis have only recently started to come to light (Baker *et al.* 2020), and this metabolic diversity could potentially play a role in the

stability of archaeal communities during hurricane conditions. Further studies focusing on increased metagenomic sequencing depth and on a greater number of samples will help to identify trends in archaeal genes and genomes across disturbances such as hurricanes.

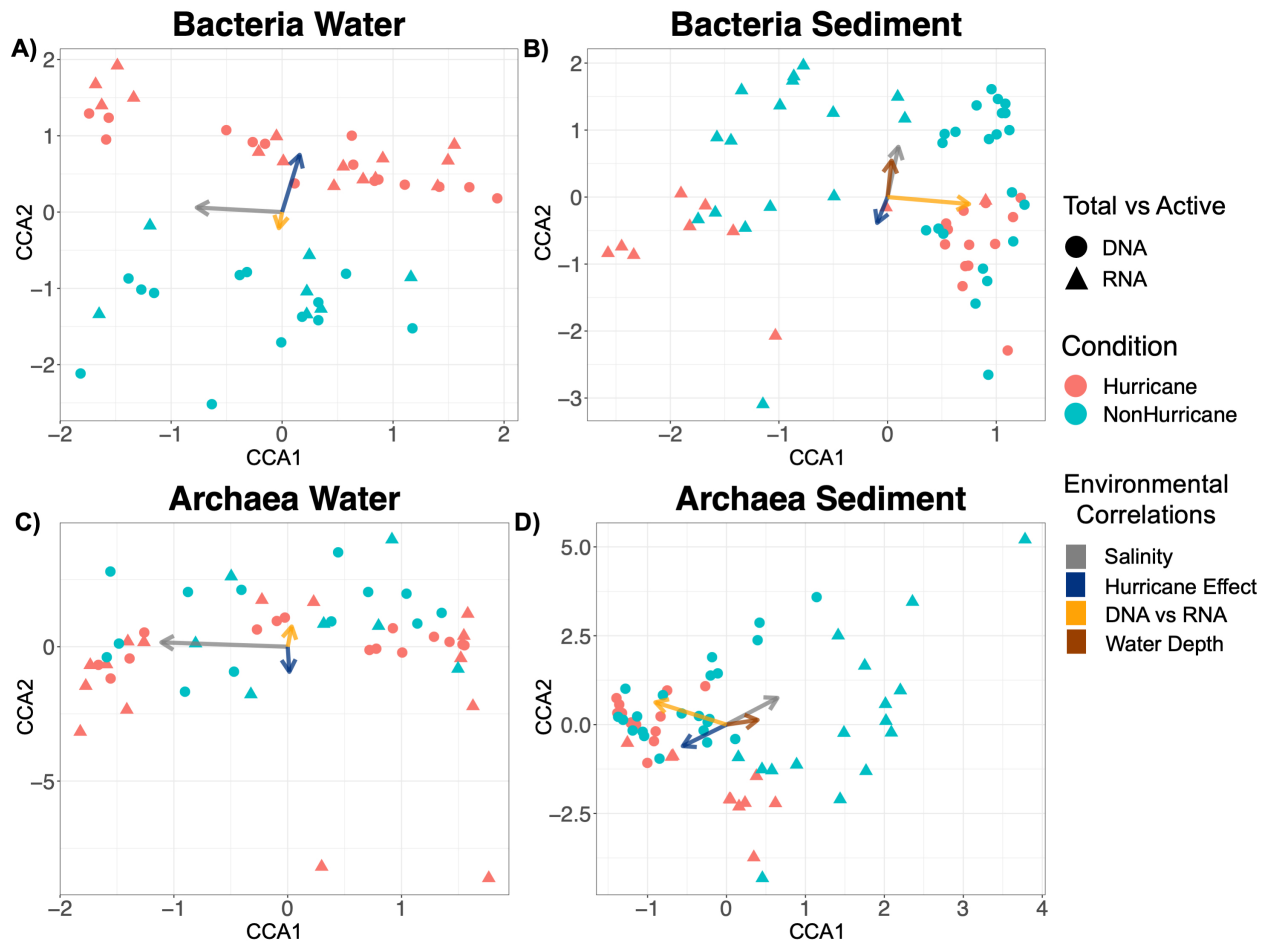


Fig. IV.2. Canonical Correspondence Analysis plots with data points representing individual microbial community samples, and distance between points representing degree of similarity between each microbial community composition. Arrows represent influence from environmental variables, and length of arrow represents strength of correlation. **A)** Hurricane conditions led to significant differences from non-hurricane conditions within water column bacterial communities (ANOSIM test: $R = 0.185$, $p < 0.001$), with community composition significantly correlated with salinity and DNA vs RNA (PERMANOVA test: Salinity: $R^2 = 0.357$, $p < 0.05$; Hurricane effect: $R^2 = 0.105$, $p < 0.05$; DNA vs RNA: $R^2 = 0.102$, $p < 0.05$); **B)** Bacterial sediment communities were

most strongly correlated with DNA vs RNA, followed by salinity and water depth (DNA vs RNA: $R^2 = 0.31$, $p < 0.05$; Salinity: $R^2 = 0.059$, $p < 0.05$; Water depth: $R^2 = 0.043$, $p < 0.05$); **C**) Archaeal surface water communities were correlated only with salinity ($R^2 = 0.377$, $p < 0.05$); and **D**) Archaeal sediment communities were most strongly controlled by DNA vs RNA, followed by salinity and water depth (DNA vs RNA: $R^2 = 0.305$, $p < 0.05$; Salinity: $R^2 = 0.152$, $p < 0.05$; Water depth: $R^2 = 0.060$, $p < 0.05$).

The overall trends in bacterial and archaeal response to extreme storms in this study are further confirmed by RNA-based microbial profiling. Hurricane affected bacterial community compositions derived from DNA in the water column were significantly different than those derived from RNA (ANOSIM test: $R = 0.104$, $p = 0.03$) (Fig. IV.S1). Hurricane conditions likely contributed to certain bacterial taxa being selected for active growth and cell proliferation over other taxa. The additional differences seen between DNA and RNA profiles across domains and environments (Fig. IV.2A-D) suggest that not all microbial cells within the water column were active. Even so, the active bacterial members during hurricane conditions differed significantly from both total and active bacteria during non-hurricane conditions (i.e., differences in microbial community composition due to hurricane effects were not merely an artifact of inactive or dead microbial cells). Sediment microbial communities exhibited even greater significant differences between DNA and RNA, which further supports the occurrence of cell dormancy, inactivity, and endospore formation within sediments (Luna *et al.* 2002; Wörmer *et al.* 2019).

Although the active and inactive portions of the microbial communities were important determinants of functional potential during the 2018 hurricane season, the most significant effects were observed across domains (i.e., archaea vs bacteria) and

environment types (i.e., sediment vs water column). Results from this study suggest that both archaeal and sediment communities may be more resilient to hurricane conditions compared to bacterial communities within the water column. When considering what might be driving those taxonomic differences across environment types, both bacterial and archaeal communities within the water column exhibited very little similarity to those within the sediment during hurricane conditions (ANOSIM test: $R = 0.971$, $p < 0.0001$). Taxonomic classes that were more prevalent in the water column during hurricane conditions included *Flavobacteriia*, *Alphaproteobacteria*, and unclassified *Bacteroidetes*, while classes more prevalent in the sediment included *Gammaproteobacteria* and *Deltaproteobacteria* (Fig. IV.S3). Taxonomic classes that were more shared across hurricane-affected sediment and water column samples included unclassified *Proteobacteria*, *Planctomycetia*, and *Sphingobacteriia*. This suggests that taxa more unique to sediment (*Gammaproteobacteria* and *Deltaproteobacteria*) may be stronger contributors to resiliency compared to the shared taxa, while conversely taxa more unique to the water column (*Flavobacteriia*, *Alphaproteobacteria*, and unclassified *Bacteroidetes*) may be more vulnerable or less resilient compared to shared taxa during hurricane conditions. Further investigations into these taxonomic tendencies in future studies could potentially provide more detail on functional impact of extreme storms and resiliency towards environmental disturbance. Overall this study shows that microbial communities have highly variable responses to extreme storms across putative cell activity level, substrates and domains, further adding to the context-dependent nature of these disturbance scenarios. Understanding these variable contexts is important for paving the way for future studies, and inclusion of RNA-based analyses will be vital to

improving the link between phylogeny and function of hurricane-affected microbial communities.

