ABSTRACT

Title of Document:	MICROBIAL INTERACTIONS AND ECOLOGY WITHIN BLOOMS OF THE TOXIC DINOFLAGELLATE *KARENIA brevis* ON THE WEST FLORIDA SHELF.
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The dinoflagellate *Karenia brevis* is capable of significant ecological and economic impacts in Florida waters where blooms typically occur. Blooms and cultures of *K. brevis* were sampled to determine the composition, production, and possible ecological function of bacteria and virus communities associated with *K. brevis*. Bacterial communities on the West Florida Shelf (WFS) were similar inside and outside *K. brevis* blooms, but primary and secondary (bacterial) production and bacteria and virus abundances were different depending on bloom stage. Bloom stages need to be identified so that discrete sampling events can be combined to characterize an entire bloom event. Within an initiating bloom bacterial production and mortality was high and viral abundance was low, suggesting that viral genomes were either within host cells or bacterial mortality was due to mixotrophic grazing by

K. brevis or heterotrophic nanoflagellates. In a maintenance phase bloom the bacterial community was metabolically stressed, subject to increased viral infection, and most likely not being subjected to mixotrophic grazing. Bacterial communities associated with healthy *K. brevis* were dominated by the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex. As *K. brevis* shifted to stationary or senescing growth communities had higher proportions of *Alphaproteobacteria*. The SAR406 group, typically found in deep waters, was present in the surface waters of the WFS which supports existing *K. brevis* bloom formation hypotheses involving upwelling of deep waters from the mid to outer shelf. The CFB complex of bacteria also need to be further investigated as the consistent presence of CFB bacteria in both blooms and cultures of *K. brevis* suggest CFB bacteria are capable of numerous interactions with *K. brevis*. Furthermore, such interactions may be a vector of bloom control through viral infection; a high proportion of CFB bacteria would be ideal for density-dependent viral infection which could disrupt interactions between bacteria and *K. brevis*. Inoculating cultures of *K. brevis,* which included associated bacteria, with viral concentrates from the WFS showed differences in bacterial production and growth which indicate viruses are acting upon the bacterial community and not the dinoflagellates. Interactions between bacteria and *K. brevis* need to be further elucidated and explored for a better understanding of the role of each in dynamics of this harmful algal species. There may be a natural community succession amongst bacteria during blooms: utilizing certain indicator species to indicate bloom stage and transition between stages may aid in bloom forecasting and detection efforts.

MICROBIAL INTERACTIONS AND ECOLOGY WITHIN BLOOMS OF THE
TOXIC DINOFLAGELLATE *KARENIA brevis* ON THE WEST FLORIDA SHELF.

By

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Preface

Since I was in second grade "marine biologist" was always a career I was interested in pursuing; this despite growing up in St. Louis (nowhere near an ocean) and not seeing an ocean until I was 8 years old. When deciding to go to college not only did this field continue to intrigue me but it also gave me the opportunity to go "somewhere with a beach." Not only did I love the marine sciences but I became deeper entrenched in them. I originally began my graduate career having previously worked on shallow macroalgae and fisheries with the expectation I would be studying zooplankton. Instead, I ended up studying bacteria, viruses, and toxic dinoflagellates. I had no experience with any of these and even less knowledge of the methods I would need, how to use them, and how to make heads from tails out of the results. Many people have pushed, listened, or dragged me along the way and I would not be anywhere without their help and encouragement. This work is a result of this near-lifelong passion and I am the better for having been given the chance to explore this amazing field that still fascinates me like it did when I was a little boy.

Dedication

This work is dedicated the mentors in my life. They provided guidance, support, and a kick in the rear when needed to help me on a career path I loved while keeping me in only moderate amounts of trouble. First, my parents Robert L. and Jean-Marie Meyer who provided a loving home with ample encouragement and support. Second, to my high school science teachers Richard Nolte and Patricia Lathrop who first began to cultivate my love of science into a career path. The Coast Guard officer Chris O'Neil (A Lieutenant when I knew you but I'm sure you have since been promoted) who taught me leadership, humility, and perseverance. To Dr. Kevin Beach, who gave me a start in marine research, and scientific writing and presentation. To Amanda Colton, M.S. who encouraged me through graduate school to excel, push myself, and learn how to build furniture. You have all been with me during different points of my life, but you have all helped me to be the person I am today.

Lastly, to the best big brother imaginable, Robert R. Meyer, "Rob" who has been there with me though every adventure possible. You may not know it, but you've been a bigger influence on me than even I thought possible and I love you for it all.

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to nucleic acid synthesis (TdR production) within a given population, and therefore how hostile a particular environment may be for growth (del Giorgio et al., 2011). 213

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Chapter 1: Introduction

Background

Harmful Algal Blooms are a well documented occurrence throughout human history

There are a vast number and variety of phytoplankton in aquatic systems throughout the world. Of the approximately 5,000 known species of phytoplankton, 300 are documented as capable of reaching numbers sufficient to discolor water (Granéli and Turner, 2006). Large, single-species, blooms of algae discoloring water have been well documented throughout history. Reports detailing fish kills due to harmful algae along the Florida coast (Steidinger et al., 1998), and the Texas and Mexican coasts (Steidinger, 2009) date back to 1542 and 1648, respectively. Charles Darwin wrote of a large bloom in 1832 on his circumnavigation of the globe aboard the *H.M.S. Beagle*. Noting,

"The whole surface of the water, as it appeared under a weak lens, seemed as if covered by chopped bits of hay, with their ends jagged. … Their numbers must be infinite: the ship passed through several bands of them, one of which was about ten yards wide, and, judging from the mud-like colour of the water, at least two and a half miles long. In almost every long voyage some account is given of these confervae" (Darwin, 1839).

Darwin called this phenomena "Sea-saw dust" but it was actually the cyanobacteria *Trichodesmium erythraeum*, as Darwin would learn after discussion with a colleague. This cyanobacterium is known to form large blooms, similar to those documented in <u>The Voyage of the *Beagle*,</u> and is the organism from which the Red Sea gained its name (Darwin, 1839). The number and coverage of such accounts demonstrate that algal blooms are not a new phenomenon, nor are some of the secondary effects associated with certain species.

Some of the 300 species of algae that discolor water have been given the descriptor Harmful Algal Blooms (HABs) and are associated with secondary environmental effects such as: toxicity (toxin production), alteration of habitat (hypoxia, anoxia, turbidity), physiological dysfunction (decreased respiratory or reproductive capacity), or alteration of trophic structure (Fisher et al., 2003). HABs are considered especially detrimental, when compared to algae that only discolor waters when blooming, because of these secondary effects associated with blooms.

Secondary effects are capable of directly and indirectly impacting coastal ecosystems. Direct impacts include fish and aquatic organism kills and a variety of clinical symptoms in humans exposed to toxins, ranging from headache to death (Table 1.1, Hallegraeff, 2004). Indirect impacts include: loss of work days and medical treatment of exposed individuals, community expenses cleaning up dead organisms from beaches and rehabilitating protected species, and loss of revenue through fisheries closures, decreases in tourism, and reduced consumer consumption of seafood (Fisher et al., 2003). In the United States alone it has been estimated that

$30-$70 million is lost annually as a result of HABs (Fisher et al., 2003). Within U.S. waters, annual blooms of the dinoflagellate *Karenia brevis* along the west coast of Florida are of particular concern.

Karenia brevis **taxonomy and description**

The dinoflagellate *Karenia brevis* (Davis) G. Hansen et Moestrup is 18-45µm in diameter and athecate (Daugbjerg et al., 2000, Steidinger, 2009). It is dorso-ventrally flattened, with a rounded epitheca adorned with a domelike protrusion, and slightly bi-lobed hypotheca (Figure 1.1, Steidinger and Tangen, 1997). *K. brevis* has a round nucleus that is located in the lower left quadrant of the cell and is easily visible by light microscope (Steidinger, 2009). *K. brevis* was originally described in 1947 as *Gymnodinium brevis* by Davis after a report of "…mass mortality of marine animals on the lower West Coast of Florida which was associated with a yellow or yellow-green discoloration of the water…" (Davis, 1948). Following this initial description, the name was changed to *Ptychodiscus brevis*, returned to *Gymnodinium brevis*, and then finally changed to *Karenia brevis* after recognizing the phylogenic independence of the *Karenia* genus and in honor of Florida red tide researcher Karen Steidinger's contributions to science (Daugbjerg et al., 2000).

Reproduction of *K. brevis* is typically via binary fission with a division rate of 0.2 to 1 day^{-1} (Steidinger et al., 1998). In laboratory experiments gamete pairing was observed in 5-10% of the cells in *K. brevis* cultures (Walker, 1982), but this sexual

Table 1.1: Causative organisms, clinical symptoms, and available treatments for described fish and shellfish poisoning. Neurotoxic shellfish poisoning within Gulf of Mexico and West Florida Shelf waters is primarily due to *Karenia brevis* with mild symptoms more prevalent (or more reported). Modified from Hallegraeff (2004).

Type of poisoning	Causative Organism	Symptoms		Treatment
		Mild case	Extreme case	
Paralytic shellfish poisoning (PSP)	*Alexandrium catenella, A. minutum, A. tamarense, Gymnodinium catenatum, Pyrodinium bahamense var. compressum*	**Within 30 minutes: Tingling sensation or numbness around lips, face and neck; prickly sensation in fingertips and toes; headache, dizziness, nausea, vomiting, diarrhea**	**Muscular paralysis; pronounced respiratory difficulty; choking sensation; death through respiratory paralysis may occur within 2-24 hours after ingestion**	**Patient has stomach pumped and is given artificial respiration. No lasting effects.**
Diarrhetic shellfish poisoning (DSP)	*Dinophysis acuminata, D. acuta, D. fortii, D. norvegica, Prorocentrum lima*	**30 min to <12 hours: diarrhea, nausea, vomiting, abdominal pain.**	**Chronic exposure may promote tumor formation in the digestive system.**	**No treatment available: all individuals naturally recover after 3 days.**
Amnesic shellfish poisoning (ASP)	*Pseudo-nitzschia multiseries, P. pungens* (some strains), *P. pseudodelicatissima, P. australis, P. seriata, P. delicatula*	**After 3-5 hours: nausea, vomiting, diarrhea, abdominal cramps.**	**Decreased reaction to deep pain; dizziness, hallucinations, confusion; short-term memory loss; seizures.**	**No treatment available.**
Neurotoxic shellfish poisoning (NSP)	*Karenia brevis, K. papilionacea, K. selliformis, K. bicuneiformis*	**After 3-6 hours; chills, headache, diarrhea; muscle weakness, muscle and joint pain; nausea and vomiting.**	**Tingling, pricking or numbness of the skin; reversal of hot and cold; difficulty in breathing, double vision, throat constriction.**	**No treatment available.**
Ciguatera fish poisoning	*Gambierdiscus toxicus*	**After 12-24 hours of eating toxic fish: gastrointestinal symptoms: diarrhea, abdominal pain, nausea, and vomiting.**	**Neurological symptoms: numbness and tingling; reversal of hot and cold, loss of balance; low heart rate and blood pressure; rashes. Extreme cases death through respiratory failure.**	**No targeted treatment available: symptoms may last months to years. Calcium and mannitol may help relieve but do not prevent recurrence.**
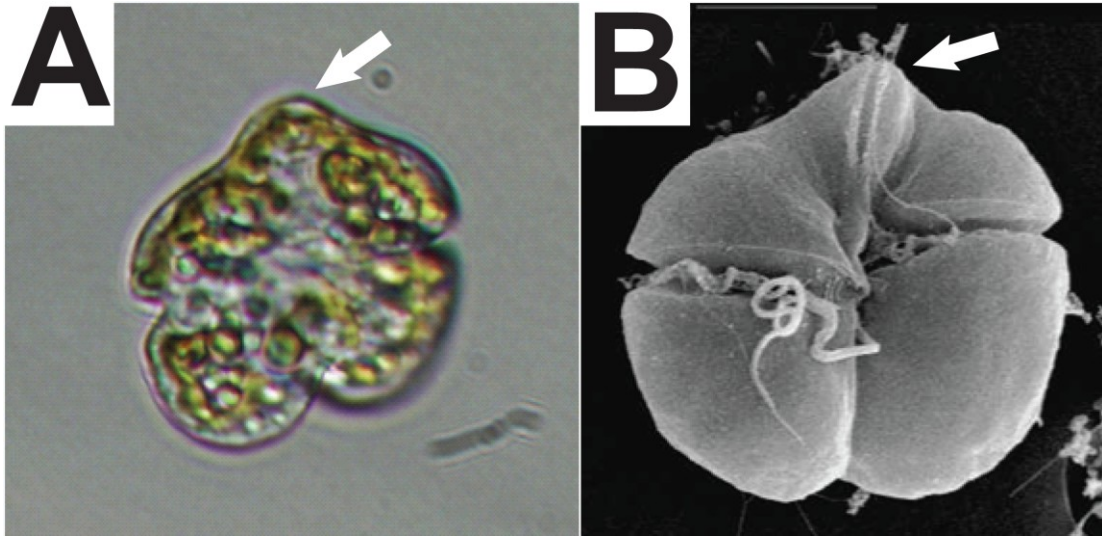
Figure 1.1: The dinoflagellate *Karenia brevis* as viewed by a light microscope (A) and scanning electron microscope (B). The rounded epitheca with domelike protrusion can clearly been seen (arrows). The light microscope (A) exhibits oil globules within the cell that is indicative of a late-stage *K. brevis* bloom cell (C. Heil, personal communication). The electron micrograph (B) displays the two flagella all dinoflagellates possess as well as the dominant bi-lobed hypotheca. From: Florida Fish and Wildlife Research Institute.

cycle has not been fully resolved; the primary means of reproduction in bloom situations appears to be asexual (Walker, 1982). In addition to its photosynthetic capabilities *K. brevis* has been documented to graze upon the cyanobacterium *Synechococcus* sp. (Glibert et al., 2009, L. Procise, pers. comm.) classifying it as a mixotroph (capable of heterotrophy and autotrophy, Burkholder et al., 2008). Depending on the clone of *K. brevis*, optimal salinity ranges for growth can be 20-25 or 37.5-45; a minimum salinity of 17.5 is required for growth and blooms tend to occur in high-salinity areas (> 35, Maier Brown et al., 2006). It also appears that *K. brevis* can be both light- (Schaeffer et al., 2007) and shade-adapted (Walsh et al., 2006).

