CONSILIENCE OF IRON IN THE ECOLOGY OF *VIBRIO* BACTERIA

by

JASON ROBERT WESTRICH

(Under the Direction of Erin K. Lipp)

ABSTRACT

Vibrio are common heterotrophic bacteria found in marine waters around the world. *Vibrio* have a large repertoire of metabolic potential and are able to mount a rapid response to new sources of nutrients, they therefore play an important, but poorly defined role in nutrient cycling in the ocean. Members of this genus are also important pathogens able to cause disease in humans and many marine organisms. Key to *Vibrio* survival, both in the environment as well as in an animal host is the ability to acquire the essential trace element iron. In a series of studies I investigated the importance of iron in the survival and growth of both environmental and pathogenic strains of *Vibrio*, demonstrating that heterotrophic bacteria, like *Vibrio* play a key role in iron cycling in marine ecosystems. Most investigations have predominantly focused on autotrophic phytoplankton because of the high demand of iron in photosynthetic processes; therefore, there is a paucity of methodologies for directly studying iron limitation in marine heterotrophic bacteria. The initial efforts of this dissertation work were to develop a novel iron limited artificial seawater system in which to study *Vibrio* response to iron limitation as well as their response to various sources of iron. The remainder of the dissertation demonstrates the importance of heterotrophs, like *Vibrio*, in processing iron from Saharan dust. Because dust blown from the Sahara is a major source of iron to downwind marine communities, I investigated

the effects of this ephemeral pulse of iron both in manipulative and *in situ* investigations in the Tropical Mid Atlantic and Caribbean. In this dissertation, I show the critical importance of iron in the growth and survival of *Vibrio*, having important implications in marine biogeochemical cycling of iron as well as disease potential.

INDEX WORDS: *Vibrio*, Saharan Dust, Iron, Marine Biogeochemistry, Heterotrophic Iron Limitation

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DEDICATION

Without the love and support of my immediate and extended family none of this work would have been possible. I therefore dedicate it to each and every one of them, especially, to my children Isabella, Clara and Amatus and my wife Lisa.

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CHAPTER 1

INTRODUCTION

Foreword

Consilience is a term recently resurrected from antiquity by the renowned evolutionary ecologist and sociobiologist, E.O. Wilson in his work, *Consilience: The Unity of Knowledge*. Derived from Latin, consilience literally means, "the jumping together" and refers to a convergence of evidence. In principle, consilience posits that evidence from multiple, often unrelated sources converge to form a strong conclusion, a key concept that emerged from Enlightenment thinking. Wilson proffered that though this principle forms the bedrock of modern scientific endeavors, today both natural and social sciences often develop independently in disciplinary silos. Wilson champions the viewpoint that what is currently most needed is a synthesis of knowledge to more efficiently draw together discoveries across the scientific realm. This dissertation represents my attempt at the convergence of evidence for the importance of iron in the ecology of the ubiquitous marine bacterial genus *Vibrio*.

Members of the genus *Vibrio* are a diverse group of marine heterotrophic bacteria found globally in coastal and open ocean surface waters (Thompson et al. 2004). Though ubiquitous in marine water, *Vibrio* typically constitute <1% of the total bacterioplankton community (Thompson and Polz 2006). *Vibrio* spp. have a broad genomic and metabolic capability allowing them to survive nutrient-limiting conditions yet rapidly bloom in response to new resources

(Eilers et al. 2000; Polz et al. 2006). Despite this versatility, little is known about their role in cycling of biologically important nutrients in marine ecosystems (Takemura et al. 2014; Thompson and Polz 2006).

Vibrio spp. are most notable for their role in disease. There are 24 species that are known pathogens in plants and animals, including the human pathogens, *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Grimes et al. 2009). *V. cholerae*, the causative bacterial agent of the severe diarrheal disease cholera has been a human scourge since ancient times and is still a significant source of disease (Reidl and Klose 2002). There have been six historical cholera pandemics, including a recent outbreak in Haiti, which killed over 8,000 people (Chin et al. 2011; Reidl and Klose 2002). *V. parahaemolyticus* and *V. vulnificus* together are a significant source of water-borne infections and are responsible for most cases of fatal seafood poisoning (Lipp and Rose 1997). *Vibrio* pathogens also cause disease in a variety of marine organisms such as shrimp, fish and corals (Kustusch et al. 2011; Thompson et al. 2004). Recently both human and marine infection rates have been on the rise (Newton et al. 2012; Rosenberg et al. 2007). Therefore, considerable interest exists for understanding the drivers of *Vibrio* population dynamics in the environment.

Vibrio populations are known to be influenced by environmental gradients like temperature, salinity and plankton abundance, but this does not fully account for their distribution (Takemura et al. 2014; Turner et al. 2009). From a clinical point of view, *Vibrio*'s success as a pathogen is in part due to their ability to obtain iron, which is withheld in host organisms as a form of nutritional defense against invading bacteria (Parrow et al. 2013). All of the major *Vibrio* pathogens have a wide array of iron acquisition and transport mechanisms that facilitate host colonization (León-Sicairos et al. 2015; Wright et al. 1981; Wyckoff et al. 2007).

Because some *Vibrio* pathogens have been shown to maintain free-living populations in the environment, and do not require human hosts for survival, these iron acquisition mechanisms presumably play a role in *Vibrio* growth and survival in the ocean.

Iron is a limiting factor in biological production over a large part of the world's oceans (Boyd et al. 2007). This is especially true in the oligotrophic open ocean but also includes some coastal systems, traditionally considered to have higher amounts of terrestrial iron input (Öztürk et al. 2002). Heterotrophic bacteria, like *Vibrio*, play a central role in nutrient cycles in these systems, including iron cycling, yet specifics of heterotrophic use of environmental iron sources remain poorly resolved (Azam and Malfatti 2007; Strzepek et al. 2005).

This dissertation is a "convergence of evidence" drawing together the biological oceanography understanding of heterotrophs as important players in biogeochemical cycles in marine systems with the clinical viewpoint of *Vibrio* as capable iron scavengers. I reasoned that similar mechanisms probably drive *Vibrio* dynamics in the world's oceans where iron can be a limiting nutrient as in the iron-limiting host environment. The third chapter of this dissertation outlines development of a novel iron-limited medium, for investigating the effects of iron-limitation and heterotrophic usage of environmental sources of iron, using *Vibrio* as a model heterotroph. The fourth and fifth chapters investigate coastal and open ocean *Vibrio* response to Saharan dust, a major source of iron to downwind surface waters, showing an increase in *Vibrio* within 24 hours and an increase in the detection of pathogenic species in waters that received dust. Together, these findings suggest that environmental iron flux contributes to *Vibrio* population distribution and dynamics, shedding light on heterotrophic iron cycling in the ocean as well as discovering an important driver in the distribution of a genus that has important health implications for humans and wildlife.

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CHAPTER 2

LITERATURE REVIEW

VIBRIO DYNAMICS

The genus *Vibrio* is a member of the Vibrionaceae family encompassing 142 currently described species of a highly diverse group of heterotrophic marine bacteria whose common ancestor is estimated to have been present 600 million years ago (Sawabe et al. 2007; Sawabe et al. 2013). Over this long evolutionary history *Vibrio* have evolved to inhabit a wide range of ecological niches including benthic sediment, free-living in coastal and open ocean environments and most notably in association with a multitude of organisms including algae, marine animals and humans (Thompson et al. 2004). The biogeography, ecology, and genetics of *Vibrio* have been an important area of investigative research, primarily because of their relevance in human and marine animal disease (Grimes et al. 2009). Infection by *Vibrio*, or vibriosis, is characterized in humans primarily by diarrhea, primary septicemia, or wound infection (Newton et al. 2012). There are at least 12 *Vibrio* species known to be human pathogens, with *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* accounting for the majority of human vibriosis worldwide and *V. alginolyticus* emerging as an important human and fish-associated pathogen (Grimes et al. 2009; Kustusch et al. 2011; Newton et al. 2012).

The study of *Vibrio* biogeography and population dynamics has predominately focused on the distribution of disease species (Takemura et al. 2014). Populations in coastal and estuarine waters have been extensively studied because of the relevance of these systems for

recreation, fishing and transportation. Though *Vibrio* have been isolated from open ocean environments, little is known about the ecology and biology of these species (Thompson and Polz 2006). Most surveys show, that though *Vibrio* are among the most abundant culturable bacteria, culture-independent methods indicate they are rarely dominant in surface water communities, typically numbering less than 1% of total bacterioplankton (Thompson and Polz 2006; Yooseph et al. 2010). It is emerging that *Vibrio* are capable of significant blooms in the water column during which they can become a dominant member of the community, though the drivers of *Vibrio* bloom formation are not well constrained (Gilbert et al. 2012; Takemura et al. 2014).

Temperature and salinity have been shown to be important factors correlating with *Vibrio* growth and persistence in the environment (Takemura et al. 2014). *Vibiro* species generally thrive in salinities from 5-25 in warmer water temperatures (> 15° C) (Thompson et al. 2004). Temperature has been especially regarded as an important determinant in *Vibrio* abundance. A recent long-term retrospective analysis in North Sea coastal waters has supported this relationship over a multi-decadal time scale, demonstrating an increase in *Vibrio* with the rise of surface seawater temperature and showing a causal relationship with increased bathing infections (Vezzulli et al. 2012). Though these bulk environmental variables are useful for explaining the potential range of *Vibrio* distribution, they do not fully resolve the distribution of this genus, nor address the basic resource needs necessary to support bloom-levels of growth. This is complicated by the fact that *Vibrio* are considered generalists and can meet their nutritional demands through a wide variety of resources.

Multiple characteristic traits of *Vibrio* suggest that they are well adapted to survive in a wide range of nutrient conditions and can rapidly exploit new nutrient substrates or resources in

the environment (Takemura et al. 2014; Thompson and Polz 2006). *Vibrio* have been shown to persist for long periods under conditions of nutrient limitation (Eilers et al. 2000). Adaptations evolved to starvation pressures include reduction of cell volume (Denner et al. 2002), yet maintenance of a high number of rRNA copies to rapidly ramp up protein synthesis when conditions become more favorable (Eilers et al. 2000; Polz et al. 2006). Characterized as "opportunitrophs," *Vibrio* have a broad genomic and metabolic repertoire allowing them to utilize a wide range of substrates, and seek out nutrient "hot-spots" through chemotaxis and flagellar motility (Polz et al. 2006; Reidl and Klose 2002). *Vibrio* have primarily been characterized as particulate-associated organisms, often found attached to plankton and detritus (Turner et al. 2009) where they can produce a broad range of extracellular enzymatic proteins such as chitinases, proteases, and lipases (Thompson and Polz 2006) to access nutrient resources. Accumulating evidence suggests this particulate association is only part of their dynamic life cycle, and free-living members can thrive in the bacteioplankton community (Takemura et al. 2014; Worden et al. 2006). Free-living *Vibrio* have also shown explosive growth response to nutrient enrichment (Eilers et al. 2000; Thompson and Polz 2006) demonstrating > 4 doublings per day in the absence of predation (Worden et al. 2006).

This adaptive "feast or famine" life strategy allows exploitation of spatially and temporally variable resources leading to bloom conditions but also subjects them to "kill the winner" top down controls, such as grazing and viral lysis (Campbell et al. 2011), both of which tightly control *Vibrio* populations (Beardsley et al. 2003; Suttle 2007; Worden et al. 2006). This bloom-bust cycle presumably could have large implications on the biogeochemical cycles in marine ecosystems.

Much remains to be determined about the drivers of *Vibrio* population distribution and bloom formation. Although heterotrophic bacteria play a key role in nutrient cycling in marine systems (Azam and Malfatti 2007), there has been little investigation into the role of *Vibrio* in the cycling of biologically important nutrients. Furthermore, there is little understanding of how important trace nutrients might in turn control the population dynamics of these metabolically flexible bacteria.

IMPORTANCE OF IRON

Iron is a great biological capacitor because of its broad redox potential and is therefore utilized by almost all life on earth. It is arguably one of the most important trace metals in biology as a cofactor in many enzymatic and metabolic processes that are essential to life, such as respiration, photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, the trichloroacetic acid (TCA) cycle, oxygen transport, gene regulation and DNA biosynthesis (Andrews et al. 2003). Though iron is the fourth most abundant element in the Earth's crust, it is found in vanishingly low amounts in marine surface waters around the world due to low external supply and limited bioavailability.

Iron bioavailability and chemistry in seawater is highly complex due to physiochemical and biological processes (Boyd and Ellwood 2010). It has been a common practice to measure dissolved iron (traditionally defined as <0.2 μm filterable iron) as a proxy for bioavailability, as dissolved iron is the most readily utilized form of iron (Baker and Croot 2010). Iron solubility is dependent on pH and redox chemistry, which influences its speciation between Fe^{2+} and Fe^{3+}. In aerobic environments above pH 4.0, iron exists mainly as Fe^{3+} and is extremely insoluble and thus less biologically available. Therefore in the oxic, alkaline (pH ~ 8) surface waters of the

world's oceans, iron is rapidly oxidized becoming insoluble and biologically limited. Iron chemistry in marine waters is further complicated by the observation that 99.9% of the dissolved iron is bound to poorly defined organic ligands (Hunter and Boyd 2007; Rue and Bruland 1995). A portion of the stronger binding ligands are thought to be bacterially derived iron chelators called siderophores that enhance the solubility and uptake of Fe^{3+} (Boyd and Ellwood 2010).

The importance of iron availability in marine systems is most resolutely demonstrated by the iron hypothesis of John Martin that proposes biological productivity in a vast portion of the world's open oceans, specifically High Nutrient Low Chlorophyll (HNLC) regions are limited solely by iron (Martin 1990). In the last 25 years, many artificial seeding experiments have largely confirmed that iron supply limits primary production and has important impacts on phytoplankton in a large portion of the world's oceans (Boyd et al. 2007; Breitbarth et al. 2010). Though this is especially true in the oligotrophic open ocean, biological iron limitation can also occur in coastal systems traditionally considered to have higher amounts of terrestrial iron input (Öztürk et al. 2002). Coastal microbes have evolved a higher dependence on iron compared to their pelagic counterparts (Wells and Trick 2004), yet face limitation of bioavailable iron because of intense competition and rapid physical removal processes like salt flocculation and humic precipitation (Boyd and Ellwood 2010; Öztürk et al. 2002). Consequently, iron is an important factor in understanding marine production on a global scale, in both coastal and pelagic systems. Taken together, due to low solubility, high competition and low external input some estimates propose that 50% of global marine production is limited by iron (Moore et al. 2001).

IRON IN MARINE HETEROTROPHIC PROCESSES

Marine heterotrophic bacteria have a higher iron per biomass quota than many phytoplankton (Tortell et al. 1999) accounting for up to 80% of the total planktonic uptake in some systems (Schmidt and Hutchins 1999). Iron plays a keystone role in the ability of heterotrophic bacteria to metabolize carbon substrates and crucial macronutrients like N and P, setting limits on their growth (Fourquez et al. 2014; Kirchman et al. 2003; Yong et al. 2014). Some heterotrophs, including some *Vibrio* spp. are capable of fixing nitrogen, which requires extremely high amounts of iron as a co-factor (Chimetto et al. 2008; Criminger et al. 2007). Iron limitation also limits C metabolism and growth in oceanic, and to an even greater degree, coastal heterotrophic bacteria (Fourquez et al. 2014). This is especially important in light of the fundamental contribution of heterotrophic bacteria to the cycling of C in marine ecosystems (Azam and Malfatti 2007).

Heterotrophic bacteria, play a key role in modulating iron solubility in marine water through secretion of high-affinity iron-chelating siderophores (Granger and Price 1999; Strzepek et al. 2005). Iron-siderophore complexes allow active uptake into the bacterial cell, but are also a usable exogenous source of solubilized iron for phytoplankton uptake (Hopkinson and Morel 2009; Soria-Dengg et al. 2001). Similarly, heterotrophic bacteria mutualistically associated with algae can provide siderophore-bound iron, that undergoes photolysis in support of the photosyntheic needs of the algal host (Amin et al. 2009).

Vibrio and other rapidly responding heterotrophs likely play a pivotal, but as yet poorly understood role, in coupling iron flux with C and nutrient cycling in marine food webs, ultimately influencing global climate processes and food web dynamics (Adly et al. 2015; Azam and Malfatti 2007).

IRON IN VIBRIO ECOLOGY

Despite the key role of heterotrophic bacteria in marine biogeochemical iron cycling, few investigations have examined the direct effect of iron availability on the environmental population dynamics of *Vibrio*. In a recent meta-analysis comparing environmental correlates to *Vibrio* presence and abundance, only one study over the past 25 years had examined the potential of iron to influence *Vibrio* distribution (Takemura et al. 2014). Although this single study found no correlation with iron, the parameter measured was ferrous iron Fe^{2+} that would expectedly be low since ferrous iron rapidly oxidizes to ferric iron hydroxides in the seawater that was analyzed (Baker and Croot 2010; Blackwell and Oliver 2008).

Most of what we currently know about iron in the ecology and biology of *Vibrio* is driven from a clinical perspective with a focused interest on pathogenic strains, especially, *V.cholerae*, *V.vulnificus*, *V.parahaemolyticus,* and *V.alginolyticus* (Crosa and Payne 2004; Kustusch et al. 2011). The ability of pathogenic bacteria to cause disease is dependent upon competing with the host organism for iron. Host defense strategies have evolved to sequester iron as a primary line of defense to prevent bacterial invasion (Parrow et al. 2013; Weinberg 2009). Successful pathogens are able to circumvent these host-withholding strategies by utilizing a broad array of iron acquisition mechanisms and virulence factors that liberate iron from host tissue (Crosa and Payne 2004; Litwin and Calderwood 1993). The importance of iron in host susceptibility to *Vibrio* infection is most clearly illustrated by *V. vulnificus*, the deadliest seafood borne pathogen with a mortality rate of approximately 50% (Blackwell and Oliver 2008). Infections by *V.vulnificus* have been highly correlated with liver disease and hemochromatosis, resulting in poor host control of iron-serum levels and implicated in the lethality of this species (Wright et al. 1981).

Arguably, one of the most highly investigated disease-causing microbes is *V. cholerae*, the causative agent of the deadly diarrheal disease cholera. *V.cholerae* has an absolute requirement for iron and must obtain this element in the diverse environments in which it has been found; including free-living, algal and zooplanktonic association and in human and animal hosts (Reidl and Klose 2002; Wyckoff et al. 2007). *V.cholerae* has over 50 iron acquisition and transport genes spread across its two chromosomes with high redundancy for ferrous, ferric and biologically complexed iron uptake (Wyckoff and Payne 2011). *V.cholerae* as well as most other *Vibrio* species are capable of directly transporting heme, an important iron-rich compound found in photosynthetic and animal cells (including the hemoproteins cytochrome and hemoglobin) (O'malley et al. 1999). It has been suggested that virulence factors like hemolysins, found in a range of *Vibrio* spp. [thermostable direct hemolysin (TDH) in *V.parahaemolyticus* and those coded by the genes *HlyA* and *vvhA* in *V.cholerae* and *V. vulnificus* respectively], are mechanisms to liberate iron from heme-containing proteins (Crosa and Payne 2004). Indeed, in *V. parahaemolyticus* iron limitation induces production of TDH (León-Sicairos et al. 2015), giving some support to this idea. In contrast to the role of heme utilization in *Vibrio* pathogenesis, iron and heme uptake are also important in supporting symbiotic colonization of bioluminescent *V. fischeri* in the light organ of bobtail squid (Septer et al. 2011), demonstrating the key role of iron in a range of host-*Vibrio* interactions.

A common iron scavenging strategy found in all *Vibrio*, both in the host and in the environment is the production of siderophores (Wyckoff et al. 2015). Siderophores have a high affinity constant for ferric iron that can effectively "steal" iron from host iron-storage proteins like ferritin and transferrin as well as chelate iron from environmental sources (Tortell et al. 1999; Wyckoff et al. 2007). Presumably, in the marine environment, similar ferritin storage

proteins found in algal phytoplankton and diatoms could also be a target for *Vibrio* siderophore scavenging, though this has yet to be investigated (Botebol et al. 2015; Marchetti et al. 2008). *V. cholerae* can also transport and use iron from siderophores produced by other bacteria, a common trait among all pathogenic *Vibrio* species (León-Sicairos et al. 2015; Wyckoff et al. 2015).

Taken together, it is clear that *Vibrio* are adept at accessing iron in the well studied realm of host association where iron is actively sequestered, what is less clear is the role of iron availability in controlling the environmental prevalence of *Vibrio*. Their ubiquity in the environment and the fact that many of the pathogenic species are not reliant on host association, but can be found in a free-living state (León-Sicairos et al. 2015; Reidl and Klose 2002), demonstrate that *Vibrio* can also be successful planktonic scavengers of iron in the often iron-limiting conditions of marine water. Because of the importance of iron in heterotrophic processing of carbon and macronutrients, ultimately affecting growth, an influx of newly available iron could presumably be an important driver of *Vibrio* growth in environmental waters.

ATMOSPHERIC DUST IRON

Atmospheric dust deposition is a major supply mechanism of iron to the global ocean (Duce and Tindale 1991), estimated to deliver over 3-times more dissolved iron than riverine input (Duce and Tindale 1991). The Sahara Desert of Africa is the source of 2/3 of global atmospheric dust (Mahowald et al. 2009). Dust originating in northern Africa is swept along easterly trade winds to downwind surface waters in the Mediterranean Sea, Atlantic Ocean and tropical Caribbean, providing up to 87% of the dissolved iron (the most bioavailable form) to

marine surface water communities (Conway and John 2014; Mahowald et al. 2009). Atmospheric processing (acidification and photo-reduction) during long-range transport along with wet deposition reportedly produce highly soluble species of iron (Baker and Croot 2010). The full biological response to Saharan dust deposition is uncertain, but many studies have predominately focused on autotrophic primary production response because of the high iron demand of photosynthetic processes and the large climatic consequences to carbon cycling (Boyd et al. 2010; Lenes et al. 2001).

Evidence is growing that marine heterotrophs may play a key role in the rapid cycling of deposited dust nutrients (Pulido-Villena et al. 2008). The comparatively few studies that have specifically examined heterotrophic bacterial response to dust-associated nutrients have monitored bulk parameters such as bacterial production and respiration (Herut et al. 2005; Pulido-Villena et al. 2008) with little taxonomic resolution of the key players. There is clear need to partition the response to dust nutrients; dust associated iron stimulation of primary production potentially increases the efficiency of the biological pump in the sequestration of atmospheric CO_2, however, it also has the potential to drive the microbial loop, enabling heterotrophic respiration to respire any organic carbon present in the system resulting in little biological draw-down of CO_2. Considering the central role of heterotrophic bacteria in iron cycling, it is likely that dust deposited iron can affect the structure and functioning of the whole microbial food web and have broad ecological implications (Pulido-Villena et al. 2014). Rapid responding "opportunitrophic" bacteria like *Vibiro* could serve as an important model responder of opportunistic heterotrophic response to dust iron.

AIMS AND OBJECTIVES

While much is known about *Vibrio* pathogenicity, less is known about their role in marine nutrient cycling and the environmental and the nutritional resources that determine their growth and distribution in marine waters. The goals of the following studies are to test whether environmental iron flux can drive *Vibrio* population dynamics. Further, the biological response to dust-associated pulses of nutrients is not fully known. Using *Vibrio* as a model, the following investigations examine the role of heterotrophic bacteria in processing Saharan dust iron in coastal and open ocean systems, with broad implications for marine productivity and environmental and human health.