Lower Shannon diversity, lower phylogenetic evenness and increased abundance of copiotrophs at hurricane-affected offshore marine sites

Previous studies involving extreme storm impacts on coastal ecosystems seldom report comparisons to offshore sites, and this study provides new evidence for ecosystem disruptions beyond the coastline (Sinigalliano *et al.* 2007; Steichen *et al.* 2020; Ares *et al.* 2020). Microbial communities at offshore marine sites (>25 ppt) exhibited lower Shannon diversity (Mann-Whitney U test: $W = 7$, $p < 0.01$) and lower phylogenetic evenness ($W = 0$, $p < 0.001$) within the water column after hurricane conditions compared to non-hurricane conditions (Fig. IV.3). There was no difference in microbial community richness between conditions. Offshore marine sites seemed to maintain significant disruptions to the microbial community longer than nearshore estuarine sites. These observed differences could be due to delayed advection of nutrients from inland sources towards endpoints at further distances. In contrast, nearshore estuarine sites likely benefitted from tidal cycles facilitating faster ecosystem recovery via consistent flushing of seawater, nutrients, and microbes in and out of the system.

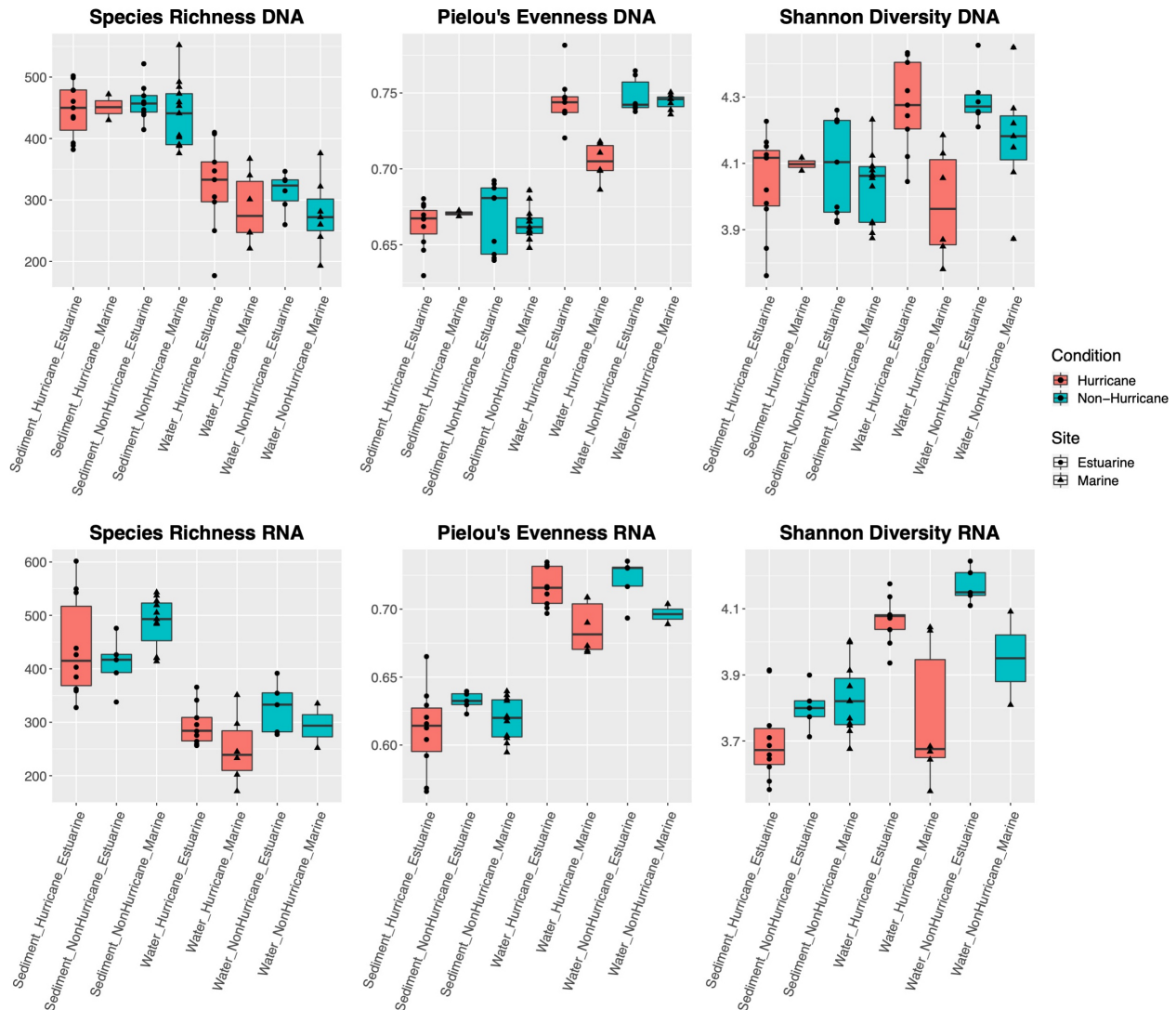


Fig. IV.3. Boxplots of microbial community diversity indices. Microbial communities at offshore marine sites (>25 ppt) exhibited lower Shannon diversity (Mann-Whitney U test: $W = 7$, $p < 0.01$) and lower phylogenetic evenness ($W = 0$, $p < 0.001$) within the water column after hurricane conditions compared to non-hurricane conditions.

The results presented here suggest that phylogenetic richness may return to pre-storm levels within time periods of this study (77 days from first storm), while other community dynamics (i.e., Shannon diversity, phylogenetic evenness, and overall community composition) could still be disrupted depending on the severity of the storm and the local ecosystem characteristics (i.e., proximity to urban environments,

topography of the local landscape, and local tidal dynamics). Conversely, previous studies have shown correlations between extreme storms and increased phylogenetic richness (Ares *et al.* 2020). Depending on the severity of the storm event, the Neuse River Estuary is known to act as either a “processor” of increased nutrient loads (i.e., C, N, P) from floodwater discharge, or as a “pipeline” for transporting the increased nutrients to the Albemarle-Pamlico Estuary (Paerl *et al.* 2020). The low-lying characteristics of Eastern North Carolina can also cause additional releases of organic carbon from surrounding wetlands into the coastal estuarine system following prolonged periods of riverine flooding (Paerl *et al.* 2020). Hurricane Florence in particular has been shown to cause “pipeline” conditions with phytoplankton blooms delayed over 3 months post-storm due to an exceptionally slow recession of the high river flow (Paerl *et al.* 2020). Our results suggest that this “pipeline” may extend beyond nearshore estuarine environments with evidence of microbial community disruptions occurring at offshore marine sites near the continental shelf and Gulf Stream. The extension of this “pipeline” to even further distances offshore implies a realistic potential for these extreme weather events to impact nutrient cycling on a global scale.

We acknowledge that sampling for this study was conducted within different seasons of each year (i.e., Spring 2016, Fall 2018, and Summer 2019), and environmental factors associated with seasonality (e.g., length of day) could have therefore impacted microbial community dynamics. Previous studies have demonstrated such an effect on marine microbial communities (Gilbert *et al.* 2012), although they found a peak in microbial diversity during winter months with shorter lengths of day,

which is opposite of the findings in this study. Other studies have found minimal seasonal patterns, but rather strong spatial patterns in microbial communities within coastal North Carolina estuarine environments (Bullock *et al.* 2017). We propose that for our geographical region of study, perhaps tropical storms and hurricanes drive microbial seasonality dynamics to a stronger degree than temperature or length of day, similar to findings by Angly *et al.* (2016).

Geochemical variables of interest (i.e., total suspended solids, dissolved organic carbon, total dissolved nitrogen, $\delta^2\text{H}$ and $\delta^{18}\text{O}$) were measured by Mitra *et al.* (2020), and tested for correlations with bacterial community composition within the water column. Communities were significantly correlated with $\delta^2\text{H}$ measurements and strongly correlated with dissolved organic carbon, while $\delta^{18}\text{O}$ and total suspended solids had weak correlations (PERMANOVA test: $\delta^2\text{H}$: $R^2 = 0.022$, $p < 0.05$; Dissolved organic carbon: $R^2 = 0.021$, $p = 0.058$; $\delta^{18}\text{O}$: $R^2 = 0.014$, $p = 0.159$; Total suspended solids: $R^2 = 0.01$, $p = 0.37$) (Fig. IV.S2). For comparison, salinity was negatively correlated with dissolved organic carbon and positively correlated with $\delta^2\text{H}$ and $\delta^{18}\text{O}$. Note that geochemical measurements were not available for any 2016 samples nor for several of the 2018 and 2019 samples. Interestingly, these geochemical measurements did not exhibit significant correlations with hurricane conditions (Mitra *et al.* 2020), suggesting that microbes are either more sensitive to extreme storm disturbances or are affected for a longer period of time than water chemistry.