The primary distribution of *K. brevis* is throughout the Gulf of Mexico and the southeast coast of the U.S. in both oceanic and estuarine waters to salinities as low as 17.5 (Fig. 1.2, Steidinger, 2009, Steidinger and Tangen, 1997, Maier Brown et al., 2006). Annual blooms typically form on the West Florida Shelf (WFS, Fig. 1.3) between Tarpon Springs and Ft. Myers, Florida, USA; this range also happens to contain the major population centers of the western Florida Coast (Fig. 1.3, Steidinger, 2009, Derby et al., 2003). The epicenter of blooms has been noted to shift from the northern to southern ends of this range on a decadal cycle with Charlotte Harbor currently being the focal point (C. Heil, K. Steidinger, pers. comm.). These blooms have been documented to exceed 10^6 cells L^{-1} and cover an area greater than 2000 km^2 (Heil and Steidinger, 2008).
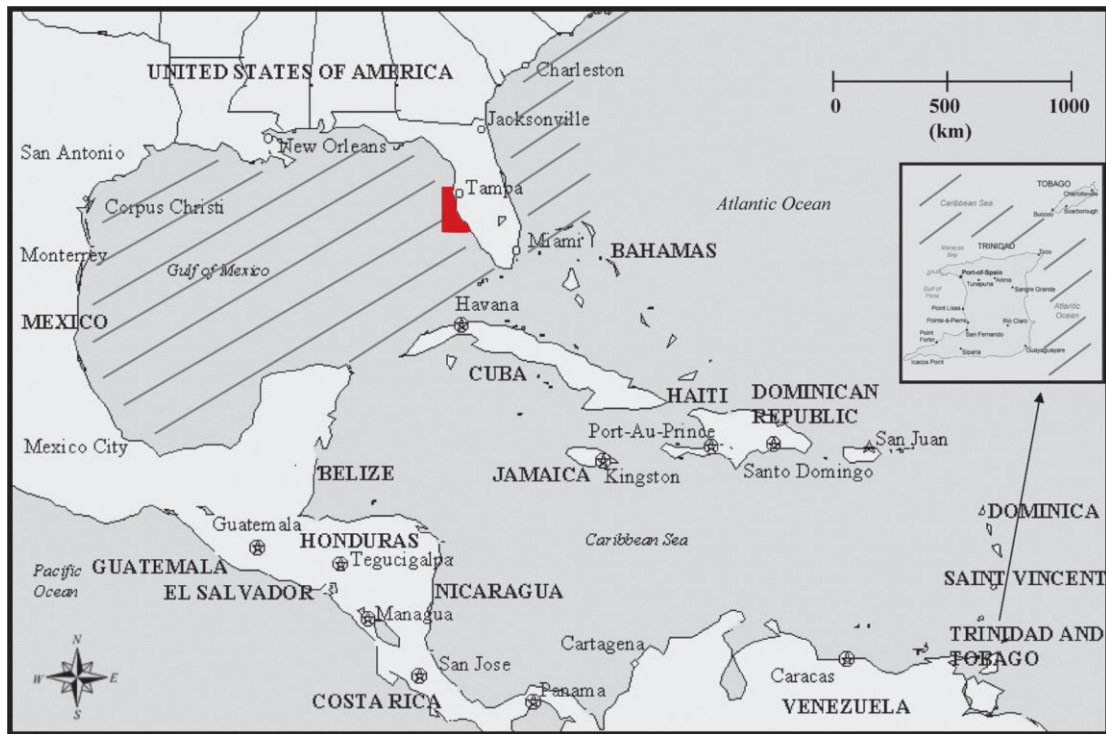
Figure 1.2: Known distribution of *Karenia brevis* in the western Atlantic Ocean, Caribbean Sea, and Gulf of Mexico. Insert: distribution of *K. brevis* surrounding Trinidad and Tobago. There have been confirmations of *K. brevis* in Jamaican waters (not shown). The red box represents the West Florida Shelf where *K. brevis* blooms almost annually and a majority of studies of naturally occurring blooms have taken place. From: Steidinger, 2009.
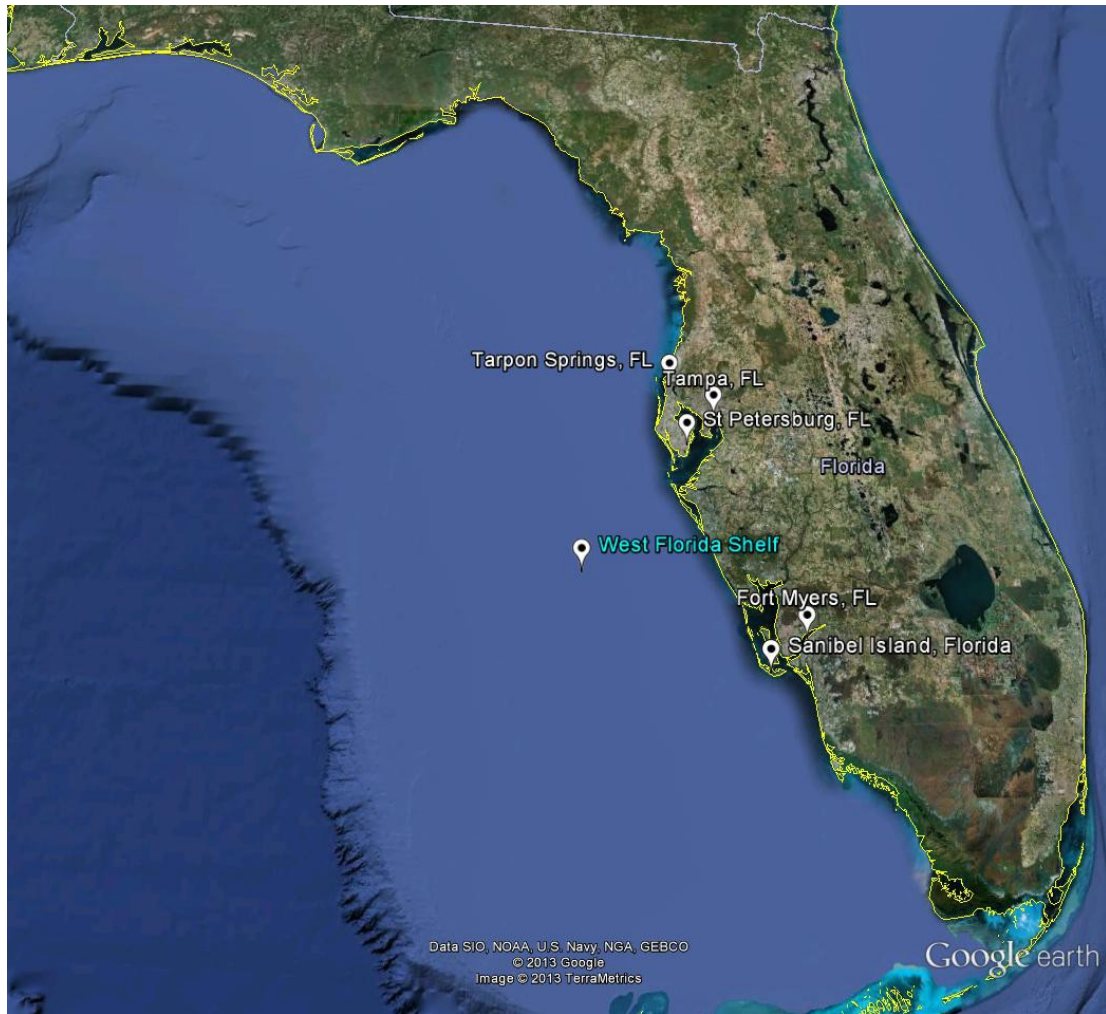
Figure 1.3: The West Florida Shelf which extends over 220 km off Central Florida's Gulf Coast. Blooms of *Karenia brevis* almost annually bloom between Tarpon Springs, FL and Sanibel Island, FL. The Ecology of Harmful Algal Blooms: *Karenia* (ECOHAB: *Karenia*) project conducted research and sampling inside and outside of *K. brevis* blooms from outside Tampa Bay to south of Sanibel Island. Map from Google, 2013.

What makes *Karenia brevis* a toxic species?

Blooms of *K. brevis* are associated with the appearance of brevetoxins (PbTxs), a suite of lipid-soluble neurotoxins (Schneider et al., 2003). Linkage between the presence of *K. brevis* and the production of PbTxs has been recent (Snyder et al., 2005). Brevetoxins are polyketides, and must be assembled by polyketide synthase (PKS) enzymes. The genes AT2-10L and AT2-15, both PKS genes, were isolated and amplified solely from *K. brevis*. These PKSs are complex and fit the operational definition of Type I PKSs, which demonstrates *K. brevis* has the capability to partially synthesize PbTxs. As a complete operon for PbTx synthesis has not been identified, more work is necessary to demonstrate that *K. brevis* can be solely responsible for brevetoxin production (Snyder et al., 2005).

The most effective competitive action for a slow growing species like *K. brevis* is predator deterrence (Flynn, 2008) rather than the production and release of compounds that inhibit competitors (Kubanek et al., 2005). Production and release of PbTxs by *K. brevis* has been found to be allelopathic on a species specific basis only (Prince et al., 2008), primarily helping *K. brevis* outcompete other algae for resources at high densities where resources are limited (Flynn, 2008). This is supported by data showing that PbTx levels are higher in stationary growth phase, making aged, high density blooms the largest source of PbTxs (Maier Brown et al., 2006). *K. brevis* also produces different concentrations of PbTx, which are partitioned differently within their environment: PbTx-3 and -6 (less potent derivatives of PbTx-1 and -2 respectively) are the most common extracellular PbTxs (Lekan and Tomas, 2010). This implies that PbTxs are not a significant factor in the development of *K. brevis*

blooms, but instead contribute to bloom maintenance (Kubanek et al., 2005, Kubanek et al., 2007) and that aged blooms are more likely to be toxic (Landsberg et al., 2009).

PbTxs have been observed to be predator deterrents, having detrimental effects on the growth, reproduction, and motor function of the copepods *Calanus pacificus* and *Paracalanus parvus* (Turner and Tester, 1997). However, *K. brevis* does not appear to be directly allelopathic to many potential grazers as those grazers instead seek out alternate food sources and therefore assist in the elimination of competing phytoplankton, many of which can metabolize PbTx (Turner and Tester, 1997). For example, the copepod *Acartia tonsa* would not consume *K. brevis* under any conditions (Speekman et al., 2006). Rotifers also showed negative preference for *K. brevis*; exhibiting decreased consumption of *K. brevis* as the proportion of other food sources increased (Kubanek et al., 2007).

The diatoms *Skeletonema costatum* and *Thalassiosira sp.*, and *Asterionellopsis glacialis*, dinoflagellate *Prorocentrum minimum*, and cryptophyte *Rhodomonas lens*, all endemic to the Gulf of Mexico, have been documented to metabolize waterborne PbTx-2 (Myers et al., 2008). *Crassostrea virginica* (eastern oyster) has also been shown to metabolize PbTxs, being able to eliminate PbTx-1, -2, -3, and -9 within 2 weeks of exposure (Plakas et al., 2004). Interestingly, Lekan and Tomas (2010) found PbTx-1 and -2, the most potent forms of PbTx, are the dominant forms within intact cells while PbTx-3 and PbTx-6 were found to be the most common extracellular PbTxs. Thus, many organisms are capable of eliminating the most common intracellular form of PbTx, which would be expected to be abundant when blooms terminate and not when blooms are in the maintenance phase.

Considering these data, it would appear that PbTxs are not the primary grazing deterrents for potential predators of *K. brevis*. Grazers instead display a negative preference for *K. brevis* regardless of toxicity which leads to speculation on what benefit PbTxs are to *K. brevis*.

One possibility is that brevetoxins facilitate the maintenance or development of nutrient pools of a large enough magnitude to maintain large *K. brevis* blooms. Blooms of Medium classification (Table 1.2) are capable of killing fish (Heil and Steidinger, 2008, Heil and Steidinger, 2009), and have been documented to kill up to 100 tonnes of fish per day (Kirkpatrick et al., 2004). Most of these fish will remain in the water (not washed up on beaches) and can therefore serve as a nutrient pool for *K. brevis* as the tissues decay (Walsh et al., 2006). Form PbTx-2 is known to be converted to PbTx-3 in the environment (Pierce and Henry, 2008) and PbTx-3 and PbTx-6 were not metabolized by *C. virginica* (eastern oyster, Plakas et al., 2004). Cysteine conjugates and cysteine sulfoxides of PbTx-1 and -2 (Fig. 1.4) were highly abundant, and detectable, up to six months after exposure (Plakas et al., 2004). Thus, within medium to large blooms dominated by *K. brevis* at least four types of PbTx (PbTx-3, PbTx-6, cysteine conjugate(s) and cysteine sulfoxides) remain in the water column for extended periods of time, increasing the potential for PbTxs to kill fish and supplement nutrient pools.

Toxins may also facilitate mixotrophic feeding of *K. brevis* by debilitating or disabling prey to supplement inorganic nutrient pools with organic nutrient pools (Stoecker et al., 2006b). The use of brevetoxins most likely allows for *K. brevis*, a

Table 1.2: The classification system used by the State of Florida for monitoring and managing blooms of *Karenia brevis* based on cell concentrations. Very Low was previously divided into sub-classifications "Very Low-a" and "Very Low-b." The previous classifications were delineated by the closure of shellfish beds in and around locations where cell counts reached or exceeded 5×10^3 cells L^{-1}. Furthermore, Low was previously divided into "Low-a" and "Low-b" to mark the concentration at which blooms contain enough chlorophyll *a* to be detected by satellite (5×10^4). Modified from Heil and Steidinger, 2009.

Bloom classification	*Karenia brevis* cell concentration (cells L^{-1})	Commonly reported exposure symptoms	Environmental Impacts	Management/ regulatory action taken
Present	$\leq 1 \times 10^3$	None		
Very low	$> 1 \times 10^3$ to 1×10^4	Respiratory irritation		Commercial shellfish beds closed in areas with $> 5 \times 10^3$ cells L^{-1}
Low	$> 1 \times 10^4$ to 1×10^5	Respiratory irritation	Possible fish kills	Satellite imagery used to track and monitor bloom ($>1 \times 10^5$)
Medium	$> 1 \times 10^5$ to 1×10^6	Respiratory irritation	Fish kills	
High	$> 1 \times 10^6$	Respiratory irritation	Fish kills and water discoloration	
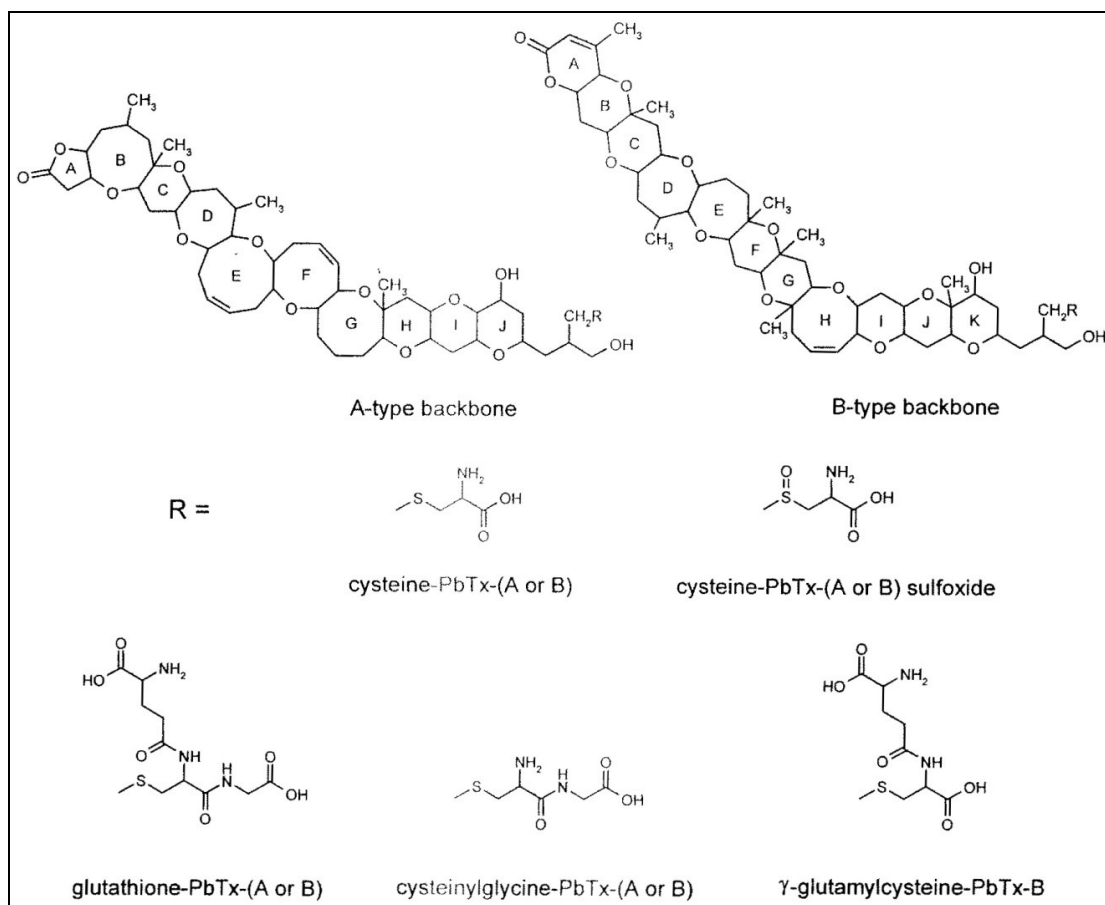
Figure 1.4: Base structure of Type A and B brevetoxins (top) and structure of cysteine and cysteine-peptide adducts of the parent brevetoxins found within tissues of *Crassostrea virginica* (eastern oyster) as idenfitied using liquid chromatography-tandem mass spectrometry (LC/MS(/MS)). From Plakas et al. (2004).

slow growing or K selected species, to supplement nutrient requirements while mortality due to predators selecting food sources other than *K. brevis* remains low (Stolte and Garcés, 2006) This will promote accumulation of large cell numbers that can be described as a bloom (Stolte and Garcés, 2006).

Parent toxins PbTx-1 and PbTx-2 are highly abundant within intact cells, reaching concentrations that could be detrimental to normal cellular function, but no function for PbTxs within cells has been discerned (Lekan and Tomas, 2010). Sodium ion channels are kept open by PbTxs, which could prevent cells from maintaining osmotic balance, yet cell death through osmotic-lysis has seldom been observed. This is most likely due to the presence of brevenal, a natural inhibitor of PbTx also produced by *K. brevis* (Bourdelais et al., 2004, Lekan and Tomas, 2010). Brevenal acts on Site 1 of voltage-sensitive sodium channels which out-competes the effect of PbTxs acting on Site 5 of the same channels (Lekan and Tomas, 2010). The production of brevenal has not been observed to match that of PbTx, and is therefore insufficient to neutralize the effect of PbTx (Lekan and Tomas, 2010). It may be that the brevenal is necessary for *K. brevis* to maintain osmotic balance and is only produced at required levels to maintain cellular osmotic balance while *K. brevis* is releasing PbTx to the environment to provide new or maintain nutrient pools required for growth.

Brevetoxin chemical properties and physiological effects

Brevetoxins consist of eleven rings, each containing one oxygen, fused in the trans- conformation (Lin et al., 1981). Since their initial description multiple PbTx types and derivatives have been isolated and described (Fig. 1.5, Nakanishi, 1985). All brevetoxins are based upon two main backbone structures, called Type 1 (Type A) and Type 2 (Type B, Fig. 1.5, Baden, 1989). Each of these backbones can have multiple PbTx derivatives which differ in the structure of one functional group attached to the last ring (Roth et al., 2007).