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CHAPTER 3

IRON-LIMITED ARTIFICIAL SALTWATER MEDIUM FOR THE STUDY OF IRON PROCESSING IN MARINE HETEROTROPHIC BACTERIA

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Abstract

Iron, an essential co-factor in photosynthesis, respiration, and nitrogen fixation, is a key determinant of biological productivity in much of the global ocean. Iron has a complex chemistry in seawater, and its availability and biological processing are not fully understood. Heterotrophic bacteria play a central role in iron cycling, yet specifics of heterotrophic use of environmental iron sources remain poorly resolved. An artificial medium specifically designed to study heterotrophic iron dynamics in seawater is needed but is currently lacking. To date such studies have relied on defined media predominantly adapted from studies examining autotrophic response to iron, with media components that are optimized for phytoplankton growth. Additionally, other formulations use natural seawater as a base, but availability, lack of defined components, and the need for time-consuming elimination of viruses make them disadvantageous. Here we present a simple, inexpensive, defined saltwater medium, VibFeL, optimized for examining heterotrophic growth, using the cosmopolitan genus *Vibrio*. We establish that VibFeL effectively restricts growth of *Vibrio alginolyticus* (a common surface water heterotroph), compared to VibFeL supplemented with $FeCl_3$. Next, we next demonstrate that growth of cultured and environmental strains of *Vibrio* are completely restricted in VibFeL alone, but increases by up to 18 fold in VibFeL supplemented with iron from Saharan dust material, a major source of iron to the Atlantic Ocean. This system provides a model for studying iron limitation and iron resource utilization in heterotrophic bacteria, which ultimately could be used to elucidate physiological and molecular targets of heterotrophic iron processing in more biologically complex environmental waters.

Introduction

The trace metal iron is a limiting factor in biological production over a large part of the world's oceans (Boyd et al. 2007), especially in the oligotrophic open ocean but also in coastal systems traditionally considered to have higher amounts of terrestrial iron input (Öztürk et al. 2002). Coastal microbes have evolved a higher dependence on iron compared to their pelagic counterparts (Wells and Trick 2004), yet they face limitation of bioavailable iron because of rapid physical removal processes like salt flocculation and humic precipitation (Boyd and Ellwood 2010; Öztürk et al. 2002). Consequently, iron is an important factor in understanding marine production on a global scale, in both coastal and pelagic systems. Although heterotophic bacteria are known to play a key role in nutrient processing in marine systems (Azam and Malfatti 2007), a defined and adaptable system in which to study their role in iron processing and utilization of environmental iron sources are lacking. Here, we describe the preparation of an iron-limited saltwater medium, referred to as VibFeL. This formulation was created specifically to study heterotrophic response to iron limitation and heterotrophic ability to process various environmental iron sources, using *Vibrio* as a model heterotroph.

Seawater media commonly used for heterotrophic experimentation have several limitations. They are typically based on defined formulations originally created for phytoplankton studies, containing many unnecessary constituents (adding cost, complexity, and increased potential for iron contamination during preparation). Conversely, others have relied on a base of 0.2- or 0.45-μm filtered natural seawater (Hopkinson et al. 2008; Kirchman et al. 2003; Weaver et al. 2003). Additional ultrafiltration (0.02 μm) or other processing is needed to remove viruses from natural waters, which could impact evaluation of bacterial growth. Furthermore,

accessibility to natural seawater is not always possible. Formulations are also typically reliant on the addition of complex (undefined) organic compounds (e.g., peptone and casein) to support bacterial growth (Hopkinson et al. 2008).

The VibFeL formulation described here is a defined medium, partially based on the artificial seawater medium Aquil (Price et al. 1988/1989) used in phytoplankton growth studies. The composition has been optimized for heterotrophic bacterial growth conditions, eliminating many of the non-essential components in Aquil that are specific to phytoplankton growth (silica, selenium, B-vitamins, etc.), but utilizing similar trace-metal chelation and sterilization techniques. This medium is also modular, allowing for manipulation of the type and concentration of individual constituents. For example, in the case of *Vibrio*, which have among the highest doubling rates observed in prokaryotes (<10 min) (Maida et al. 2013), inorganic nutrients (P and N) and carbon sources can be added at highly replete levels to ensure that iron is the limiting factor for bacterial growth.

Materials and procedures

Preparation overview

All manipulations were made using trace-metal clean techniques (Bowie and Lohan 2009). Storage vessels, filters, and equipment used in medium synthesis and experimentation were acid washed in 1 N HCL (Fisher Trace Metal Grade; A500) and rinsed at least three times in high-quality, pure water to remove iron contamination and residual acid. In these studies Milli-Q water (Millipore, resistivity of 18.2 MΩ) was used for rinse and medium preparation. All procedures were performed in a laminar flow hood (class 100) to minimize contamination. Reagent grade salts and nutrients can be used in the synthesis of this medium (preferably <

0.001% Fe contamination), but if economically feasible, it is worthwhile to purchase the highest-grade reagents possible (for example Sigma Ultra or Sigma Trace SELECT, ≥99.999%). This is especially true for chemicals that are naturally higher in iron contamination (i.e., phosphate containing reagents) as well as any additions that are not purified of trace-metal contamination (in this study sucrose, $MgSO_4$, and $CaCl_2$).

The procedure of VibFeL synthesis is divided into three parts: 1) basal salt nutrient preparation (including chelation of contaminating trace metals and microwave sterilization), 2) trace metal-EDTA preparation, and 3) "VibFeL Complete" preparation by adding the following filter-sterilized pre-experimental additions to the basal medium: carbon source, additional salts prone to precipitation ($MgSO_4$ and $CaCl_2$), trace metals-EDTA, and the iron source to be tested (Fig. 1).

Basal Salt and Nutrients

Basal salts and nutrients were dissolved in a total volume of 1 L Milli-Q water to create a 5x stock solution yielding constituent concentrations as shown in Table 1. The solution was made and stored in an acid washed, Milli-Q rinsed, 1 L Pyrex bottle. The combined 5x stock contained K_2HPO_4 and $Na_2HPO_4 \bullet 7H_2O$ as sources of K and PO_4 as well as providing medium buffering. Of particular note, KH_2PO_4 was suspected to be the source of a white precipitate forming after microwave sterilization and was replaced with K_2HPO_4 in this formulation and for all experimental manipulations described in this study. Salinity was adjusted with the addition of NaCl. The medium contained NH_4Cl as a source of nitrogen, because it has been shown that some *Vibrio* species have poor growth efficiency when grown on NO_3 as the sole nitrogen source when compared to ammonium, especially in low-iron conditions (Kirchman et al. 2003). Final

experimental concentration of basal salts and nutrients (Table 1) were obtained by diluting 200 ml of the 5x stock into 1 L final volume of Milli-Q water in a trace metal clean Pyrex bottle, producing the following constituent concentrations: NaCl (420 mM), K_2HPO_4 (0.1 mM), NH_4Cl (18.6 mM), and $Na_2HPO_4 \bullet 7H_2O$ (42 mM).

The basal medium was purified of trace metals by passing through a column containing Chelex-100 ion exchange resin (Sigma Aldrich; 95621). Chelex has a high affinity for transition metals, even in highly concentrated salt solutions. Before the Chelex resin was loaded onto the column, it was purified following the method of Price et al. (1988/1989) to remove organic metal-binding ligands that can potentially leach from the resin and enter the solution. Briefly, 30 g of resin was alternately soaked in the following reagents, followed by thorough rinsing in Milli-Q water before the subsequent soak: 1) methanol soak for 3-4 h to remove residual organics, 2) overnight soak in 1 M HCl (Trace Metal Grade) to convert the resin to the hydrogen form, 3) overnight soak in 1 M NH_4OH (Fisher Optima; A470) to convert the resin to the NH_4^+ form, and 4) a final 10 min soak in 0.1 M HCl (Trace Metal Grade) immediately before column loading. After the final Milli-Q rinse, the resin was mixed as a slurry in 300 ml of basal medium. Chelex binding of transition metal cations, including iron, is optimized at a pH 6.5 or higher. Accordingly, the Chelex-basal-medium solution was adjusted to pH 7.6-8.0 by adding several drops of 50% NaOH. The slurry of Chelex-basal-medium was then transferred to an acid cleaned Pyrex chromatography column (VWR 26307). The basal medium was allowed to run at a rate of 4.5 ml min^{-1} by adjusting the fluid head above the resin packed column (~10 cm), allowing for slow kinetics of exchange with the resin. Several bed volumes of basal medium were passed through the column (~250 ml) into a trace metal clean 500 ml Pyrex collection bottle to ensure adequate packing of the Chelex resin in the column. The initial effluent was used as a rinse for

the collection bottle and then discarded. The rinsed bottle was then used to collect the remaining "Chelexed" (trace-metal chelated) basal medium.

Sterilization by autoclave may introduce steam-associated trace metal contamination; therefore, the Chelexed basal medium was microwave sterilized based on the procedure of (Keller et al. 1988). The medium was brought to a boil 3x in a 1250 W microwave and kept near boiling by microwaving every 15 min for 1 h. Reduction of the power level to half during the entire process allowed sterilization to occur more slowly without rapid boil-over. The sterile, trace-metal free, basal medium was then allowed to cool overnight before pre-experimental additions (carbon source, trace metals, Ca^{2+} and Mg^{2+} salts, and iron-source) were added.

Trace Metal- EDTA Preparation

Individual trace metal (TM) stock solutions of cobalt, copper, manganese, molybdenum, and zinc were prepared in 0.01 M HCl (Fisher Trace Metal Grade) and allowed to equilibrate for several days (concentrations between 10^{-2} and 10^{-3} M as listed in Table 1). A combined TM stock solution, buffered with sodium EDTA was created by adding 5 x 10^{-2} M of Na_2EDTA salt to Milli-Q water followed by additions of each individual TM stock (1000-fold dilutions). Addition of EDTA maintains a computable pool of trace metals by preventing precipitation or adsorption and minimizes the effect of contaminating metals inadvertently introduced during medium preparation. The final combined TM solution was allowed to equilibrate for at least 24 h and then 0.2-µm sterile-filtered before being added to the basal medium.

VibFeL Complete

It is important to add 0.2-μm sterile-filtered stocks of the carbon source, magnesium and calcium salts, TM-EDTA, as well as the iron source to the basal medium after the medium has been microwave sterilized to prevent degradation and precipitation of these constituents (Fig. 1). High-grade sucrose (> 99 % purity, Sigma ReagentPlus; S0389) stock (20% w/v) was added as a carbon source (0.4% w/v final medium concentration) (Table 1). In the original Aquil formulation, both calcium and magnesium species were supersaturated and easily precipitated out of solution, especially after autoclave sterilization (Morel et al. 1979). Additionally, in the present formulation, phosphate provides most of the buffering capacity and has been shown to have limited usefulness in buffering Ca^{2+} and Mg^{2+} species (Sambrook and Russell 2001). We therefore reduced the final medium concentration of $CaCl_2 \bullet 2H_2O$ and $MgSO_4$ salts to 0.1 mM and 2.5 mM respectively (Table 1), adding sterile-filtered stocks of these salts to the basal medium after it had been microwave sterilized. Finally, the combined TM-EDTA stock was added at least 24 h before experimental manipulation to allow time to re-equilibrate (Morel et al. 1979). The TM-EDTA provided 50 μM EDTA in the final VibFeL complete medium (Table 1). This excess EDTA was sufficient to prevent iron precipitation in iron-replete controls (*see Assessment*). Inorganic $FeCl_3$ (1000x stock solution, prepared in 0.01 M HCl, was used to provide ~ 4 μM iron in control cultures.

Culture conditions for assessment experiments

Frozen cell stocks of commercially available *Vibrio* strains, *V. alginolyticus* (American Type Culture Collection [ATCC] strain 33839) and *V. cholerae* (ATCC 39315), and an environmental *Vibrio* surface water isolate from the Dry Tortugas (Florida, USA), were

recovered in sterilized artificial seawater (Instant Ocean; RC3) with a salinity of 30, amended with 1% peptone and 0.5 % yeast extract (ASW+PYE). Recovered cells were incubated for 12 h at 30°C, sub-cultured in 5 ml fresh ASW+PYE (1:100 dilution), and allowed to grow to log phase as monitored by measuring the optical density at 600 nm (OD_{600}) on a spectrophotometer (Eppendorf Biophotometer; 6131). Cells were washed twice and re-suspended in Vib-FeL to eliminate iron carry-over. The experiment was initiated at time zero by inoculating washed cells (1:100) into 5 ml of the appropriate culture conditions (VibFeL alone, VibFeL with $FeCl_3$ as a control, or VibFeL with the environmental iron source to be tested), and incubated for 24 h at 30°C on a shaking platform to maintain aeration. *Vibrio* abundance was determined by OD_{600} or by plating aliquots from each culture onto ASW+PYE agar plates at the appropriate time point. Spread plates were incubated at 30°C for 24 h, and colony forming units (CFU) were enumerated and compared.

Assessment

VibFeL induces iron limitation in Vibrio

Initial experiments were performed to validate the use of various carbon sources in VibFeL as well as the applicability of using $FeCl_3$ as an iron-replete control. We then demonstrated that VibFeL could successfully limit heterotrophic *Vibrio* growth by inducing iron limitation compared to an iron-replete control. Culturing was done as described previously, inoculating cells into VibFeL alone or VibFeL with 4 μM $FeCl_3$. Growth was monitored spectrophotometrically by measuring the OD_{600}.

Saccharides (sugars) in natural ocean waters can affect the solubility and bioavailability of iron, above the level observed for inorganic iron alone (Breitbarth et al. 2010; Hassler et al. 2011). Media formulations used in other heterotrophic growth studies have used complex organic sources of carbon (e.g., peptone or casein) (Granger and Price 1999; Hopkinson et al. 2008) potentially confounding iron chemistry in the media. We therefore used a defined carbon source, opting for the least complex forms: the monosaccharide glucose and the disaccharide sucrose. In preliminary tests we compared growth of *V. cholerae* on sucrose versus glucose in iron-replete (4 μM Fe) VibFeL medium. After 24 h, a nominal difference between growth in sucrose and glucose was observed (OD_{600} 2.5 and 2.4, respectively), demonstrating similar robust growth characteristics with both sugars. Because glucose is a reducing sugar and the solubility and bioavailability of iron is highly dependent upon its redox state, we chose to use the potentially less reactive sucrose, which is a non-reducing sugar, for all further experiments described in this study. Additionally, all strains examined in these experiments were sucrose fermenters, although some *Vibrio* species lack the ability to ferment sucrose.

Inorganic iron has been shown to be highly available to marine bacteria (as demonstrated with *V. natriegens*), in both field and laboratory experiments (Weaver et al. 2003). Accordingly, we used inorganic $FeCl_3$ as a replete control, providing 4.4 μM of Fe (as measured by ICP-MS), similar to iron-replete conditions found in other studies (Hopkinson et al. 2008). There was concern that this relatively high concentration of iron might exceed EDTA-buffering capacity, resulting in a precipitated substrate for *Vibrio* growth, potentially confounding growth interpretations. Consequently, we used a chemical equilibrium model, Visual MINTEQ (version 3.0) (http://vminteq.lwr.kth.se) for the calculation of metal speciation and solubility saturation at pH 7.6 (pH at experiment initiation after the addition of cells). Ferrihydrite and other highly

soluble Fe species (Khanna et al.) were found to be under-saturated (negative saturation index value) in this medium formulation, and not likely to precipitate (Appendix Table 2). The bioavailable free metal cations and weak Fe complexes, iron prime (F'), was calculated to provide at least 3.52 pM of highly labile iron for *Vibrio* growth after accounting for non-bioavailable iron-EDTA complexes (Appendix Table 3).

After establishing an appropriate carbon source and a replete control for *Vibrio* growth, we then examined the ability of VibFeL to effectively restrict growth of *V. alginolyticus* through iron limitation. *V. alginolyticus* was chosen as a test strain because it is a predominant *Vibrio* species in coastal and oligotrophic marine waters (Kustusch et al. 2011). Cultures of *V. alginolyticus* grown in VibFeL alone were compared to iron-replete VibFeL (4 μM Fe) by monitoring culture growth (OD_{600}) at 0, 8, 18, 24 and 48 h (Fig. 2). VibFeL effectively restricted *V. alginolyticus* growth up to 48 h. In contrast, there was a logarithmic increase in growth between 8 and 18 h in the Fe-replete control. At 24 h, the Fe-replete control was visually turbid compared to no observable growth in VibFeL alone (Fig. 2).

Establishing Vibrio *growth response to an environmental iron source using VibFeL*

The *Vibrio*-VibFeL model system was used to determine if heterotrophic bacteria could process an environmental source of iron to support growth. Because of its importance as a source of iron in the world's oceans, we evaluated Saharan Desert dust as our environmental iron source. Saharan topsoil, rich in insoluble iron oxides like hematite and goethite is entrained into the atmosphere at its source. This atmospheric dust is transported great distances across the Atlantic Ocean (Mahowald et al. 2009). During transport, some fraction of the iron becomes more soluble due to physical and chemical processes like ultra-violet reduction and atmospheric

acidification (Baker and Croot 2010). Dust deposition into downwind marine waters is suspected to provide an ephemeral pulse of nutrients and iron to surface water communities. The biological response to this iron deposition has not been fully resolved, with most investigation focused on autotrophic organisms (Boyd et al. 2010). Recent evidence suggests that *Vibrio* and other fast-responding heterotrophs might play an important role in processing Saharan dust iron in marine surface waters (Westrich et al. in review). Yet, much remains to be determined, such as the physiological and molecular mechanisms at play in heterotrophic processing of dust-iron (i.e., siderophores) potentially exerting control of iron bioavailability to the autotrophic community.

In our model *Vibrio* system, Saharan dust, as the sole source of iron, was able to alleviate iron limitation in VibFeL. Saharan source material collection, manipulation, and characterization are reported in detail elsewhere (Westrich et al. in review). Briefly, the U.S. Geological Survey (USGS) provided Saharan source material collected from a highly weathered dune in Morocco. Saharan material was processed to simulate atmospheric processing by size fractionation (sieving out large particulates), UV irradiation, and acidification with a weak acid. The processed source material was measured to contain 1 μM Fe by ICP-MS analysis. Growth of *V. alginolyticus*, *V. cholerae*, and a *Vibrio* isolate from the Florida Straits (an area routinely exposed to Saharan dust deposition) was tested individually with Saharan source material added to VibFeL (1:150) as well as in VibFeL alone. Growth was monitored by measuring OD_{600} at 0, 18, 24 and 48 h. All *Vibrio* tested were able grow with the addition of Saharan material, indicating that this could act as a sufficient source of iron to support growth (Fig. 3). *V. alginolyticus* exhibited similar growth dynamics as the previous tests using $FeCl_3$ as a source of iron, with a peak response between 18-24 h. Growth of *V. alginolyticus* was completely inhibited in VibFeL alone (lacking

any iron source). *V. cholerae* and the Florida isolate also exhibited a robust growth response using Saharan dust, but their growth in VibFeL alone was not entirely restricted, showing a minimal response up to 24 h and declining thereafter (Fig.3). There are many potential causes for this slight growth in the iron-restricted cultures. There could have been some iron contamination from culture carry-over or accidentally introduced during the procedure. Also, some *Vibrio* species, like *V. cholerae,* have been shown to have a very robust array of iron acquisition mechanisms (Wyckoff et al. 2007) possibly allowing them to be more efficient at taking advantage of very minute amounts of iron contamination, including iron scavenging during cryptic growth from dead or dying cells in the culture.

V. alginolyticus and *V. cholerae* growth in response to the addition of iron from Saharan dust was also examined using culture methods. The growth of both strains was tested under three separate conditions: 1) VibFeL alone, 2) VibFeL amended with Saharan source material and 3) VibFeL with $FeCl_3$ (as detailed in previous experiments). At the initiation of the experiment, time zero (T_0), and at peak response time (T_n) (18 or 24 h), 100 µl from each culture was spread-plated onto ASW+PYE agar plates and incubated at 30°C for 24 h. Colony forming units (CFU) were enumerated and compared (Fig. 4). CFU ml^{-1} was normalized among experiments by comparing the ratio of CFU ml^{-1} at T_n to that at T_0 for each strain. Vib-FeL alone effectively restricted growth of both test strains. The addition of Saharan dust led to significant growth of both *V. cholerae* and *V. alginolyticus*, compared to VibFeL controls (13 and 18 times as great, respectively; ANOVA, $P < 0.05$; $df = 5$). The proportional growth for the Saharan amendments was not significantly different from that noted for the $FeCl_3$ controls, which had 20 and 23 times as much growth for *V. cholerae* and *V. alginolyticus*, respectively, compared to VibFeL alone (Fig. 4).

Discussion

Heterotrophic bacteria play a significant role in the biogeochemical cycling of iron in the marine environment (Strzepek et al. 2005). Heterotrophic bacteria have a high demand for iron especially in their electron transport system during respiration (Kirchman et al. 2003). Indeed, heterotrophs have been reported to have a higher demand by biomass than most phytoplankton, accounting for up to 80% of iron uptake in some systems (Schmidt and Hutchins 1999). Because of the dynamic complexity between microorganisms and iron chemistry in natural marine waters, there is a need to study heterotrophic processing of iron flux in a more controlled environment. We have developed a successful model system for examining heterotrophic response to iron in seawater using VibFeL. VibFeL was specifically optimized for studying heterotrophic processing of iron, and differs from other defined formulations that were originally optimized for phytoplankton (Hopkinson et al. 2008; Price et al. 1988/1989). Our results show that heterotrophic *Vibrio* spp. could be successfully iron-limited in VibFeL. The VibFeL system demonstrated that several *Vibrio* strains could use iron from Saharan dust for growth and survival. Further application of the *Vibrio*-VibFeL model system (though beyond the scope of the assays reported here) could be to profile molecular and physiological response of other heterotrophs to iron limitation as well as environmental iron enrichment. These targeted studies would aid in elucidating specific heterotrophic genomic, transcriptomic and proteomic pathways that may be observed in more complex community analyses of natural waters.

The formulation of VibFeL also differs from other reported media used for heterotrophic growth studies in that it does not rely on a natural seawater base. The complexity of natural seawater and its inherent variability from source to source can challenge experimental result

repeatability. Additionally, many fast-responding heterotrophs like *Vibrio* have been reported to be susceptible to predation and viral lysis in natural waters (Worden et al. 2006); therefore, a natural seawater base specific for heterotrophic analysis might require time consuming ultra-filtration (<0.02 μm pore size).

Comments and recommendations

Some studies have used the fungal siderophore desferriferrioxime-B (DFB) to progressively decrease the biological availability of iron in seawater (Wells and Trick 2004). We caution against this methodology because of a homologue siderophore produced by *Vibrio* that is similar in structure to DFB (Martinez et al. 2001). Instead we recommend, as demonstrated in this study, using the well-established technique of chelating contaminating iron from experimental medium using Chelex, followed by controlled complexation of trace metals and iron with EDTA.

As a defined modular medium, components can be adjusted to simulate alternative environmental conditions. For example, because salt flocculation plays an important role in iron dynamics (Boyd and Ellwood 2010; Boyle et al. 1977), comparative studies could examine the salinity differential between estuarine and open ocean conditions on iron uptake by adjusting the salt (NaCl) content of Vib-FeL. Additionally, though many carbon substrates can be used for heterotrophic growth, differential growth on other carbon sources should be considered and compared. For example, the strains in this study were sucrose fermenting *Vibrio*, found to grow equally well on both sucrose and glucose. However, non-sucrose fermenting *Vibrio* (for example *V. parahaemolyticus or V.vulnificus*) might vary in growth rate when sucrose is the sole

carbon source. Also, when using this medium to study iron mechanisms in open ocean heterotrophic bacteria, a close consideration of carbon source is necessary given that oceanic strains have been shown to have differential carbon metabolism when compared to coastal strains when iron limited (Fourquez et al. 2014).