The decrease in diversity at offshore marine sites was analyzed further via rRNA gene copy number. Marine sites affected by the hurricane exhibited higher average rRNA (*rrn*) copy number compared to estuarine sites affected by the hurricane and all sites from non-hurricane conditions (Mann-Whitney U-test: $W = 120$, $p < 0.001$) (Fig. IV.4). Microbes with higher rRNA copy numbers have been shown to have higher growth rates and exhibit a lifestyle that can be classified as copiotrophic (Klappenbach *et al.* 2000; Stevenson & Schmidt 2004; Yano *et al.* 2013; Roller *et al.* 2016). This suggests increased abundance of copiotrophic microbes at offshore marine sites following the hurricanes in 2018. This shift in metabolic lifestyle for marine sites could potentially be due to increases in organic nutrient concentrations at these sites caused by inland flooding of rivers and wetlands and subsequent transport of nutrients offshore. The increase in rRNA copy number ultimately provides additional support to the results above showing general microbial community disruption from the 2018 hurricanes, and it aligns even more specifically with decreased community evenness and Shannon diversity for offshore marine sites. An increase in copiotrophs could easily lead to competition conditions unfavorable for community members with contrasting metabolic lifestyles.

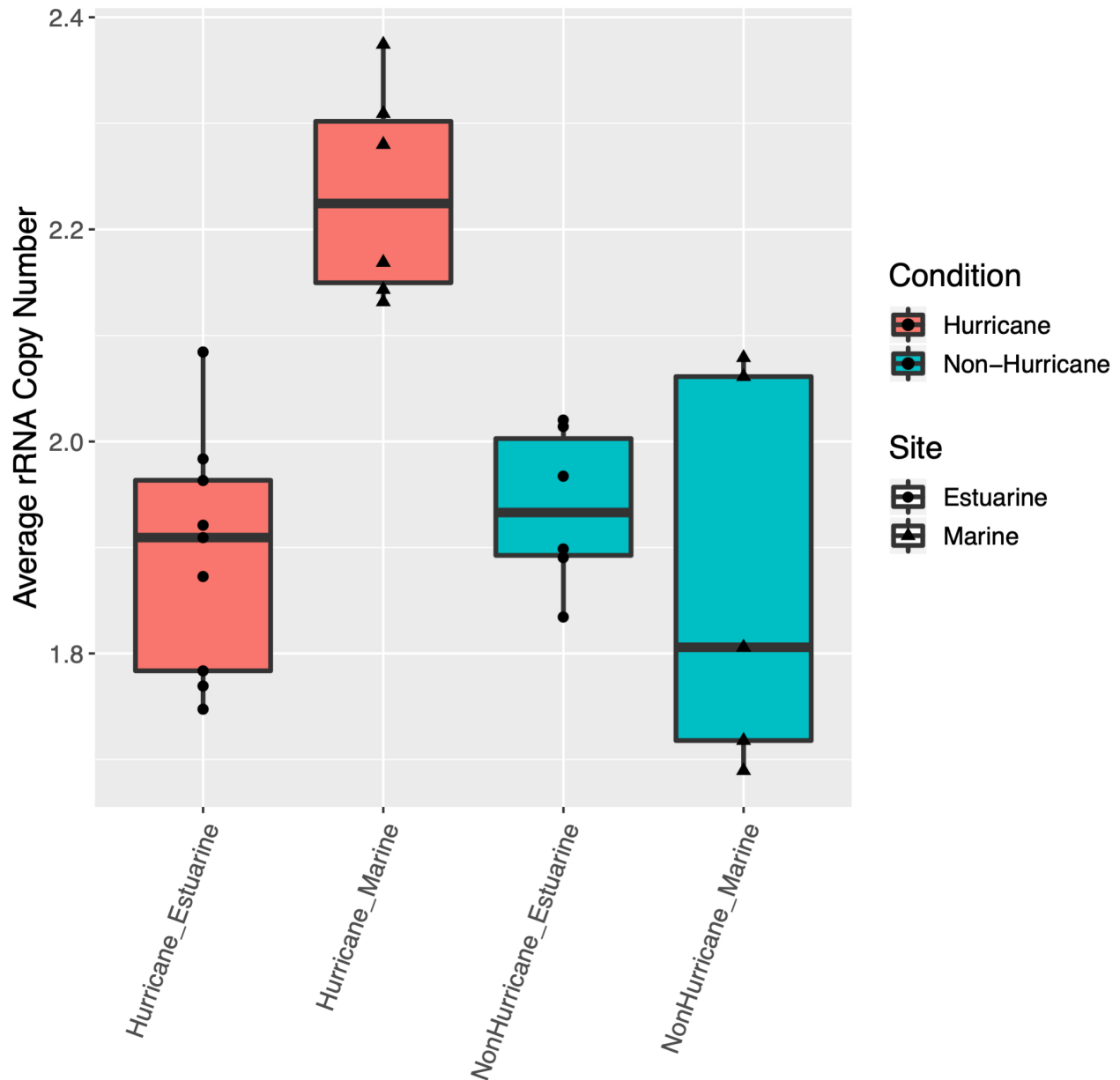


Fig. IV.4. Boxplots of average rRNA (*rrn*) copy number across samples. Hurricane-affected marine sites had higher average copy number compared to hurricane-affected estuarine sites and all sites from non-hurricane conditions (Mann-Whitney U-test: $W = 120$, $p < 0.001$).

Investigating community disturbances on a taxonomic level, hurricane-affected offshore marine sites had higher relative abundance of *Flavobacteriia*, *Gammaproteobacteria*, and unclassified *Bacteroidetes* while having lower relative

abundance of *Alphaproteobacteria* and unclassified *Proteobacteria* in the water column compared to non-hurricane conditions (Fig. IV.5). These trends were further confirmed with SIMPER analysis showing the most influential classes in determining community composition between hurricane and non-hurricane samples at marine sites (*Flavobacteriia*: 15.62%, *Gammaproteobacteria*: 10.88%, unclassified *Bacteroidetes*: 9.77%, *Planctomycetes*: 6.26, *Alphaproteobacteria*: 6.21%, *Sphingobacteria*: 4.45%). *Flavobacteriia* are common marine heterotrophs that have the ability to breakdown a wide range of simple and complex carbohydrates (Thomas *et al.* 2012; Pollet *et al.* 2018; Liu *et al.* 2019; Kappelmann *et al.* 2019) and would likely benefit from exposure to copiotrophic conditions. Their relative abundance has also been positively correlated with increased nutrients and subsequent phytoplankton blooms, with active expression of enzymes involved in polymer and particulate organic matter degradation (Teeling *et al.* 2012). Decreases in relative abundance of the dominant marine *Alphaproteobacteria* taxa make sense due to their often-streamlined genome with metabolic functions enabling them to thrive in oligotrophic conditions (Morris *et al.* 2002; Giovannoni *et al.* 2005; Luo *et al.* 2013; Luo & Moran 2015). Marine *Gammaproteobacteria* have highly diverse functional roles but have been linked to carbohydrate metabolism within biofilms (Edwards *et al.* 2010), increased relative abundance under elevated nutrient conditions (Lawes *et al.* 2015), and even resilience to disturbance events in marine environments (McKew *et al.* 2011). The change in relative abundance of *Flavobacteriia*, *Alphaproteobacteria*, and *Gammaproteobacteria* after the hurricanes would be consistent with a shift from nutrient-poor to nutrient-rich eutrophic conditions favoring copiotrophic metabolisms at these marine sites.