The only effects known to be specifically caused by PbTxs are toxicity and death of marine organisms (Steidinger, 2009) and neurotoxic shellfish poisoning (NSP) of humans (Table 1.1). In laboratory tests, PbTx was distributed to the liver, muscle, and gastrointestinal tract of mice within thirty seconds (Baden, 1989). A very small amount (< 1.5%) of PbTx was distributed to the brain, lungs, or spleen implying that PbTx is concentrated on peripheral rather than central systems of the body (Baden, 1989). The biochemical pathway PbTx impacts are sodium ion channels, which PbTx keeps open, leading to uncontrolled flux of sodium ions into cells (Kirkpatrick et al., 2004). Studies using fish have demonstrated that PbTx exposure causes lack of muscle coordination, paralysis, convulsions, and death through respiratory failure (Kirkpatrick et al., 2004). Human PbTx exposure via ingestion is typically accompanied by minor effects (chills, headache, diarrhea, weakness, pain, nausea, and vomiting) after 3 or more hours (Table 1.1, Hallegraeff, 2004). In extreme cases, exposure to high levels of PbTx can lead to altered

Type A
brevetoxins:

		Nominal mass of MH$^+$
PbTx-1	R = CH$_2$C(=CH$_2$)CHO	867
PbTx-7	R = CH$_2$C(=CH$_2$)CH$_2$OH	869
Oxidized PbTx-1	R = CH$_2$C(=CH$_2$)COOH	883

Open A-ring Forms
of Type A
brevetoxins:

		Nominal mass of MH$^+$
Open A-ring PbTx-1	R = CH$_2$C(=CH$_2$)CHO	885
Open A-ring PbTx-7	R = CH$_2$C(=CH$_2$)CH$_2$OH	887
Open A-ring oxidized PbTx-1	R = CH$_2$C(=CH$_2$)COOH	901

Type B
brevetoxins:

		Nominal mass of MH$^+$
PbTx-2	R = CH$_2$C(=CH$_2$)CHO	895
PbTx-3	R = CH$_2$C(=CH$_2$)CH$_2$OH	897
PbTx-9	R = CH$_2$C(=CH$_3$)CH$_2$OH	899
Oxidized PbTx-2	R = CH$_2$C(=CH$_2$)COOH	911
PbTx-2 adduct	putatively PbTx-2 methanol adduct	927

Open A-ring Forms
of Type B
brevetoxins:

		Nominal mass of MH$^+$
Open A-ring PbTx-2	R = CH$_2$C(=CH$_2$)CHO	913
Open A-ring PbTx-3	R = CH$_2$C(=CH$_2$)CH$_2$OH	915
Open A-ring oxidized PbTx-2	R = CH$_2$C(=CH$_2$)COOH	929

Figure 1.5: Structure of Type A and B brevetoxins including open A-ring forms and derivatives. Derivatives differ in the structure of the R group attached to the J- or K-ring (for Type A and B brevetoxins respectively). The toxicity of Type A brevetoxins is generally higher than that of Type B. Modified from Roth et al., 2007.

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perception of hot and cold, difficulty breathing, double vision, and constriction of the throat and airway which could lead to death (Table 1.1, Hallegraeff, 2004).

Most commonly, brevetoxins in the environment lead to deaths of marine organisms and respiratory irritation in humans by inhalation of aerosolized toxins (Hoagland et al., 2009, Kirkpatrick et al., 2011). Blooms with concentrations of *K. brevis* greater than 5×10^3 cells L^{-1} are considered toxic, and are often reported with extensive fish kills that can persist for months (Table 1.2, Heil and Steidinger, 2008). Fish can be exposed to PbTxs through absorption of PbTx across gill membranes or direct ingestion of contaminated prey; depending on the vector of exposure and concentration of PbTx fishes can either bioaccumulate PbTx or suffer death through acute toxicity (Landsberg et al., 2009). Shellfish are capable of being killed by PbTxs but are more often found to survive exposure through sequestration of PbTx within tissues (Landsberg et al., 2009). Sequestration of PbTxs by shellfish leads to varying concentrations of PbTx within shellfish, even within the same location, which shortens the bioaccumulation pathway and makes shellfish an efficient vector for trophic transfer of PbTxs (Landsberg et al., 2009). Marine mammals have been killed by PbTx, but it is believed that the mortality was due to ingestion of PbTxs from the environment; on several occasions *Tursiops truncatus* (bottlenose dolphin) have been observed feeding on dead fish within a *K. brevis* bloom (M. Garrett, pers. comm.).

Brevetoxins are a large compound with a complex structure. A prominent functional group on PbTxs which differs between types of PbTx changes the toxicity of the compound. This makes PbTxs a compound of concern for multiple reasons because toxicity can be transported, and persist, through multiple vectors in the

environment. This makes *K. brevis*, the causative organism for PbTx in the environment, a significant ecological and human health issue for the state of Florida and other states in which blooms occur.

Epidemiology of *Karenia brevis* blooms in the state of Florida

With multiple pathways for human exposure to PbTxs and nearly annual blooms in Florida waters, the State of Florida has an established "Red Tide" monitoring program which includes *K. brevis* as a HAB species of interest. This program accounts for up to date concentrations of HAB species in Florida waters as well as bloom boundaries and reported effects (fish kills, fisheries closures, beach closures, etc.). Being a well-known HAB on the WFS makes certain monitoring requirements easy for officials, while other aspects of monitoring and management remain challenging.

Deaths of some (e.g. larger) marine organisms are relatively straightforward to monitor; records from the present day State of Florida date back to 1542 describing blooms and fish kills of varying size and magnitude (Steidinger et al., 1998). In 2005, a bloom of *K. brevis* lasting more than a month was credited with fish kills and mortality of benthic organisms over 2,000 km^2 as well as manatee, dolphin, seabird, and sea turtle mortality and stranding (Heil and Steidinger, 2008).

As Florida is a coastal-dominated state with a long-standing commercial fishery it is no surprise that there are extensive records of fish kills. Epidemiology of NSP, and therefore exposure to PbTx, is more difficult to track. In studies of closely-

monitored individuals asthma-like symptoms (wheezing, lower airway constriction, persistent coughing) were reported over a wide range of *K. brevis* bloom categories (cells L^{-1}, Table 1.2, Heil and Steidinger, 2008). More severe exposure is associated with gastrointestinal irritation (diarrhea, abdominal pain, vomiting, tingling sensations, Table 1.1, Kirkpatrick et al., 2010) which is much harder to track in affected populations. The difficulty with NSP epidemiology lies in the large number of unreported cases; from both individuals who do not seek medical treatment for "minor things" such as diarrhea or vomiting and uninformed medical professionals not properly diagnosing NSP in individuals who are admitted to emergency rooms for respiratory or gastrointestinal irritation (Kirkpatrick et al., 2010).

Contraction of NSP by Floridians can be documented all the way back to 1880, in which one account from a Tampa Bay resident describes cases of shellfish-related illness that match symptoms of neurotoxic shellfish poisoning (Steidinger, 2009). At the time of that report *K. brevis* and brevetoxins had not been described and residents of affected areas only correlated the discolored water with humans becoming sick (e.g., people who ate shellfish became ill while people who ate other foods did not, Steidinger, 2009). Until the description of *K. brevis* in 1947, and its association with NSP, word of mouth may have been the only thing preventing large numbers of people from contracting NSP in the 19th and well into the 20th century.

The only documented case of a definitive NSP diagnosis is a report of three family members admitted to an emergency room in 1996 (Poli et al., 2000). This was a post-discharge diagnosis however; the diagnosis was confirmed through radioimmunoassay and receptor binding assay tests after the family had been

stabilized and discharged from the hospital (Poli et al., 2000). While this was a correct diagnosis of PbTx-induced NSP it is not known whether the affected individuals were informed of the cause of their illness and educated as to how to prevent future exposure. This public health concern led to the development of multiple systems to assist in the identification and education of HAB related symptoms and illness. The Centers for Disease Control and Prevention established the Harmful Algal Bloom Illness Surveillance System HABISS) which assists in tracking reports from existing, but disparate, information networks used by individuals and organizations in the state of Florida and elsewhere so that local health care providers are connected to information and potential partnerships in other local, state, and federal entities (Reich et al., 2009).

Blooms of *Karenia brevis* have substantial impacts on the State of Florida's economy

Most Florida Gulf Coast residents will be able to describe blooms of *Karenia brevis* as "Florida Red Tide," as they are aware that discolored water is associated with dead fish (Table 1.2, Heil and Steidinger, 2009). What most residents are not aware of, however, is that blooms occur and are toxic at concentrations well below those that color the water (Table 1.2, Heil and Steidinger, 2009). A survey of beachfront restaurant managers demonstrated that when "red tides" were noted by mangers or beachgoers cell counts averaged over 5×10^5 cells L^{-1} (Morgan et al., 2009), which is classified as a medium bloom (Table 1.2, Heil and Steidinger, 2009).

While they may be unaware of the presence of a *K. brevis* bloom, data show that the presence of red tide still affects decisions of beach visitors, workers, and businesses.

Beach attendance decreased by approximately 50,000 visitors each month a bloom was present (Backer, 2009). As much of Florida's economy revolves around tourism fostered by beaches and other aquatic recreation, a decrease in beach visitors has a cascading effect on other aspects of the economy. A 2007 study found red tides reduced Florida coastal hotel income by $2.3 million (Backer, 2009). Another study focusing on beachfront restaurants estimated a 13.7%-15.3% reduction in daily sales while *K. brevis* blooms were present, representing a daily loss of $868-$3734 depending on restaurant size (Table 1.3, Morgan et al., 2009).

The budget of beachfront towns is also affected by *K. brevis* and effects of blooms. In 1971 beach cleanup of dead or stranded organisms cost the city of St. Petersburg $200,000 and private individuals at least $20,000 (Backer, 2009). It is further estimated that Sarasota County, FL spends $500,000 to $4 million annually depending on the severity of blooms (Hoagland et al., 2009). As the severity of blooms is difficult to predict, and varies annually, local governments may have difficulty allocating appropriate funds or justifying the expense to taxpayers. Without a better understanding leading to accurate long-term forecasting of *K. brevis* blooms, budgeting appropriate bloom-response funds will not be possible. This therefore leaves the problem of bloom mitigation costing a significant amount of money to local governments, which ultimately hurts the economy of affected areas.

The State of Florida has Monitoring and Management programs to reduce the impact of *Karenia brevis* blooms

While there have been no reported human deaths from PbTx exposure in published literature, humans could still contract NSP from the consumption of contaminated shellfish (Burkholder et al., 2006) and respiratory irritation from exposure to aerosolized PbTxs (Kirkpatrick et al., 2004). The State of Florida has an extensive monitoring program in place to minimize the risk of human exposure and NSP (Heil and Steidinger, 2009). In addition to protecting human health, the goals also serve to directly or indirectly protect living marine and natural resources key to the economic stability and future of the state (Heil and Steidinger, 2009).

Currently, the Florida Fish and Wildlife Research Institute (FWRI) and the Florida Department of Agriculture and Consumer Services have a system to continuously monitor for *K. brevis* as well as a variety of other potential HAB species so that shellfish fisheries can be managed to prevent transmission of toxins to humans via shellfish consumption. This monitoring program involves: volunteers to collect samples, FWRI personnel trained in phytoplankton taxonomy and identification, an extensive database, and timely dissemination of information (Heil and Steidinger, 2009). This monitoring program has further been augmented through the expansion of sample collection, in terms of both spatial and resources, the creation of a toll-free

Table 1.3: Averaged environmental data as reported by managers of three beachfront restaurants located within 16 m of the water's edge of the Gulf of Mexico in Southwest, Florida. Individual reports were totaled and averaged for each year in each of the three categories. It should be noted the number of rainy days were 5-fold higher than red tide days. Rainy days also reduced sales by 23.0%-27.0% while red tides decreased sales 13.7%-15.3% in two of the three restaurants. The year 1998 only includes data from November and December. Modified from Morgan et al., 2009.

Year	"Red Tide" Noted	Rain	Tropical storm/hurricane
1998	0	2	1
1999	1	34.3	2
2000	0	39.3	1
2001	9	25.3	2
2002	4.7	50	0.3
2003	1	34.7	1
2004	1	50	4.3
2005	37	43.3	3
1999-2005			
Total	53.7	278.9	14.6
Average	6.7	34.9	1.8

hot-line for public reporting of fish kills (and fish-kill database), and the development of bloom modeling and forecasting systems.

Currently management strategies for *K. brevis* include precautionary, controlling, and educational components. Precautionary components are designed to lessen or prevent impacts of established *K. brevis* blooms so that humans and economies are not significantly exposed or harmed. The precautionary approach is largely reactionary as it depends on collection of samples and identification and quantification of *K. brevis* in samples before the presence and location of blooms can be disseminated to managers and the public at large. Control measures primarily deal with regulation of shellfish harvest and cleanup of animals killed by bloom toxins which are also reactionary in nature as little is done when no blooms or toxin are present (Heil and Steidinger, 2009). Properly designed education efforts have the capacity to help create new and effective reactive and proactive programs. Reactive programs include fast distribution of information and monitoring data in easy to understand formats while proactive programs seek to educate the population (both local and transient) to the dangers of *K. brevis* blooms, where to find information, and how to limit personal exposure. Combined, these programs have been effective but room for improvement exists, especially in bloom detection and forecasting, which can further limit the environmental and economic impacts of *K. brevis* blooms in the state of Florida.

The Florida Red Tide and the ECOHAB: Karenia *project*

Why is the West Florida Shelf a hotspot for *Karenia brevis* blooms?

While blooms of *K. brevis* have been documented in several places within the
Gulf of Mexico and along the east coast of the United States, the nearly annual
blooms along the WFS suggest this area is more conducive to bloom formation and
maintenance than other areas. As the WFS circulation is subject to wind forcing, the
Loop Current, and estuarine inputs, different populations of phytoplankton appear to
be specific to different physical conditions (Walsh et al., 2006). *K. brevis* is common
during weak upwelling (Walsh et al., 2006), which brings seed populations from mid-
shelf waters to the surface (Lenes and Heil, 2010) and retains them within fronts
along the WFS between Tampa Bay and around Sanibel Island, FL (Walsh et al.,
2003). Once within surface waters, nutrients are provided by aeolian deposition and
estuarine outflow (Walsh et al., 2003). The estuarine outflow further provides high
concentrations of colored dissolved organic matter (CDOM) which provided
protection from light-inhibition to the shade-adapted *K. brevis* (Walsh et al., 2006).

Additionally, the bloom forming cyanobacteria *Trichodesmium* spp. may play
a significant role in the initiation of blooms (Lenes et al., 2001, Lenes and Heil,
2010). The mid-shelf waters in which *K. brevis* is entrained are oligotrophic, and
therefore require significant external inputs to provide the nutrient required for a large
bloom to occur (Lenes and Heil, 2010). As a diazotroph, *Trichodesmium* spp. can
utilize aeolian dust, rich in iron, to fix nitrogen which is passed to *K. brevis* through

excretion and remineralization of decayed or lysed cyanobacterial biomass (Mulholland et al., 2006, Lenes and Heil, 2010). It is hypothesized that *Trichodesmium* spp. blooms occur when offshore transport is limited, nutrients are adequate for growth, and competition with other bacteria or phytoplankton is minimized (Lenes and Heil, 2010); these conditions are also conducive for blooms of *K. brevis* (Walsh et al., 2006). It has been well documented that these blooms co-occur, with *Trichodesmium* spp. preceding *K. brevis*; it is further believed that the strength of the *Trichodesmium* spp. bloom is related to the physical conditions outlined above, with emphasis placed on the concentration of iron in aeolian dust, and subsequently to the magnitude and duration of *K. brevis* blooms that follow (Heisler et al., 2008, Lenes et al., 2001). *Trichodesmium* spp. therefore may serve as a bridge between normal community composition and blooms of *K. brevis*.

Once initiated, a bloom of *K. brevis* is then subject to the physical processes of the WFS, which may serve to promote, maintain, transport, or terminate a bloom (Figueiras et al., 2006). Many studies have documented the inhibition or termination of HABs subject to turbulence and shear forces in the water column (Thomas and Gibson, 1990, Juhl et al., 2001, Stoecker et al., 2006a), it follows that *K. brevis* in turbulent or shear conditions will lose cellular functions (loss of flagella and inhibition of cell division, Thomas and Gibson, 1990) and therefore terminate, while *K. brevis* in calm conditions will be able to maintain optimal growth if nutrient pools are adequate. As horizontal mixing is more dominant in oceanography, *K. brevis* has a high probability of being either retained along the WFS by eddies (Tester and Steidinger, 1997) or transported along the Florida coast; northward intrusion of the

Loop Current (Wiseman and Dinnel, 1988) is believed to be the cause behind *K. brevis* appearance along the Atlantic Coast of Florida (Tester and Steidinger, 1997, Figueiras et al., 2006). Thus, initiation and termination of *K. brevis* blooms are strongly tied to physical conditions along the WFS, yet bloom magnitude and duration are more dependent upon nutrient conditions which will dictate community composition and physiological state (Heil et al., 2007).