Finally, most heterotrophic studies using defined media have included vitamins, following the recipes of phytoplankton specific studies (Granger and Price 1999; Morel et al. 1979). B-vitamins are often used as a substitute in enzymatic co-factors during iron stress in marine microorganisms, especially in oligotrophic waters (Giovannoni 2012) . Our desire was to isolate the specific effect of iron on physiological response, because many prokaryotes synthesize their own B-vitamins we excluded them from our *Vibrio* specific formulation. If adapting this medium to open ocean heterotrophs, consideration should be taken for re-introducing B-vitamins into the formulation.

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Table

Table 3.1. VibFeL Formulation.

Basal Salts & Nutrients	**Combined 5x Stock (M)**	**Final Concentration (M)**
K_2HPO_4	5.0×10^{-4}	1.0×10^{-4}
NH_4Cl	9.3×10^{-2}	1.86×10^{-2}
$Na_2HPO_4 \bullet 7H_2O$	2.1×10^{-1}	4.2×10^{-2}
NaCl	2.1	4.2×10^{-1}

Additional Salts	**Individual Stock (M)**	**Final Concentration (M)**
$CaCl_2 \bullet 2H_2O$	1.0×10^{-1}	1.0×10^{-4}
$MgSO_4$	1	2.5×10^{-3}
Carbon Source		
Glucose or Sucrose	20% (w/v)	0.4% (w/v)

Trace Metals (TM)	**Stock (M)**	**Combined TM (M)**	**Final Concentration (M)**
Na_2MoO_4	1.1×10^{-2}	1.1×10^{-4}	1.1×10^{-7}
$MnCl_2 \cdot 4H_2O$	2.3×10^{-2}	2.3×10^{-4}	2.3×10^{-7}
$CuSO_4 \cdot 5H_2O$	9.8×10^{-3}	9.8×10^{-6}	9.8×10^{-9}
$CoCl_2 \cdot 6H_2O$	2.5×10^{-3}	2.5×10^{-5}	2.5×10^{-8}
$ZnSO_4 \cdot 7H_2O$	3.9×10^{-3}	3.9×10^{-5}	3.9×10^{-8}
$Na_2EDTA \cdot 2H_2O$	–	5.0×10^{-2}	5.0×10^{-5}

Figures

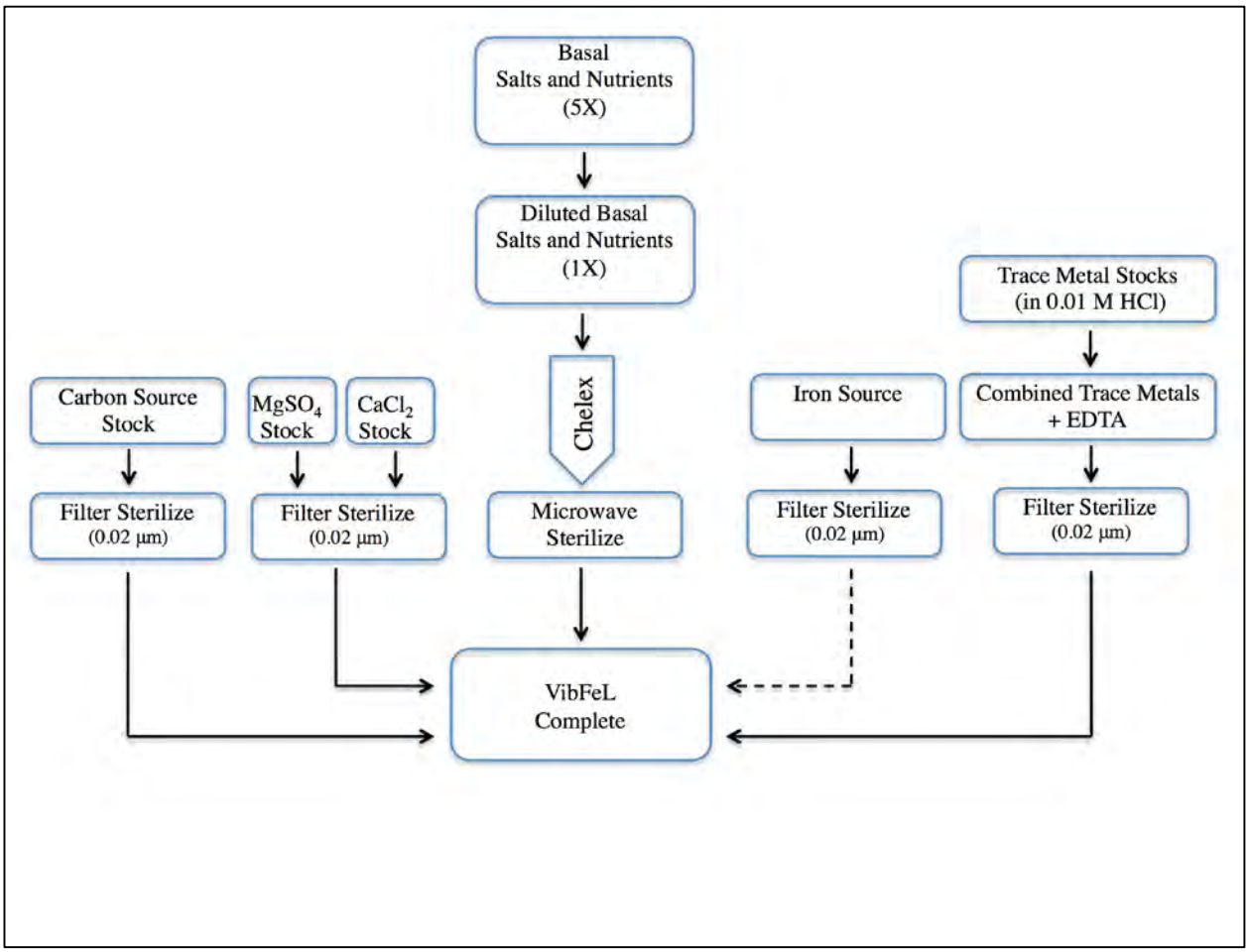

Fig. 3.1. Schematic outline of VibFeL preparation. Dashed line indicates preparations with iron additions (eliminated for iron-depleted experimentation).

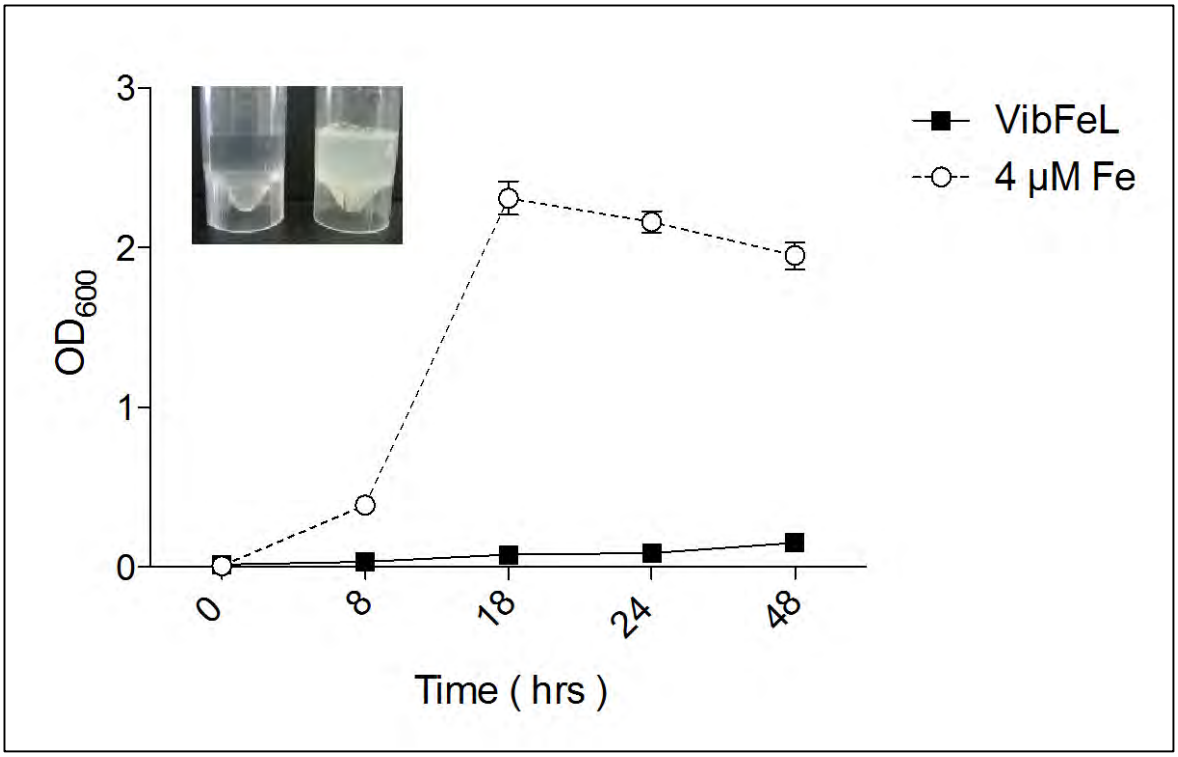

Fig. 3.2. *Vibrio* growth limitation in VibFeL. Growth of *Vibrio alginolyticus* measured by optical density at 600 nm (OD600) (mean ± SEM) in VibFeL alone and VibFeL supplemented with 4 µM iron (Fe) (n=4 for all time points, except 8 h, n=1). Photo inset shows turbidity difference at 24 h between cells growing in VibFeL alone (left) and VibFeL supplemented with iron (right).

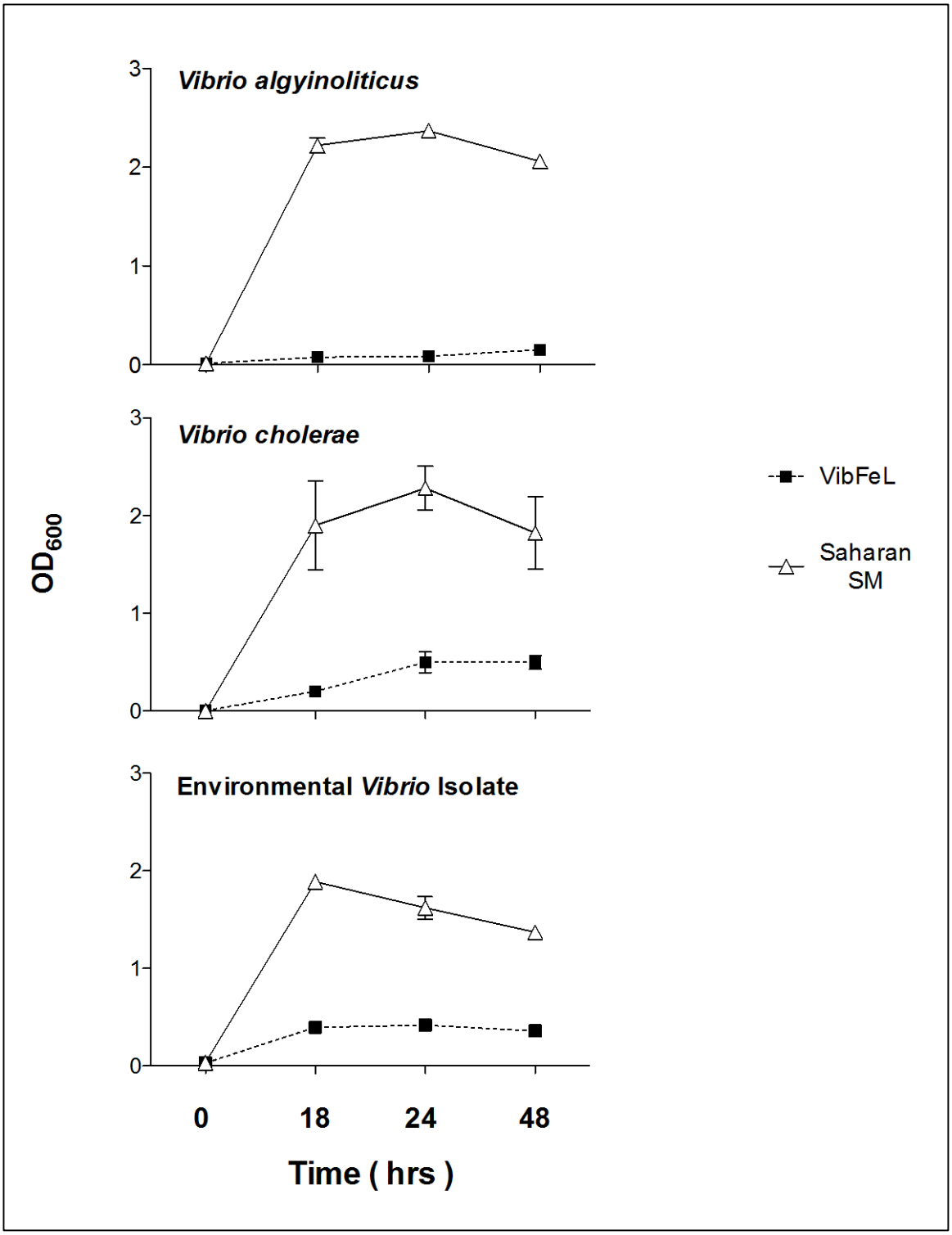

Fig. 3.3. *Vibrio* growth using iron from Saharan Dust. Growth measured by optical density at 600 nm (OD600) (mean ± SEM) of *Vibrio alginolyticus* (n=4), *Vibrio cholerae* (n=5 for 0 and 24

h; n=3 for 18 and 24 h) and an environmental isolate from the Dry Tortugas (n=2) in VibFeL alone (filled squares) compared to VibFeL supplemented with Saharan source material (SM) (open triangles).

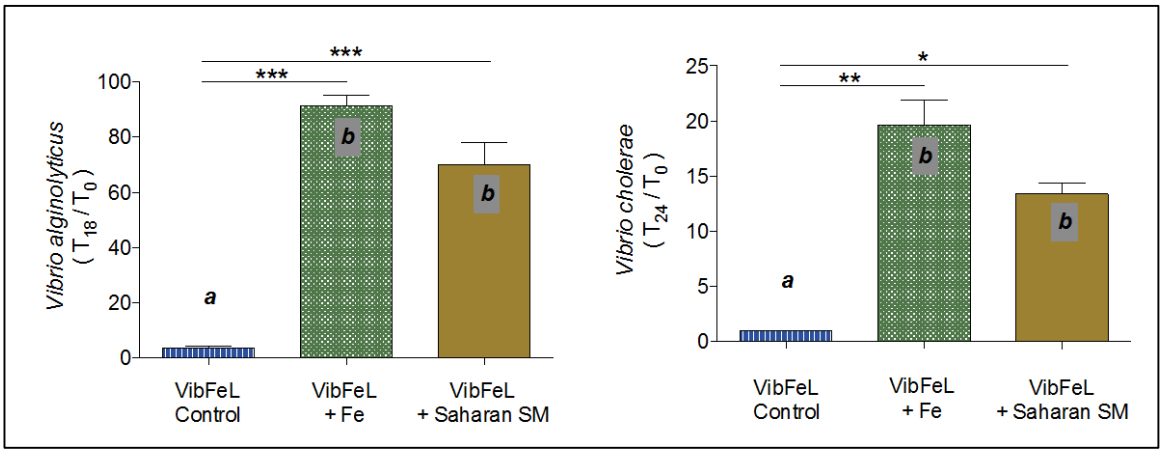

Fig. 3.4. Culturable *Vibrio* growth response to iron and Saharan source material. Growth of *Vibrio alginolyticus* (*n=3*) and *Vibrio cholerae* (*n=2*) in VibFeL alone, amended with 4 µM iron ($FeCl_3$) and amended with Saharan source material (SM) containing ~1 µM Fe. Colony forming units (CFU) ml^{-1} were normalized among experiments by comparing the ratio of CFU ml^{-1} at T_n to that at T_0, where T_0 represents time zero and T_n represents time n (e.g., 24 h) (mean ± SEM). Asterisks denote a significant effect of Fe and Saharan SM-associated Fe in VibFeL when compared to growth in VibFeL alone (ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). There was no significant difference between treatment with Saharan SM and treatment with Fe as indicated by Tukey's post-hoc test where different letters indicate means that are significantly different from each other.

CHAPTER 4

SAHARAN DUST NUTRIENTS PROMOTE *VIBRIO* BLOOM FORMATION IN MARINE SURFACE WATERS

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Abstract

Vibrio is a ubiquitous genus of marine bacteria, typically comprising a small fraction of the total microbial community in surface waters, but capable of becoming a dominant taxon in response to poorly characterized factors. Iron (Fe), often restricted by limited bioavailability and low external supply, is an essential micronutrient that can limit *Vibrio* growth. *Vibrio* species have robust metabolic capabilities, an array of Fe-acquisition mechanisms, and are able to respond rapidly to nutrient influx, yet *Vibrio* response to environmental pulses of Fe remains uncharacterized. Here we examined the population growth of *Vibrio* following natural and simulated pulses of atmospherically transported Saharan dust, an important and episodic source of Fe to tropical marine waters. As a model for opportunistic bacterial heterotrophs, we demonstrated that *Vibrio* proliferate in response to a broad range of dust-Fe additions. During *in situ* field studies, arrival of Saharan dust coincided with high levels of dissolved Fe, followed by up to a 30-fold increase of culturable *Vibrio* over background levels within 24 h. The relative abundance of *Vibrio* increased from 1.4% to 20% of the total microbial community. Abundance of pathogenic isolates, *Vibrio parahaemolyticus*, *V. cholera*e, and *V. alginolyticus*, increased in surface waters that received dust. Strains of *V. cholerae* and *V. alginolyticus* were further shown to directly utilize Saharan dust-Fe to support growth. This study is the first to describe *Vibrio* response to Saharan dust nutrients, having implications at the intersection of marine ecology, Fe biogeochemistry, and both human and environmental health.

Significance

Atmospherically transported dust from the Saharan desert provides pulses of biologically important nutrients, including iron, to ocean surface waters. The biological response to these

ephemeral events is not fully known, especially among the heterotrophic microbial community. Here we use the well-characterized *Vibrio* genus as a model for heterotrophic bacterial response. We demonstrate that Saharan dust nutrients, deposited in tropical marine waters, can promote *Vibrio* bloom formation and suggest that dust associated iron is an important driver of *Vibrio* population dynamics. This work shows not only the role of fast-acting heterotrophs in the biogeochemical cycles of environmental pulses of iron, but also highlights an important factor in the growth of bacteria that can cause disease in humans and marine organisms.

Introduction

Vibrio is a globally distributed marine bacterial genus that typically makes up a minor portion of coastal microbial assemblages (Thompson and Polz 2006; Yooseph et al. 2010). Yet, like other opportunistic heterotrophic bacteria, *Vibrio* can have disproportionately large impacts on carbon and nutrient processing because of their ability to reproduce rapidly and bloom in response to pulses of newly available resources (Eilers et al. 2000; Takemura et al. 2014; Thompson and Polz 2006; Worden et al. 2006). Characterized as "opportunitrophs," *Vibrio* have a broad genomic and metabolic repertoire (Polz et al. 2006), allowing them to compete in highly variable nutrient environments ranging from the open ocean to pathogenic associations with animal hosts (Kustusch et al. 2011; Takemura et al. 2014). This genus includes many well-known pathogens of marine organisms and humans, and disease incidence has risen sharply in the last 20 years (Newton et al. 2012; Rosenberg et al. 2007). Common human pathogens include the causative agent of the severe diarrheal disease cholera (*V. cholerae*) (Chun et al. 1999), shellfish associated gastroenteritis (*V. parahaemolyticus* and *V. vulnificus*) (Panicker et al. 2004), and seawater associated wound infections (*V. vulnificus* and *V. alginolyticus*) (Kustusch et

al. 2011). Studies examining the environmental drivers and distribution of *Vibrio* have largely focused on the role of *Vibrio* in disease, generally overlooking the importance of *Vibrio* in the biogeochemical cycling of key nutrients and trace metals (Takemura et al. 2014; Thompson and Polz 2006). Iron (Fe) is an essential micronutrient for *Vibrio* growth in the environment as well as during host invasion, where it is actively sequestered by the host to prevent bacterial colonization (Kustusch et al. 2011). *Vibrio* have evolved to be adept scavengers of Fe in a variety of conditions (Wyckoff et al. 2007). Despite this importance, there has been little characterization of the effects of environmental Fe enrichment on *Vibrio* population dynamics and the role of these bacteria in Fe cycling in marine systems.

Iron can be a limiting micronutrient in marine primary and secondary production (Strzepek et al. 2005; Sunda 2012). As an essential co-factor in many metabolic processes, including aerobic respiration, photosynthesis, and nitrogen fixation, its availability can be a determinant in the cycling of carbon (C) and biologically important macronutrients, like nitrogen (N) and phosphorous (P) (Lenes et al. 2001; Sunda 2012; Yong et al. 2014). However, dissolved Fe (dFe), believed to be the most biologically available fraction of Fe, is present in vanishingly low amounts in marine systems around the world, especially due to the low solubility of Fe (Khanna et al.) in seawater (Baker and Croot 2010). Heterotrophic bacteria, including *Vibrio*, play a key role in Fe cycling (Adly et al. 2015; Strzepek et al. 2005; Tortell et al. 1999) in part by modulating Fe solubility through secretion of high-affinity Fe-chelating siderophores (Granger and Price 1999; Poorvin et al. 2011). Fe-siderophore complexes not only allow active uptake into the bacterial cell (Granger and Price 1999), but are also a usable exogenous source of Fe for phytoplankton (Hopkinson and Morel 2009; Soria-Dengg et al. 2001). Although most studies of Fe enrichment in marine systems have focused on autotrophs (Boyd et al. 2007; Lenes et al.

2001), heterotrophic bacteria have been shown to have a higher Fe per biomass quota than many phytoplankton (Tortell et al. 1999), accounting for up to 80% of the total planktonic uptake in some systems (Schmidt and Hutchins 1999).

Atmospheric dust deposition is a major supply mechanism of Fe to the global ocean (Mahowald et al. 2009), and is estimated to deliver over triple the amount of dFe as riverine inflow (Duce and Tindale 1991). Globally, the Saharan desert is the largest source region of this atmospheric dust-Fe (Mahowald et al. 2009). Dust originating in northern Africa is swept across the Atlantic Ocean along easterly trade winds, producing spatiotemporal gradients of dust deposition in the Caribbean and southeastern U.S., especially during the summer months (Prospero et al. 2010; Trapp et al. 2010). Increased atmospheric processing time, associated with long-range transport as well as wet deposition (rain washout), are hypothesized to alter components of atmospheric dust and produce a soluble and highly biologically available form of Fe (Baker and Croot 2010; Mahowald et al. 2009). The full biological response to the episodic deposition of dust-Fe to ocean surface water, especially among microbial communities, has yet to be clearly elucidated. We hypothesized that opportunistic bacteria, like *Vibrio*, play an important but largely unexplored role in the cycling of Fe from dust deposition in marine systems and suggest a role of dust-Fe as a driver of *Vibrio* population dynamics.