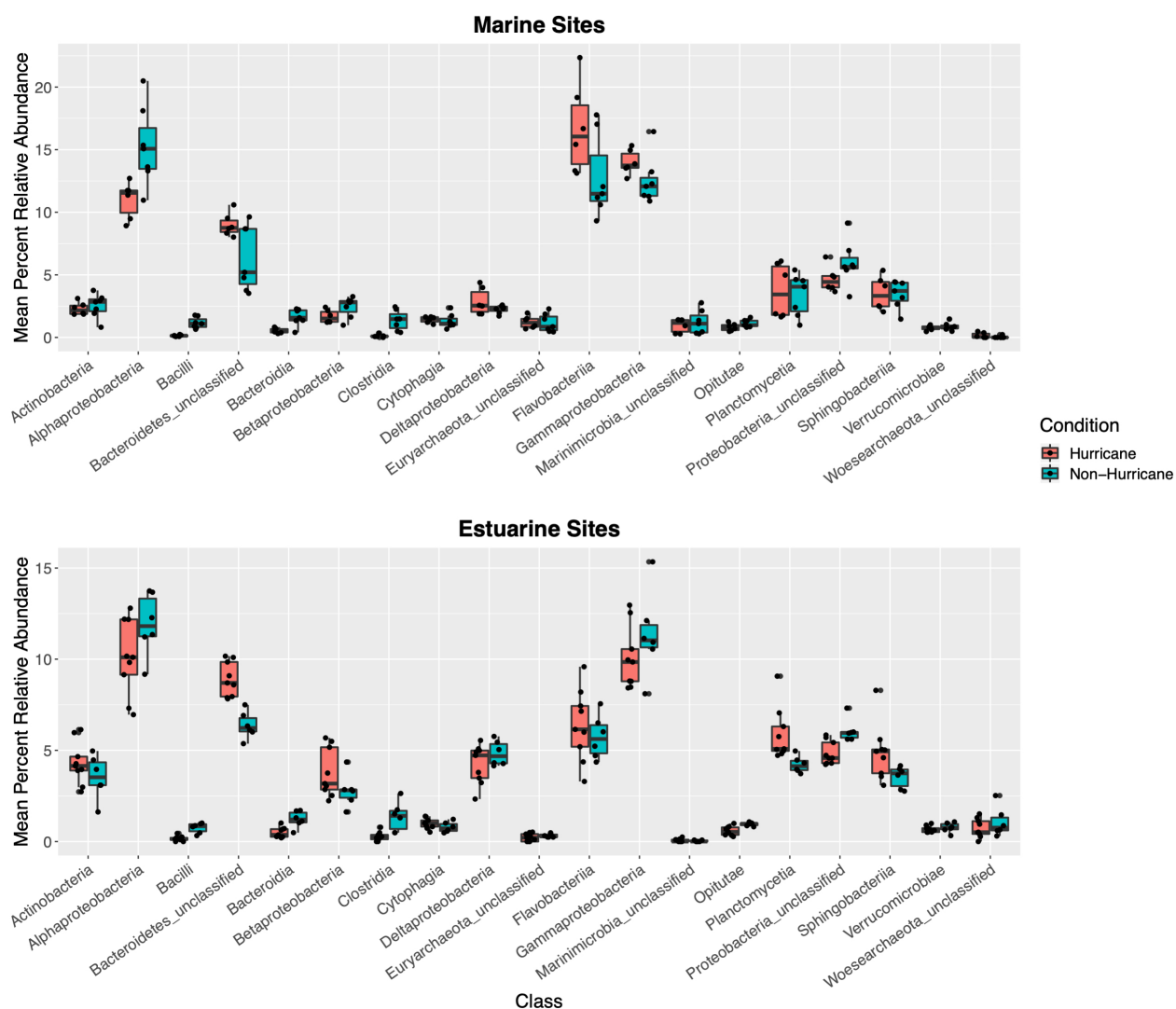


Fig. IV.5. Taxonomic boxplots of the average percent relative abundance of the most abundant microbial taxa at the class level across all sampling sites. Hurricane-affected offshore marine sites had higher relative abundance of *Flavobacteriia*, *Gammaproteobacteria*, and unclassified *Bacteroidetes* in the water column, while having lower relative abundance of *Alphaproteobacteria* and unclassified *Proteobacteria* compared to non-hurricane conditions (Fig. IV.4). Hurricane-affected estuarine sites (<25 ppt) were characterized by increased relative abundance of *Planctomycetia*, *Sphingobacteriia*, *Betaproteobacteria*, and unclassified *Bacteroidetes*, with decreased relative abundance of *Gammaproteobacteria* and unclassified *Proteobacteria* compared to non-hurricane conditions.

Hurricane-affected estuarine sites (<25 ppt) were characterized by increased relative abundance of *Planctomycetia*, *Sphingobacteriia*, *Betaproteobacteria*, and unclassified *Bacteroidetes*, with decreased relative abundance of *Gammaproteobacteria* and unclassified *Proteobacteria* compared to non-hurricane conditions. The taxonomic trends above were confirmed further with SIMPER analysis (*Gammaproteobacteria*: 9.97%, *Alphaproteobacteria*: 7.62%, unclassified *Bacteroidetes*: 7.19%, *Deltaproteobacteria*: 4.98%, *Betaproteobacteria*: 4.46%). *Planctomycetia* have been previously linked to increased relative abundance under eutrophic conditions (Li *et al.* 2020), consistent with findings in this study. *Sphingobacteriia* specialize in the degradation of complex carbon sources such as cellulose and chitin (Sack *et al.* 2014; Battin *et al.* 2016), and their increased relative abundance would be consistent with increased concentrations of organic material as a result of hurricane flooding and carbon priming. *Betaproteobacteria* are known freshwater inhabitants (Wu *et al.* 2006; Wang *et al.* 2012; Emerson 2018), also consistent with increased relative abundance from hurricane-induced river flooding. The 2018 hurricane season clearly had a large impact on microbial community dynamics and likely led to strong bottom-up regulation of nutrient cycling within this coastal ecosystem.

Shifts in water quality and prevalent metabolic pathways in hurricane-affected coastal systems

Hurricane-affected sampling sites contained significantly higher relative abundance of pathogenic taxa (i.e., *Legionella* and *Prevotella*) (mean: 1.21%; standard deviation [SD]: 1.03%) among classified genus-level taxa compared to non-hurricane

conditions (mean: 0.05%; SD: 0.15%) (Mann-Whitney U-test: $W = 261.5$, $p < 0.001$) (Fig. IV.6). This could imply an overall decrease in water quality and ecosystem health and an increase in human health risks for areas impacted by hurricanes. The distribution of these specific pathogens could be unique to the Eastern North Carolina region as a result of the high concentrations of agricultural and animal production within this rural area (Niedermeyer *et al.* 2020). Hurricane-affected estuarine sites also experienced higher relative abundance of lignin-degradation genes compared to non-hurricane conditions, although metagenomic sample numbers limit statistical significance (Fig. IV.6). This suggests an increase in carbon remineralization potential as a result of terrestrial microbes and organic matter introductions into the marine system caused by river water surging and wetland flooding.