The ECOHAB: *Karenia* project was designed to determine potential nutrient pools which could support blooms on the West Florida Shelf

Understanding how *K. brevis* was transported to the WFS did not explain the occurrence of extensive and extended blooms in an oligotrophic system. Current research suggests that Florida riverine inputs support moderate, localized, blooms of *K. brevis* (Anderson et al., 2008) with additional nutrient sources such as *Trichodesmium* spp. (Lenes and Heil, 2010), the microbial loop (Heil et al., 2007), or decaying fish (Walsh et al., 2006) allowing for blooms to increase in magnitude. This suggests that multiple nutrients sources are necessary to sustain blooms of *K. brevis*, however, few of these sources had been characterized over a spatial and temporal scale relevant to *K. brevis* blooms on the WFS (Heil et al., in press). The ECOHAB: *Karenia* project had objectives of: 1) determining the nutrient sources that promoted and sustained large *K. brevis* blooms and 2) determine the origin of such nutrient sources. The methods planned to achieve said objectives ensured multiple forms of nutrients would be investigated in addition to multiple sources, the physical

circulation within the WFS would be coupled with the chemistry and biology of nutrient dynamics, and that physiological plasticity of *K. brevis* would not be overlooked.

Blooms of *Karenia brevis* were sampled as part of the ECOHAB: *Karenia* project

The ECOHAB: *Karenia* project had four cruises along the WFS conducted aboard the R/V *Pelican* (Louisiana Universities Marine Consortium). Cruises were conducted every October from 2007-2010 and consisted of two legs, each lasting a week. The first leg was to map the physical, chemical, and biological parameters within the study area while simultaneously looking for *K. brevis* blooms (based on microscope counts of phytoplankton samples). The second leg of each cruise was to perform targeted sampling of blooms or phenomena of interest. Each day began with a primary station which was sampled just after dawn, followed by several secondary stations in a grid pattern.

In 2007 six primary stations were sampled during leg one of the cruise followed by five primary stations within a large bloom of *K. brevis* (Fig. 1.6). This bloom was determined to be in maintenance phase based on prior observation of this bloom in nearshore environments, the high biomass of the bloom, and *K. brevis* growth rates greater than one division day^{-1} (Sipler et al., in press, C. Heil, pers. comm.) and associated with fish kills. This cruise was conducted from 16 October to 26 October.

The 2008 research cruise was conducted from 2 October to 12 October 2008. There were seven primary stations in 2008 (Fig. 1.7) to obtain samples inside and outside of estuaries near the northern and southern limits of the sampling area. Four days of sampling were conducted near Sanibel Island in a bloom determined to be in initiation phase. The bloom was classified as an initiation phase bloom due to its relatively low biomass, increasing cell concentrations, movement from offshore to nearshore environments (bloom aggregation, Walsh et al., 2003), and no prior detection of this bloom by regional monitoring programs.

The seven primary stations were repeated in 2009 and there were four primary and one secondary sampling station within a *K. brevis* bloom (Fig. 1.8). The cruise was from 2 October through 12 October. This bloom was determined to be in maintenance phase due to its offshore location (bloom dispersal, Weisberg et al., in press), low to moderate concentrations of *K. brevis* present, and high numbers of lipid bodies within cells (which have been shown to indicate older cells in stationary phase, Steidinger, 1979).

The final year of sampling on the WFS was the first in which no bloom was located. The cruise was conducted from 13 October through 23 October. Six primary stations were sampled, coinciding with many of the stations from previous years (Fig. 1.9). Several additional stations were sampled which did not correspond to previous years and therefore were not include in the analysis of bloom and non-bloom stations contained within this dissertation.

27
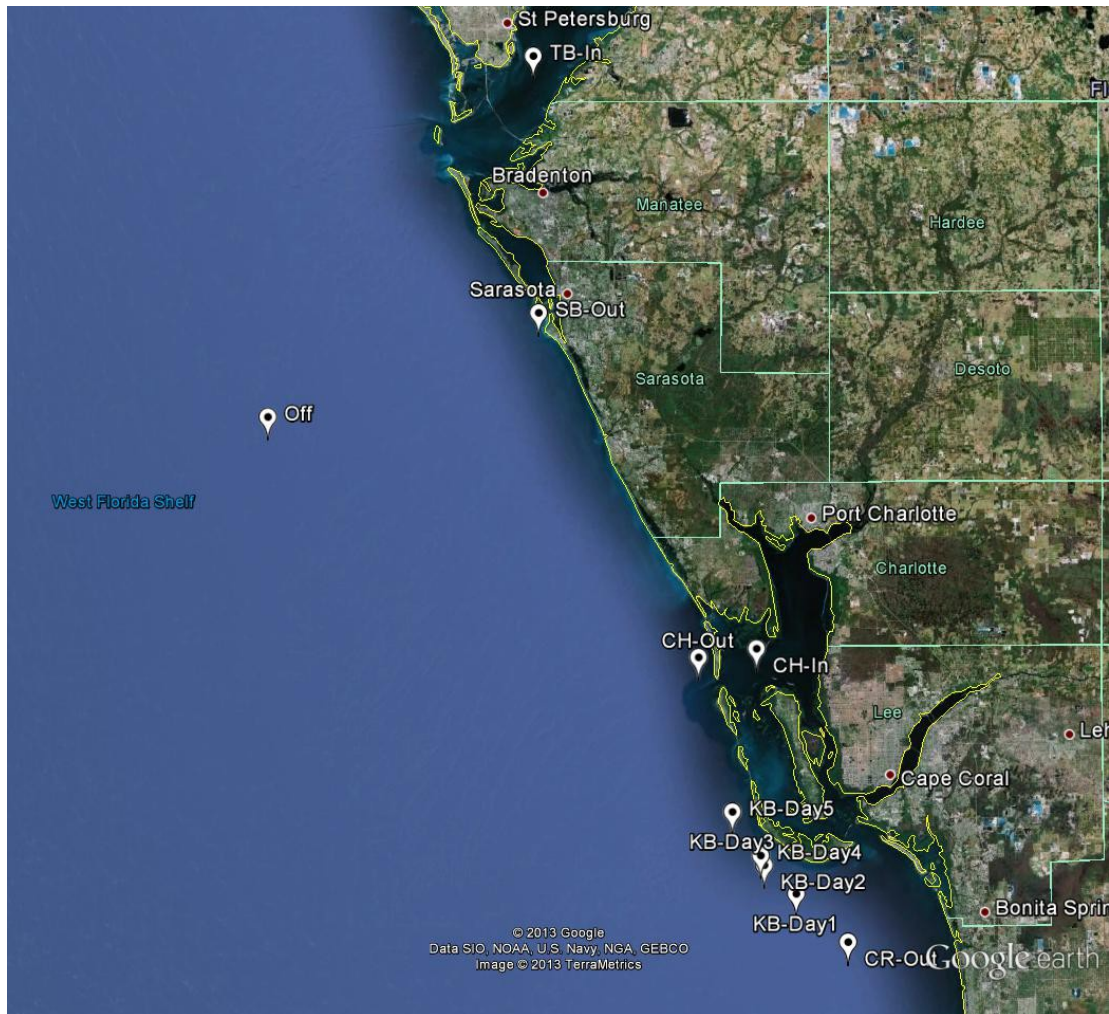
Figure 1.6: Sampling stations on the West Florida Shelf during the 2007 ECOHAB: *Karenia* project process cruise. Offshore (Off), inside Tampa Bay (TB-In), outside Sarasota Bay (SB-Out), outside and inside Charlotte Harbor (CH-Out and CH-In), in the Caloosahatchee River outflow (CR-Out), and five consecutive days sampling within a bloom of *Karenia brevis* (KB-Day1, Day2, Day3, Day4, and Day5). Google, 2013.
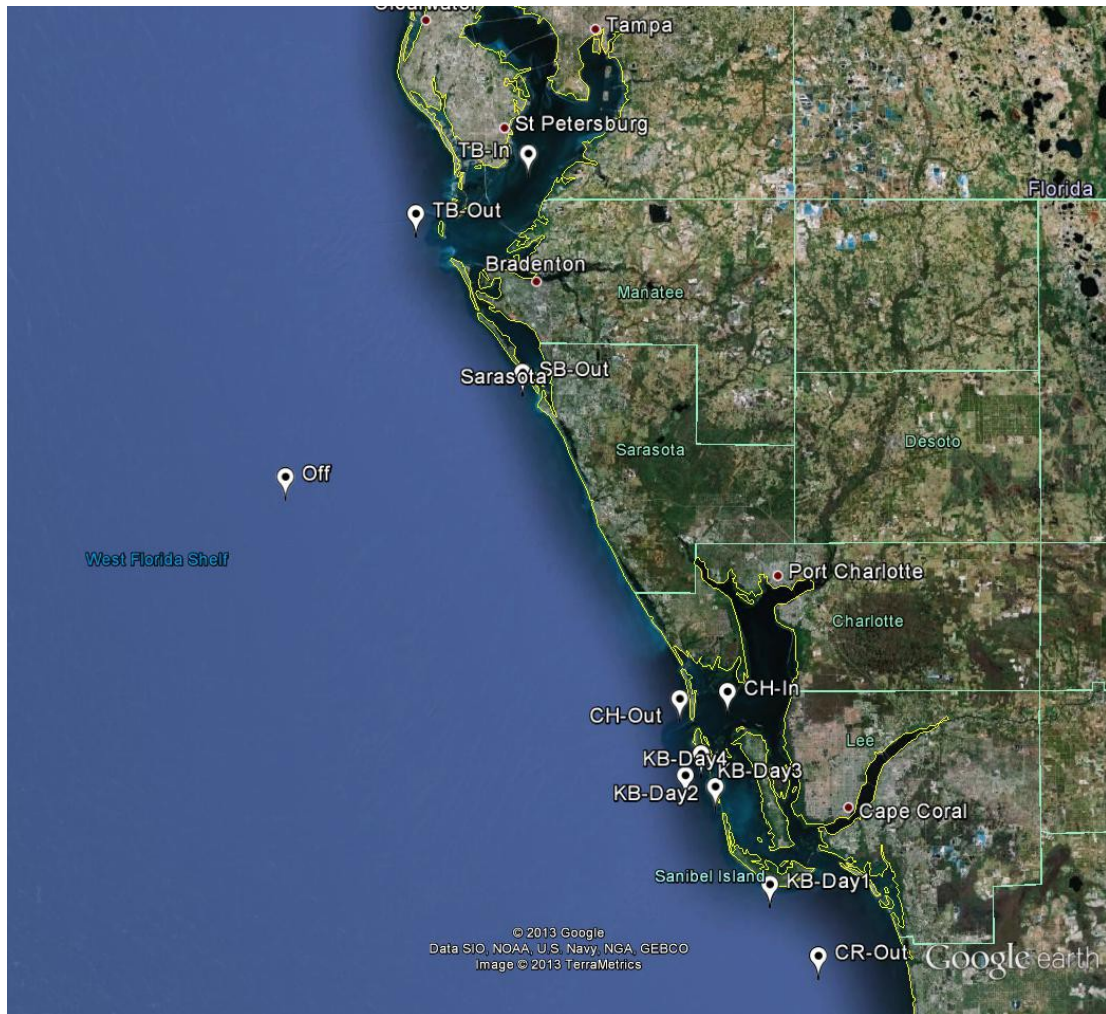
Figure 1.7: Sampling stations on the West Florida Shelf during the 2008 ECOHAB: *Karenia* project process cruise. Offshore (Off), outside and inside Tampa Bay (TB-Out and TB-In), outside Sarasota Bay (SB-Out), outside and inside Charlotte Harbor (CH-Out and CH-In), in the Caloosahatchee River outflow (CR-Out), and four consecutive days sampling within a bloom of *Karenia brevis* (KB-Day1, Day2, Day3, and Day4). Google, 2013.
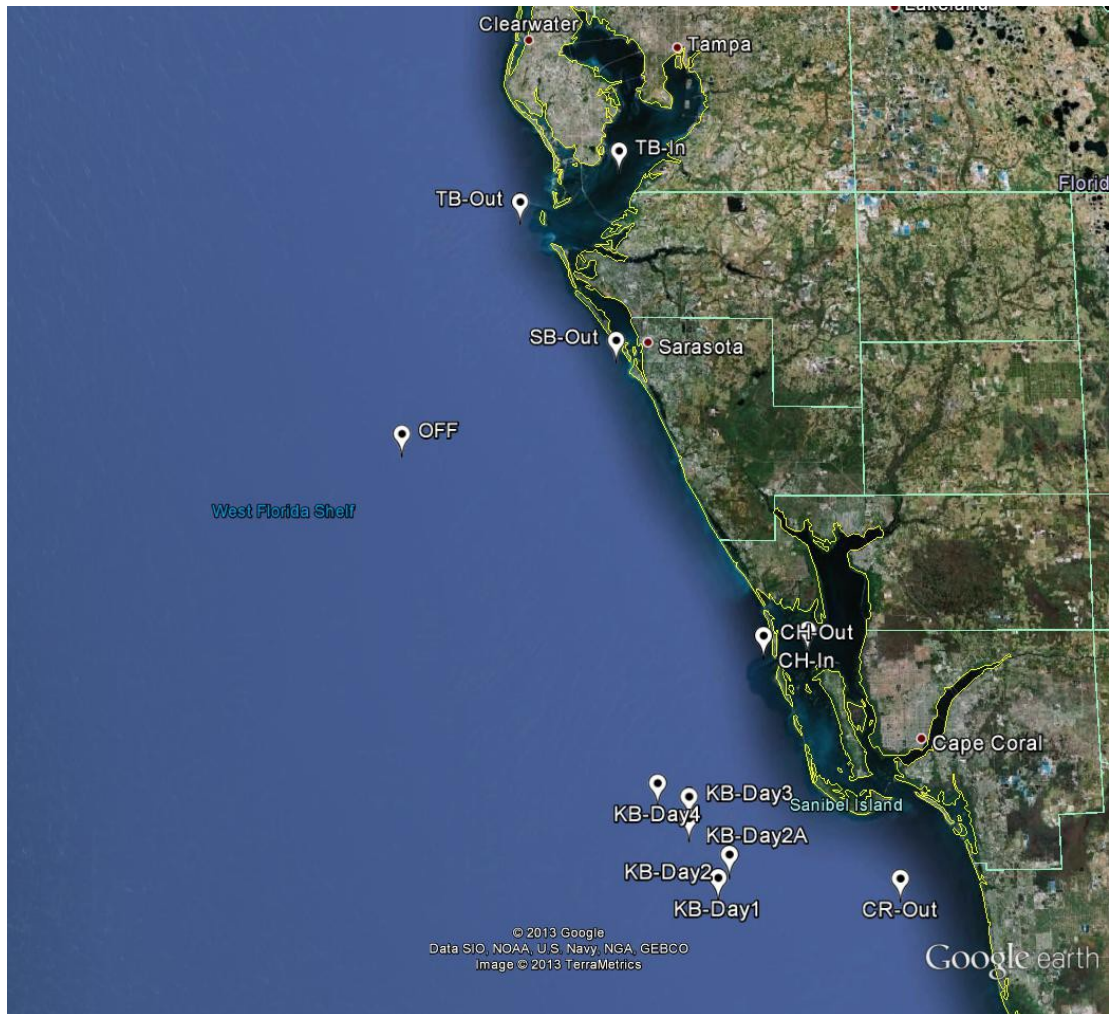
Figure 1.8: Sampling stations on the West Florida Shelf during the 2009 ECOHAB: *Karenia* project process cruise. Offshore (Off), outside and inside Tampa Bay (TB-Out and TB-In), outside Sarasota Bay (SB-Out), outside and inside Charlotte Harbor (CH-Out and CH-In), in the Caloosahatchee River outflow (CR-Out), and four consecutive days sampling within a bloom of *Karenia brevis* (KB-Day1, Day2, Day3, and Day4). Sampling done at station KB-Day2A was done on the same day as KB-Day2 but the samples were taken outside the *K. brevis* bloom. Google, 2013.
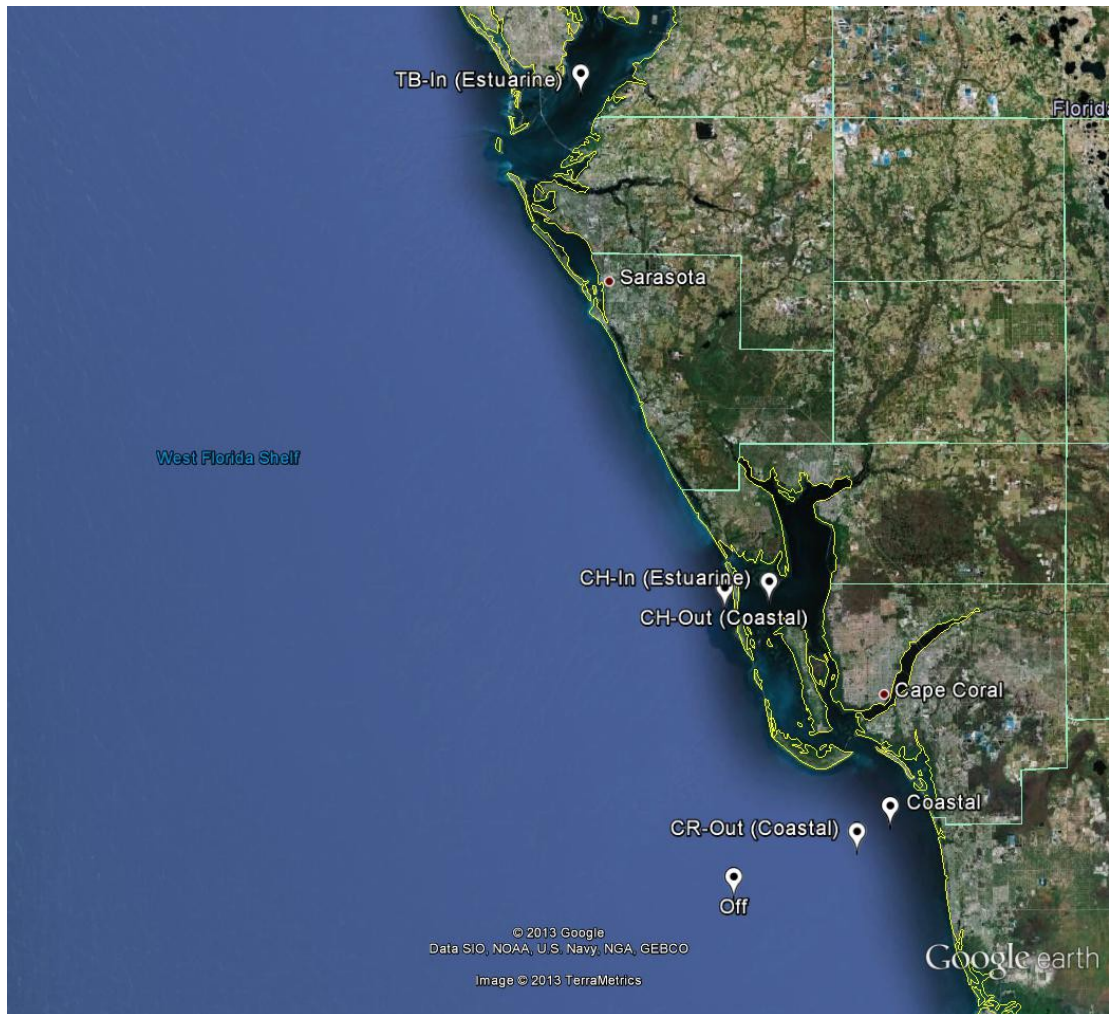
Figure 1.9: Sampling stations on the West Florida Shelf during the 2010 ECOHAB: *Karenia* project process cruise. Offshore (Off), inside Tampa Bay (TB-In), outside and inside Charlotte Harbor (CH-Out and CH-In), in the Caloosahatchee River outflow (CR-Out), and just offshore of Naples and Bonita Springs, Florida (Coastal). No *Karenia brevis* was located so stations were compared along an estuary to offshore gradient; stations were further classified by the environment in which they were located based on salinity. Google, 2013.