Results

Vibrio response to simulated Saharan dust deposition in marine surface water.

To directly determine the effect of Saharan dust nutrients on *Vibrio* growth, source material from the Saharan desert (Morocco) was added to microcosms containing natural unfiltered seawater collected in the Florida Keys (USA). Source material (characterization shown

in Table 4.1) was manipulated to simulate effects from long-range atmospheric transport and wet deposition (rain washout), and is referred to as $Dust_{SIM}$ (*Supplemental Methods*). Microcosms included surface water collected over multiple dates from three sites for each experiment, representing a cross-shelf gradient (onshore, near shore, and offshore [at Looe Key Reef in the lower Florida Keys]) (Fig. 4.1). A broad range of dust deposition scenarios (20 μg L^{-1} to 30 mg L^{-1}) was simulated. The lowest $Dust_{SIM}$ addition (20 μg L^{-1}), increased dissolved Fe (dFe) in seawater to 1.19 nM (± 0.08 SEM) above the background levels of 0.90 nM (± 0.04 SEM) found in non-amended surface water (Table 4.3). This addition provided a small but significant increase ($P<0.05$), consistent with *in situ* observations of dFe during dust events (Fig. 4.5, Table 4.3).

Following the $Dust_{SIM}$ amendments to seawater, the growth of total culturable *Vibrio* on a selective medium was normalized across experiments by calculating the ratio of colony forming units (CFU) ml^{-1} at 24 h to those at 0 h. All $Dust_{SIM}$ additions, except the 40 μg L^{-1} addition (due to high variability among replicates), had significantly higher growth compared to non-dust controls, and the response was largely dose-dependent (Fig. 4.2). At the lowest $Dust_{SIM}$ addition (20 μg L^{-1}), mean growth was six times as great as that for non-dust controls (49.0 ± 19.6 SEM and 8.5 ± 3.6 SEM, respectively, $P<0.001$). Neither the location (inshore, near shore, or offshore) nor the date of sampling had a statistically significant effect ($P>0.05$).

Due in part to the potential for the total *Vibrio* community to include human pathogens, a small subset of isolates from dust-seeding experiments was screened for those species typically associated with human illness: *V. cholerae*, *V. parahaemolyticus, V. vulnificus*, and *V. alginolyticus* (Table 4.4). Seven isolates from the pre-seeded community (representing ~82% of colonies) and 34 from dust-amended microcosms (representing 1.8 - 12% of colonies) were

recovered. Only one of the isolates from the pre-seeded community (14%) was presumptively identified as a potential pathogen, *V. alginolyticus*. Of the isolates from $Dust_{SIM}$ seeded conditions, 35% (12/34) were positive for three of the four potential pathogenic species: *V. alginolyticus, V. cholerae*, and *V. parahaemolyticus*. *V. alginolyticus* was most commonly represented in dust-amended cultures, comprising 24% of the colonies among the isolates from dust-amended seawater.

Vibrio utilization of dust-associated Fe.

To evaluate the specific effects of Fe in Saharan dust on *Vibrio* growth, cultures of individual strains were grown in a novel, Fe-limited seawater (Vib-FeL), which was replete in key macronutrients (N and P) and carbon substrate and supplemented with all essential trace elements needed for growth, except for Fe (*Supplemental Methods*). The relative growth of individual strains of *V. alginolyticus* and *V. cholerae* were tested in three separate culture conditions for each strain: 1) Vib-FeL alone, 2) Vib-FeL with $Dust_{SIM}$ (providing 0.89 μM Fe) and 3) Vib-FeL with $FeCl_3$ (providing 4.37 μM Fe), as a positive control. After overnight incubation (18 - 24 h), the proportional growth was compared for all treatments (Fig. 4.3). Vib-FeL alone effectively restricted growth of both test strains. The addition of $Dust_{SIM}$ led to significant growth of both *V. cholerae* and *V. alginolyticus*, compared to non-dust controls (13 and 18 times as great, respectively; $P<0.05$). The proportional growth for the $Dust_{SIM}$ amendments was not significantly different from that noted for the $FeCl_3$ controls, which had 20 and 23 times as much growth for *V. cholerae* and *V. alginolyticus*, respectively, as did non-dust controls (Fig. 4.3).

Iron concentrations across dust deposition events are highly variable (Lawrence and Neff 2009; Mahowald et al. 2009); therefore, we evaluated growth of *V. alginolyticus* in Vib-FeL across a broad range of scenarios, providing dFe at concentrations from 5 nM to 836 nM. All $Dust_{SIM}$ additions increased the proportional growth of *V. alginolyticus* (Fig. 4.4). The lower additions of 5, 10, and 21 nM dFe resulted in approximately four times the growth of no-addition controls. The higher dFe amendments, 201 nM and 836 nM, resulted in growth that was eight to 11 times that of controls ($P<0.001$), respectively (Fig. 4.4).

Vibrio growth in response to natural Saharan Dust Events.

Offshore sampling sites at Looe Key Reef, in the lower Florida Keys (USA), and Ragged Point, Barbados, were chosen based on a 30-year dataset of atmospheric dust sampling, demonstrating strong seasonal pulses of African dust arriving at these locations almost entirely in the summer months (June - September) (Prospero et al. 2010; Trapp et al. 2010). Satellite and aerosol-modeling products were used to monitor evolution of individual dust events from the coast of Africa (typically every five to 10 days, July through August) and their transit across the Atlantic (Fig. 4.5). Field collections were conducted in the Florida Keys (summer 2013 and 2014) and Barbados (summer 2014) during three separate Saharan dust events (SDE).

In July 2014, one week prior to the arrival of dust, dFe in surface waters in the lower Florida Keys was 0.90 nM (± 0.08 SEM), increasing to 3.24 nM (± 0.04 SEM) with the arrival of a SDE ($P<0.001$) (Table 4.3). Similarly, the highest dFe during the August 2014 study period in Barbados (2.22 nM ± 0.20 SEM) was measured during the arrival of a SDE. Dissolved Fe levels were not measured in 2013. Within 14 to 24 h of the arrival of Saharan dust (and concomitant dFe increase), surface water concentrations of culturable *Vibrio* increased significantly over

background levels in all three sampling campaigns at levels ranging from five to 30 times the non-dust levels (Fig. 4.5). At the peak of the *Vibrio* blooms in 2014, a 1.6- and 3.6-fold decrease of dFe was measured in Barbados and Florida, respectively (Fig. 4.5). *Vibrio* bloom conditions were transient, and levels returned to baseline within 24 - 48 h of the measured peak. As the *Vibrio* bloom expired, an increase of dFe was measured, followed by a tapering decline in dFe over the following 1 - 4 days to ~1.6-fold less dFe than dust arrival values.

Bacterial community composition changes in response to SDE.

As part of a co-occurring study at Looe Key Reef, microbial community data were obtained for an additional four surface water samples between February 2012 and July 2013 using Illumina-based sequencing of the community 16S rDNA. Three of the samples were collected outside of a dust event or well outside of the typical summer dust season. The final sample coincided with the dust event captured in July 2013. The abundance of operational taxonomic units (OTU) in the *Vibrio* genus was significantly greater during this event than during all other sampling dates ($P<0.001$). The relative abundance of *Vibrio* increased from less than 1.4% during non-dust conditions to 20% of the total bacterial community during the SDE (Fig. 4.6). Additionally, the relative abundance of other genera including *Pseudoalteromonas* and *Acinetobacter*, belonging to the same class of bacteria as *Vibrio* (γ-proteobacteria), also increased significantly ($P<0.05$).

Discussion

Iron acquisition is essential for *Vibrio* fitness and survival, both in the environment and in a host during infection (Kustusch et al. 2011; Wyckoff et al. 2007). *V. cholerae*, which is arguably the most studied of the *Vibrio* species and has a very well characterized genome, has

over 50 Fe acquisition genes spread across its two chromosomes for ferrous, ferric, and biologically-complexed Fe uptake (Wyckoff et al. 2007). This genetic repertoire suggests that *Vibrio* are effective competitors for Fe in many different environmental niches. Fe plays a keystone role in the ability of *Vibrio* species and other heterotrophic bacteria to metabolize carbon substrates and crucial macronutrients like N and P, setting limits on their growth (Fourquez et al. 2014; Kirchman et al. 2003; Yong et al. 2014). Fe limitation has been demonstrated to limit C metabolism and growth in oceanic, and to an even greater degree, coastal heterotrophic bacteria (Fourquez et al. 2014). This is especially important in light of the fundamental contribution of heterotrophic bacteria to the cycling of C in marine ecosystems (Azam and Malfatti 2007; Fourquez et al. 2014). *Vibrio* and other rapidly responding heterotrophs likely play a pivotal, but as yet largely unexplored role, in coupling Fe flux with C cycling in marine food webs, ultimately influencing global climate processes (Adly et al. 2015; Azam and Malfatti 2007; Tortell et al. 1999). In this study, we demonstrated that *Vibrio* rapidly exploit Fe and potentially other nutrients provided by Saharan dust, driving a temporary population bloom. Furthermore, the data suggest that dust-Fe can support this bloom across a wide range of dust and Fe deposition scenarios.

Although the response of marine autotrophs to dust deposition has been investigated (Langlois et al. 2012; Lenes et al. 2001), evidence is growing that heterotrophic microbes may play key roles in processing deposited minerals and nutrients (Pulido-Villena et al. 2008). Opportunistic bacteria, including γ-proteobacteria, that are normally minor components of the microbial community have been shown to experience rapid population growth in response to Saharan dust (Laghdass et al. 2011; Marañón et al. 2010). These opportunistic heterotrophs may be among the first responders to episodic delivery of dust-Fe, although to date taxon-level

analysis has been poorly resolved. In this study, *Vibrio*, a model for rapidly responding γ-proteobacteria response, showed a robust bloom within 24 h of the arrival of Saharan dust and concomitant high dFe concentrations. During the peak of the bloom, *Vibrio* species increased their proportion in the community by over an order of magnitude. These results indicate that this is not an exogenous import of bacteria, but rather an autochthonous response to the addition of biologically necessary nutrients like dFe. Although it has been reported that some bacteria can travel associated with dust aerosols, genus level profiling of Saharan dust has not revealed the presence of *Vibrio* (Griffin 2007). During the 2014 field studies, the drawdown of dFe to the pre-dust level of 0.9 nM (Fig. 4.5, Table 4.3) at the peak of the bloom also indicates utilization of the majority of introduced dFe by blooming *Vibrio* and other similar taxa. Indeed, other rapidly responding γ-proteobacteria, like *Pseudoalteromonas* and *Acinetobacter,* exhibited an increase in relative abundance after the arrival of dust.

As a genus, *Vibrio* is one of the most highly investigated groups of environmental microbes, aided in part by their ease of culturability (Takemura et al. 2014), making them an excellent candidate model to examine opportunistic responses and roles in larger ecosystem functioning (Polz et al. 2006). *Vibrio*, typically comprising <1% of the ocean surface bacterioplankton community (Thompson and Polz 2006), could be considered conditionally rare taxa, a term used to describe rare bacterial taxa that are subject to dramatic blooms, potentially playing an outsized role in the ecology of a system (Campbell et al. 2011; Shade et al. 2014; Yooseph et al. 2010). *Vibrio* have shown explosive growth in response to nutrient enrichment (Eilers et al. 2000), demonstrating >4 doublings day^{-1} in the absence of predation (Worden et al. 2006). This adaptive feast-or-famine life strategy allows exploitation of spatially and temporally variable resources, leading to bloom conditions. This strategy also subjects *Vibrio* to kill-the-

winner top-down controls, such as grazing and viral lysis (Campbell et al. 2011), both of which tightly control γ-proteobacteria and *Vibrio* populations (Beardsley et al. 2003; Suttle 2007; Worden et al. 2006). The population declines observed in the field 24 - 48 h after Saharan dust induced *Vibrio* blooms could be attributed to these top-down control pressures. The bloom decline corresponded with a spike in dFe concentrations, supporting the notion of lysis and bacterivory in the release of dFe, warranting further investigation. The ecosystem consequences of such a large turnover of dFe on biogeochemical cycles also remains to be determined. Bacterial grazing experiments have demonstrated that 90% of Fe remains in the dissolved fraction after 24 h (Strzepek et al. 2005). Additionally, Fe released by viral lysis of heterotrophic bacteria (as demonstrated in *V. alginolyticus*) is highly bioavailable to the plankton community, including autotrophic diatoms, with the bioavailability of this released Fe exceeding that of siderophore-bound Fe (Poorvin et al. 2011). This dFe is capable of supporting up to 90% of primary production in some systems (Poorvin et al. 2004). Taken together, the bloom-bust cycle of *Vibrio* population growth in response to Saharan dust could have an important role in trophic transfer of labile Fe to primary producers.

Vibrio can overcome predation pressure by forming close associations with marine organisms and plankton (Matz et al. 2005). In this work, the population bloom of *Vibrio* appeared transient, but further investigation is needed to determine if planktonic-associated hot spots exist after bloom termination. This is particularly salient in the case of disease causing species like *V. cholerae* or *V. alginolyticus*, both shown to be highly responsive to dust-Fe. These species are known to associate with zooplankton and marine organisms, directly influencing their survival in the environment and routes of transmission to potential hosts (Kustusch et al. 2011; Matz et al. 2005; Takemura et al. 2014).

This study is the first to demonstrate that *Vibrio* species, as a model of a rapidly responding opportunistic heterotroph, are highly responsive to Saharan dust-Fe and associated nutrients and indicate a role for these bacteria in processing dust-Fe in marine ecosystems. The Sahara and Sahel are particularly vulnerable to further drying due to changes in climate and land-use patterns (Held et al. 2005), potentially increasing dust export from this region. Coupled with the fact that dust-Fe fertilization of marine systems has been suggested as a driver of past paleoclimatic change (Lamy et al. 2014), a mechanistic understanding in the modern ocean is critical to making predictions about future oceanic production and climate scenarios. The discovery of dust-Fe as a factor in *Vibrio* population dynamics is an important first step that warrants further investigation to inform future predictions about *Vibrio*-related disease and *Vibrio* impacts on global biogeochemical cycles.

Methods

Dust$_{SIM}$ Experimental Additions. The U.S. Geological Survey (USGS) provided mineralogical and elementally characterized Saharan source material (*Supplemental Methods*) collected from a highly weathered dune in Morocco (exact location was not specified). Saharan source material was manipulated in the lab to simulate atmospheric processing and wet deposition and is referred to as Dust$_{SIM}$ (*Supplemental Methods*). Natural surface water from the Florida Keys and artificial Vib-FeL microcosm experiments were seeded with a broad range of Dust$_{SIM}$ additions because of the high spatial and temporal heterogeneity of dust deposition in any single event, as well as the high variability in dust concentrations resulting from rain washout, which is the dominant mode of deposition in Florida (~80%) (Prospero et al. 2010). To quantify Dust$_{SIM}$ loadings for experimental additions, Dust$_{SIM}$ was added (1:150 v/v) to 5 ml of ultrapure Milli-Q

water using trace metal clean chemistry techniques (Bruland et al. 1979), followed by further 1:2 serial dilutions in Milli-Q water. Dissolved (<0.2 μm) iron (dFe) was measured using ICP-MS elemental analysis (as described below) for triplicate samples of each dilution. The Fe content of trans-Atlantic transported dust has been measured to be close to the upper crustal value of 3.5% (Prospero et al. 2010; Trapp et al. 2010); we therefore quantified $Dust_{SIM}$ amendments by Fe content to allow for an approximate calculation of dust loading (mg L^{-1}) for each addition considering a fractional solubility of the Moroccan source material to be 4.39% (*Supplemental Methods*). $Dust_{SIM}$ additions for seeding experiments were calculated to be: 0.02, 0.04, 0.18, 0.37, 0.78, 7.3 and 30 mg L^{-1} of dust (Table 4.2). The higher values include representative amounts of deposition in regions closer to African source areas (e.g., Mediterranean), and the lowest values were environmentally relevant for the Caribbean and Florida Keys (e.g., during a wet deposition event) (Lawrence and Neff 2009; Prospero et al. 2010).

$Dust_{SIM}$ seeding experiments in surface water microcosms. Surface water, without filtration, was collected at sites in the lower Florida Keys (Fig. 4.1) and was used in seeded microcosm experiments. The offshore site was ~10 km from shore, over the spur and groove reef at Looe Key Reef Sanctuary Preservation Area in the Florida Keys National Marine Sanctuary. The nearshore (1 km from shore) and inshore (500 m from shore) sites were located near the Mote Tropical Research Laboratory on Summerland Key and were sampled by kayak. Grab samples of surface water were collected in the first meter at each sampling site in three replicate experiments (March 2011, May 2012, and May 2013). Samples were collected in the late spring to avoid the influence of naturally occurring Saharan dust deposition, which is typically heaviest in the summer months (Prospero et al. 2010). Samples were collected in sterile 1-L polyethylene

bottles prepared using trace-metal clean protocols (Bruland et al. 1979). Each bottle was further rinsed three times with surface water at the collection site immediately before collection. Samples were transported to the laboratory in an ambient temperature cooler with a min-max thermometer, and experimental testing was initiated within 3 h of collection. No significant deviation from ambient temperature was observed in any experiment.

$Dust_{SIM}$ dilutions were created in the same manner as described above in 5 ml of sample seawater from each site followed by further 1:2 serial dilutions, providing calculated dust additions between 0.02 and 30 mg L^{-1}. A natural seawater (non-dust) control was included for each site and experiment. Cultures were incubated for 24 h at 30°C (a typical temperature for these sites during dust season) on a nutating mixer at 24 rpm (Fisher Scientific) to maintain aeration. Culturable *Vibrio* species were detected after spread plating aliquots from each microcosm onto a *Vibrio*-selective medium, thiosulfate-citrate-bile salt-sucrose agar (TCBS; Oxoid), in triplicate, at experimental time 0 and at experimental time 24 h. Spread plates were incubated at 30°C for 24 h, and CFU were enumerated per ml and compared. Isolates from the March 2011 dust-seeding experiments were screened by polymerase chain reaction (PCR) to identify potential pathogenic species of *Vibrio* (*Supplemental Methods*).

Iron Limited Seawater (Vib-FeL) Experiments. To investigate the specific effect of Fe from Saharan dust on *Vibrio* growth dynamics, experimentation was done in an Fe-limited medium, referred to as Vib-FeL (*Supplemental Methods*). Frozen cell stocks of *V. alginolyticus* (American Type Culture Collection [ATCC] strain 33839) and *V. cholerae* (ATCC 39315), were recovered in sterilized artificial seawater amended with peptone and yeast extract (ASW+PYE). Recovered cells, incubated for 12 h at 30°C, were sub-cultured in 5 ml fresh ASW+PYE (1:100

dilution) and allowed to grow to log phase as monitored by optical density at 600 nm (OD_{600}) on a spectrophotometer. Cells were washed twice and re-suspended in Vib-FeL to eliminate Fe carry-over. The experiment was initiated at time zero by inoculating washed cells (1:100) into 5 ml of the appropriate culture conditions: Vib-FeL alone; Vib-FeL with $FeCl_3$; $Dust_{SIM}$ added to Vib-FeL (1:150 v/v), and incubated for 24 h at 30°C on a shaking platform to maintain aeration. *Vibrio* abundance was determined by spread plating aliquots from each culture onto ASW+PYE agar plates at time 0, 18, and 24 h. Spread plates were incubated at 30°C for 24 h, and CFU were enumerated and compared. Because dust deposition is highly variable (Lawrence and Neff 2009), we also evaluated growth of *V. alginolyticus* (following the same cell preparation methodology) across a broad range of Fe-deposition scenarios by diluting $Dust_{SIM}$ as described above (*$Dust_{SIM}$ Experimental Additions*) into Vib-FeL, providing dFe at concentrations from 5 nM to 836 nM dFe (Table 4.2).

***In Situ* Response to Natural Saharan Dust Events.** Saharan dust events (SDE) were monitored using satellite and modeling products from NASA (https://worldview.earthdata.nasa.gov/) and the Naval Research Laboratory (http://www.nrlmry.navy.mil/aerosol/), allowing for virtual real-time monitoring of SDE arrival and passage at two study sites: Ragged Point, Barbados (easternmost point of the island) (13.1667° N, 59.4333° W) and Looe Key Reef, FL (24.5475° N, 81.4067° W). In late July of 2013 and 2014, samples were collected, 20 h and 14 h, respectively, after arrival of dust in the lower Keys (Fig. 4.5). Samples were collected 24 h post-dust arrival for Barbados in August of 2014 (Fig. 4.5). All surface water samples were collected in triplicate and kept at ambient temperature until processing (within 1 h). Sampling collection continued, at 24-h intervals, for 3 - 5 days after the modeled arrival of dust. *Vibrio* abundance was determined

by spread plating each surface water sample and replicates on *Vibrio* selective TCBS medium, in triplicate. Spread plates were incubated at ambient temperature (~30°C) for 24 h, and CFU were enumerated and compared. Background non-dust associated summer (May-August) *Vibrio* levels at Looe Key were determined by routine *Vibrio* monitoring using similar culture methods on TCBS at Looe Key 2012 - 2014. Background levels at Barbados were determined in April 2013 (pre-dust season) in the same manner. Field collections for SDE occurred in the summer rainy season, and isolated local showers were common; however, no precipitation data were available for the specific collection sites. Rain (very light) was only noted during or immediately before sampling at Barbados (11 and 12 August 2014). No rain was observed during sampling at Looe Key Reef.

ICP-MS Fe analysis. All sample collection and trace-metal analyses were performed using trace metal clean chemistry techniques (Bruland et al. 1979). Samples were filtered through 0.2-µm track-etched polycarbonate filters and acidified to pH ~1.8 (0.024 M HCl). Concentrations of dFe were determined by isotope dilution inductively coupled plasma-mass spectrometry (ICP-MS) described in (Milne et al. 2010). Natural SDE surface water samples were collected in acid-washed 125-ml collection bottles just below the surface over Looe Key Reef, FL, and at Ragged Point, Barbados. Samples were frozen (within 30 min) for 1.5 months before filtration to prevent Fe sorption to the bottle walls until sample analysis could be completed.

SDE Community Analysis. DNA was extracted from surface water samples and sequenced using Illumina MiSeq PE 250 sequencing of V4 hyper-variable region of the 16S rRNA gene (Table 4.5) (Caporaso et al. 2011). (*Supplemental Methods*).

Statistical Analysis. Estimates of growth were normalized among experiments by comparing the ratio of CFU ml^{-1} at T_n to that at T_0, where T_0 represents time zero and T_n represents time n (e.g., 24 h). Results were presented as mean ± SEM. Proportional growth (T_{24} / T_0) measurements from Florida Keys surface water amended with $Dust_{SIM}$ were first log transformed to meet normality assumptions. Statistical comparisons were done with PROC MIXED using a split-plot design with the factors of dust amendment, date, and location (SAS V9.3). Type III tests of fixed effects determined that neither location or date, nor the interactions among effects were significant ($P>0.05$). Dust amendments were compared using post-hoc pairwise comparisons. The Tukey-Kramer adjustment for multiple comparisons was used to control experiment-wise error rates, with $P<0.05$ chosen to denote significant differences. All other statistical comparisons were made using ANOVA and Tukey's multiple comparison post-hoc test (GraphPad 5.0.F). For all tests, significance was declared when $P<0.05$.