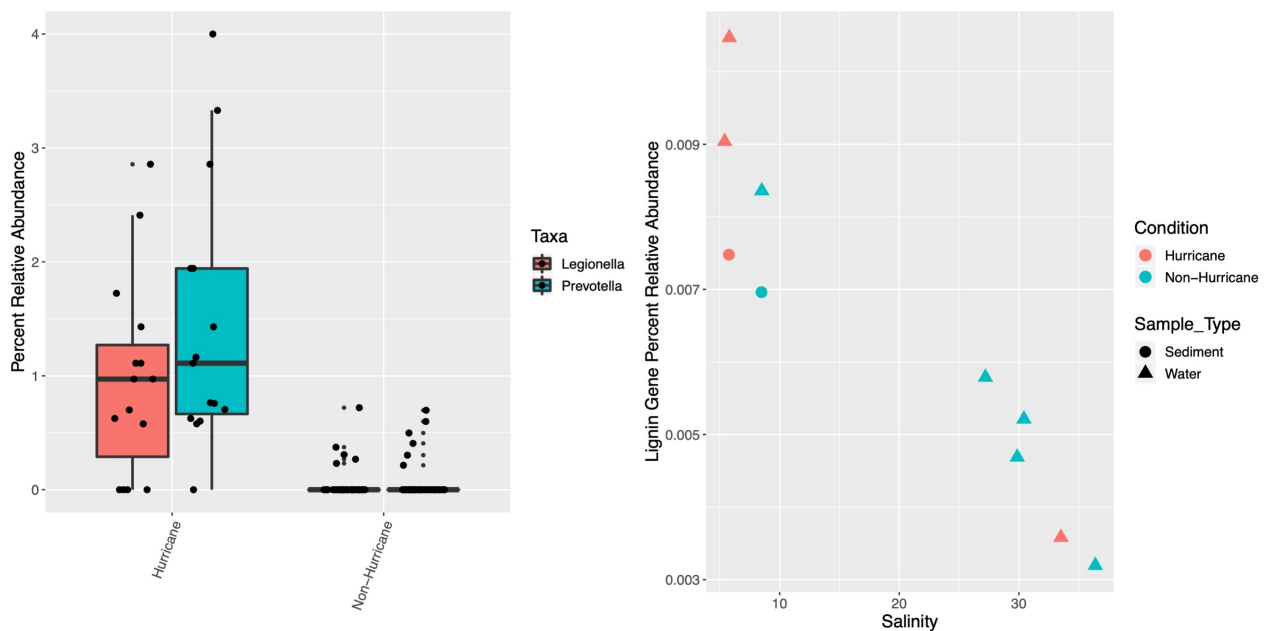


Fig. IV.6. Boxplot of percent relative abundance of potential pathogens *Legionella* and *Prevotella* among classified genus-level taxa (left), and scatterplot of percent relative abundance of potential lignin-degradation genes across metagenomic samples (right). Hurricane-affected samples contained significantly higher relative abundance of *Legionella* and

Prevotella (mean: 1.21%; standard deviation [SD]: 1.03%) compared to non-hurricane conditions (mean: 0.05%; SD: 0.15%) (Mann-Whitney U-test: $W = 261.5$, $p < 0.001$).

Hurricane-affected marine sites also had decreased relative abundance of genes involved in the glycolysis pathway, citrate cycle, glyoxylate cycle, reductive pentose phosphate cycle, reductive acetyl-CoA pathway, and ammonia oxidation pathways (Fig. IV.7). Meanwhile, hurricane-affected estuarine sites showed an increase in genes related to nitrification, denitrification, and nitrogen fixation pathways (Fig. IV.8). These shifts in metabolic pathways during hurricane conditions further support disruptions within microbial communities compared to non-hurricane conditions. Shifts in nitrogen pathways are consistent with an overall increase in nitrogen-based inputs to the coastal marine system during hurricane conditions, likely due to riverine and wetland flooding. Shifts in pathways involved in the breakdown of simple sugars and carbohydrates via the glycolysis pathway and citrate cycle could indicate a shift towards increased breakdown of more complex carbohydrates such as lignin, or a general shift towards alternative biosynthesis pathways. Lignin-based carbon sources could potentially persist in marine systems for extended time periods due to its recalcitrance and the complex degradation process required for its removal (Crawford & Crawford 1980; Brown & Chang 2014; Kamimura *et al.* 2019). This persistence could be one avenue leading to microbial community disruptions without a significant difference observed for geochemical measurements taken during this study (Mitra *et al.* 2020).

Marine sites also had fewer genes associated with the glyoxylate cycle, which some bacteria utilize for mineralization of organic matter via acetate as an intermediate

carbon source and has been linked to increased particulate carbon concentrations, reduced oxygen levels, and increased relative abundance of SAR11, the most abundant marine heterotroph (Zhuang *et al.* 2019). The lower relative gene abundance for this pathway at offshore marine sites could be a result of injections of terrestrial-based microbes into the system causing an imbalance within the microbial community, or of shifts toward communities dominated by copiotrophs (i.e., within the *Flavobacteriia* and *Gammaproteobacteria* clades) rather than oligotrophs such as SAR11 (*Alphaproteobacteria*). Further, reductions in genes associated with the reductive acetyl-CoA and ammonia oxidation pathways suggest potential shifts to metabolic pathways with alternative nitrogen sources such as nitrate reduction. Finally, less recovered genes for reductive pentose phosphate cycle, an important pathway for photosynthesis (Bassham 1979), potentially suggest less primary production occurring at hurricane-affected offshore marine sites compared to non-hurricane conditions. This would be consistent with an overall shift within offshore sites from autotroph dominated to heterotroph dominated metabolisms or towards a carbon cycle state characterized as an overall net source of atmospheric CO₂ within the time frame of this study (77 days from 1st storm, 50 days since 2nd storm). We do acknowledge that metabolic pathway data from metagenomic sequencing represents DNA only, and future studies on hurricane-affected metabolic pathways should include RNA-based transcriptomics analyses in order to better understand active functional roles of these pathways.

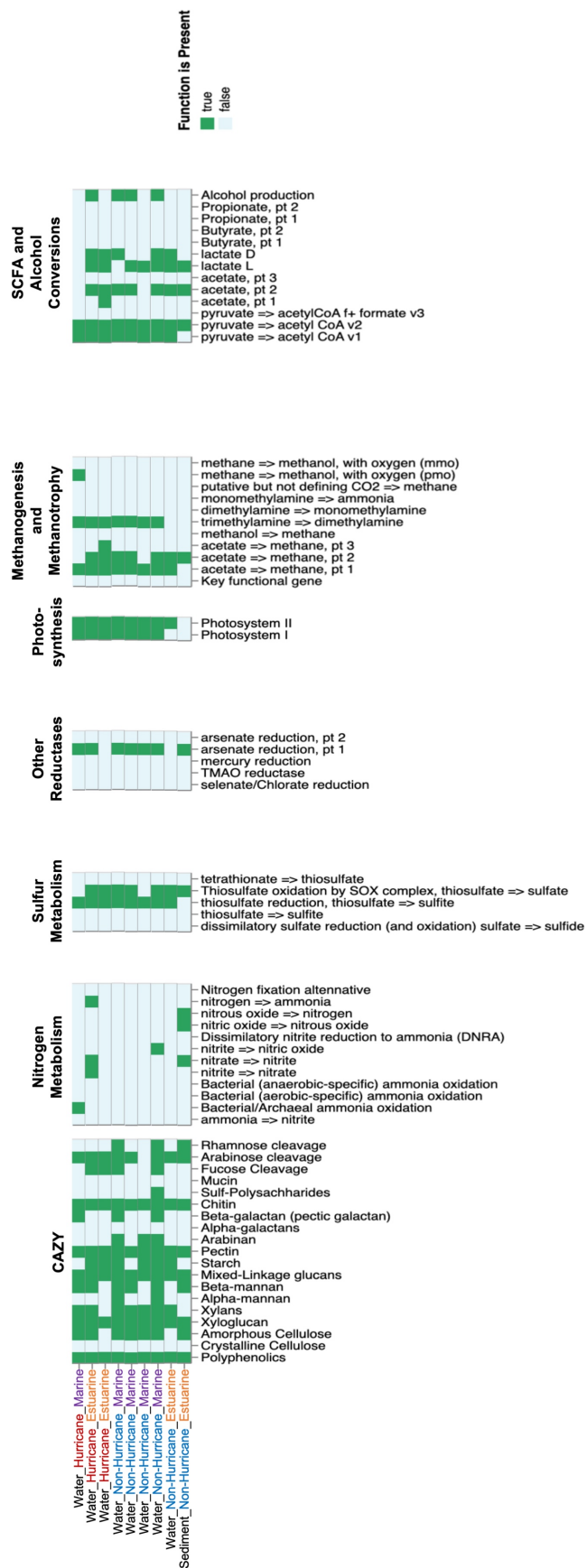


Fig. IV.8. Heatmap indicating presence or absence of metabolic pathways present across metagenomic samples. Hurricane-affected estuarine sites showed an increase in genes related to nitrification, denitrification, and nitrogen fixation pathways.