Bacterial and viral interactions within* Karenia brevis *blooms are important, but overlooked, components of bloom dynamics

Bacteria and viruses are important components of oceanic ecosystems

Marine microbes are essential components of biogeochemical cycling of chemicals and elements in the oceans (Konopka, 2009). Bacteria are able to utilize dissolved organic matter as a nutrient source to increase biomass and are able to return energy to higher tropic levels through grazing on the bacterial community (Azam et al., 1983). The classic pathway by which bacteria recycle organic matter and return energy to the food web is the transfer of organic matter through bacteria to flagellates and then microzooplankton; this pathway is called the microbial loop (Azam et al., 1983). The microbial loop can lead to heterotrophic production within a system exceeding primary production through the recycling of the same carbon, biomass, or metabolic products (Fuhrman, 1992). The augmentation to primary production turns over very fast in marine systems (days to weeks, Strom, 2008) which highlights the importance of marine microbes in rapid responses to environmental changes, especially in regard to nutrient inputs and cycling. Combining knowledge of the composition on the microbial community and community functions assists in nutrient budget and tropic transfer calculations. Correlations between bacterial biomass and phytoplankton biomass as well as bacterial abundance and chlorophyll *a* further demonstrate the effect marine microbes can have on the production and biogeochemical cycling of ecosystems (Simon et al., 1992).

Viruses are the most abundant biological entity on the planet, representing a pool of approximately 200 Mt of carbon (Bratbak et al., 1992). Marine viruses are capable of infecting both autotrophic and heterotrophic plankton and thus have a substantial effect on marine food webs by short-circuiting the flow of nutrients from higher trophic levels to pools of dissolved and particulate organic matter (Hereafter this mechanism will be referred to as the viral loop, Fig. 1.10). Viruses have an impact on marine food webs similar to zooplankton grazing, but instead of contributing to overall ecosystem productivity they increase community respiration and decrease the efficiency of carbon transfer (Suttle, 2005).

Viral infection follows one of two pathways: lytic or lysogenic infection. Lytic infection is easier to understand: a virus invades a host, hijacks its metabolic functions to replicate, and eventually kills the host releasing new viral particles in the process. Lysogenic infection is considered to be a symbiotic relationship in which the virus incorporates its genome into that of the host; this virus is now called a prophage (Paul, 2008). The prophage genome is replicated along with host DNA, passing the viral genome to daughter cells. Given the right stimulus the prophage switches to a lytic cycle making prophages "molecular time bombs" which can alter the structure and function of microbial communities (Fig. 1.11, Paul, 2008). An estimated 20% of the water column ambient bacterial community is lysed daily via lytic infection (Suttle, 1994) and 0-100% contains prophage genomes (Paul, 2008).
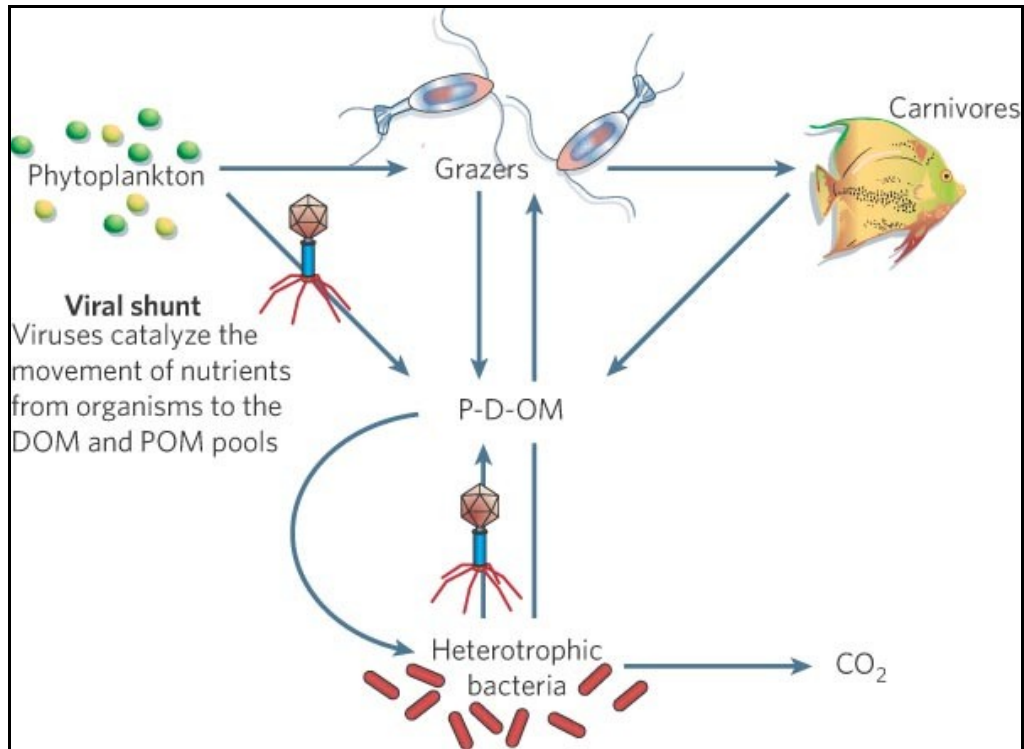
Figure 1.10: The viral loop (or viral shunt) as understood in euphotic aquatic systems. Lysis of phytoplankton and bacterioplankton feeds back into the particulate and organic matter pool which prevents transfer of said matter to higher trophic levels. This increases respiration and decreases trophic efficiency, ultimately limiting the productivity of the ecosystem. From Suttle, 2005.
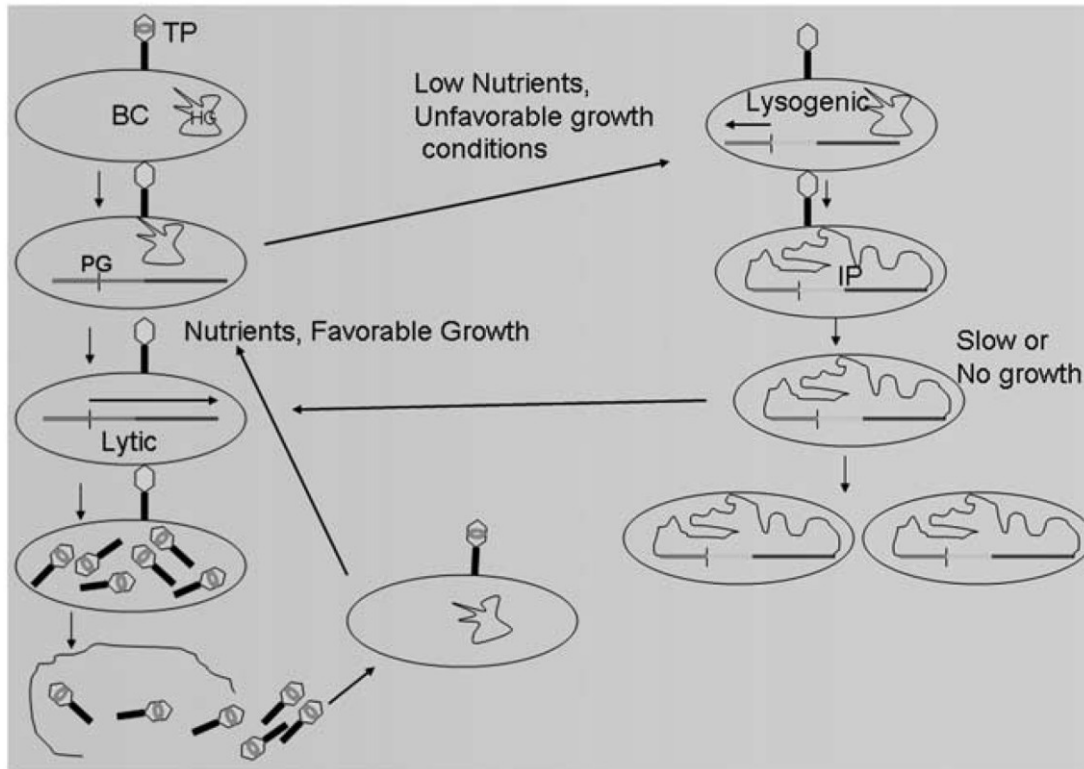
Figure 1.11: The two types of viral infection. Lytic infection is a process in which viruses infect a host simply for replication of itself, killing the cell in the process. Given certain stimuli, typically hostile environment, viruses will not lyse the host but insert their genome into that of the host (lysogenic infection). The host will then replicate the incorporated phage (called a prophage) when dividing. At some point, the phage will activate a lytic cycle and use the host for phage construction, making prophages "ticking time bombs" (from Paul, 2008).

Maintenance of such astonishing numbers of viruses requires a large or continuous production of new viruses through the lysis of host cells (Wilhelm and Suttle, 1999) due to high decay rates of viruses in euphotic waters (8-10% h[-1], Noble and Fuhrman, 1997). Viral abundances were observed to correlate with bacterial production in time and space (Wilhelm and Suttle, 1999) which suggests three things: 1) there is a high percentage of microbial mortality due to viral infection, 2) new virus particles must be produced at a high rate to replace lost or destroyed viruses and maintain viral abundance, and 3) most viruses within a marine system are produced within that system (Bratbak et al., 1993, Fuhrman and Noble, 1995, Fuhrman, 1999, Wilhelm and Suttle, 1999, Hewson et al., 2001, Suttle, 2008).

Cells infected by lytic-stage viruses will lyse, burst, or otherwise die in a manner conducive to the release of newly formed viral particles located within the infected cell while simultaneously returning their biomass to the dissolved organic matter (DOM) pool (Fuhrman, 1999). The DOM pool can be directly consumed (Fig. 1.10, Suttle, 2005) or extracellular enzymes can further digest lysed cellular material, and possibly phages, before being taking up by bacteria (Bratbak et al., 1990). The viral loop decreases the transport efficiency of carbon to higher trophic levels (Suttle, 2005), and increases bacterial respiration and production (Fuhrman, 1999). As a result, a system in which 50% of bacterial mortality is due to viral lysis could lead to a 7% decrease in macrozooplankton production, a 27% increase in bacterial respiration and production (Fuhrman, 1999), and a global loss of 2-3% of primary production back to the DOM pool (Suttle, 1994).

Combined, marine bacteria and viruses form pathways through which nutrients are recycled, potentially relieving the nutrient limitation of portions of the plankton community (Gobler et al., 1997). In oligotrophic ecosystems, such as the WFS, the microbial and viral loops are important pathways to the overall productivity of the ecosystem. Nutrient budgets and production measurements will be largely incomplete without the inclusion of these key components of the biogeochemical cycle.

Research into the relationship between bacteria and *Karenia brevis* has been limited in scope

The potential importance of bacteria in *K. brevis* bloom dynamics was first suggested when unidentified bacteria were observed to be dominant only in the presence of *K. brevis* (Collier, 1958). Further studies found multiple bacterial species were dominant at different time periods before, during, and after blooms of *K. brevis*, but no species-specific differences were observed in direct bacterial counts due to overlapping cycles of bacterial growth (Evans, 1973).

A clone library analysis of the bacterial community from a *K. brevis* bloom which occurred on the WFS in 2001 revealed that the associated bacterial community could be separated into distinct groups based on *K. brevis* concentrations (Jones et al., 2010). Bacterial groups present were significantly different when ≤ 20 cells mL^{-1} or ≥ 500 cells mL^{-1} *K. brevis* were present. Bacterial production, measured by ^{3}H-Leucine incorporation, was also observed to be significantly higher within the bloom,

suggesting the microbes were highly active in nutrient cycling (Heil et al., 2004).

Enumeration of bacteria surrounding this bloom also revealed a range of bacterial

concentrations outside and inside the bloom which corresponded with published

concentrations for oligotrophic and mesotrophic waters, respectively (Jones et al.,

2010).

Looking at *K. brevis*-bacteria interactions from a different aspect, experiments

were designed to determine if bacterial communities were affected by the presence of

brevetoxins (Sipler, 2009). Bacterial communities from locations with frequent *K.*

brevis blooms, relatively few blooms, and no documented blooms at all, were

exposed to varying concentrations of PbTx. Bacterial communities with a historical

exposure to PbTx (WFS communities) were least affected by PbTx. In these

communities there was no significant decrease in live bacterial cells observed, and a

concentration of 200 µg PbTx L^{-1} was required to alter the community composition

(Sipler, 2009). On the other hand, communities with limited or no previous exposure

to PbTx showed a significant decrease in live bacterial cell numbers with the addition

of only 25 µg PbTx L^{-1}. These communities also became more similar as the PbTx

concentration increased (Sipler, 2009).

More recently the primary focus of studies investigating *K. brevis*-bacteria

interactions has been bloom termination facilitated by algicidal bacteria (Mayali and

Doucette, 2002, Roth et al., 2008a and b). Isolated from the Gulf of Mexico, the

bacteria *Flavobacteriaceae* strain S03 and *Cytophaga* strain 41-DBG2 were identified

as algicidal and cultured for use in multiple laboratory studies. In two different

experiments, it was found that an addition of 10^5 cells mL^{-1} algicidal bacteria was

sufficient to induce lysis of *K. brevis* after 3 and 7 days of exposure to S03 and 41BDG2 respectively (Roth et al., 2008b). Previously, *Flavobacteriaceae sp.* had been found to produce algicidal chemicals at cell concentrations $>10^6$ cells mL^{-1} in Japan (Fukami et al., 1991, Fukami et al., 1992). It was also determined that another *Flavobacteriaceae* strain, D38BY, was antagonistic towards strain S03, providing a concentration-dependent level of algicidal resistance to *K. brevis* (Roth et al., 2008a). Despite extensive laboratory evidence of bacterial-mediated lysis of *K. brevis*, such interactions have not been observed in the field and remain effectively unknown (Kodama et al., 2006, Roth et al., 2007).

These studies demonstrate that the bacterial community surrounding *K. brevis* blooms is dynamic but not well understood. More research is necessary, both in the field and in culture collections used for laboratory experiments, to determine if prior results represent true relationships between bacteria and *K. brevis* or just isolated observations unique to the bloom or culture being studied.

The role of viruses in *Karenia brevis* bloom dynamics has not been discerned

As viral infection is primarily density-dependent (phages need to encounter hosts in order to infect them), certain algal blooms represent an ecosystem in which viral host encounter rates are higher than usual, promoting viral production while also controlling the bloom (Fuhrman, 1999). Large blooms of the marine coccolithophore *Emiliania huxleyi* have been show to be susceptible to viral infection, leading to 25-

100% mortality (Bratbak et al., 1993) and a population shift from diploid to haploid phases of *E. huxleyi* to escape viral infection (Frada et al., 2008). Alternately, viruses can lyse up to 20% of the bacterial community daily (Suttle, 1994) and cause a release of 9.2 μmol C L^{-1} d^{-1} (Bratbak et al., 1992). If viruses are lysing large portions of the bacterial community, algal growth could be promoted through the loss of bacterial competitors and the additional nutrients released via bacterial lysis.