Supplemental Methods

Saharan Source Material Analysis. The U.S. Geological Survey (USGS) provided Saharan source material collected from a highly weathered dune in Morocco (exact location was not specified). Elemental composition analysis was coordinated by the USGS and followed USGS Geochemical Landscape Project protocols (Smith et al. 2013). Compositional analysis of 37 elements was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using USGS methodology (Briggs 2002) (Table 4.1A). A Perkin Elmer 2400 Carbon, Hydrogen, Nitrogen Analyzer (CHN) was used to determine the total carbon and nitrogen content in 91.21 mg of Moroccan source material (Table 4.1A). Particle size was determined

using a Malvern Mastersizer 2000 laser analyzer, followed by textual classification according to USGS protocols (Table 4.1B).

Dust$_{SIM}$ Preparation. Atmospheric processing and wet deposition of Saharan dust were simulated in the lab using Saharan source material. The material was manually dry sieved through trace metal clean 335-µm nylon mesh followed by 150-µm mesh (Aquatic Eco-Systems Inc.) to remove coarse sand grains. To simulate high altitude UV exposure and oxidize organic contaminates (including microbial contaminants), sieved material was exposed to UV irradiation (300 watt Hg-lamp ~254 nm) in a class 100 laminar flow hood in six 1-h increments with hand mixing of the material between exposures. Atmospheric acidification processes from anthropogenic aerosols (containing organic, nitric, and sulfuric acid species) are reported to render dust-Fe more soluble during atmospheric transport (Baker and Croot 2010; Mahowald et al. 2009). Dust passage and entrainment in the low pH environments of clouds and rain, as well as deposition by precipitation washout, were simulated by mixing 1 g of sieved UV irradiated dust into 100 ml of 0.025 M HCl Optima (Fisher). HCl was used because seawater is already high in chloride and results in minimum elemental alteration of the sample matrix during experimentation as well as provides maximal Fe dissolution. To eliminate large particulates that might promote adhesion and particle-supported bacterial growth, coarse material was allowed to settle out of the suspension and the top 90 ml of the mixture were removed and filtered through an acid-washed, Milli-Q-rinsed, 0.2-µm pore-sized polycarbonate membrane (Whatman). The resulting stock material used in experimental manipulations was termed Dust$_{SIM}$. The dissolved Fe (dFe) fraction of Dust$_{SIM}$ that passed through the 0.2 µm membrane, considered the most bioavailable fraction (Baker and Croot 2010), was 7.42 ppm (± 0.12 SEM) as determined by

ICP-MS. The fractional solubility of Moroccan source material Fe (16,900 ppm; Table 4.1) was calculated to be 4.39%. $Dust_{SIM}$ stock was confirmed to be free of culturable bacteria by direct spread plating of 0.1 ml $Dust_{SIM}$ stock on experimental culture media: artificial seawater amended with peptone and yeast extract agar (ASW+PYE) and the *Vibrio* selective media, thiosulfate-citrate-bile salts-sucrose agar (TCBS; Oxoid). No growth was observed. ASW+PYE was prepared by dissolving Instant Ocean (Spectrum Brands) in Milli-Q water to a specific gravity of 1.023 and adding 1% Peptone (Fluka Analytical), 0.5% Yeast Extract (Oxoid), and 1.5% Bacto agar (Difco).

Surface Water $Dust_{SIM}$ Seeding - PCR Identification of *Vibrio* spp. Isolates from the March 2011 Florida Keys dust-seeding experiments were screened for potential pathogenic species of *Vibrio*. Genomic DNA was extracted from green (non-sucrose fermenting) and yellow (sucrose fermenting) colonies isolated on TCBS from both the pre-seeded community and dust-amended cultures (24 h) using DNeasy tissue kits (Qiagen). Extracted DNA was screened using PCR for *V. alginolyticus, V. cholerae, V. parahaemolyticus,* and *V. vulnificus* (primary species associated with vibriosis in humans) (Table 4.4). For each reaction, 1 µl of sample DNA (~10 ng) was added to a 24-µl master mix (5-PRIME). All reactions contained 1 X PCR buffer (providing 1.5 mM Mg^{2+}), 0.2 µM deoxynucleoside triphosphates (*d*NTPs), 1.25 µM of each primer, and 1.25 units of *Taq* polymerase. A no-template negative control composed of 24 µl of reaction mixture amended with 1 µl of sterile nuclease-free PCR water was used in all experiments. Positive controls comprised of 24 µl of reaction mixture amended with 1 µl of purified DNA from an American Type Culture Collection (ATCC) control culture known to harbor the desired gene target (Table 4.4). A standard PCR protocol was used as follows: initial denaturation of the

template DNA at 95°C for 120 s, followed by 35 cycles of amplification of the template DNA, in which each cycle consisted of denaturation at 95°C for 60 s, primer annealing at 60°C for 60 s, and primer extension on the template DNA at 72°C for 60 s. A final extension step at 72°C for 5 min was included in all reactions. Following amplification, amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The bands were visualized and photographed using a Gel Logic 200 Imaging System (Kodak).

Iron Limited Seawater (Vib-FeL). To investigate the specific effect of Fe from Saharan dust on *Vibrio* growth dynamics, experimentation was done in an Fe-limited medium, referred to as Vib-FeL, which was a modified version of the artificial saltwater medium, Aquil (Price et al. 1988/1989). Components of Vib-FeL were chosen with the lowest Fe content available. A solution of salts and nutrients was dissolved in Milli-Q water (18.2 MΩ x cm) containing NaCl (420 mM), $MgSO_4$ (2.5 mM), K_2HPO_4 (0.1 mM), $CaCl_2$ (0.1 mM), NH_4Cl (18 mM), and Na_2PO_4 (42 mM). To remove potential contaminating Fe and other trace metals, the medium was passed through a column of Chelex 100 (Sigma) ion-exchange resin, according to the procedure of Price et al. (Price et al. 1988/1989), followed by microwave sterilization. After cooling (24 h), trace metals (50-µM EDTA-buffered solution of 2.5 X 10^{-8} M Co, 9.8 X 10^{-9} M Cu, 2.3 X 10^{-7} M Mn, 1.1 X 10^{-7} M Mo, 3.9 X 10^{-8} M Zn) and a carbon source of sucrose (Sigma) (0.4% w/v) were added from 0.2-µm sterile-filtered stocks. All manipulations were performed in a laminar flow hood to minimize contamination.

***In Situ* Community Analysis.** Water was collected with a sterile syringe (up to 60 ml) approximately one meter above the reef (~2 m below the surface) and held on ice (< 3 h).

Aliquots (2 ml) were centrifuged at ~13,000 x *g* for 20 min at 4º C. The supernatant fluid was discarded and the bacteria-containing pellet was stored at -20°C. The protocol of Boström and colleagues (Boström et al. 2004) was used to extract environmental DNA from frozen pellets, with slight modifications. Lysis buffer [175µl of 400 mM NaCl, 750mM sucrose, 20 mM EDTA (ethylenediaminetetraacetic acid), 50 mM Tris-HCL (pH 9.0)] and lysozyme (1µl of 10 mg ml^{-1}) were added to the pelleted sample. Following incubation at 37°C for 30 min, proteinase K (100 µg ml^{-1} final concentration) and SDS (1% w/v final concentration) were added and tubes incubated at 55° C for 16-18 h. To aid in the precipitation of DNA, tRNA (50 µg) was used as a carrier molecule. Precipitation of DNA was initiated by adding 20 µl of 3M NaAc and 120 µl isopropyl alcohol and incubating for an hour at -20°C. Samples were centrifuged (~13,000 x *g* for 20 min) and the supernatant fluid decanted, retaining the pelleted DNA in the original tube. Samples were then washed with 500 µl EtOH (70%), centrifuged (~13,000 x *g* for 20 min), and supernatant fluid discarded. A SpeedVac (Eppendorf Concentrator 5301) was used to dry the DNA pellet, which was then resuspended in 50 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Next-Generation Sequencing. The bacterial community was sequenced by targeting the hypervariable V4 region of the 16S rRNA gene using primers containing barcodes and Illumina adaptors (Table 4.5) as described by Caporaso and colleagues (Caporaso et al. 2011). PCR reactions (triplicate for each sample) included 12.5 µl Qiagen Taq PCR Master Mix, 0.5 µl forward and reverse primers (2 µM final concentration), 2 µl extracted DNA, and commercial sterile nuclease free water added to a final volume of 25 µl. Amplified product (253 bp) was confirmed by gel electrophoresis; each band was excised and purified (MoBIO's UltraClean® GelSpin® DNA Extraction Kit). PCR products were pooled and normalized into one sequencing

sample based on the lowest sample concentration and mixed with 10% PhiX. Sequencing was completed with Illumina MiSeq PE250 (UGA Georgia Genomics Facility, Athens, GA).

Sequence Analysis. Sequences were demultiplexed and barcodes removed with the Illumina software (MiSeq Control Software 2.2.0.2 & 2.3.0.8). Standard processing of the sequences was completed to remove sequences with poor quality and trim ends if under a quality score of 30, then only retaining sequences with at least 100 bp and 95% of bases with a quality score of $\geq$20 (Lohse et al. 2012) (Gregory Hannon; http://hannonlab.cshl.edu/fastx_toolkit/index.html). The QIIME pipeline was used for all further sequence processing and data analysis, selecting default options (Caporaso et al. 2011). Mitochondrial sequences were removed by creating a corresponding sequence file from GenBank against which to BLAST sample sequences; matches to mitochondrial sequences with an e-value of $\geq 1^{-10}$ and 97% alignment were subsequently removed from analyses. Chimera Slayer was used to detect and remove chimeric sequences (Haas et al. 2011). Sequences that were not taxonomically classified within the Bacterial kingdom were removed; these were Archaea, chloroplasts, and unclassified sequences. The remaining OTUs were picked using an open reference frame and taxonomy was assigned with the green genes database (gg_13_5.fasta). The most abundant sequence for each out, excluding singletons, was selected to create a representative set of sequences to align and create a phylogenetic tree (Caporaso et al. 2011). To analyze changes in the abundance of bacteria, proportions of each genus were square root arcsine transformed to approximate a normal distribution and compared by sample date using ANOVA.

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Tables

Table 4.1. **Analysis of Moroccan Source Material. (A)** ICP-AES analysis of elemental concentration of Moroccan source material (ppm) (200 mg was analyzed). * Total concentrations of C and N were measured with a Perkin Elmer 2400 elemental (CHN) analyzer (91.21 mg was analyzed). **(B)** Mineralogical makeup and size analysis of Moroccan source material (% of total).

A.

Element	Concentration (ppm)	Element	Concentration (ppm)	Element	Concentration (ppm)
Ag	<1	**Fe**	16900	**Rb**	52.3
Al	37200	**Ga**	7.6	**Sb**	0.8
As	6.7	**K**	17200	**Sc**	5.9
Ba	437	**La**	15.9	**Sr**	104
Be	0.9	**Li**	20.1	**Th**	4.48
Bi	0.16	**Mg**	9160	**Ti**	1850
Ca	29900	**Mn**	254	**U**	1.2
Cd	0.09	**Mo**	0.33	**V**	48.9
Ce	30.3	**Na**	7150	**Y**	10.8
Co	5.8	**Nb**	8.1	**Zn**	32.8
Cr	37.4	**Ni**	10.8		
Cs	1.8	**P**	496	**N %**	0.008 *
Cu	14.4	**Pb**	7.88	**C %**	0.875 *

B.

Sand	Silt	Clay
87.09	7.09	5.83

PM 2.5[a]	PM 10[a]	< 20 μm	< 63 μm	< 2 mm
4.16	8.65	11.21	12.91	100

[a] PM indicates particulate matter below the listed cutoff of 2.5 or 10 μm

Table 4.2. Analysis of dFe in $Dust_{SIM}$ and calculated equivalent dust loading. $Dust_{SIM}$ was serially diluted into ultrapure Milli-Q water. Dissolved (< 0.2 μm) iron (dFe) was measured using ICP-MS elemental analysis (mean ± SEM); *n*=3 replicates for each dilution. Dust loading (mg L^{-1}) was calculated based on Fe crustal content of 3.5% found in downwind dust and 4.39% fractional solubility of Moroccan source material.

dFe (nM) Mean (± SEM)	Calculated Dust Loading (mg L^{-1})
835.82 (7.21)	**30**
200.92 (0.94)	**7.3**
21.31 (0.31)	**0.78**
10.18 (0.19)	**0.37**
4.93 (0.02)	**0.18**
1.10 (0.03)	**0.04**
0.61 (0.17)	**0.02**

Table 4.3. Analysis of dFe in Florida Keys seawater (July 2014). Mean concentration (± SEM) (n = 3) of dissolved iron (dFe) measured by ICP-MS elemental analysis of **(A)** Florida Keys surface water prior to the arrival of a Saharan Dust Event (SDE), **(B)** Pre-SDE water seeded with the lowest $Dust_{SIM}$ (20 μg L^{-1}) experimental addition and **(C)** Keys Surface water on July 27, 2014 coinciding with the arrival of a Saharan Dust Event (SDE). Statistical significance is represented by asterisks, where *** represents statistically higher ($p < 0.001$) than **(A, B)** and * represents significantly higher ($p < 0.05$) than **(A)** (ANOVA; Tukey's post-hoc test).

A. Pre-SDE July 21-25, 2014 *(n=3)*	**B.** Pre-SDE Addition of $Dust_{SIM}$ (20 μg L^{-1}) *(n=3)*	**C.** SDE Arrival July 27, 2014 *(n=3)*
0.90 (0.04)	**1.19** (0.08) *	**3.24** (0.04) ***

Table 4.4. Targets for PCR identification of *Vibrio* species among isolates.

***Vibrio* sp.**	**Amplification Target**	**Reference**	**Positive Control Strain**
V. cholerae	ITS (intergenic spacer region)	(Chun et al. 1999)	ATCC 39315
V. parahaemolyticus	*tlh* (thermolabile hemolysin)	(Panicker et al. 2004)	ATCC BAA-238
V. vulnificus	*vvh* (*V. vulnificus* hemolysin)	(Panicker et al. 2004)	ATCC 27562
V. alginolyticus	*gyrB (gyrase B)*	(Zhou et al. 2007)	ATCC 33839

Table 4.5. 16S rRNA gene hypervaiable V4 region using primers containing barcodes and Illumina adaptors. All reactions used the same forward (F) primer, with unique bar codes associated only with the reverse (R) primer.

Primer	V4 PCR primer (5' to 3')	Adaptor (5' to 3')	Linker	Illumina Sequencing Primer (5' to 3')
F	AATGATACGGCGACCACCGAGATCTACAC	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
R *	CAAGCAGAAGACGGCATACGAGAT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT

Sample Name	*R-Primer Barcode
Aug 2011-1 H2O	ACGAGACTGATT
Aug 2011-2 H2O	ATCACCAGGTGT
Aug 2011-3 H2O	ATCGCACAGTAA
Aug 2011-4 H2O	ATCCTTTGGTTC
Aug 2011-5 H2O	ACCGGTATGTAC
Aug 2011-6 H2O	AGTCGAACGAGG
Aug 2011-7 H2O	TATACCGCTGCG
Feb 2012-1 H2O	GAATACCAAGTC
Feb 2012-2 H2O	TAACGTGTGTGC
Feb 2013-1 H2O	AGTTACGAGCTA
Feb 2013-2 H2O	ACCATAGCTCCG
Feb 2013-3 H2O	GAACACTTTGGA
July 2013-1 H2O	TTGGGTACACGT
July 2013-2 H2O	TCGGAATTAGAC
July 2013-3 H2O	TACTACGTGGCC
July 2013-4 H2O	CTATCTCCTGTC
July 2013-5 H2O	GTCGACAGAGGA

Figures

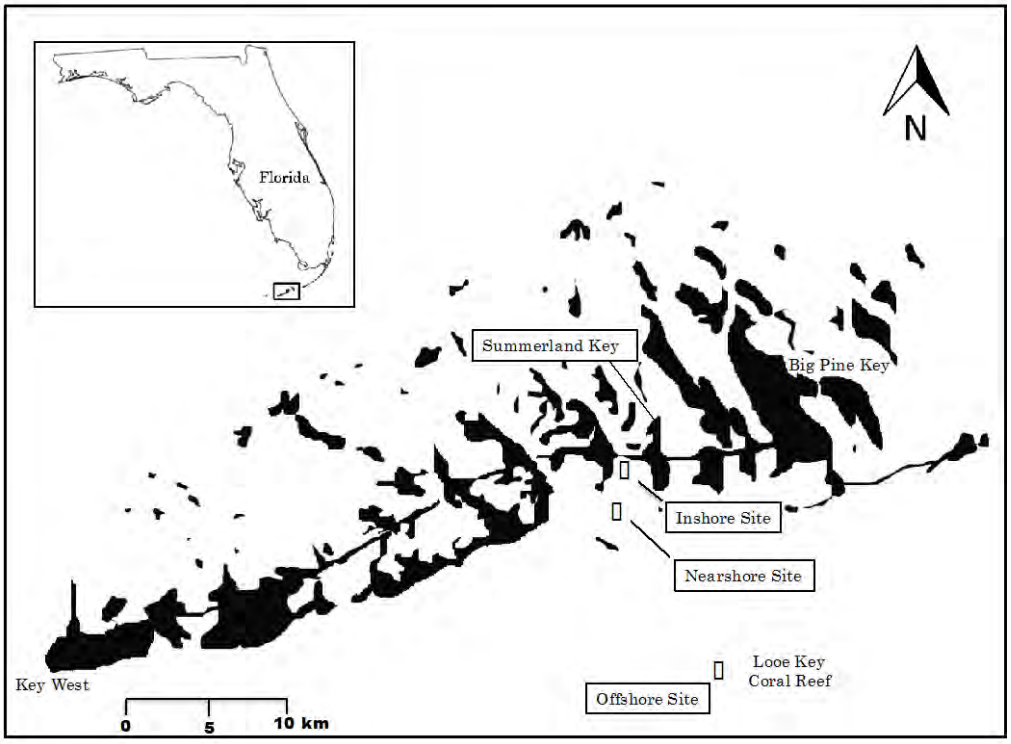

Fig. 4.1. Sampling locations for Dust$_{SIM}$ seeded experiments (Florida Keys, USA). Inshore (500 m from Mote Marine Lab, Summerland Key, Florida), Nearshore (1 km from shore), Offshore (10 km from shore), Looe Key Coral Reef.

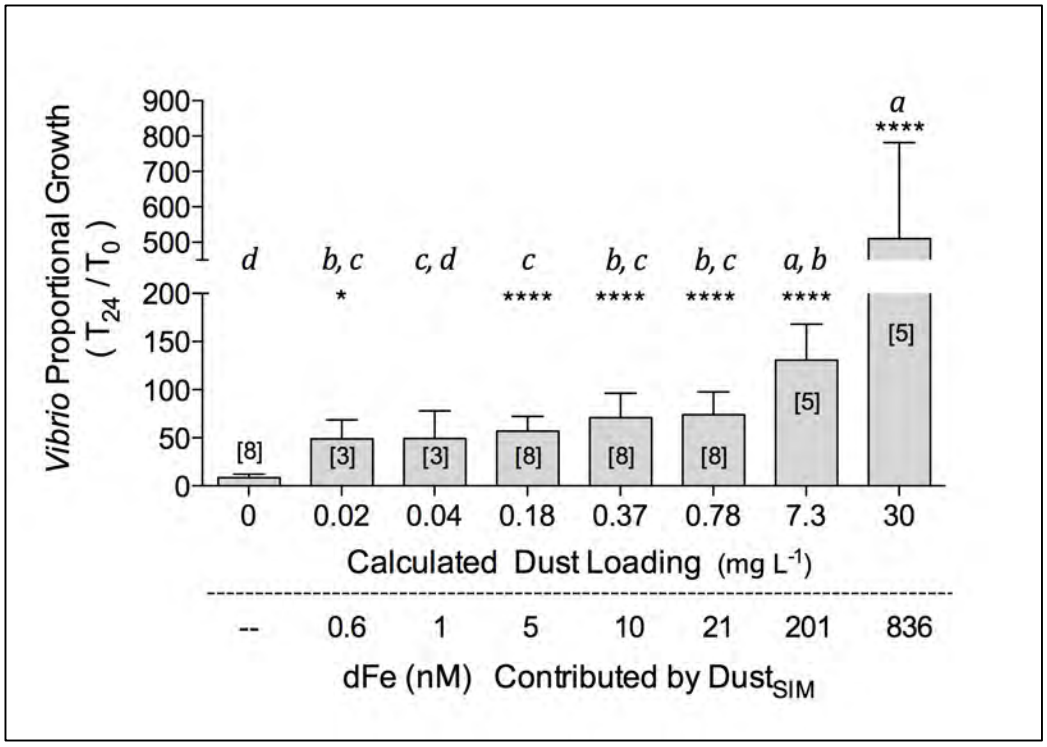

Fig. 4.2. Florida Keys surface water *Vibrio* response to a range of Saharan Dust$_{SIM}$ additions. *Vibrio* growth was normalized among experiments by comparing the ratio of colony forming units (CFU) ml^{-1} 24-hours after Dust$_{SIM}$ addition (T_{24}) to time zero (T_0) (error bars represent mean of replicate additions [n] ± SEM). Amendments in which growth was significantly different than the no-dust (0 mg L^{-1}) control are represented by asterisks; different letters indicate additions that were statistically different from each other (Mixed Linear Model; Tukey's post-hoc test; * $p < 0.05$; **** $p < 0.0001$).

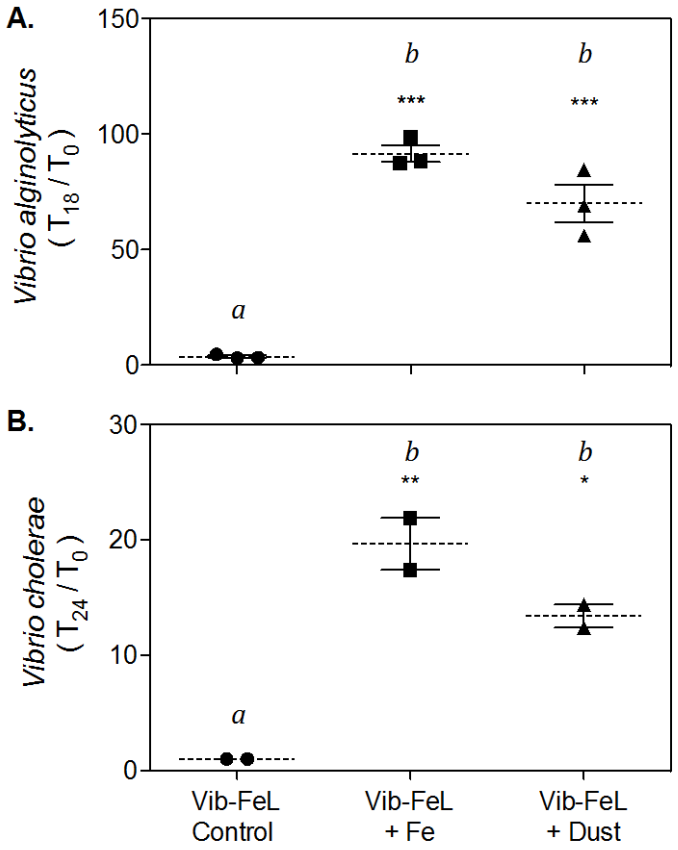

Fig. 4.3. *Vibrio* spp. response to Fe and Dust. Growth of **(A)** *Vibrio alginolyticus* (*n=3*) and **(B)** *Vibrio cholerae* (*n=2*) in Fe-limited seawater (Vib-FeL) alone, amended with 4 µM $FeCl_3$ (Fe) and $Dust_{SIM}$ (Dust) containing 0.89 µM Fe. Colony forming units (CFU) ml^{-1} were normalized among experiments by comparing the ratio of CFU ml^{-1} at T_n to that at T_0, where T_0 represents time zero and T_n represents time n (e.g., 24 h) (mean ± SEM). Asterisks denote the existence of a significant effect of Fe and Dust-associated Fe in Vib-FeL when compared to growth in Vib-FeL alone (ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). There was no significant difference between treatment with Dust and treatment with Fe as indicated by Tukey's post-hoc test where different letters indicate means that are significantly different from each other.