Conclusion:

This study highlights the impact of extreme storms on coastal microbial communities and suggests that these impacts are highly dependent on the geographical characteristics of the study site. Other studies have shown rapid recovery of microbial communities to pre-storm conditions within several days (Ares *et al.* 2020), whereas our results show significant disruptions lasting up to 77 days after the first storm (Hurricane Florence) and 50 days after the second storm (Hurricane Michael). It is possible this could be attributed to the long residence times characterized by the Outer Banks, NC coastal area (Paerl *et al.* 2001). Of particular importance, we found contrasting responses among habitats (i.e., water column vs sediment), phylogenetic domains (i.e., bacteria vs archaea), and putative cell activity (i.e., DNA vs RNA). These hurricane-impacted communities were also characterized by a decrease in phylogenetic evenness and Shannon diversity, as well as shifts towards individual taxa and metabolic pathways associated with increased nutrient concentrations, decreased water quality, and shifts from autotroph dominated to heterotroph dominated. The increased relative abundance of copiotrophs and pathogenic taxa, as well as shifts in carbon and nitrogen cycling genes further suggests that the differences in microbial community dynamics observed between years were influenced more strongly by extreme storms rather than other seasonal-related variables. The weak correlations evident between microbial community composition and water chemistry imply that microbes are either more sensitive to

extreme storm disturbances or are affected for a longer period of time compared to geochemical tracers measured in this study. More importantly however, the lasting impacts on microbial communities from hurricanes likely cause significant disruptions to nutrient cycling within a geographical area that fuels primary production in the global oceans (i.e., The Gulf Stream). Significantly altered marine microbial communities as a result of extreme storms likely have important consequences for local and global nutrient cycling with the potential to create a positive feedback loop for atmospheric CO₂ for a period of time, further exacerbating the issue of high concentrations of atmospheric CO₂. These analyses methods and results should ideally be used as building blocks for future studies aimed at interpreting and predicting the microbiological impact of increased intensities and frequencies of extreme storms accompanying a warming global climate. As we expand our understanding of microbial responses to extreme weather disturbances, we can begin to improve our knowledge of the potential fate of future global oceans.

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Supplemental Materials:

Table IV.S1. Descriptions of sampling sites

Site	Sampling Date	Latitude	Longitude	Salinity (ppt)	Water Depth (m)	Temp (°C)	Surface Water DNA Analysis	Surface Water RNA Analysis	Sediment DNA Analysis	Sediment RNA Analysis
1	10/25/18	35.3766	-75.8046	21.3	5.9	N/A	Y	Y	Y	Y
2	10/25/18	35.4877	-75.6641	16.9	5.6	17.9	Y	Y	Y	Y
3	10/25/18	35.6484	-75.6230	11.7	4	16.1	Y	Y	Y	Y
4	10/26/18	35.7030	-75.6575	11.8	4.1	15.4	Y	Y	Y	Y
5	10/26/18	35.7606	-75.5875	19.8	3.4	15.6	Y	Y	Y	Y
6	10/26/18	35.7710	-75.5380	29.9	3.8	18.3	Y	Y	Y	N
7	10/26/18	35.7536	-75.5275	29.3	1.4	19	Y	Y	Y	N
8	10/26/18	35.7949	-75.6221	10.7	2.1	15.7	Y	Y	Y	Y
9	10/26/18	35.8782	-75.7144	5.8	2.4	15.4	Y	Y	Y	Y
50	11/30/18	35.8065	-75.5879	4.2	1.9	7.5	Y	Y	Y	Y
51	11/30/18	35.7817	-75.5284	5.4	4.8	7.4	Y	Y	Y	N
52	11/30/18	35.8794	-74.8322	33.5	35.3	14.6	Y	Y	N	N
53	11/30/18	35.9033	-75.0908	32.4	32	13.9	Y	Y	N	N
54	11/30/18	35.8994	-75.1953	31.4	37.8	13.6	Y	Y	N	N
55	11/30/18	35.8779	-75.3627	30.2	25	13.2	Y	Y	N	N
100	7/2/19	35.8781	-74.8375	29.9	356	25.4	Y	N	N	N
101	7/2/19	35.8802	-75.0141	29.7	44.2	25.7	Y	N	N	N
102	7/2/19	35.8684	-75.1331	29.8	30.5	25.8	Y	N	Y	Y
103	7/2/19	35.8066	-75.4487	26.7	12	24.7	Y	N	Y	Y
104	7/2/19	35.7976	-75.5171	27.2	11.4	24.3	Y	N	Y	Y
105	7/3/19	35.4560	-75.7683	20.1	5.9	28.4	Y	N	Y	Y
106	7/3/19	35.4856	-75.6656	18.7	5.6	28.4	Y	Y	Y	Y
107	7/3/19	35.6440	-75.6269	15.9	4	28.7	Y	Y	Y	N
108	7/3/19	35.7596	-75.5910	14.7	2.7	28.1	Y	Y	Y	N
109	7/3/19	35.7636	-75.5307	14	1.3	N/A	Y	Y	Y	N
110	7/3/19	35.8591	-75.6852	8.5	3	28.7	Y	Y	Y	Y
ML1	3/17/16	35.0498	-75.2631	36.4	98	20	Y	Y	Y	Y
MM2	3/18/16	35.1378	-75.1010	36.4	230	21	N	N	Y	Y
XS1	3/22/16	35.7696	-75.4736	30.4	20	9.3	Y	Y	Y	Y
DD11	3/22/16	35.5609	-74.8669	34.2	55	10	N	N	Y	Y

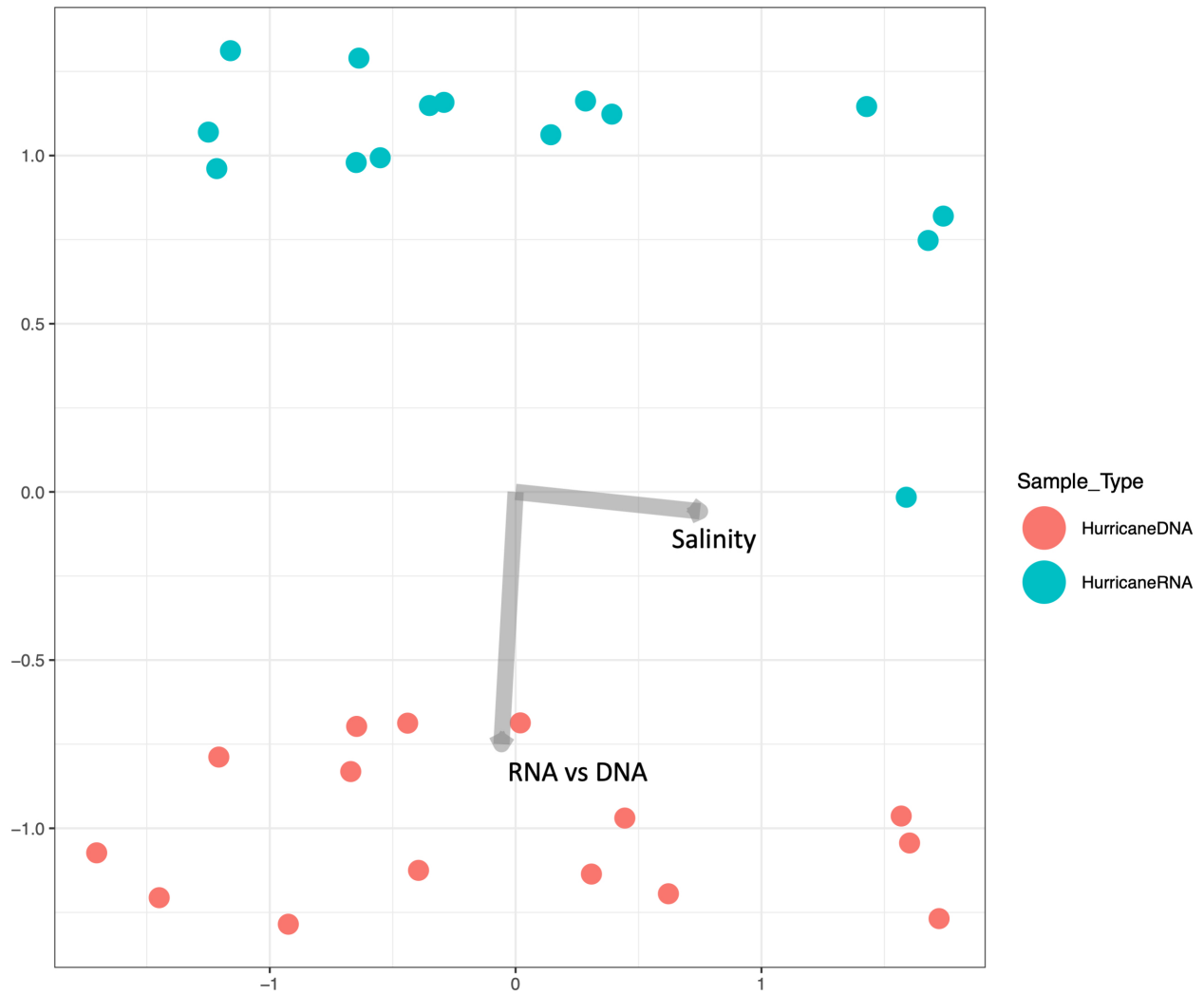


Fig. IV.S1: Canonical Correspondence Analysis plot showing water column bacterial community compositions derived from DNA compared to RNA in hurricane affected conditions. Hurricane affected bacterial community compositions derived from DNA in the water column were significantly different than those derived from RNA (ANOSIM test: $R = 0.104$, $p = 0.03$).