There have been several attempts to determine if blooms of *K. brevis* are influenced by viruses native to the WFS ecosystem. In one study, addition of a viral concentrate resulted in lysis of *K. brevis* cultures, yet transmission electron microscopy (TEM) was unable to find evidence of viruses lysing *K. brevis* cells (Paul et al., 2002). Another attempt to lyse *K. brevis* cultures through the addition of a viral concentrate did not achieve lysis of the cultures (I. Hewson, pers. comm.). Thus, it remains unclear whether there are viruses specific to *K. brevis,* or if lysis of bacteria associated with *K. brevis* causes termination of cultures (Paul et al., 2002). A better understanding of the role viruses play in *K. brevis* blooms will provide further insight into bloom dynamics, specifically controls on the magnitude of blooms and timeline for bloom formation or termination.

Research Objectives

Characterize the function of bacteria within natural blooms of *Karenia brevis* on the West Florida Shelf through *in situ* measurements

As a HAB species *K. brevis* has been well studied, however a majority of research has focused on aspects directly relating to the dinoflagellate, its toxicity, and bloom forecasting and management. Little research has focused on the role of bacteria within blooms despite the importance of bacteria in oligotrophic ecosystems and evidence to suggest a large role within blooms (Heil et al., 2004). In order to determine the role of bacteria during blooms of *K. brevis* direct measurements of both the bloom (Chapter 2) and associated bacterial community were undertaken as part of the ECOHAB: *Karenia* project (Chapters 3 and 4). Primary production and cell abundance of *K. brevis* were measured in addition to three key aspects of the bacterial community: growth, abundance, and community composition. These parameters were measured inside and outside of observed blooms so that differences due to the presence of *K. brevis* could be differentiated across location, year, and bloom stage.

Growth was measured using tritiated Leucine and Thymidine (Chapter 3), which measure protein and DNA synthesis, respectively (del Giorgio et al., 2001). Use of both isotopes allows for measurements of metabolic function (Leucine uptake) and cellular replication (Thymidine uptake): combined, these allow for a determination of the energetic demands upon the microbial community which will

ultimately determine the amount of production available for recycling via the microbial loop.

Abundance is an easy parameter to measure that also provides significant information, primarily growth and mortality (Chapter 3). Given that *K. brevis* is a mixotroph, bacteria represent both a potential food source and a nutrient source (via nutrient recycling through the microbial loop). Obtaining measurements inside and outside blooms on the WFS will provide information regarding the distribution of bacteria within the WFS ecosystem, and within blooms of *K. brevis*.

Multiple technologies exist to determine bacterial community composition, many of which have been developed so that they are fast and have a high probability of accuracy (Chapter 4). Understanding "who" is present in the microbial community is just as important as determining what they are doing. Evidence for this has been presented in prior studies which found certain bacteria to be dominant when *K. brevis* was present or when exposed to PbTxs produced by *K. brevis* (Collier, 1958, Evans, 1973, Jones et al., 2010, Sipler, 2009). Understanding how individual components of the bacterial community respond to the presence of *K. brevis* on the WFS could lead to a better understanding of how blooms form, maintain bloom concentrations for extended periods, or terminate.

The three-pronged approach for studying the bacterial community on the WFS was designed to cover many aspects of microbial community structure and function so that any potential relationships between bacteria and *K. brevis* could be discerned. Given that many of these relationships have not been studied in-depth, they could be

a missing component to the current understanding of *K. brevis* bloom dynamics and better improve modeling and forecasting capabilities.

Determine if viruses are playing a role in *Karenia brevis* bloom dynamics, and if so, if viruses are acting directly on *K. brevis* or on associated bacteria (Chapter 5)

The most widely known investigation of virus-*Karenia* interactions failed to fully achieve their objective: cultures of *K. brevis* were terminated, but the vector of viral termination was not determined (Paul et al., 2002). This study sought to build upon the knowledge gained from that study by further characterizing viruses within naturally occurring blooms of *K. brevis* and within cultures of *K. brevis* and bacteria obtained from cultures of *K. brevis*.

Production of viruses needs to remain high to maintain viral abundances within the ocean (Suttle, 1999), however viral abundances do fluctuate and changes provide a significant amount of information. A decrease in viral abundance could represent degradation of viral particles, a decrease in available hosts (exposing viruses to vectors of degradation for longer), or that most viral genomes are contained within cells and therefore not counted by traditional techniques which focus on planktonic phages. An increased in viral abundances could represent a large production event and therefore a significant release of material via cell lysis. Tracking viral abundances on the WFS inside and outside blooms of *K. brevis* allows any such

events to be observed so that more targeted studies can be undertaken to determine the most likely reason for changes in viral abundance.

Since no study has directly confronted the inconclusive results of the virus-*Karenia* study, a targeted experiment was designed to expose different components of *K. brevis* cultures to naturally occurring viruses from the WFS. Determining if viruses are capable of infecting *K. brevis* could lead to new avenues of bloom management. Understanding which component(s) of blooms are being affected by viruses will also improve nutrient budgets and subsequently models and forecasting of bloom development and termination.

Classify the bacteria communities within cultures of *Karenia brevis* which are maintained for scientific research (Chapter 6)

Algal cultures are a useful tool for oceanographers: they allow for the ecosystem or environment to be closely controlled so that causation can be confirmed through experimental manipulation. Several strains of *K. brevis* are maintained at various laboratories throughout the U.S.A. for scientific experimentation and while many of these cultures are different in some aspects (strain, toxicity, media, growth conditions, etc.) there is one common denominator in currents cultures: they all contain bacteria (K. Steidinger, pers. comm.). This is perplexing considering that algal cultures without bacteria, axenic cultures, are considered "pure cultures." Efforts to culture *K. brevis* axenically have all failed (C. Heil, pers. comm.). This suggests a key relationship between bacteria and *K. brevis* (Amin et al., 2012) but

also questions why no efforts have been made to characterize these communities, which have been subjected to the same conditions and restrictions used to isolate and maintain the dinoflagellate.

Sampling the bacterial communities within cultures of *K. brevis* will provide valuable information for both laboratory studies and field observations. The "bottle effect" (response of bacterioplankton to confinement) has been scientifically defined for over twenty years, with the landmark publication noting that culturable bacteria become dominant the longer bacterioplankton are confined or exchange of cells is restricted (Ferguson et al., 1984). This raises many questions regarding the composition of bacterial communities present within cultures of *K. brevis*, which can further be expanded to include growth and productivity that may also have an effect on the viability of the dinoflagellate in culture. Given that most cultures are maintained for targeted experiments, direct sampling of the bacterial communities within these cultures was undertaken to better understand how the composition and function of cultured bacteria differ from those found on the WFS and within naturally occurring blooms of *K. brevis*.

Synthesize information regarding bacteria and virus community structure along the West Florida Shelf, within blooms of *Karenia brevis*, and within cultures of *Karenia brevis* maintained for scientific experimentation

Scientific understanding of marine bacteria and viruses has been rapidly advancing since fluorescent stains first allowed oceanographers to accurately count bacterio- and virioplankton. Unfortunately, these key components of marine food

webs have been largely overlooked in harmful algal bloom research due to the more pressing concerns of toxins, human health, seafood management, and bloom forecasting. With the advent of the ECOHAB: *Karenia* project to investigate nutrient sources to initiate and maintain blooms, a thorough investigation of the bacterial and viral communities was warranted so that no potential nutrient sources or key interactions were overlooked. As important as the samplings themselves, is the synthesis of results and conclusions so that relationships between a HAB species and co-occurring bacteria and viruses can be utilized in education, management recommendations, and bloom forecasting. This dissertation will focus on independent components of such relationship before ultimately synthesizing that information for a more complete picture of *K. brevis* bloom dynamics on the WFS and how those dynamics may be impacted within experimental cultures used for more targeted studies of this toxic dinoflagellate.

Chapter 2: Primary Production of *Karenia brevis* blooms in initiation and maintenance phase on the West Florida shelf

Abstract

Size-fractionated primary production was measured on the West Florida Shelf during 3 stages of October blooms of the dinoflagellate *Karenia brevis* which occurred from 2007 to 2009. Primary production (mg C m^{-3} h^{-1}) ranged from 0.20-62.26 with no bloom present, 30.57-93.99 in an initiating bloom, and 3.87-35.85 in a bloom in maintenance phase. Primary production was positively correlated ($p < 0.05$) with chlorophyll *a* (Chl*a*) in the >3.0μm size fraction, which included *K. brevis*. Blooms sampled in this study were entrained in frontal zones which prevented *K. brevis* from entering estuaries and river outflows: other phytoplankton outside frontal zones had production rates as high as the blooms of *K. brevis*. The 2008 bloom in the initiation phase and the 2009 bloom in the maintenance phase had similar levels of chlorophyll-specific primary production in both total (>0.2 μm) and >3.0 μm size fractions, with averages of 4.56 and 6.07 mg C mg chl*a*$^{-1}$ h^{-1} in 2008, and 2.58 and 5.17 mg C mg chl*a*$^{-1}$ h^{-1} in 2009 (for total and >3.0μm respectively). The 2007 bloom was associated with large fish kills, which could have supplied additional nutrients to the bloom. It is likely that the transition between *K. brevis* bloom phases is a function of the need to better utilize available nutrient sources, which change as bloom duration increases. If older blooms need to rely more on heterotrophy it would come

at the cost of photosynthetic capabilities and primary production would decrease.

Each bloom may be characterized by different rates of primary production depending upon bloom phase; which may have significant impacts on the carbon budget of the West Florida Shelf. It is therefore necessary to include *K. brevis* blooms in carbon budgets of this area, with particular attention placed on bloom phase, location, and duration.

Introduction

Numerous studies have investigated the productive capabilities of blooms of the red tide dinoflagellate *Karenia brevis* (Davis) G. Hansen & Møestrup (Vargo et al., 1987, Bendis et al., 2004, Heil et al., 2004, Walsh et al., 2009, Hitchcock et al., 2010) along the West Florida Shelf (WFS). However, none of these studies have attempted to relate the physiological differences in the initiation, maintenance, and termination phases of blooms to the magnitude of primary production. This study compares 3 consecutive years (2007-2009) in which two bloom phases were identified and sampled: bloom initiation (2008), and bloom maintenance (2007 and 2009). Given that daily production rates within blooms can be 2-5 times greater than non-bloom communities, blooms can have a large impact on the carbon budget of the WFS (Vargo et al., 1987). This study investigated multiple stages of *K. brevis* blooms to determine if differences existed in the magnitude of production among bloom stages, and the impact of each stage on the carbon budget of the WFS.

The dinoflagellate *Karenia brevis*, formerly *Ptychodiscus brevis* and *Gymnodinium breve*, (Daugbjerg et al., 2000), blooms almost annually on the WFS, typically between Tampa Bay and Ft. Myers (Walsh et al., 2009). *K. brevis* produces brevetoxins (Lin et al., 1981), a suite of lipid soluble neurotoxins (Nakanishi, 1985) that have harmful effects on humans (Poli et al., 2000) and fish (Kirkpatrick et al., 2004, Anderson et al., 2005). *K. brevis* blooms originate offshore and are transported inshore within bottom Ekman layers (Walsh et al., 2006). Near the coast, blooms can become entrained in thermal and salinity frontal zones (Steidinger and Haddad, 1981, Vargo et al., 2004, Walsh et al., 2006) which promote bloom formation by concentrating *K. brevis* within defined water masses near nutrients sources that may also limit or exclude predators and competitors (Steidinger and Haddad, 1981). A physically constrained bloom creates a population stock (Donaghay and Osborn, 1997) that can subsequently be transported to new nutrient sources (Bendis et al., 2004) or exported to the Gulf of Mexico and up the Atlantic Coast of the U.S.A. (Steidinger and Haddad, 1981, Carlson and Clarke, 2009, Weisberg et al., 2009).

The WFS, where *K. brevis* blooms commonly occur, is an oligotrophic ecosystem in the central Gulf that is critical to the economy of Florida. The Gulf Coast of Florida was responsible for 31% of state employment and 2,209,215 jobs in 2004, which generated $67.8 billion in wages and $163.7 billion in Gross State Product (Kindow et al., 2009). Industries such as commercial and recreational fisheries, tourism, coastal development, seafood processing, merchant shipping, commercial cruise activity, and maritime vessel construction are tied to the Gulf; fisheries in particular are critically linked to the health and productivity of the Gulf

ecosystem (Adams et al., 2009). From 2000-2010 fisheries efforts in Florida were 56% commercial and 44% recreational, and harvested over 128 million pounds of fish per year (NMFS, pers. comm.) worth nearly $120 million annually (Adams et al., 2009). This fishery is supported by an average primary production of 0.06-2.0 g C m^{-2} d^{-1} (Vargo et al., 1987) and chlorophyll a (Chl$a$) biomass of 0.47-4.56 µg L^{-1} (Bendis et al., 2004).

Primary production within blooms is highly variable due to differences in bloom density, geographic extent, duration, and physiological and toxicological differences (Table 2.1, Schofield et al., 2006, Heisler et al., 2008, Lenes and Heil, 2010). Between log and stationary growth phase (defined as a 32% decrease in growth rate) Johnson et al. (2012) observed that *in vitro*, 29% of *K. brevis* transcriptomes changed: photosystems were re-structured to less efficient energy pathways and DNA repair mechanisms decreased, which could allow for DNA damage to increase to a threshold required for programmed cell death. Brevetoxin concentrations have been found to be higher in senescing blooms, making aged, high density blooms a greater threat to ecosystems and human health (Maier Brown et al., 2006). Lipids and waste products begin to accumulate in the periphery of cells, which can help identified aged blooms, even in field populations (Steidinger and Haddad, 1981).

Given the changes in *K. brevis* physiology and bloom toxicity as blooms age, concomitant changes in productivity as blooms age are not unexpected. To date,

Table 2.1: Primary production rates (measured and calculated) for the West Florida Shelf when *K. brevis* is blooming and not blooming. Years are given when multiple blooms were sampled within the same study individually: [a] Assumed a 12-h photoperiod and a photosynthetic quotient (mol O_2 evolved mol C fixed[-1]) of 1, [b] Assumed a 12-h photoperiod, [c] Modified from Lohrenz et al., 1999, [d] Calculated by multiplying the production at the 47% light level by the water column depth from which each sample was taken, [e] mg C m^{-3} h^{-1}. Modified from Vargo et al. (1987).

Source	Daily (g C m^{-2} d^{-1})
No-bloom	
Curl, 1956 [a, c]	2.76 – 3.84
El Sayed, 1972	0.06 – 0.08
Bittaker, 1975 [c]	0.03 – 0.44
El Sayed and Turner, 1977 [c]	0.20 – 1.6
Sklar and Turner, 1981 [b]	0.12 – 2.23
Ortner et al., 1984 [c]	0.17 – 0.74
Randall and Day, 1987 [a]	0.00 – 9.60
Vargo et al., 1987	
Nearshore (<20m)	0.3
Offshore (<100m)	0.4
Madden, 1992 [c]	0.00 – 2.50
Pennock et al., 1994 [c]	0.20 – 1.70
Gilbes et al., 2002	0.25 – 2.0
Bendis et al., 2004 [d]	0.73 – 6.4
Heil et al., 2004 [e]	0.68 – 1.76
This study (>0.2µm)	0.08 – 4.38
This study (>3.0 µm)	-0.01 – 2.02
Bloom	
Odum et al., 1955	1.6
Vargo et al., 1987	
Measured 1980	0.8 – 1.8
Calculated 1978 [b]	0.5 – 3.8
1978	0.7 – 2.7
1980 [b]	1.8 – 2.9
1980	1.1 – 1.8
1982	1.7 – 2.7
Bendis et al., 2004 [d]	0.35 – 10.0
Heil et al., 2004 [e]	19.2 – 127.3
This study: Initiating Bloom (>0.2µm)	2.02 – 4.10
This study: Initiating Bloom (>3.0 µm)	2.75 – 5.08
This study: Maintenance Bloom (>0.2µm)	0.88 – 3.86
This study: Maintenance Bloom (>3.0 µm)	0.46 – 1.42

primary production measurements have not been differentiated based on bloom phases when a bloom is present. This study sampled the WFS when blooms were not present, and during bloom initiation and maintenance phases over 4 consecutive years with the objectives of examining primary production in different bloom phases and to determine the potential impact the measurement from individual phases verses total bloom production may have on the carbon budget of the WFS.

Methods

Station sampling and bloom classification

Two-week research cruises were conducted from 2007 to 2009 from 16-26 October, 2007; 2-12 October, 2008; and 2-12 October, 2009 aboard the R/V *Pelican* (LUMCON). The first week of each cruise (leg 1) involved sampling established stations to map the distribution of nutrients, primary production, phytoplankton, biomass and community composition, and *Karenia brevis* in representative estuarine, coastal, and offshore environments on the WFS. The second week of each cruise (leg 2) focused on sampling bloom populations and identifying bloom stage based on bloom monitoring data, location, cell abundance, and growth.