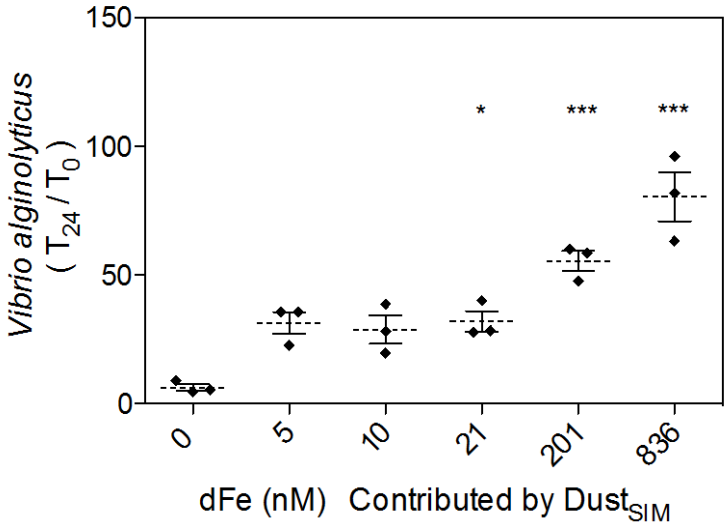

Fig. 4.4. Proportional growth of *Vibrio alginolyticus* to a range of $Dust_{SIM}$ dFe concentrations in Fe-limited artificial seawater (Vib-FeL). *Vibrio* growth was normalized among experiments by comparing the ratio of colony forming units (CFU) ml^{-1} of *V. alginolyticus* 24-hours after $Dust_{SIM}$ addition (T_{24}) to time zero (T_0). Data shown are the mean (dashed line) ± SEM (n = 3 independent experiments). Amendments in which growth was significantly different than the 0-$Dust_{SIM}$ control are represented by asterisks (ANOVA; Tukey's post-hoc test; * $p < 0.05$; *** $p < 0.001$).

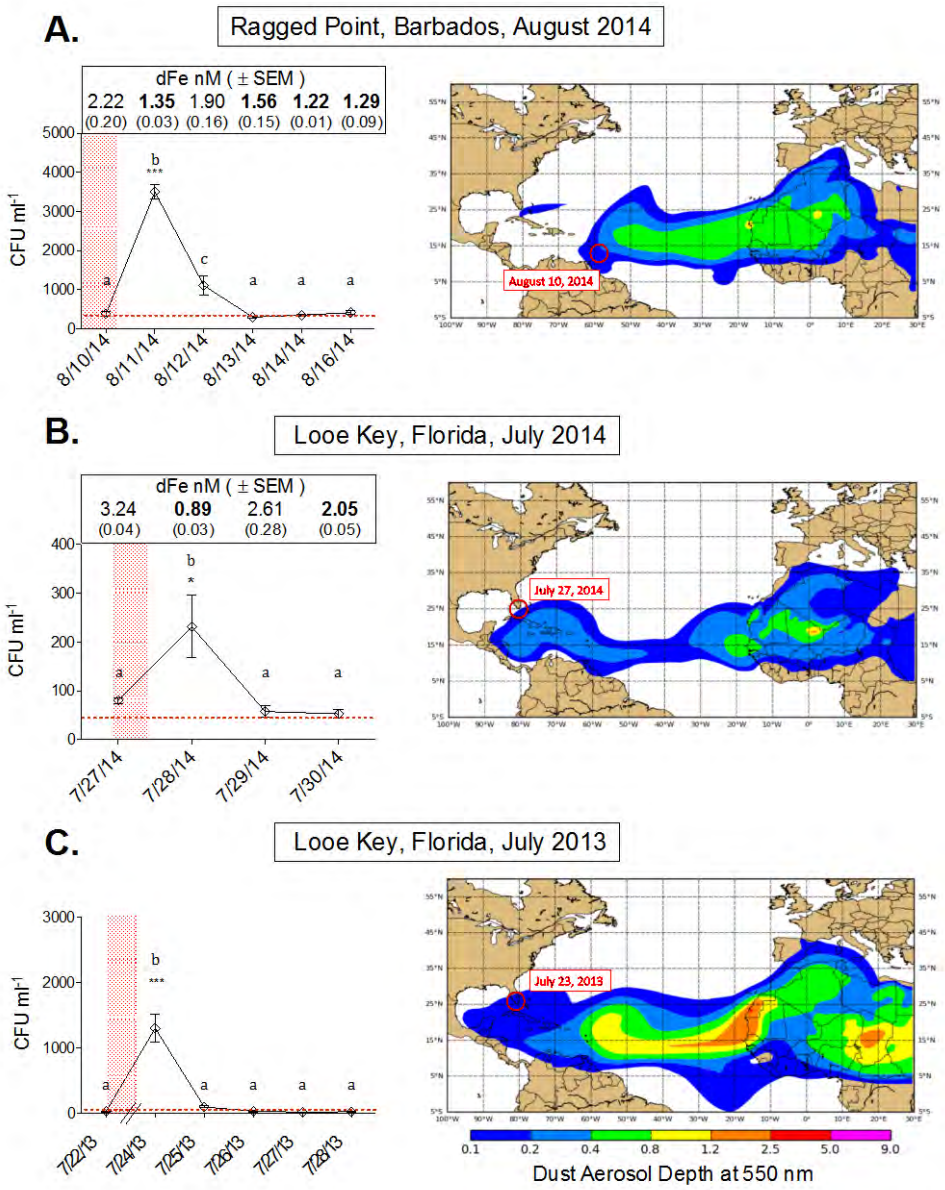

Fig. 4.5. *Vibrio* response to Natural Saharan Dust Events (SDE). Panels on the left show the mean (± SEM) of *Vibrio* concentrations (CFU ml^{-1}) (n = 3) in surface waters during the arrival (shaded bar) of SDE and 3-5 days post-dust arrival in **(A)** Barbados and **(B & C)** the Florida Keys. Modeled dust aerosol depths (Naval Research Laboratory (http://www.nrlmry.navy.mil/aerosol/), are shown in the accompanying panel to the right

(study site indicated by red circle). Dashed lines represent baseline surface water *Vibrio* counts for **(A)** Barbados (348 ± 122 SEM, n=3), and **(B & C)** the FL Keys (45.4 ± 2.5 SEM, n=42), determined during the dust season (April-August) but not associated with a SDE. The *Vibrio* concentration on the date of peak response in each graph is significantly different from all other dates (one-way ANOVA *P < 0.05, ***P < 0.001; different letters are significantly different by Tukey's post-hoc test). Double hash indicates non-consecutive sampling date **(C)**. All dissolved Fe (dFe) concentrations **(A & B)** are the mean of triplicate water samples analyzed by ICP-MS (± SEM); bold values indicate significantly lower dFe than the dust arrival date (ANOVA with Tukey's post-hoc test; P < 0.05).

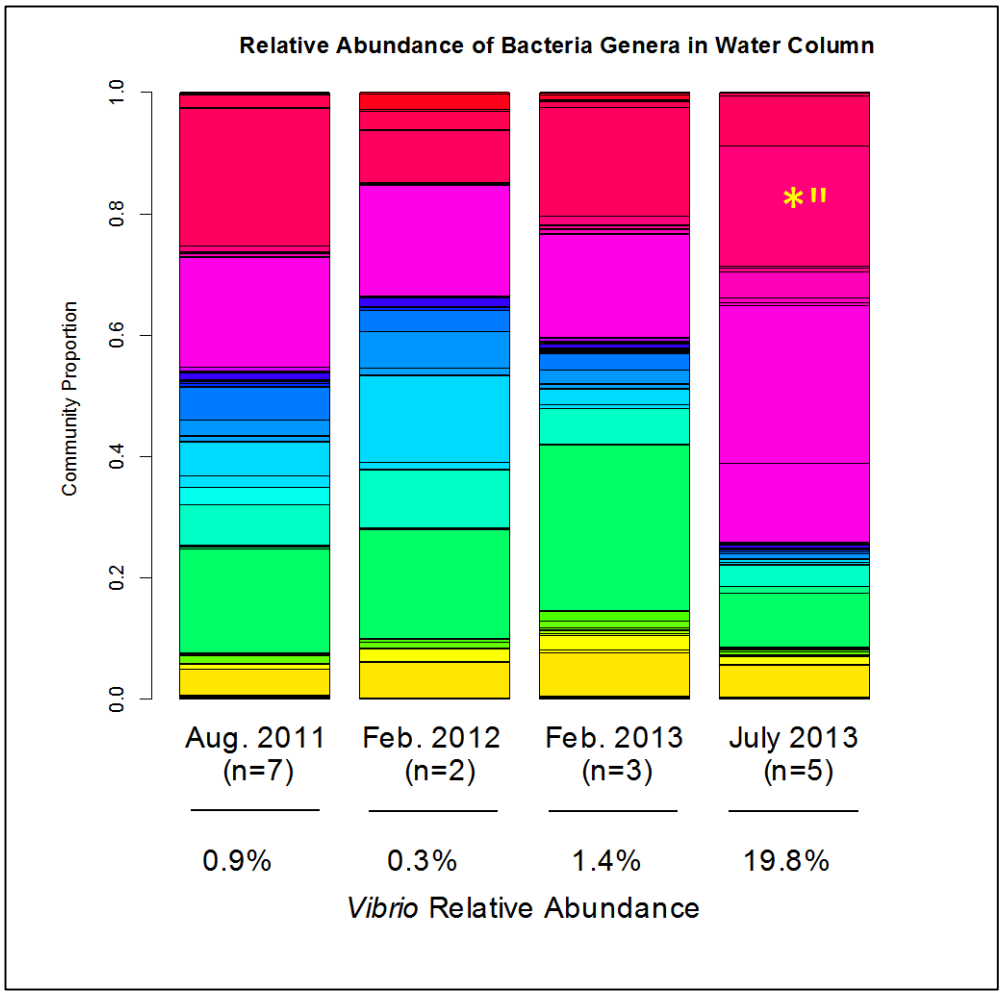

Fig. 4.6. Bacterial community response to Saharan Dust Event *in situ*. Composition of the bacterial community in surface water by date at Loee Key reef, Florida using 16S rRNA bacterial community analysis. *Vibrio* are denoted by ***** (too low for the first 3 dates to be discernable on the figure). The relative abundance of *Pseudomonas* and *Acinetobacter* also expanded during the 2013 SDE.

Fig. 4.7. Genera represented in bacterial community analyses (Legend for Fig. 4.6).

CHAPTER 5

VIBRIO POPULATION DYNAMICS IN MID-ATLANTIC SURFACE WATERS DURING SAHARAN DUST EVENTS

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Abstract

Vibrio is a cosmopolitan genus of marine bacteria, mostly associated with coastal and estuarine environments, where they play an important role in biogeochemical cycles. *Vibrio* have been isolated in low numbers from pelagic waters, yet little is known about the ecology of these oligotrophic species. In this study we examined the change in bacterial abundance and the population dynamics of *Vibrio* in the tropical North Atlantic, in response to the arrival of pulses of Saharan dust, a major source of biologically important nutrients for downwind marine surface waters. Aerosol and surface water samples were collected daily between 5 October and 4 November 2011. Four dust events were sampled and occurred roughly every 3-4 days. Total bacterial counts increased by 1.6 fold with arrival of Saharan dust. Virus-like particles also followed this trend and were correlated with bacterial counts ($r=0.66$; $p=0.01$). Concentrations of *Vibrio* ranged from below detection limits (5.6 cell equivalents (CE) ml^{-1}) to a high of 1058 CE ml^{-1} with the arrival of dust ($r=0.58$; $p=0.03$). *Vibrio* exhibited bloom-bust cycles over the course of the study, which could potentially be attributed to selective viral lysis or post-bloom depletion of organic carbon for growth. This work is one of the few studies to examine the ecology of *Vibrio* in the open ocean, whose feast or famine adaptive strategy and bloom-bust lifestyle is likely a contributing factor in the flow of nutrients and energy in pelagic ecosystems.

Introduction

The pelagic ocean is one of the largest habitats on earth. Surface water microbes in the ocean drive the majority of biogeochemical cycling and are a critical linkage in the ocean-atmosphere exchange of gases that affect global climate (Falkowski et al. 2008). An understanding of microbial community composition and function - who is doing what - is a key aspect in marine microbial ecology research (Shilovaa et al. 2014) (Brown et al. 2014; Lauro et al. 2009). Through metagenomic analysis of surface ocean prokaryotes, two different adaptive strategies have been identified (Polz et al. 2006; Yooseph et al. 2010). Globally, the most abundant group, adapted to nutrient poor conditions of the open ocean, is characterized by small size and streamlined genomes, resulting in slow but sustained growth with little metabolic plasticity and low capability to respond to nutrient influx (Yooseph et al. 2010). An opposing adaptive strategy is characterized by the rare opportunistic group, with greater genomic and metabolic diversity, able to survive in low numbers, but capable of rapidly exploiting spatially and temporally variable nutrient resources, thus occasionally becoming highly abundant in the community (Yooseph et al. 2010). This latter group of conditionally rare taxa (CRT) can have a disproportionate affect on ecosystem functioning during periodic bloom conditions by providing important ecosystem services that are disproportionate to their initial low abundance (Shade et al. 2014). Their susceptibility to predation and viral lysis also make them important players in food web dynamics and biogeochemical cycling (Yooseph et al. 2010), effectively influencing the trophic transfer of key elements and trace metals. Because of their rarity in ocean surveys, little is known about CRT and what environmental drivers induce an increase in their abundance, especially in remote pelagic systems.

The genus *Vibrio* includes a group of heterotrophic marine bacteria with a worldwide distribution, but characterized as conditionally rare taxa because of their infrequent detection and low numerical abundance in the surface ocean (Yooseph et al. 2010). *Vibrio* have a broad metabolic and genomic potential, are able to take advantage of ephemeral pulses of nutrients through chemotaxis and motility, and show rapid growth in response to nutrient addition (Takemura et al. 2014; Worden et al. 2006). *Vibrio* are often found in close association with plankton and marine particulates where they degrade a broad range of substrates with extracellular enzymatic proteins such as chitinases, proteases, and lipases (Thompson and Polz 2006), but can also survive in a free-living state (Hunt et al. 2008; Worden et al. 2006). *Vibrio* tend to be biogeographically associated with nutrient-rich, copiotrophic environments of near-shore systems (Takemura et al. 2014). Although they can be isolated from oligotrophic open ocean environments, little is known about the ecology of these pelagic species and their population dynamics (Thompson and Polz 2006).

In a recent study *Vibrio* were shown to be highly responsive to nutrient and trace metal inputs through atmospheric deposition of Saharan dust delivered to downwind coastal surface waters in the Caribbean (Westrich et al. submitted). Saharan dust is entrained in the atmosphere from source regions in northern Africa and is blown across the Atlantic Ocean and deposited, providing bio-essential nutrients and trace metals, like iron, to marine ecosystems (Boyd and Ellwood 2010; Mahowald et al. 2009). Several studies suggest that fast responding heterotrophs in oligotrophic waters can out compete autotrophic organisms for Saharan dust associated nutrients and the degree of response increases with the degree of oligotrophy (Marañón et al. 2010; Pulido-Villena et al.

2008). We hypothesized, that *Vibrio*, as a representative of CRT, would increase in abundance with the arrival of Saharan dust in the open ocean of the tropical North Atlantic.

Materials and Methods

Sample Collection

All samples were collected during the Integrated Ocean Drilling Program Expedition 336 (September 16 - November 16, 2011) aboard the R/V JOIDES *Resolution*. Surface water samples used in this study were collected when on station at the North Pond area on the western flank of the Mid-Atlantic Ridge at 22°45' N and 46°05' W (Fig. 5.1) during the period of 5 October – 5 November 2011. Surface water grabs (~50 mL) were collected daily between 1045 and 1200 h from a port/bow location (under the bridge) at a maximum depth of ~0.25 m using a sterile 50 mL tube and weight attached to a string. For each sample, two 1 mL subsamples were centrifuged at ~13,000 x g for 20 min; after centrifugation, the supernatant fluid was removed and discarded using a micropipette. These samples were stored at -80°C for qPCR analyses for *Vibrio* spp. Two 10 mL subsamples were fixed in formalin and used for bacteria and virus like particle direct counts. The remaining aliquot of surface water for each sample date was stored at -80°C.

DNA Extraction

DNA was extracted from the pelleted seawater samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for Gram-positive bacteria using AE buffer as the elution medium. Two duplicate elutions of 25 µL each were combined for a final eluent volume of 50 µL. Extracts were stored at -80 °C until use.

Construction of Quantitative PCR (qPCR) Standard

PCR standards were prepared from genomic DNA extracted from *V. alginolyticus* (American Type Culture Collection strain 33839). Briefly, a culture was grown overnight in LBS (Miller Broth [Fisher, BP1426] supplemented with 2% NaCl and 0.05 M Tris Buffer) then pelleted by centrifugation for 10 min at 8,000 x g. Supernantant fluid was discarded and the genomic DNA (gDNA) in the final pellet was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for Gram-negative bacteria. A PCR product was generated from the gDNA using *Vibrio* group specific primers targeting a variable region of the 16S rRNA gene (rDNA), 567F, 5'GGCGTAAAGCGCATGCAGGT-3' and 680R, 5'-GAAATTCTACCCCCCTCTACAG-3, (the PCR cycling conditions were described by Thompson et al. and outlined below) (Thompson et al. 2004). The PCR amplicon was cleaned (QIAquick PCR purification kit; Qiagen) and checked on an agarose gel to verify it was a single amplicon of the correct size. The amplicon was inserted into a PCR-4 vector and cloned into *E. coli* using a TA-TOPO kit (Life Technologies Grand Isle, NY). The plasmid was extracted (QIAquick Spin Miniprep kit; Qiagen) and the cloned region

sequenced to verify the correct insert. The plasmid was linearized with NotI (Roche, Indianapolis, IN) after cleanup (QIAquick PCR purification kit; Qiagen) and was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Grand Island, NY). The linearized standard was serially diluted in AE and run with each qPCR assay.

Quantitative PCR

Total *Vibrio* concentrations were quantified using a SYBR green qPCR method utilizing the *Vibrio* specific primers 567F and 680R (described above) (Thompson et al. 2004). DNA template (5 µl) was added to 10 µl SYBR SELECT PCR Master Mix (Applied Biosystems, Foster City, CA), 4.76 µl of PCR-grade water (Thermo Fisher Scientific), and primers were added to a final concentration of 0.16 µM in a total reaction volume of 20 µl. All reactions were run on a StepOne real-time PCR system (Life Technologies, Grand Isle, NY) in fast mode with the following cycling conditions: 2 min at 50 °C for UDG activation and 95 °C for 2 min to activate AmpliTaq polymerase and UP, followed by 40 cycles of 95 °C for 3 s for denaturation and 60 °C for 30 s for annealing and extension. Each run was followed by a dissociation step (95 °C for 15 s and 60 °C for 1 min and 95 °C for 15 s) to determine a melt curve for analysis of specificity. Each sample was analyzed in triplicate (technical replication) and at least two replicate positive controls and two replicate negative (no template) controls were included with every run. PCR inhibition tests were conducted by the addition of 5×10^3 gene copies of linearized standard to each sample. If inhibition was detected, the sample was diluted 1:10 and amplification was repeated. Samples that continued to show inhibition were excluded from the analysis, resulting in a total of 32 surface water samples analyzed by qPCR. All

qPCR assays compared cycle threshold (C_T) values to standard curves (equivalent to 10^1 -10^6 gene copies per reaction volume). Linearized standards could be detected to a level of 2 copies μl^{-1} corresponding to a C_T value of 37. Samples were considered not detectable if the mean C_T of technical replicates for each sample was above this value. *Vibrio* concentrations in seawater were expressed as number of cell equivalents (CE) by dividing the sample copy number by 9 (the average 16S rDNA copy number in *Vibrio*) (Acinas et al. 2004). Final concentrations were reported as CE ml^{-1} of seawater sampled. The final assay detection limit was 5.6 CE ml^{-1}.

Aerosol Analyses

A ParticleScan Pro (IQAir, La Mirada, CA) was used to determine aerosol particle counts daily at 10 min intervals. Measurements were taken on the roof of the bridge of the ship, ~10 meters above sea level. A daily average was calculated. High-volume membrane filtration samples were collected at 24 h intervals for daily composite aerosol analyses (Haley et al.). The unit flow rate was 0.57 m^3 min^{-1}. Collection began at ~0700 h each day using a Staplex TF1A filtration unit (Staplex, Brooklyn, NY) and Whatman TFAGF41 glass fiber filters. Each filter was weighed daily and stored in a labeled zip-top bag at -80° C. Filters were visually inspected for color and other features. In particular, filters with an orange tint, indicative of a heavy Saharan dust event, and those with a black to grey tint, indicative of diesel or other smoke, were noted.

Aerosol Modeling Analysis

The NAAPS (Navy Aerosol Analysis and Prediction System [http://www.nrlmry.navy.mil/aerosol/]) analysis surface-level dust and aerosol optical depth (AOD3), a vertical integral of total dust (unitless), was downloaded for the IODP sampling location (22° N and 46°W) during the sampling time period of October 5 – November 4, 2011. The daily analyses (based on 4 intervals) were averaged for each day providing one AOD value per day for comparative analysis purposes.

Bacterial and Viral Direct Counts

Total bacterial and viral-like particle were enumerated following the protocol first described by Noble et al. (1997) and modified by (Griffin et al. 2001; Noble and Fuhrman 1997). In short, 10 ml aliquots of surface water (collected daily at ~1300 h) were filtered through Whatman Anodisc 0.02-µm pore-sized, 25 mm diameter membranes, in duplicate. Material was stained by placing the membrane sample-side up on top of a drop of diluted SYBR Gold nucleic-acid stain (Molecular Probes, Eugene, OR) (97.5 µl of 0.02-µm filtered water + 2.5 µl of a 1/10 dilution of SYBR Gold) and incubated at room temperature in the dark for 10 - 15 minutes. Twenty-seven microliters of Prolong Antifade (Molecular Probes, Eugene, OR) was placed on a coverslip, and the coverslip placed over the filter on a glass slide. Fifteen fields per slide were counted at 1000 x magnification under oil immersion using a Carl Zeiss, Inc. (Jena, Germany) Axioskop 40 epifluorescent microscope. For negative controls, 10 mL of autoclaved 0.02-µm filter-sterilized deionized water was filtered and stained as described above.

Statistical Analysis

Aerosol particulates, bacterial and viral direct counts, and *Vibrio* cell equivalent counts were compared with and without lag factors using the Pearson correlation analysis, calculating a two-tailed P-value for each analysis. Samples with non-detectable *Vibrio* levels were assigned a value of one-half of the detection limit for statistical analyses (detection limit was 5.6 CE ml^{-1}). For all tests, significance was declared when $P \leq 0.05$. All tests were conducted in GraphPad Prism 5.0.