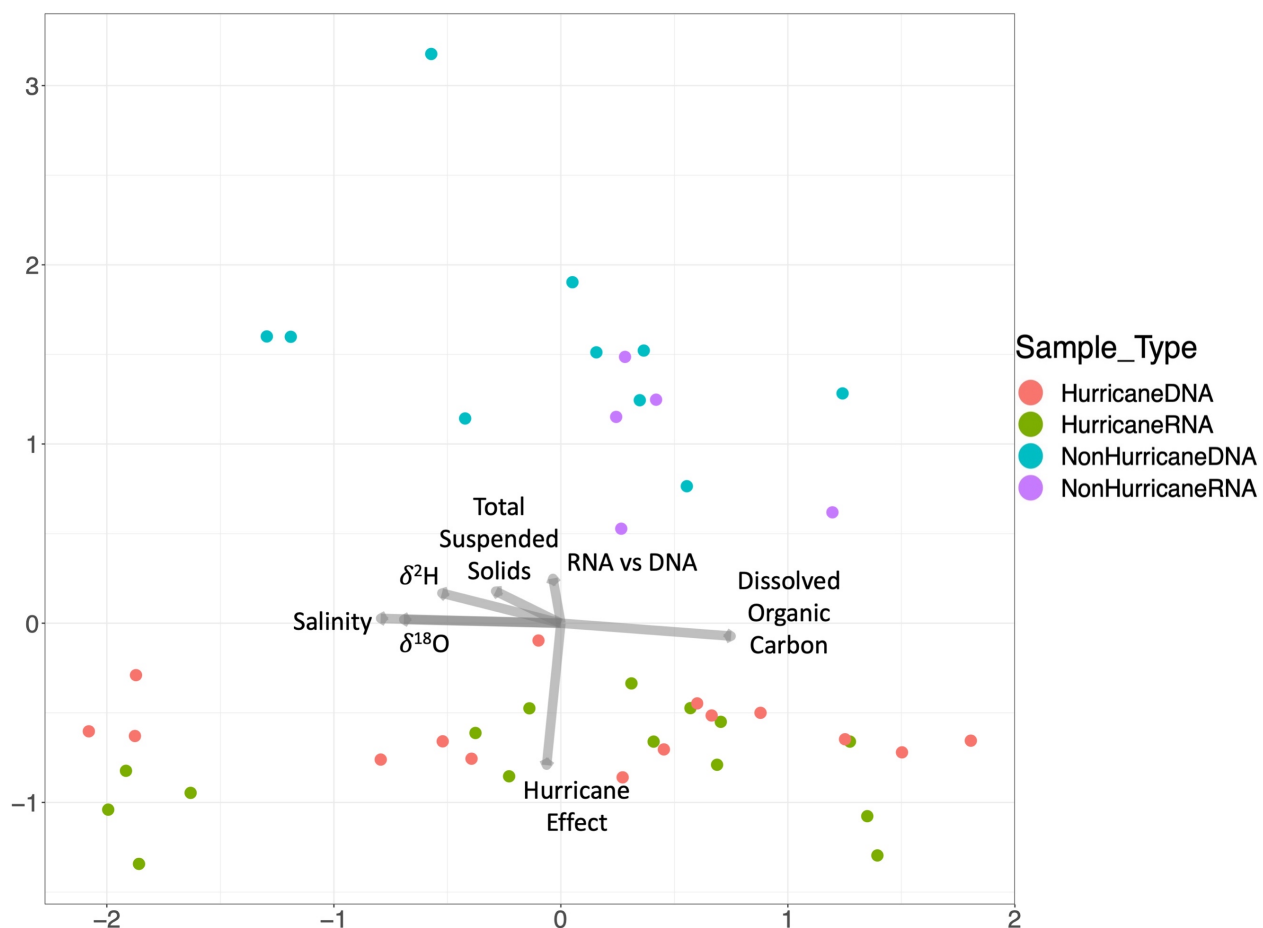


Fig. IV.S2: Canonical Correspondence Analysis plot showing bacterial community compositions in water column samples with influence of additional geochemical variables included. All of the 2016 samples and several 2018 and 2019 samples are omitted due to lack of geochemical measurements. Communities were significantly correlated with $\delta^2\text{H}$ measurements and strongly correlated with dissolved organic carbon, while $\delta^{18}\text{O}$ and total suspended solids had weak correlations (PERMANOVA test: $\delta^2\text{H}$: $R^2 = 0.022$, $p < 0.05$; Dissolved organic carbon: $R^2 = 0.021$, $p = 0.058$; $\delta^{18}\text{O}$: $R^2 = 0.014$, $p = 0.159$; Total suspended solids: $R^2 = 0.01$, $p = 0.37$).

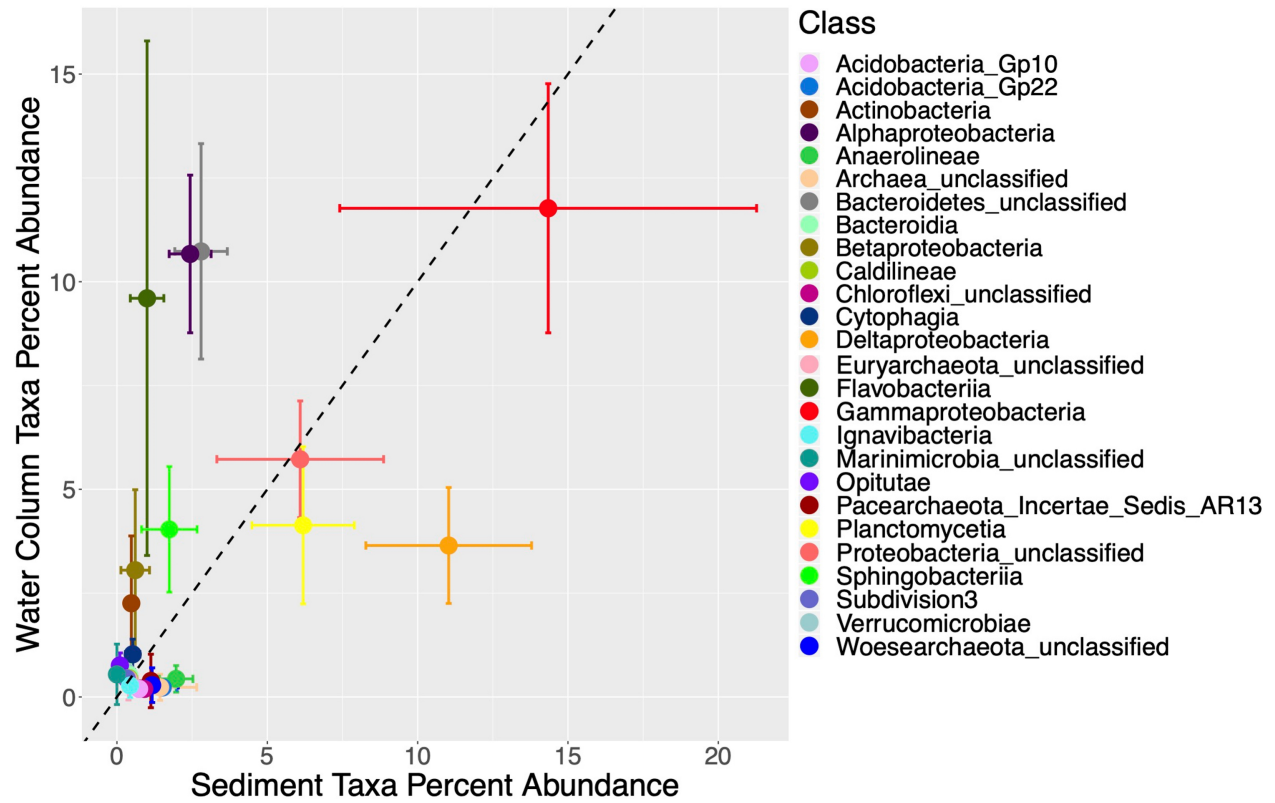


Fig. IV.S3: Sediment taxa abundance plotted against water column taxa abundance at the class level during hurricane conditions indicated that *Flavobacteriia*, *Alphaproteobacteria*, and unclassified *Bacteroidetes* were more prevalent in the water column while *Gammaproteobacteria* and *Deltaproteobacteria* were more prevalent in the sediment. Taxonomic classes that were more shared across hurricane-affected sediment and water column samples (closest to the 1:1 ratio line) included unclassified *Proteobacteria*, *Planctomycetia*, and *Sphingobacteriia*. Data points represent average relative abundance and error bars represent standard deviation across samples.