A CTD cast was conducted each day within an hour of dawn and water samples were collected at specified depths using 20 L Niskin bottles. Sample depths were chosen on the basis of the vertical fluorescence profile and physical structure of

the water column. A 4 L volume was drained into an acid-cleaned polycarbonate carboy and immediately subsampled for determination of Chl*a*, primary productivity, and *K. brevis* concentration. Additional samples were also fixed in Lugol's Iodide for *K. brevis* enumeration according to the Florida Fish and Wildlife Research Institute Red Tide Monitoring Program.

Primary production

The $H^{14}CO_3$ uptake method was used to measure primary production (Parsons et al., 1984). Replicate water samples were directly siphoned from Niskin bottles into acid and sample rinsed 125mL PTEG bottles. All bottles were kept in the dark during sampling to minimize light exposure and dark replicates were wrapped in two layers of aluminum foil before isotopes were added at a concentration of approximately 10 $\mu Ci\ L^{-1}$. All treatments were placed in on-deck flow-through incubators in neutral-density screening mesh bags which reduced ambient light to levels similar to a depth of 0.5m, where *K. brevis* is typically found (Schofield et al., 2006). Size-fractionated uptake was determined at termination of incubations by filtering samples onto 0.2, 0.7, or 3.0 μm nucleopore filters. Alkalinity of water samples was determined according to Parsons et al. (1984).

Rates were standardized to a 12 hour light period, as in previous works, because the day length in Florida is near 12 hours in length in October (Vargo et al., 1987, Lohrenz et al., 1999, Heil et al., 2004). Vertical integration was done to the

bottom depth at each station as the water column was well mixed at all stations and a majority of stations were in shallow (<10 m) water.

Statistical analysis

All data were tested for normality; non-normal data were Log-transformed before further analysis. The relationship between production and chlorophyll *a* concentration was tested with a correlation analysis. Differences in production between bloom types and size classes were tested with a two-way ANOVA at a significance level of $\alpha = 0.05$. All statistics were done using SAS software, Version 9.2 (SAS Institute Inc., Cary, N.C., U.S.A.).

Results

Bloom phases

Karenia brevis blooms were identified on cruises in 2007, 2008, and 2009. A combination of cell abundance and biomass, previous bloom monitoring history, and cellular structures within cells from each bloom were used to classify bloom phase (K. Steidinger, pers. comm.). The 2008 bloom was defined as being in initiation phase due to: relatively low biomass, increasing cell concentrations, the bloom moving from offshore to nearshore (physical environment aggregating the bloom, Walsh et al., 2003), and no prior detection of blooms by any regional monitoring

programs. The 2007 and 2009 were classified as being in maintenance phase. In 2007 the bloom had been monitored in nearshore environments prior to sampling, had a high biomass, and growth rates greater than one division day^{-1} (Sipler et al., in press). The 2009 bloom was defined as a maintenance/stationary bloom due to its offshore location (serving to disperse the bloom, Weisberg et al., in press), low to moderate concentrations of *K. brevis* present, and high numbers of lipid bodies within cells (which have been shown to indicate older cells in stationary phase, Steidinger, 1979). This still allowed for comparison between *K. brevis* blooms in initiation and maintenance phases.

Primary production

Carbon uptake (g C m^{-2} h^{-1}) was positively correlated with chl*a* concentration for blooms in both initiation and maintenance phases. A bloom in initiation phase (2008) had a linear relationship that was only significant for the larger size fraction (>3.0 μm, Figure 2.1), with a Pearson's Correlation Coefficient of 0.69 ($p < 0.05$). The 2009 *K. brevis* bloom in maintenance phase had a significant correlation between carbon uptake and chl*a* in both size classes (Fig. 2.1, Pearson's 0.61 and 0.72 for Total and >3.0 μm respectively). The 2007 maintenance phase bloom had a significant correlation in the Total size fraction only (Fig. 2.1), Pearson's 0.73.

Bloom concentration and spatial extent differed each year of this study, which was evident in primary production values which differed between bloom types and could identify the type of bloom present. In both Total and >3.0 μm size fractions, the
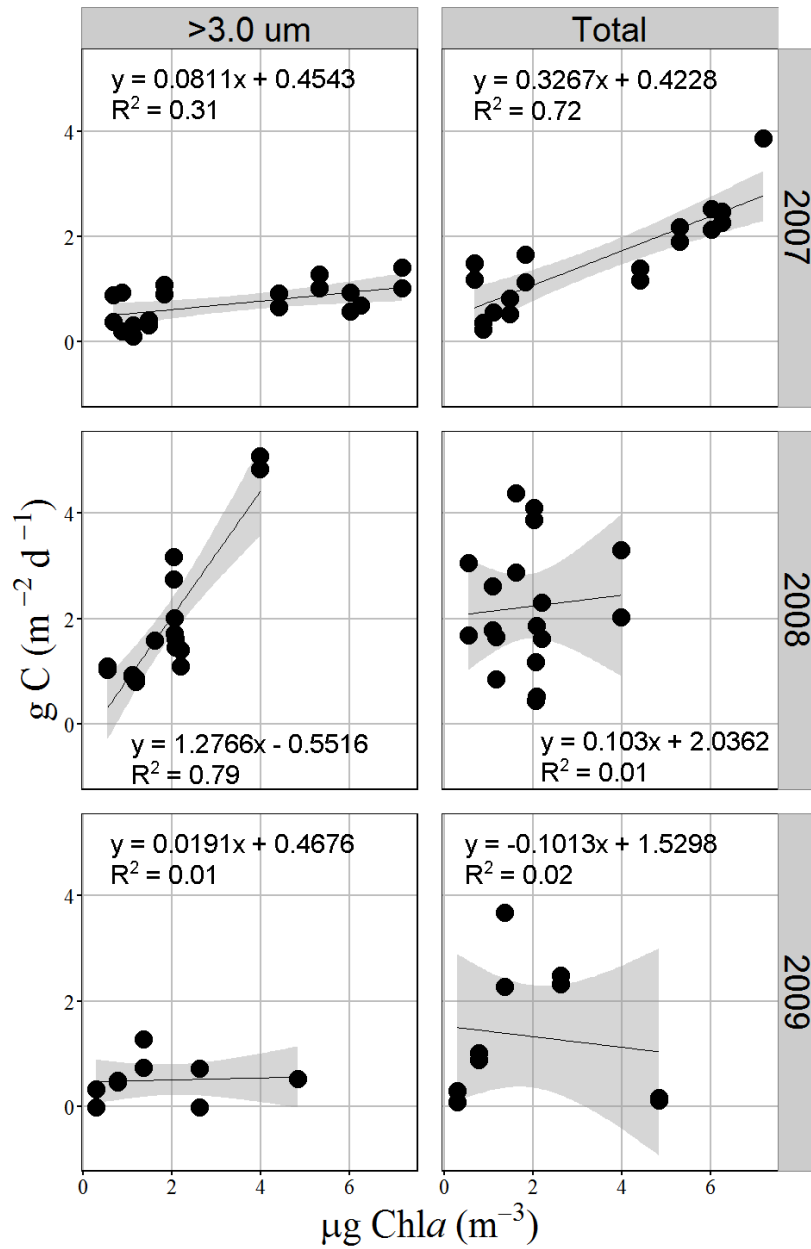
Figure 2.1: Net primary production (g C m^{-3} day^{-1}) per unit chlorophyll a (µg L^{-1}) in each year of this study size fractionated into "total" (>0.7 µm) and >3.0 µm size fractions. Linear correlations (solid line), standard error (shaded regions), and equations are displayed for each environment.

highest carbon uptake rates were found in an initiating bloom (Table 2.1, Fig. 2.2). As the bloom was increasing in magnitude, as indicated by chl*a* concentration, production rates increased more rapidly than observed in maintenance phase. The relationship between production and chl*a* was only significant for the >3.0 μm size fractions. Smaller cells (0.7-3 μm in 2007 and 0.2-3 μm in 2008 and 2009) had more variable production that was not dependent on chl*a* concentration. Neither bloom in maintenance phase exhibited a strong correlation between primary production and chl*a*, however, total production was lower than that observed in an initiating bloom at equal chl*a* concentrations (Fig. 2.1). High rates of production corresponding with high chl*a* concentrations were in the initiating blooms; *K. brevis* blooms in maintenance phase had more variable production than in an initiating bloom.

Data were separated in four inshore-to-offshore zones so that production could be compared among regions. Correlation coefficients between production and chl*a* concentration were calculated for each zone. The four zones were estuarine, nearshore (inside the 45 m isobath and <18 km from shore, Heil et al., 2001), offshore (beyond the 45 m isobath and >18 km from shore), and bloom; all blooms took place in a nearshore environment. All environments had similar production when standardized for chl*a* concentration (Fig. 2.2). There was no correlation between production and chl*a* in the offshore environments ($p = 0.22$ and 0.10 for Total and >3.0 μm size fractions respectively, Fig. 2.3) and production was lowest in offshore stations in all years ($p < 0.05$, Fig. 2.2). When production levels were normalized to chl*a* concentrations, all environments were statistically similar (Fig. 2.2). Nearshore,
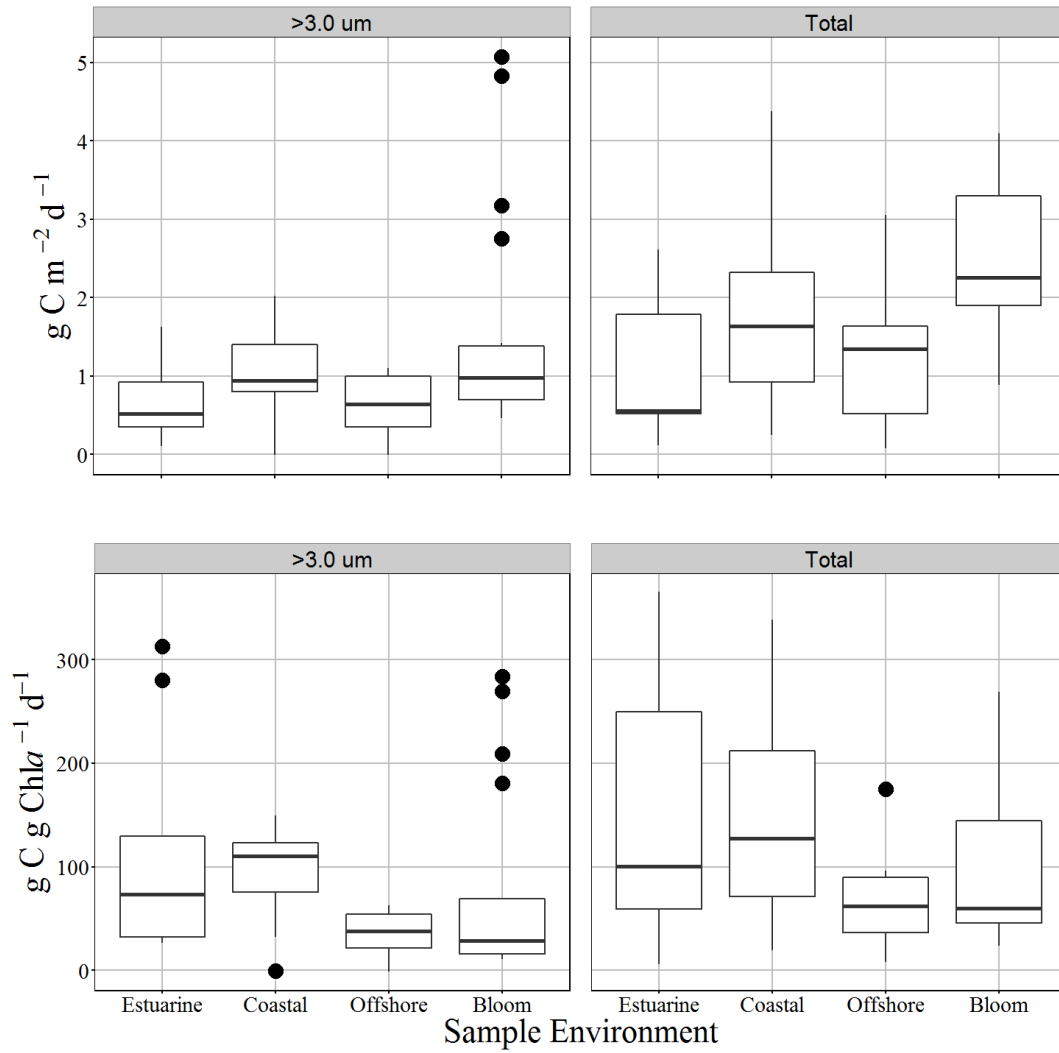
Figure 2.2: Net primary production (g C m^{-2} d^{-1}, Top) and NPP per unit chlorophyll *a* (mg C mg chl*a*$^{-1}$ d^{-1}, Bottom) for each of the geographic zones: Estuarine, coastal/nearshore, offshore, and bloom stations (which were all in the coastal/nearshore environment). Measurements were done two size fractions: >3.0 μm and Total (>0.7 μm). The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Measurements outside these quartiles (as determined by a Tukey test) are represented as points.
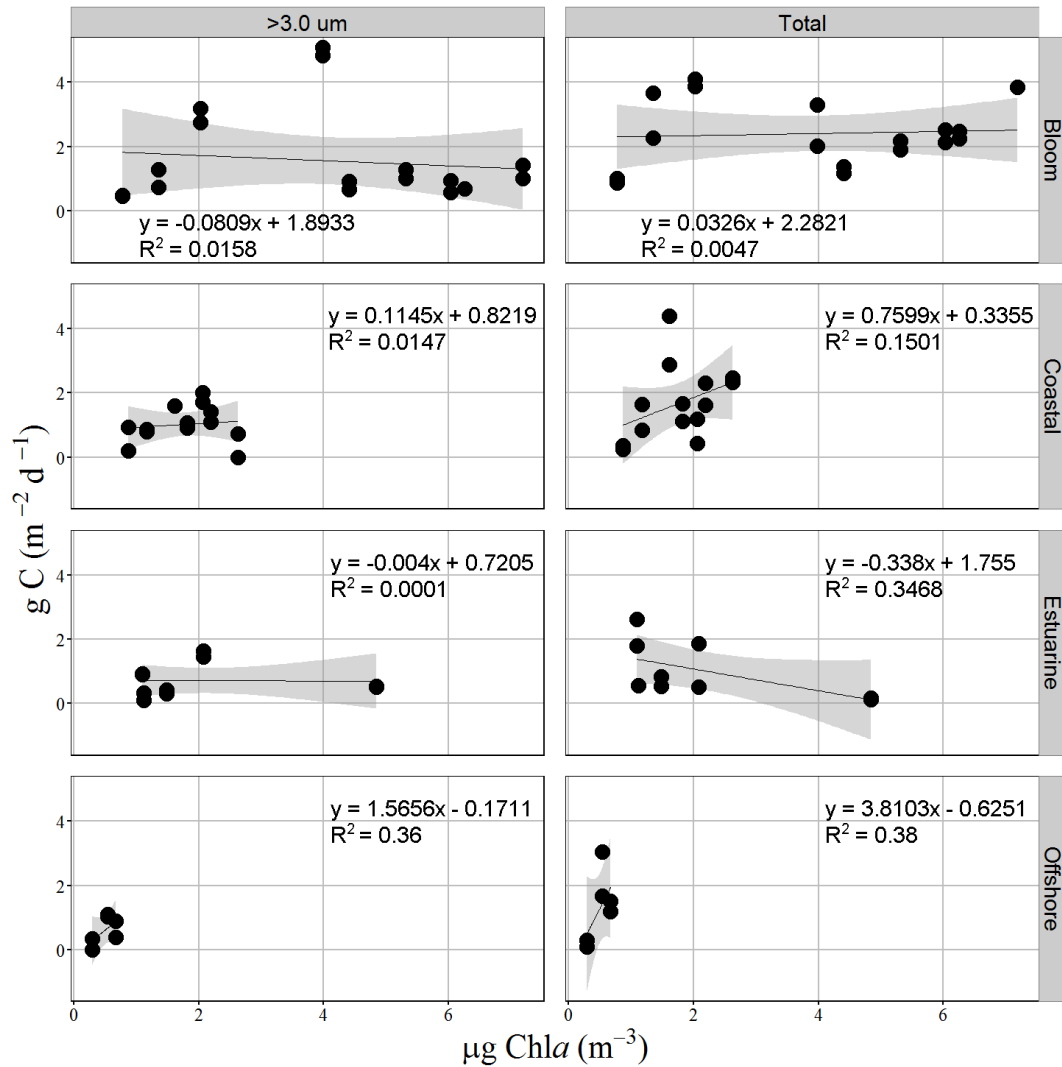
Figure 2.3: Regressions between net primary production (mg C m^{-2} d^{-1}) and chlorophyll *a* concentration (μg m^{-3}) for four geographic zones along the West Florida Shelf: Estuarine, coastal/nearshore, offshore, and bloom stations (which were in the coastal/nearshore environment). Data were size fractionated into >3.0 μm and "total" (>0.7 μm) size fractions with linear correlations (solid line), standard error (shaded region) and equations displayed for each environment.

bloom (nearshore), and estuarine environments were statistically similar for both production and production standardized for chl*a* ($\alpha = 0.05$). Bloom production measurements were the highest, but the WFS was also a highly variable environment making instances of high production not statistically different from estuarine or nearshore environments. Production within blooms was correlated to chl*a* in the > 3.0 µm size fraction (Pearson's correlation coefficient 0.81, $p < 0.05$), the only environment or size class with a significant relationship (Fig. 2.3). Production offshore was consistently lower than nearshore, estuarine, and bloom environments, however when standardized for chl*a* production there were no significant differences between any of these environments during cruises undertaken.