Results

Aerosol particle counts and optical density

Aerosol particle counts were used as a proxy for the presence of Saharan dust particulate matter in the immediate vicinity of sampling. Daily counts during the period of analysis averaged 1.89 x 10^7 particles L^{-1}, with a range from 1.82 x 10^6 particles L^{-1} on October 5 to 4.88 x 10^7 particles L^{-1} on October 22. After visual inspection of concurrently collected HV filters, days with notable black particulates and little to no wind (indicating a high likelihood of originating from diesel exhaust from the ship) were noted to occur from October 5 - 11. Conversely, a notable orange coloration was observed on HV filters October 14, 15, 17, 21, 22, and 23 (Fig. 5.2). The high dust load during these dates was also confirmed by NAAPS surface level modeling with a peak dust level October 22-23 (~80 μg m^{-3}) (Fig. 5.3).

Vibrio specific qPCR

A total of 54 surface water samples were collected for *Vibrio* specific qPCR. After elimination of samples that exhibited PCR inhibition, or were chronologically

isolated (making determination of *Vibrio* population dynamics difficult to interpret), a total of 32 samples were used for comparative analysis, providing a consecutive month long daily data set (October 5 – November 5, 2011). A daily average of 418 *Vibrio* CE ml^{-1} were measured during this time period. The lowest *Vibrio* concentrations of the study were measured on October 5, 6 and 7 with non-detectable, 11 and 23 CE ml^{-1}, respectively. These dates immediately preceded the first increase in aerosol particle counts. *Vibrio* detection of 1,058 CE ml^{-1} was measured on November 1, following a period of above average aerosol particle counts (3.06×10^7 particles L^{-3}; October 29-31) (Fig. 5.4). For the entire dataset, *Vibrio* CE ml^{-1} were directly associated with aerosol particle counts with a 24 h lag, to account for a one day delay in *Vibrio* growth ($r=0.47$; $p=0.01$; $n=32$) (Fig. 5.4). There was no significant correlation with particle counts when incorporating 48 and 72 h lag times.

Because of the likely influx of diesel exhaust during October 5 – 11, analyses between *Vibrio* and aerosol particulate counts were also examined excluding this period. Between October 12 – 25 dust and non-dust days could be conclusively differentiated. During this period, *Vibrio* showed a higher correlation with aerosol particle counts than noted over the full range of dates ($r=0.56$; $p=0.04$). Additionally, *Vibrio* concentrations increased with NAAPS aerosol optical depth (AOD) ($r= 0.58$; $p=0.03$) (Fig. 5.5).

Direct Counts of total bacteria and virus-like particles

Bacterial and virus-like particle (VLP) direct counts were also analyzed for the high dust period, October 12 - 25 (Fig. 5.6). Total bacterial counts averaged 3.59×10^5 ml^{-1} and ranged from 2.85×10^5 ml^{-1} on October 14 to a high on October 22 of 4.60×10^5

ml^{-1}, associated with an influx of dust (NAAPS dust model Fig. 5.5). VLP were almost an order of magnitude higher than bacterial counts, averaging 2.66 x 10^6 ml^{-1}, and ranged from 1.66 x 10^6 ml^{-1} on October 12 to a high of 3.37 x 10^6 ml^{-1} on October 23, 24 h following the peak bacterial count. Bacterial concentrations increased with aerosol particle counts, with a 24 h lag response ($r=0.76$; $p=0.001$). VLP concentrations were also significantly correlated with aerosol particle counts, with a 24 h lag ($r=0.37$; $p=0.02$). VLP also increased with bacterial concentration ($r=0.67$; $p=0.01$) (Fig. 5.6). *Vibrio* were not significantly correlated with either the total bacterial nor VLP direct counts with or without a time lag.

Discussion

Studies of *Vibrio* biogeography and environmental population dynamics have focused mostly on nutrient-replete coastal systems. In this study we show that though conditionally rare in the tropical open-ocean surface water of the mid-Atlantic (Yooseph et al. 2010), an increase in *Vibrio* concentrations is closely associated with the influx of Saharan dust, a known source of biologically important nutrients (Jickells et al. 2005; Mahowald et al. 2009). *Vibrio* population fluctuated from undetectable levels (below 5.6 CE ml^{-1}) to 1,058 CE ml^{-1}, following the arrival of Saharan dust. A 24 h lag in growth response is consistent with previous findings in near coastal regimes where *Vibrio* response to Saharan dust iron was seen 14 - 24 h post dust arrival (Westrich et al., in revision). This is believed to be an endogenous response to nutrients, as it has been reported that though microbes can be transported in Saharan dust, genus level profiling has not revealed the presence of *Vibrio* (Griffin 2007).

The dominant adaptive strategy of bacteria in pelagic systems is typically characterized by a streamlined existence of slow growing, small cells containing minimal genetic plasticity to respond to newly available nutrient sources (Yooseph et al. 2010). Here we have shown that in contrast to this minimalistic strategy, *Vibrio* with larger genomes and biomass, have the metabolic capacity to survive in low numbers in the open ocean, and can mount a rapid growth response to an ephemeral nutrient pulse. *Vibrio* have evolved adaptations to starvation pressures and have been shown to persist for long periods under conditions of nutrient limitation (Eilers et al. 2000). *Vibrio* have been shown to reduce cell volume in response to starvation (Denner et al. 2002; Thompson and Polz 2006) yet maintain a high number of rRNA copies to rapidly ramp up protein synthesis when conditions become more favorable (Eilers et al. 2000; Polz et al. 2006). This metabolic flexibility could be ecologically advantageous in the open ocean environment with varying nutrient regimes. Though not directly measured in this study, we suggest that Saharan dust associated nutrients, especially iron, drive the observed *Vibrio* growth response (e.g., Westrich et al. in revision).

Saharan dust is the dominant source of iron in the North Atlantic, providing up to 87% of the dissolved iron (most bioavailable form) to marine surface water communities (Conway and John 2014; Mahowald et al. 2009). *Vibrio* spp., like other heterotrophs, have a high intracellular demand for iron in enzymatic and metabolic processes, especially in their respiratory electron transport chain (Fourquez et al. 2014). Some *Vibrio* spp. are capable of fixing nitrogen, which requires extremely high amounts of iron as a co-factor (Chimetto et al. 2008; Criminger et al. 2007). Iron availability has a direct effect on *Vibrio* growth and ability to metabolize carbon and other biologically important

macronutrients like N and P (Fourquez et al. 2014; Kirchman et al. 2003; Sunda 2012). Furthermore, *Vibrio* can utilize dust leachate filtered through a 0.2 μm membrane (Westrich et al. in revision), suggesting that these often particle-associated microbes are not necessarily reliant on dust particulates for growth, but can utilize dust nutrients in a free-living state. A recent survey of microbial assemblages from the western tropical Atlantic (near the Mid-Atlantic Ridge) found significant increases in *Vibrio* operational taxonomic units (OTU) and that iron acquisition genes were the dominant functional response during turbulence driven nutrient enrichment (Moreira et al. 2015) further supporting the importance of iron in heterotrophic fitness in Mid-Atlantic pelagic communities.

Heterotrophic bacteria may play a much larger role in the connections between dust and the ocean carbon cycle than previously recognized (Pulido-Villena et al. 2008). The bloom-bust cycles of *Vibrio* seen in this study could be attributed to both bottom-up and top-down controls. The temporary release from nutrient-limitation with dust arrival could directly drive the *Vibrio* increase observed with a resulting crash after exhaustion of organic carbon sources. Alternatively, the increase in virus like particles, closely associated with the increase in total bacterial counts, could be a source of carbon and nutrients from viral-lysed cells, indirectly supporting increased *Vibrio* abundance (Pulido-Villena et al. 2014). Top-down controls are also known to tightly regulate *Vibrio* populations and could account for the 24 - 48 h bloom to bust dynamics we observed in this study and similarly found in other systems (Suttle 2007; Worden et al. 2006). *Vibrio* and other fast responding gamaproteobacteria have been estimated to have 100 times more biomass per cell than the equivalent, globally abundant SAR 11

alphaproteobacteria (Yooseph et al. 2010). This larger biomass is believed to be a liability, especially during bloom conditions, where they have been shown to preferentially be preyed upon by eukaryotic protists and subject to viral lysis (Polz et al. 2006; Yooseph et al. 2010). The turn–over of *Vibrio* and larger-biomass members of the community, even though numerically minor, could have significant impacts on marine carbon and nutrient cycling in the pelagic ocean (Suttle 2007). Additionally, primary production could benefit from lytic turnover of lysed cellular debris that is rich in P and N (Suttle 2007). Viral lystate of *V. alginolyticus* has been shown to be a highly available source of iron supporting photosynthesis (Poorvin et al. 2011).

In conclusion this study demonstrates that *Vibrio* populations, though typically rare in pelagic waters significantly increase in abundance with the arrival of Saharan dust. We propose that dust associated nutrients like iron, essential for *Vibrio* survival and fitness, drive this response in these oligotrophic systems, extending a previous finding of iron in Saharan dust influencing coastal *Vibrio* population blooms. Ecological roles of rare microorganism are largely unknown, especially in the open ocean, but it is thought that they contribute to stability and function of an ecosystem (Shade et al. 2014). Though many uncertainties remain, identifying this response is crucial first step for more targeted studies that could have larger implications in the carbon and nutrient cycling in the oligotrophic open ocean.

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Figures

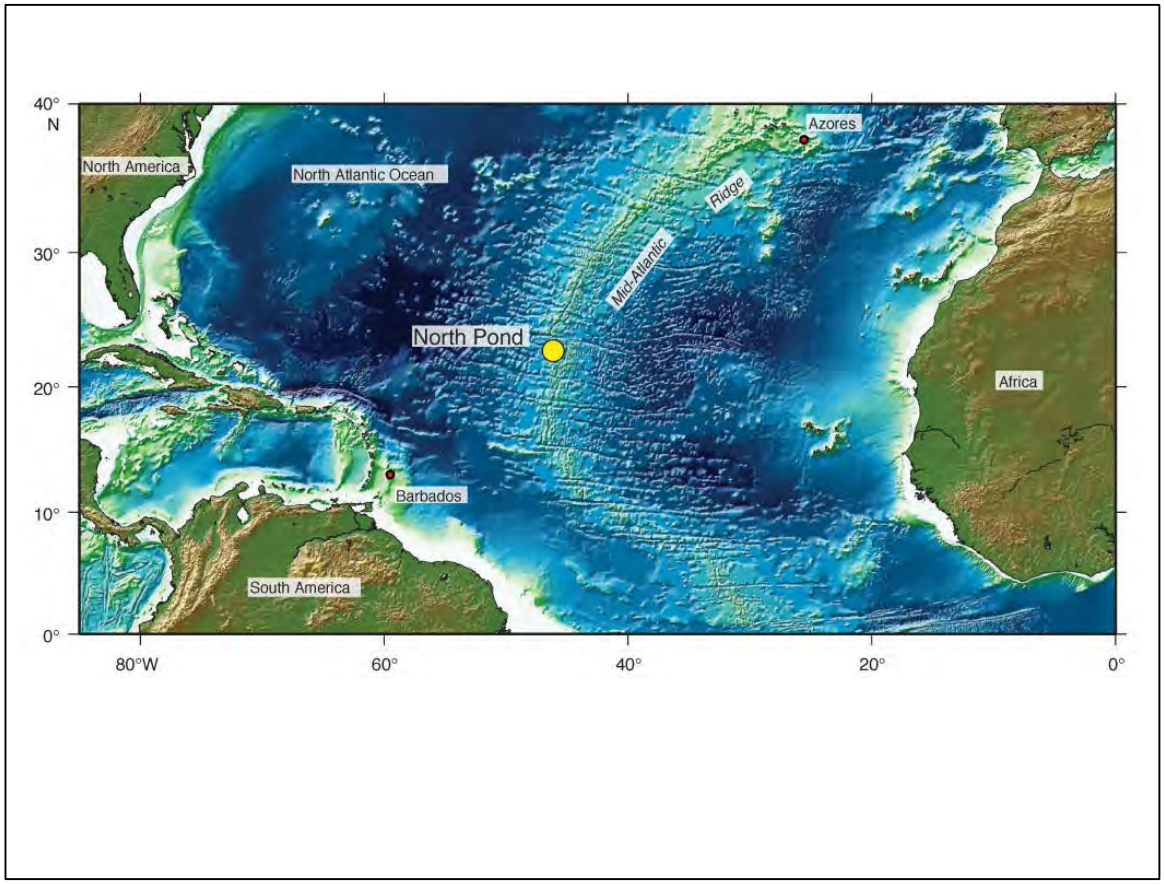

Fig. 5.1. Sample collection location at North Pond on the western flank of the Mid-Atlantic Ridge at 22°45’ N and 46°05’ W (Griffin 2011).

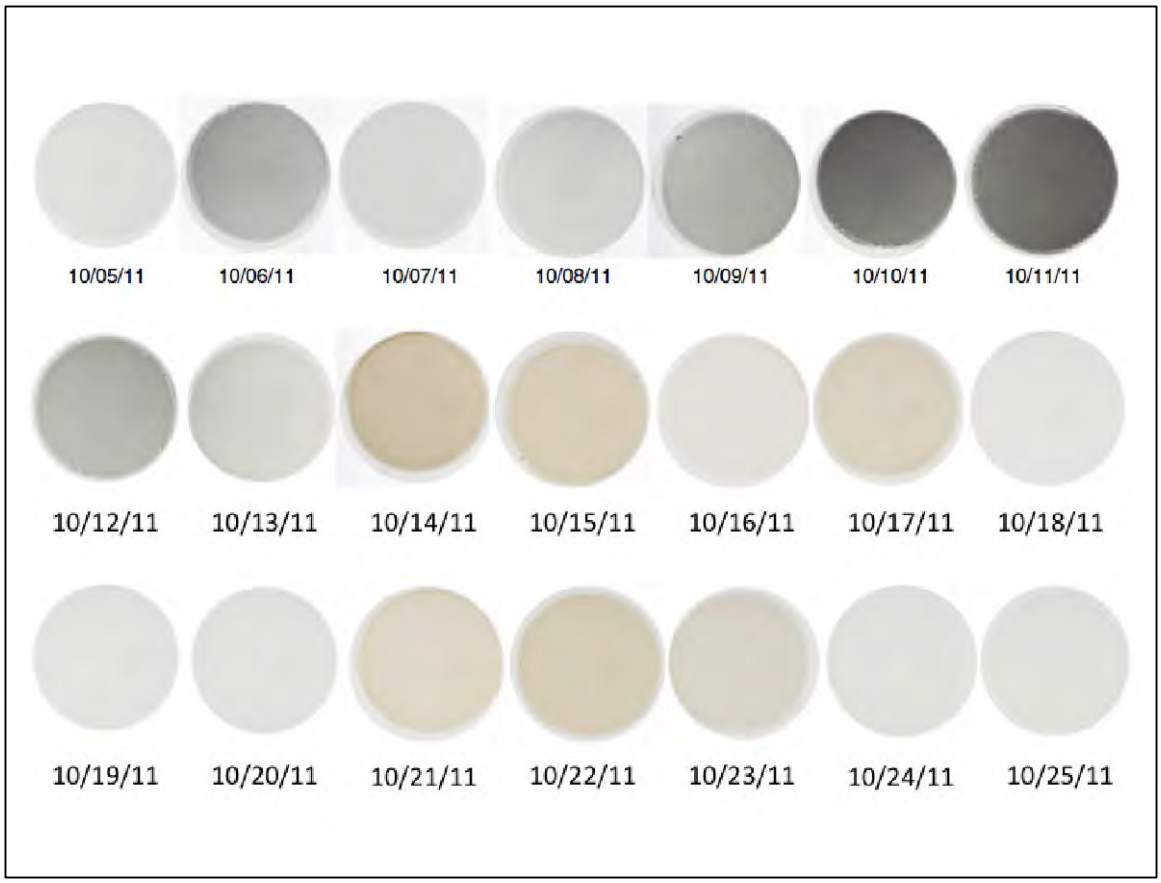

Fig. 5.2. High-volume sample filters collected daily over ~24 h. Filters are Staplex Type TFAGF41 glass fiber filters. Time period October 5 - 25 2011. Black filters were speculated to be from ship exhaust (October 5-11), orange tinted filters (October 14,15,17,21,22,23) indicated high Saharan dust loading.

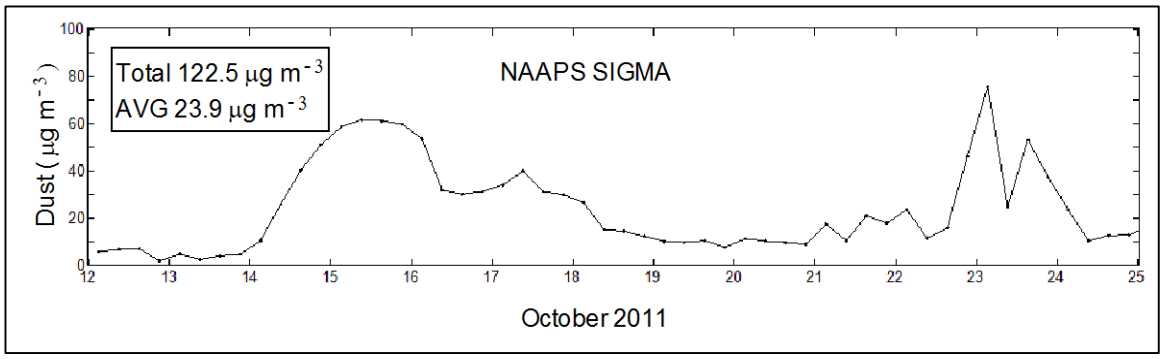

Fig. 5.3. Naval Aerosol Analysis and Prediction System (NAAPS) model of surface level dust at the Mid-Atlantic Ridge (22°45' N and 46°05' W) October 12-25, 2011.

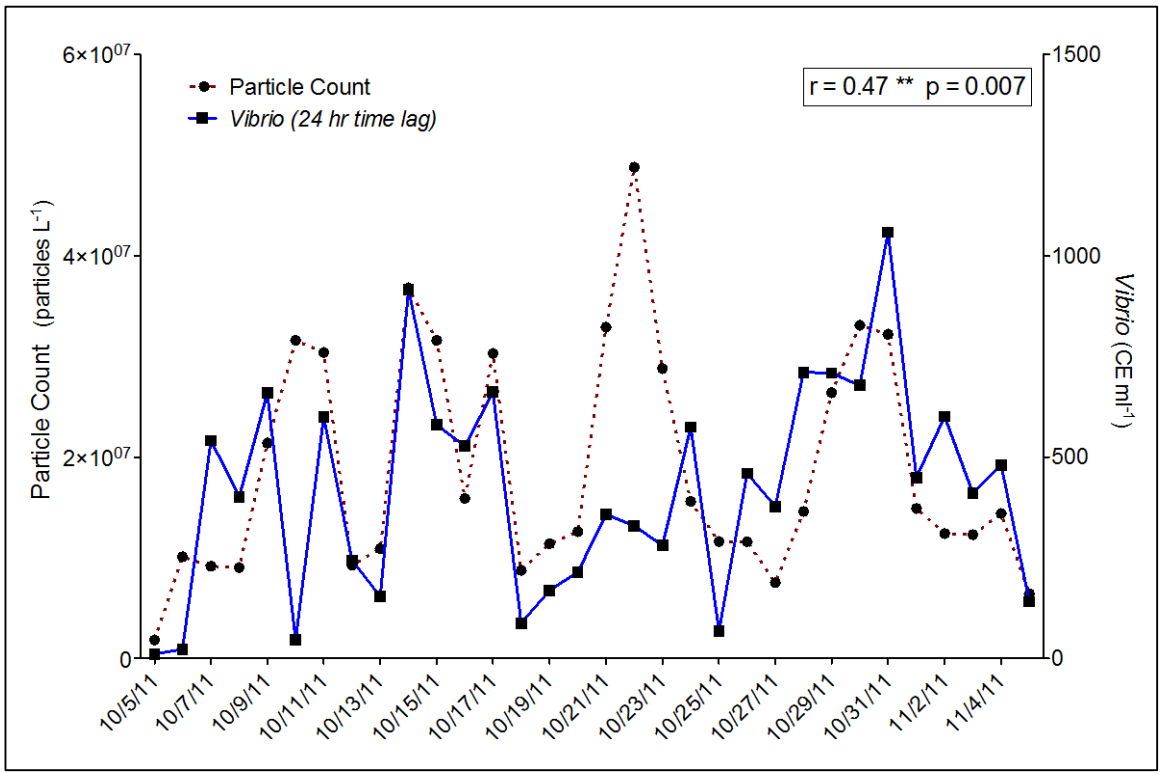

Fig. 5.4. Relationship between ship-based particle counts and *Vibrio* cell equivalents (CE) ml^{-1} (*Vibrio* counts were shifted by 24 h to incorporate a growth response lag-time). Pearson correlation r=0.47, **p = 0.007; n=32.

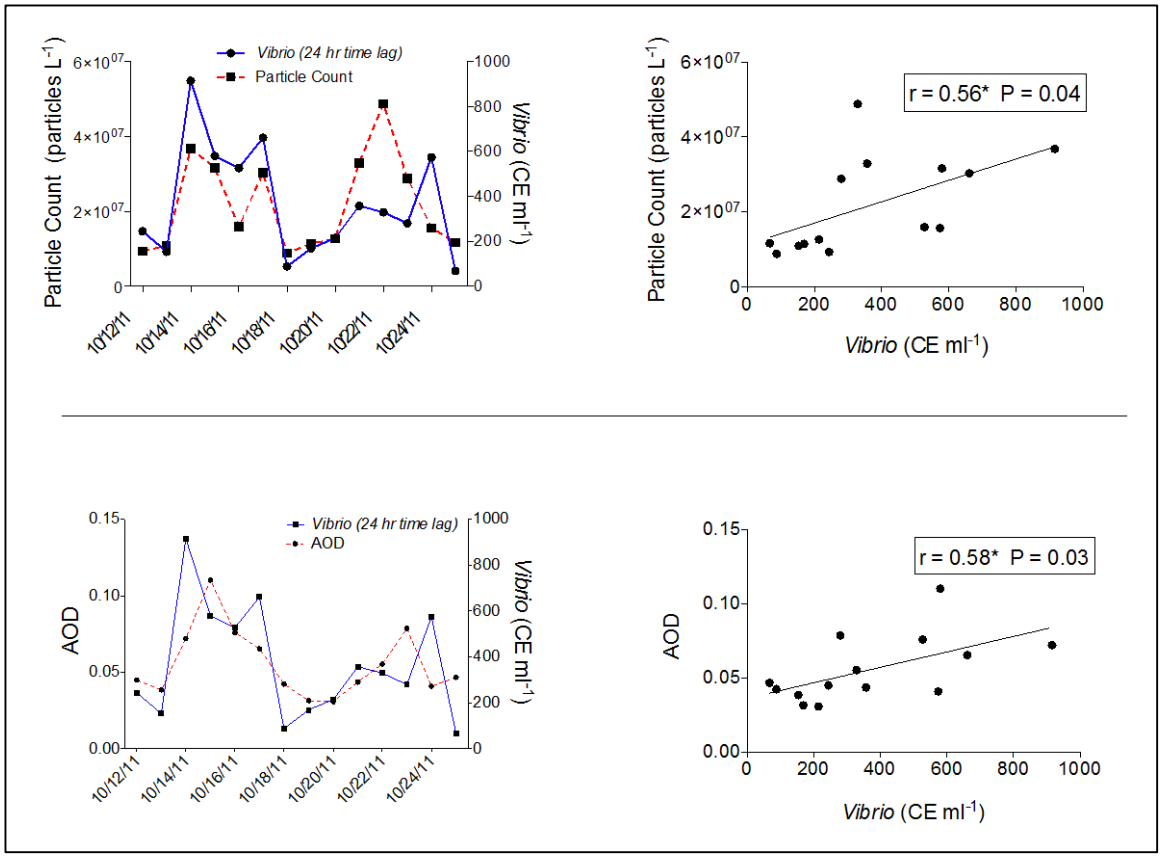

Fig. 5.5. Scatter plots (left side of figure) of *Vibrio* cellular equivalent (CE) ml^{-1} (*Vibrio* counts were shifted by 24 h to incorporate a growth response lag-time) compared to particle counts (top) and modeled Aerosol Optical Density (AOD) (bottom) for high dust days (October 12-25, 2011). Pearson correlation (right side of graph) for corresponding scatter plot (n=14).