V. CONCLUSIONS AND FUTURE DIRECTIONS

The complex responses of microbial communities to environmental change during the Anthropocene show that community composition and function adapt to the environmental conditions inflicted upon them. The historical quote by Baas Becking (1934), “Everything is everywhere, but the environment selects”, seems to hold true even in the modern world as humans continue to impact the natural environment in many ways. Environmental conditions are fully expected to continue changing in the future as human technologies and their impact on Earth’s physical and chemical characteristics continue to evolve. It is critical that we maintain observations and analysis of microbial community resiliency in all environment types as the environment changes in order to more accurately detect community trends over space and time and predict how these trends may affect global ecosystem health and function. For example, if microbial community compositions are altered by extreme weather disturbances or introductions of novel substrates and nutrient sources, then the resulting change in microbial nutrient cycling within the ecosystem may lead to nutrient imbalances within local communities impacting many other species across all domains.

Introductions of novel substrates such as steel infrastructures in aquatic environments have created novel surface environments for microbial colonization and energy acquisition on small spatial scales but long temporal scales. Iron is a much more limited element in marine environments now compared to early Earth time periods, and steel infrastructures have thus contributed to maintaining the ubiquity of microorganisms such as iron-oxidizing bacteria (FeOB). Humans have adapted in their self-interest by

creating advanced steel types such as stainless steel, made specifically for resisting biocorrosion and degradation processes of these iron-based structures. FeOB have also adapted to these evolving substrate types, and this is evident from the differential FeOB colonization abundance across stainless steel types with different chemical compositions. This differential abundance suggests a potential preference of FeOB towards 316 stainless steel rather than 304 stainless steel and is further supported by functional genes within their genome for molybdenum utilization and reactive oxygen species protection. Molybdenum is a constituent of 316 stainless steel and this gene may represent an adaptation of FeOB for utilizing this constituent as a micronutrient for growth. Genes for reactive oxygen species may represent adaptations for FeOB to oxidize steel infrastructure in highly oxygenated waters, in contrast to their typical low oxygen environments (e.g., benthic iron mats). FeOB also exhibited higher colonization abundance at higher salinities, suggesting that marine lineages of FeOB may be more adapted to these anthropogenic substrates than their freshwater phylogenetic neighbors. This evidence shows that microbes have the ability to adapt to novel substrate types affecting nutrient cycling within small spatial scales (i.e., within the geographic area of the steel infrastructure) for time periods of decades to centuries until the infrastructure is physically degraded or removed. These results suggest that other microbial taxa will likely continue to find ways of adapting to and exploiting other novel substrate constituents or substrate types in the future.

Steel infrastructures have additionally impacted ecosystem services beyond iron cycling. These structures provide general surface attachment ability in aquatic systems

where surface environments are scarce. In contrast to marine sediments which can also act as a surface environment, steel structures closer to the water surface experience higher flow rates and thus more exposure to nutrients and energy for growth (Winterwerp & Van Kesteren 2004). These surface environments allow formation of biofilms which provide sources of nutrients, habitat diversity, mutualistic relationships among colonizing microbes, and gene exchange networks. Similarities in microbial community compositions observed across multiple steel types have shown that there may be a “core steel microbiome” composed of common steel colonizers regardless of elemental composition within the steel. These common colonizers are comprised of generalist heterotrophic microbes that likely provide biofilm formation and maintenance functions that have stabilizing effects on the microbial community. Note that the corrosion-causing microbes (i.e., iron-oxidizing bacteria) can be considered to have a specialist lifestyle due to their very specific energetic growth requirements, and the functional stability of these steel communities likely rests on a delicate coexistence of specialists and generalists both benefitting each other.

Furthermore, the phylogenetic compositions of these steel microbial communities diverged with changes in salinity, while abundance of functional genes largely did not. This suggests that core steel microbiomes may be specific to certain ranges of salinity, but also that the overall functional benefits provided by these core members and the remaining community members are likely similar, regardless of shifts in phylogeny. This is important because it suggests that these adaptations are likely not confined by the chemical and physical characteristics of a given environment, but rather many different

microbial lineages may have the ability to adapt to environmental change in very similar ways, potentially an example of convergent evolution. These adaptations across phylogenies and abiotic conditions could be a result of the inherently long timescales associated with steel infrastructure introductions to aquatic environments. The longer temporal scales presumably increase the likelihood that genomic mutations within a steel microbiome will be beneficial to either an individual cell or the entire community and lead to adaptations that remain within the microbial population.

The Anthropocene time period has not only introduced novel substrate types into the environment but has also accompanied significant changes to Earth's climate and weather. High intensity tropical storms and hurricanes have become a more frequent occurrence, and likely have important consequences for microbial communities and ecosystem function across large spatial scales according to the geographic area along storm paths. Unlike novel substrate introductions however, extreme weather events have shorter temporal scales with high precipitation and winds only lasting for the length of time of the storm. Following sequential hurricanes across the Outer Banks, NC, bacterial communities within the water column exhibited shifts in composition correlated with hurricane effect (i.e., samples that were taken soon after hurricanes compared to samples taken during non-hurricane conditions on different years). In contrast, archaeal communities in the water column had no significant change in composition, and both bacterial and archaeal communities in the sediment did not exhibit shifts in composition in relation to the hurricanes. This suggests that bacterial taxa could be more responsive and more adaptable to environmental changes compared to archaea. Water column

microbial communities may also be more adaptable than sediment communities as a result of higher exposure to abiotic fluctuations. It is also possible that water column communities are more accurate representations of finer-scale environmental fluctuations, while sediment communities depict more broad-scale environmental change. The overall decreased microbial diversity and likely increase in copiotrophic lifestyles at hurricane-affected offshore sites suggests that nutrient cycles were likely impacted as a result of the hurricanes. The increased prevalence of copiotrophic microbes was likely driven by advection of high concentrations of nutrients and run-off downstream from terrestrial-based sources. The increase in abundance of several copiotrophic microbial taxa within an aquatic community can lead to declines in abundance of many other community members and therefore decreased community diversity, evenness, and overall functional stability (Wittebolle *et al.* 2009; Haig *et al.* 2015; Schmidt *et al.* 2020). Similar to the balance between generalists and specialists within an environment, balanced coexistence of copiotrophs and oligotrophs may also be necessary for maintaining functional stability within an ecosystem. It is important to improve our understanding of these shifts in microbial communities correlated with extreme storm events in order to infer overall ecosystem impacts. Additional increases in relative abundance of pathogenic bacterial taxa further suggest decreased water quality as a result of extreme storms and could imply increased human health consequences in coastal areas in future Anthropocene years.

As microorganisms continue to adapt to Anthropocene conditions, humans continue to create new environmental factors for microbes to acclimate to across many

different scales. Continued investigations into novel anthropogenic substrates will allow better understanding of microbial adaptations towards these substrates and how that might affect nutrient cycles and ecosystem health. Additional studies into microbial interactions with other man-made metal alloys, plastics, and fabrics will be critical to fully understanding microbial adaptations for colonization and degradation of novel substrates and subsequent effects on ecosystem services. Anthropogenic plastic pollution is currently a global pollution crisis in marine environments (Worm et al. 2017; Vince & Stoett 2018; Phelan et al. 2020), and if microbial cells have increased ability to degrade these various types of plastic polymers due to genomic adaptations, then it could result in significant consequences to global carbon cycling. Additional studies into the effects of Anthropocene climate and weather or microbial community functional potential will be necessary to fully predict potential reversals in atmospheric carbon feedback loops as well as the overall impact on global ecosystem stability. Microorganisms have proven resilient and adaptable to an ever-changing planet Earth, and we must continue efforts to comprehend the true limits of the seemingly endless potential of microbial life.

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