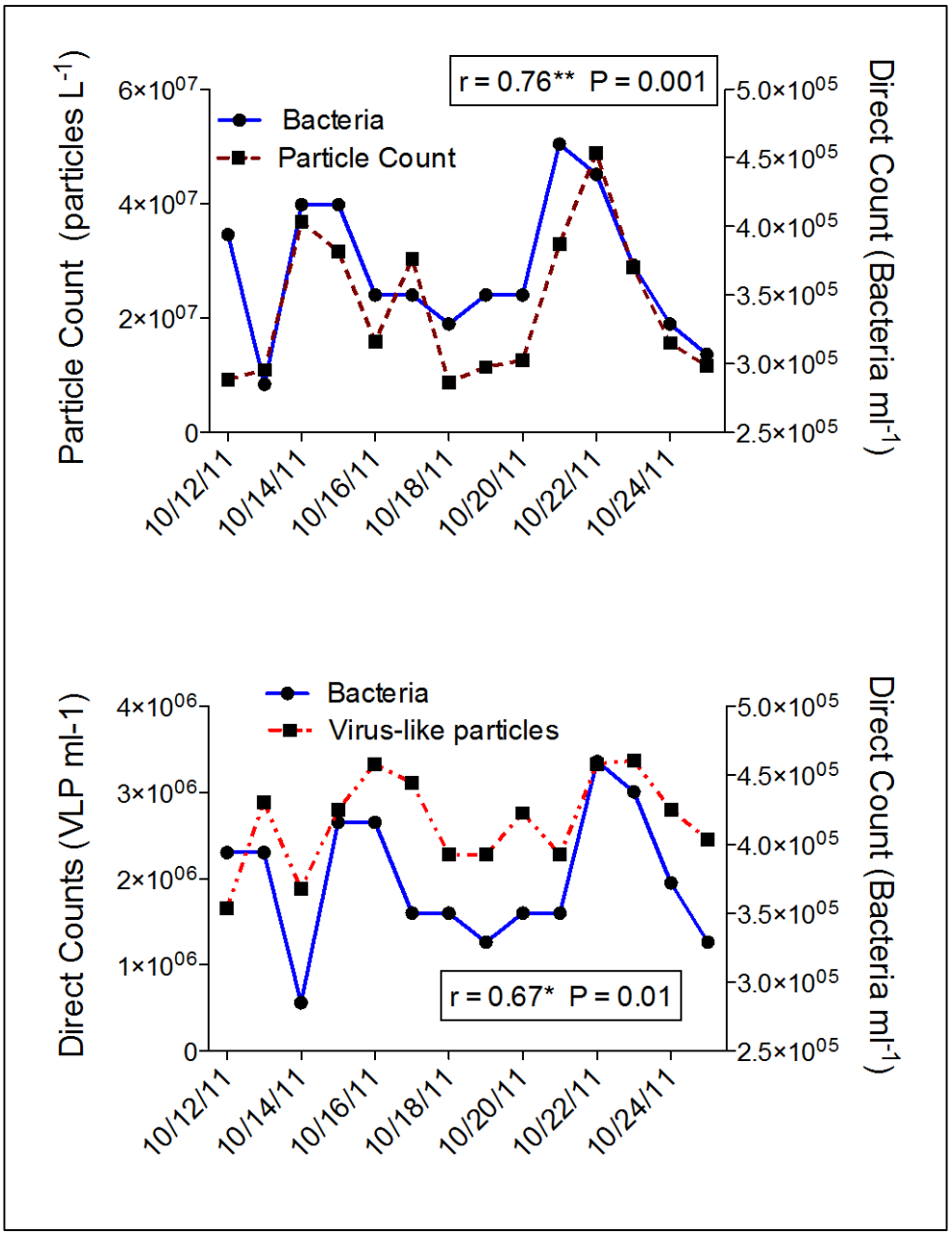

Fig. 5.6. Direct Counts of total bacteria and virus-like particles (VLP) for dust events around for (October 12-25, 2011). Total bacteria compared to particle count were shifted by 24 hrs to incorporate a growth response lag-time (top). Virus-like particles compared to total bacteria (no time shift) (bottom). Comparisons reported on the graph were made using Pearson correlation calculating a two-tailed P value (n=14).

CHAPTER 6

CONCLUSION

This work illustrates the importance of iron in the growth and ecology of the genus *Vibrio* and contributes to a fuller understanding of heterotrophic responses to Saharan dust nutrients. The investigations in this body of work attempted to address several knowledge gaps: 1) the role of environmental iron flux on *Vibrio* populations dynamics, 2) examination of the ecology of open water *Vibrio* and 3) the role of heterotrophic processing of Saharan dust iron.

In chapter 3 we introduce a novel iron-limited seawater medium in which to examine heterotrophic iron processes. In manipulative studies, using *Vibrio* as a model of heterotrophic response, we showed that this medium, VibFeL, can completely iron-restrict growth of *Vibrio*, allowing a controlled examination of heterotrophic response to environmental iron sources. Chapter 4 and 5 were a series of environmental studies investigating the response of *Vibrio* to Saharan dust nutrients in near-shore (Florida Keys and Barbados) and open ocean systems (tropical Mid-Atlantic). In both systems a growth response, ~24 hours post dust arrival was observed. Analysis of the total community demonstrated an increase in relative abundance of *Vibrio* from 1.4% to 20% of the surface water community in the Florida Keys and an increase in isolated disease-causing species, *V. alginolyticus*, *V. cholerae* and *V.parahaemolyticus*. Using Vib-FeL we demonstrated that dust-associated iron supported this growth. Little is known about open ocean Vibrio ecology and here we show that increases in their populations are significantly correlated with the arrival of dust nutrients in these otherwise nutrient-poor systems. Though

we know much about the importance of iron in *Vibrio* clinical pathology, this is one of a very few investigations into the role of iron in environmental *Vibrio* dynamics.

APPENDIX 1

VibFeL Trace metal speciation and saturation. For each mineral species, the log IAP (ion activity product) is computed as well as the saturation index for VibFeL complete medium, pH 7.6, ionic strength of 0.4803. Calculations made using Visual MINTEQ (Version 3).

Mineral	log IAP	Sat. index
Antlerite	-22.808	-31.596
Atacamite	-10.93	-18.321
Bianchite	-16.946	-15.181
Brochantite	-24.194	-39.416
Brucite	11.685	-5.415
Ca3(PO4)2 (am1)	-29.487	-3.987
Ca3(PO4)2 (am2)	-29.487	-1.237
Ca3(PO4)2 (beta)	-29.487	-0.567
Ca4H(PO4)3:3H2O(s)	-49.317	-1.367
CaHPO4(s)	-19.83	-0.555
CaHPO4:2H2O(s)	-19.83	-0.835
CaMoO4(s)	-12.655	-4.705
Chalcanthite	-20.036	-17.396
Co(OH)2 (am)	1.351	-11.743
Co(OH)2 (c)	1.351	-10.939
Co3(PO4)2(s)	-55.952	-21.265

CoCl2(s)	-14.964	-23.231
CoCl2:6H2O(s)	-14.964	-17.501
CoFe2O4(s)	4.787	8.315
CoHPO4(s)	-28.652	-9.591
CoMoO4(s)	-21.477	-13.716
CoO(s)	1.351	-12.236
CoSO4(s)	-17.299	-20.101
CoSO4:6H2O(s)	-17.299	-14.826
Cu(OH)2(s)	-1.386	-10.676
Cu3(PO4)2(s)	-64.163	-27.313
Cu3(PO4)2:3H2O(s)	-64.163	-29.043
CuMoO4(s)	-24.213	-11.137
CuOCuSO4(s)	-21.422	-31.725
Cupric Ferrite	2.05	-3.938
CuSO4(s)	-20.036	-22.975
Epsomite	-6.965	-4.838
Fe(OH)2.7Cl.3(s)	-0.729	2.311
Fe2(SO4)3(s)	-52.513	-48.779
Ferrihydrite	1.718	-1.482
Ferrihydrite (aged)	1.718	-0.972
Goethite	1.718	1.227
Goslarite	-16.946	-14.935
Gypsum	-8.477	-3.867
H2MoO4(s)	-22.827	-9.951

Halite	-1.073	-2.623
Hematite	3.436	4.854
H-Jarosite	-32.145	-26.755
Hydroxyapatite	-39.144	5.189
K2MoO4(s)	-15.443	-18.705
KCl(s)	-4.465	-5.365
K-Jarosite	-28.453	-17.453
Langite	-24.194	-41.683
Lepidocrocite	1.718	0.347
Lime	10.172	-22.527
Maghemite	3.436	-2.95
Magnesioferrite	15.121	-1.738
Melanothallite	-17.701	-23.958
Mg(OH)2 (active)	11.685	-7.109
Mg2(OH)3Cl:4H2O(s)	15.213	-10.787
Mg3(PO4)2(s)	-24.949	-1.669
MgHPO4:3H2O(s)	-18.317	-0.142
MgMoO4(s)	-11.142	-9.292
Mirabilite	-4.48	-3.366
Mn3(PO4)2(s)	-45.385	-21.558
MnCl2:4H2O(s)	-11.442	-14.157
MnHPO4(s)	-25.129	0.271
MnSO4(s)	-13.777	-16.36
MoO3(s)	-22.827	-14.827

Na2Mo2O7(s)	-31.485	-14.888
Na2MoO4(s)	-8.658	-10.148
Na2MoO4:2H2O(s)	-8.658	-9.882
Na-Jarosite	-25.06	-19.74
Periclase	11.685	-9.899
Portlandite	10.172	-12.532
Powellite	-12.655	-4.735
Pyrochroite	4.873	-10.321
Strengite	-28.284	-1.884
Tenorite(am)	-1.386	-9.876
Tenorite(c)	-1.386	-9.026
Thenardite	-4.48	-4.802
Zincite	1.704	-9.526
Zincosite	-16.946	-20.876
Zn(OH)2 (am)	1.704	-10.77
Zn(OH)2 (beta)	1.704	-10.05
Zn(OH)2 (delta)	1.704	-10.14
Zn(OH)2 (epsilon)	1.704	-9.83
Zn(OH)2 (gamma)	1.704	-10.03
Zn2(OH)2SO4(s)	-15.242	-22.742
Zn2(OH)3Cl(s)	-4.75	-19.941
Zn3(PO4)2:4H2O(s)	-54.893	-19.473
Zn3O(SO4)2(s)	-32.188	-51.102
Zn4(OH)6SO4(s)	-11.835	-40.235

Zn5(OH)8Cl2(s)	-7.796	-46.296
ZnCl2(s)	-14.611	-21.661
ZnMoO4(s)	-21.124	-10.998
ZnSO4:1H2O(s)	-16.946	-16.308

APPENDIX 2

VibFeL Trace metal total component concentration and activity. For each mineral species, the concentration is computed as well as the activity based on an ionic strength of 0.4803 at a pH of 7.6. Calculations made using Visual MINTEQ (Version 3).

Component	Concentration	Activity	Log activity
Ca(NH3)2+2	2.668E-12	6.1097E-13	-12.214
Ca+2	0.000040984	9.3854E-06	-5.028
CaCl+	9.4424E-06	6.5319E-06	-5.185
CaEDTA-2	0.000031603	7.2371E-06	-5.14
CaH2PO4+	4.5006E-07	3.1134E-07	-6.507
CaHEDTA-	8.9044E-10	6.1598E-10	-9.21
CaHPO4 (aq)	0.000014362	0.000016041	-4.795
CaMoO4(aq)	7.3636E-11	8.2248E-11	-10.085
CaNH3+2	1.881E-08	4.3076E-09	-8.366
CaOH+	1.0852E-10	7.5068E-11	-10.125
CaPO4-	2.4563E-06	1.6992E-06	-5.77
CaSO4 (aq)	6.8343E-07	7.6336E-07	-6.117
Cl-1	0.40052	0.27707	-0.557
Co(NH3)+2	1.9169E-15	4.3898E-16	-15.358
Co(NH3)2+2	1.601E-17	3.6663E-18	-17.436
Co(NH3)3+2	3.9461E-20	9.0368E-21	-20.044
Co(NH3)4+2	4.8748E-23	1.1163E-23	-22.952

Co(NH3)5+2	1.6208E-26	3.7118E-27	-26.43
Co(OH)2 (aq)	3.2257E-18	3.603E-18	-17.443
Co(OH)3-	4.1658E-23	2.8818E-23	-22.54
Co+2	6.1776E-14	1.4147E-14	-13.849
Co4(OH)4+4	1.1893E-53	3.2707E-56	-55.485
CoCl+	2.531E-15	1.7508E-15	-14.757
CoEDTA-2	2.4999E-08	5.7249E-09	-8.242
CoH2EDTA (aq)	6.9141E-19	7.7227E-19	-18.112
CoHEDTA-	5.5951E-13	3.8705E-13	-12.412
CoHPO4 (aq)	5.3754E-14	6.004E-14	-13.222
CoOH+	1.6357E-16	1.1315E-16	-15.946
CoSO4 (aq)	8.9723E-16	1.0022E-15	-14.999
Cu(NH3)2+2	2.3312E-16	5.3385E-17	-16.273
Cu(NH3)3+2	4.2595E-17	9.7544E-18	-17.011
Cu(NH3)4+2	1.5175E-18	3.4752E-19	-18.459
Cu(OH)2 (aq)	2.1668E-18	2.4202E-18	-17.616
Cu(OH)3-	5.4186E-21	3.7484E-21	-20.426
Cu(OH)4-2	5.2967E-26	1.213E-26	-25.916
Cu+2	1.1324E-16	2.5933E-17	-16.586
Cu2(OH)2+2	1.4923E-28	3.4174E-29	-28.466
Cu2OH+3	1.439E-31	5.2203E-33	-32.282
Cu3(OH)4+2	3.1168E-40	7.1375E-41	-40.146
CuCl+	2.0724E-17	1.4336E-17	-16.844
CuCl2 (aq)	9.7946E-19	1.094E-18	-17.961

CuCl3-	4.0893E-21	2.8288E-21	-20.548
CuCl4-2 1	1.7153E-23	3.9282E-24	-23.406
CuEDTA-2	9.7975E-09	2.2437E-09	-8.649
CuH2EDTA (aq)	6.9651E-19	7.7796E-19	-18.109
CuHEDTA-	2.7606E-13	1.9097E-13	-12.719
CuHPO4 (aq)	1.1577E-15	1.2931E-15	-14.888
CuHSO4+	7.3141E-26	5.0597E-26	-25.296
CuNH3+2	3.4339E-16	7.8638E-17	-16.104
CuOH+	4.7521E-17	3.2874E-17	-16.483
CuOHEDTA-3	2.2096E-12	8.016E-14	-13.096
CuSO4 (aq)	1.8884E-18	2.1092E-18	-17.676
EDTA-4	1.018E-10	2.7997E-13	-12.553
Fe(OH)2+	3.3746E-12	2.3344E-12	-11.632
Fe(OH)3 (aq)	4.679E-14	5.2262E-14	-13.282
Fe(OH)4-	6.001E-14	4.1513E-14	-13.382
Fe(SO4)2-	3.6205E-23	2.5045E-23	-22.601
Fe+3	2.2832E-20	8.2829E-22	-21.082
Fe2(OH)2(EDTA)2- 4	1.4697E-08	4.0419E-11	-10.393
Fe2(OH)2+4	5.0466E-28	1.3879E-30	-29.858
Fe3(OH)4+5	7.3712E-36	7.3544E-40	-39.133
FeCl+2	3.0264E-20	6.9306E-21	-20.159
FeEDTA-	1.5323E-06	0.00000106	-5.975
FeH2PO4+2	1.0142E-19	2.3225E-20	-19.634

FeHEDTA (aq)	7.7136E-13	8.6157E-13	-12.065
FeHPO4+	3.6392E-14	2.5175E-14	-13.599
FeOH+2	1.3751E-15	3.1491E-16	-15.502
FeOHEDTA-2	2.8083E-06	6.4312E-07	-6.192
FeSO4+	7.5595E-21	5.2294E-21	-20.282
H+1	3.6311E-08	2.5119E-08	-7.6
H2EDTA-2	1.2831E-10	2.9384E-11	-10.532
H2Mo6O21-4	1.4677E-53	4.0366E-56	-55.394
H2PO4-	0.002142	0.0014818	-2.829
H3EDTA-	1.3968E-15	9.6629E-16	-15.015
H3Mo8O28-5	1.3254E-71	1.3224E-75	-74.879
H3PO4	4.6854E-09	5.2334E-09	-8.281
H4EDTA (aq)	3.5569E-21	3.9729E-21	-20.401
H5EDTA+	4.5619E-27	3.1558E-27	-26.501
H6EDTA+2	2.6747E-34	6.125E-35	-34.213
HEDTA(ii)-3	1.7198E-08	6.2389E-10	-9.205
HMo7O24-5	6.1614E-60	6.1473E-64	-63.211
HMoO4-	1.4884E-11	1.0296E-11	-10.987
HPO4-2	0.016328	0.0037392	-2.427
HSO4-	1.2598E-09	8.7151E-10	-9.06
K+1	0.00017877	0.00012367	-3.908
K2HPO4 (aq)	6.8278E-10	7.6263E-10	-9.118
K2PO4-	2.5255E-13	1.7471E-13	-12.758
KCl (aq)	0.000015375	0.000017173	-4.765

KEDTA-3	4.2632E-14	1.5466E-15	-14.811
KH2PO4 (aq)	3.2735E-07	3.6563E-07	-6.437
KHPO4-	5.0709E-06	3.5079E-06	-5.455
KOH (aq)	7.7132E-11	8.6152E-11	-10.065
KPO4-2	9.1244E-10	2.0895E-10	-9.68
KSO4-	4.4934E-07	3.1084E-07	-6.507
Mg(NH3)2+2	1.733E-10	3.9686E-11	-10.401
Mg+2	0.0013342	0.00030554	-3.515
MgCl+	0.00048719	0.00033702	-3.472
MgEDTA-2	0.000014202	3.2522E-06	-5.488
MgHEDTA-	3.1785E-09	2.1988E-09	-8.658
MgHPO4 (aq)	0.00064538	0.00072086	-3.142
MgMoO4(aq)	6.9137E-09	7.7222E-09	-8.112
MgOH+	6.7315E-08	4.6566E-08	-7.332
MgPO4-	1.2499E-06	8.6467E-07	-6.063
MgSO4 (aq)	0.000017673	0.00001974	-4.705
Mn(NH3)2+2	2.9987E-16	6.8672E-17	-16.163
Mn(NH3)3+2	1.1714E-19	2.6826E-20	-19.571
Mn(NH3)4+2	2.4016E-23	5.4998E-24	-23.26
Mn(OH)4-2	2.663E-28	6.0984E-29	-28.215
Mn+2	2.0576E-10	4.7121E-11	-10.327
Mn2(OH)3+	2.6029E-22	1.8006E-22	-21.745
Mn2OH+3	6.1628E-23	2.2357E-24	-23.651
MnCl+	1.8873E-11	1.3056E-11	-10.884

MnCl2 (aq)	5.759E-12	6.4325E-12	-11.192
MnCl3-	7.0959E-13	4.9087E-13	-12.309
MnEDTA-2	2.2934E-07	5.2519E-08	-7.28
MnHEDTA-	6.4619E-12	4.4701E-12	-11.35
MnHPO4 (aq)	4.1972E-10	4.688E-10	-9.329
MnNH3+2	4.1224E-13	9.4405E-14	-13.025
MnOH+	6.8588E-14	4.7447E-14	-13.324
MnSO4 (aq)	2.6635E-12	2.975E-12	-11.527
Mo7O24-6	2.6922E-56	4.6632E-62	-61.331
Mo8O26-4	5.2721E-76	1.4499E-78	-77.839
MoO3(H2O)3(aq)	2.3155E-15	2.5862E-15	-14.587
MoO4-2	0.000000103	2.3587E-08	-7.627
Na+1	0.44146	0.30539	-0.515
Na2HPO4 (aq)	0.0027507	0.0030724	-2.513
Na2PO4-	3.2925E-06	2.2776E-06	-5.643
NaCl (aq)	0.037967	0.042407	-1.373
NaEDTA-3	1.2087E-09	4.3849E-11	-10.358
NaH2PO4 (aq)	0.00080835	0.00090288	-3.044
NaHPO4-	0.019394	0.013416	-1.872
NaOH (aq)	1.3798E-07	1.5412E-07	-6.812
NaPO4-2	2.2531E-06	5.1598E-07	-6.287
NaSO4-	0.00086132	0.00059583	-3.225
NH3 (aq)	0.00025689	0.00028693	-3.542
NH4+1	0.018274	0.012641	-1.898

NH4SO4-	0.000069518	0.00004809	-4.318
OH-	5.7948E-07	4.0087E-07	-6.397
PO4-3	1.7304E-06	6.2774E-08	-7.202
SO4-2	0.0015504	0.00035504	-3.45
Zn(NH3)2+2	3.6095E-16	8.2658E-17	-16.083
Zn(NH3)3+2	2.3946E-17	5.4837E-18	-17.261
Zn(NH3)4+2	7.2613E-19	1.6629E-19	-18.779
Zn(OH)2 (aq)	5.7768E-16	6.4524E-16	-15.19
Zn(OH)3-	1.1824E-19	8.1793E-20	-19.087
Zn(OH)4-2	2.2692E-24	5.1966E-25	-24.284
Zn(SO4)2-2	3.3452E-17	7.6607E-18	-17.116
Zn+2	1.3928E-13	3.1895E-14	-13.496
Zn2OH+3	1.1241E-27	4.0779E-29	-28.39
ZnCl+	3.6842E-14	2.5486E-14	-13.594
ZnCl2 (aq)	6.1782E-15	6.9007E-15	-14.161
ZnCl3-	3.1011E-15	2.1452E-15	-14.669
ZnCl4-2	1.3008E-15	2.9789E-16	-15.526
ZnEDTA-2	3.8993E-08	8.9295E-09	-8.049
ZnH2EDTA (aq)	3.4103E-19	3.8092E-19	-18.419
ZnHEDTA-	8.7271E-13	6.0371E-13	-12.219
ZnHPO4 (aq)	2.2053E-13	2.4632E-13	-12.609
ZnNH3+2	6.5413E-15	1.498E-15	-14.824
ZnOH+	1.8482E-15	1.2786E-15	-14.893

ZnOHEDTA-3	5.6779E-12	2.0598E-13	-12.686
ZnSO4 (aq)	2.218E-15	2.4774E-15	-14.606