When individual *K. brevis* blooms were compared, the bloom in initiation phase had higher production than either bloom in maintenance phase; however when production was normalized to chl*a* concentrations, different bloom stages were not significantly different (Fig. 2.4). Preliminary tests (data not shown) determined that *K. brevis* blooms in maintenance phase (2007 and 2009) had net primary production measurements that were significantly different from one another, therefore it was decided to treat each bloom as an individual event rather than grouping blooms in the same growth stage. Net primary production ($g\ C\ m^{-2}\ h^{-1}$) was significantly different among bloom types; the 2008 initiating bloom having the highest production (ANOVA, $\alpha = 0.05$, Fig. 2.4). All three blooms were statistically similar on a production per chl*a* basis in the >3.0 µm size fraction. In the Total size fraction the NPP per unit chl*a* in the 2007 maintenance phase bloom was significantly lower than
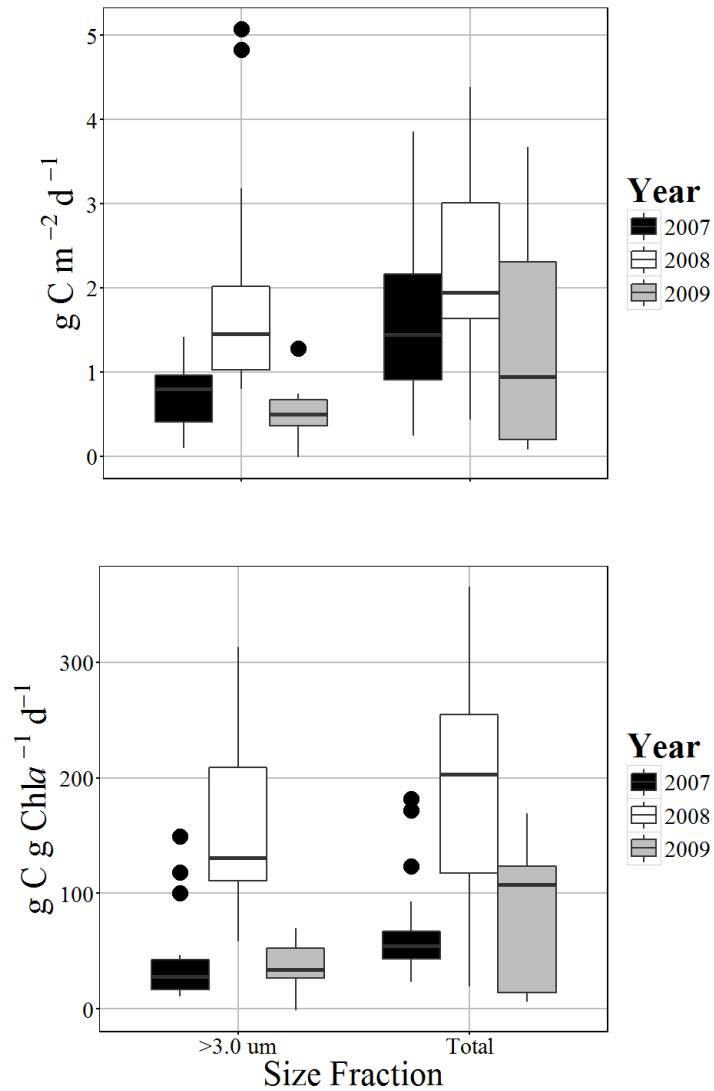
Figure 2.4: Production in different blooms stages of *K. brevis* blooms sampled in 2007 (maintenance), 2008 (initiation), and 2009 (maintenance): Top- Net primary production (mg C m^{-3} h^{-1}) and Bottom- NPP per unit chlorophyll (mg C mg chl*a*$^{-1}$ h^{-1}). The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Measurements outside these quartiles (as determined by a Tukey test) are represented as points.

the 2008 and 2009 blooms in initiation and maintenance phases, respectively (ANOVA, $\alpha = 0.05$, Fig. 2.4). Comparing individual blooms from each year of the study determined that the initiating bloom had higher production than either bloom in maintenance phase and that the 2007 bloom, in maintenance phase, had lower production per unit chl*a*, which could be an indicator of that bloom nearing a termination or senescing phase.

Discussion

Carbon uptake (g C m^{-2} h^{-1}) was measured in multiple years inside and outside of *K. brevis* blooms. Production in both phases was positively correlated to chl*a*, and when all bloom data was pooled production in the > 3.0 μm size was correlated with chl*a* (whole water measurement). As expected, production along the WFS was lower offshore than in nearshore and estuarine environments in all size classes. When production was normalized to chl*a* concentration, there were no significant differences between any environments, including blooms in nearshore environments. Production along the WFS was consistent across multiple environments and multiple years with a trend of higher production at higher chl*a* values. When normalized to chl*a* initiating blooms had higher production per unit chl*a* than those in maintenance phase. These differences between blooms in different stages highlight the importance of classifying the bloom stage so that any data or rates obtained from multiple sampling efforts can be combined to provide a complete ecosystem picture of the entire evolution of a bloom.

Primary production during the ECOHAB: *Karenia* project

Primary production measurements were taken as part of a large suite of measurements for the ECOHAB: *Karenia* project that investigated nutrient dynamics in *K. brevis* blooms along the WFS. The objective of this study was to use primary production measurements to investigate three aspects of bloom dynamics. First, whether blooms were more productive than the non-bloom phytoplankton community or environments such as estuaries and offshore communities. Second, whether changes in production varied with bloom phase and if these measurements could be used to rapidly identify the state of bloom populations. Lastly, to assess if blooms, and specific bloom phases, impact the carbon budget of the WFS.

Data collected during the ECOHAB: *Karenia* study agreed with production values and trends seen in previous studies on the WFS. Primary production in this study was calculated from samples taken at 0.5 m depth and depth-integrated for the entire water column. A depth of 0.5 m was chosed because it is below the surface-aggregated microlayer that can form within blooms of *K. brevis* during the day and the average depth of *K. brevis* cells in a given light period (Heil et al., in press), These values therefore represent a possible underestimation of maximal carbon uptake rates.

During the dry season, starting in late fall/early winter (Bidlake et al., 1996), nutrient limitation exists along the length and breadth of the WFS (Heil et al., 2007). Nutrient limitation is relieved by a combination of aeolian dust deposition and terrestrial runoff (Walsh and Steidinger, 2001, Walsh et al., 2006), which both occur during Florida's rainy season (spring through fall, Bidlake et al., 1996). Nutrient

concentrations sufficient for *K. brevis* blooms therefore occur primarily in the fall, which was when this study conducted sampling efforts. Blooms were observed three out of four years of sampling. The highest production and chlorophyll values were observed within blooms, which matched previous studies measuring both production (Vargo et al., 1987) and chlorophyll (Ault, 2006). Production within blooms was correlated with chl*a* in the > 3.0 μm size fraction, the size class that includes *K. brevis* (Steidinger and Tangen, 1997). Both production values and the relationship between production and chl*a* in the size range which contained *K. brevis* concurred with previous work along the WFS when *K. brevis* blooms were present.

Bloom production in different nearshore environments

  K. brevis blooms have been described as having high production and chl*a* concentrations during the late summer and fall (Ault, 2006) however it was unexpected to find comparable values outside the bloom in both nearshore and estuarine environments (Fig. 2.2). The WFS is an oligotrophic environment, and blooms can require a substantial quantity of nutrient inputs for both initiation and maintenance; if blooms become entrained in water masses near nutrient sources, such as river or estuary outflows, the bloom could utilize nutrients before expanding along the shelf (Anderson et al., 2008, Omand et al., 2011). Rivers along the WFS can provide nutrients to the middle shelf (Gilbes et al., 2002), so the location of *K. brevis* blooms may be critical in determining nutrient availability to non-bloom communities (Tester and Steidinger, 1997, Vargo et al., 2004).

If *K. brevis* is entrained in nearshore frontal zones (Steidinger and Haddad, 1981, Vargo et al., 2004) then non-bloom communities closer to the coast, or within estuaries, will be supplied by the same terrestrially-derived nutrients and therefore may be capable of similar levels of production (Walsh et al., 2006). Co-occurring blooms of *K. brevis* and the cyanobacterium *Lyngbya majuscula* were observed in the nearshore and estuarine environments surrounding Sanibel Island in 2006, demonstrating that *K. brevis* blooms can form within estuaries given the proper physical conditions (Paerl et al., 2008). Typically, *K. brevis* blooms are transported to nearshore environments via onshore transport associated with upwelling and then entrained in frontal zones formed through a combination of salinity and temperature gradients (Walsh et al., 2003, Vargo et al., 2004, Figueiras et al., 2006). These fronts are formed in part through estuarine outflow and therefore can prevent further up-estuary transport of *K. brevis*, allowing non-*Karenia* phytoplankton communities to use the same nutrients sources that are fueling the bloom.

The relationship between primary production and bloom phase

One additional parameter in the ECOHAB: *Karenia* study was a microscopic classification of the growth phase of individual blooms; this index provides information on the growth, distribution, and toxicity of blooms (Maier Brown et al., 2006, Johnson et al., 2012). Fortunately this study was able to sample blooms in initiation (2008) and maintenance phases (2007 and 2009). In the total size fraction, production within the initiating bloom was significantly higher than both blooms in

maintenance phase: however in the > 3.0 μm size fraction, the size fraction containing

K. brevis cells, the 2008 initiating and 2009 maintenance phase blooms were not

significantly different (Fig. 2.4). The production measured in the > 3.0 μm size

fraction may have been due to one of two factors: the 2009 bloom was in transition

between initiation and stationary phases, or the 2007 bloom was beginning to

transition to termination or senescing phase.

In a transcriptomic study of *K. brevis* the transition from logarithmic to

stationary growth was accompanied by a restructuring of photosystems to favor

Photosystem I and cyclic ATP production and a decrease in DNA repair mechanisms

(Johnson et al., 2012). Similar changes in the blooms sampled in this study could

account for the differences in production between the two maintenance phase blooms.

A restructuring of photosystems and ATP production in the 2007 bloom could

decrease production, and a lack of restructuring such systems in the 2009 bloom

could keep production high. To determine if this was the case would require sampling

to focus on transition between *K. brevis* bloom growth phases. It is likely that changes

in production and bloom state are driven by gene expression or interactions with

bacteria, which makes measures of chlorophyll or primary production non-viable

methods in determining the phase of a *K. brevis* bloom.

Carbon budgets of the West Florida shelf need to account for *K. brevis* blooms

Significant differences were seen in net primary production rates that indicate each individual *K. brevis* bloom could not only impact the carbon budget of the WFS, but that each bloom could have additional unique impacts on the WFS ecosystem. The lowest levels of production measured were within a stationary phase bloom in 2007, which had the highest concentration of *K. brevis* measured during this study (5.87×10^5 cells mL^{-1}, data not shown). One source of nutrients that can support blooms in maintenance phase are dead fish that are decaying in the water column: fish kills were widely observed in 2007 but not 2008 or 2009 (Walsh et al., 2006, 2009). Use of fish-derived nutrients, or predation on bacteria responsible for fish decay, requires *K. brevis* to expend energy maintaining feeding abilities (Stoecker et al., 2006b). The energetic requirements for up-regulation of mixotrophic capabilities may require the shift in photosystem and ATP pathways observed in transcriptomes (Johnson et al., 2012). Highly toxic *K. brevis* blooms that lead to large fish kills also serve to prevent carbon export (Kirkpatrick et al., 2004). Dead fish will be contained by the same frontal zones that maintain the position of blooms, which could provide *K. brevis* maximum exposure to this nutrient source (Steidinger and Haddad, 1981, Walsh et al., 2009). It is possible that through mixotrophy, fish kills are a means by which *K. brevis* either maintains a bloom in order to reproduce sexually (to maintain genetic diversity) or to produce cysts for future blooms, and possibly both (Steidinger and Garccés, 2006, Roth et al., 2008). *K. brevis* blooms represent a large recycling of

nutrients within the WFS ecosystem. Little evidence supports a high level of grazing on *K. brevis*, which suggests terminated blooms could sink to the bottom where nutrients from their decay could be used by marine microbes (Kubanek et al., 2007). Mixotrophy on fish-derived nutrients will also promote nutrient recycling within the system as the dead fish will no longer be exporting carbon (either through emigration or fisheries harvesting). Therefore, despite blooms having significant levels of production supported by both new and recycled nutrients, the lack of advection of bloom biomass from the system would minimize carbon export to offshore waters.

   K. brevis blooms represent periods of high production along the WFS that are ultimately entrained in the nearshore ecosystem. Production is supported by atmospheric, offshore, estuarine, and mixotrophically derived nutrients (Lenes et al., 2001, Heil et al., 2007, Anderson et al., 2008, Burkholder et al., 2008, Vargo et al., 2008, Glibert et al., 2009, Walsh et al., 2009, Lenes and Heil, 2010). As blooms age physiologically *K. brevis* can exchange photosynthetic for heterotrophic capabilities to take advantage of new nutrient sources such as dead fish (Walsh et al., 2006, 2009, Johnson et al., 2010). Blooms can be maintained in frontal zones that allow for high levels of production, as well as nutrient recycling, on both sides of a front (Steidinger and Haddad, 1981) through the duration of the bloom. Transition between stages of *K. brevis* blooms occur when the physiological capabilities of a specific bloom experience the greatest change; therefore identifying bloom stage should be determined along with any physiological data when possible. The similarities and differences observed between blooms in both initiation and maintenance stages in this study demonstrate the unique nature of each bloom as well as differences between

bloom stages. Any attempts at calculating a carbon budget for the WFS should take

K. brevis blooms into account, however emphasis needs to be placed on each stage of

a bloom (initiation, maintenance, and termination) to accurately capture the total

effect a single bloom can have on the ecosystem and its carbon budget.

Chapter 3: Microbial production along the West Florida Shelf: Responses of bacteria and viruses to the presence and phase of *Karenia brevis* blooms

Abstract

Bacterial abundance, production, protein and nucleic acid synthesis, growth, and viral abundance were measured in waters associated with three bloom stages of the "red tide" dinoflagellate *Karenia brevis* along the south West Florida Shelf (WFS). Measurements were taken: 1) when no bloom was present; 2) during the initiation stage of a bloom; and 3) during the maintenance stage of a bloom. Results indicate that the bacterial community was nutrient limited in the non-bloom period, with highest abundance and production rates occurring near and within estuaries. Abundance of virus like particles (VLPs) was higher within estuaries, but we hypothesize VLPs were not a high source of bacterial mortality, possibly due to high decay rates due to UV degradation or extracellular nucleases. High bacterial production, balanced protein to nucleic acid synthesis, and statistically similar bacteria abundances measured on consecutive days within the initiating bloom suggest a highly productive community with equally high mortality. VLP abundance declined during the first 48 hours within both bloom stages, suggesting that viral genomes were either within host cells (not evident in water column samples), or bacterial mortality was due to mixotrophic grazing by *K. brevis*. Using a conservative

grazing rate of 1 bacteria $K.\ brevis^{-1}\ h^{-1}$, *K. brevis* grazing could account for >100% of bacterial mortality during an initiating bloom. Bacterial abundance and production were significantly decreased and protein to nucleic acid synthesis became unbalanced during the maintenance phase bloom. An increase in VLP abundance during the maintenance phase was most likely the cause of bacterial mortality as mixotrophic grazing could only account for ~4% of the change in bacterial abundance. Together, these data suggest that the associated bacteria and viruses play a critical role in the formation and termination of *K. brevis* blooms.

Introduction

The bloom forming dinoflagellate *Karenia brevis* (Davis) G. Hansen & Møestrup is endemic to the Gulf of Mexico (Steidinger, 2009). Blooms of *K. brevis* are associated with the appearance of brevetoxins (PbTxs), a suite of lipid-soluble neurotoxins (Schneider et al., 2003) which affect marine biota in a variety of ways (Heil and Steidinger, 2008) and can cost the Florida economy more than 26 million dollars each year a bloom is present (Hoagland et al., 2009, Morgan et al., 2009, Nierenberg et al., 2010).

Relatively little research has focused on the potential relationships between *K. brevis* and its associated microbial (bacteria and viruses) community (Collier, 1958, Buck and Pierce, 1989, Heil et al., 2004, Jones et al., 2010). What little research that has been published highlights the importance that bacterial-algal relationships could

be having on *K. brevis* blooms, as well as plankton community dynamics along the West Florida Shelf (WFS).

The potential importance of bacteria in *K. brevis* bloom dynamics was first suggested by Collier (1958), who observed an unidentified bacterium was dominant only in the presence of *K. brevis*. Subsequently, it was hypothesized that populations of bacteria also bloom in concert with *K. brevis* when it was observed that multiple bacterial species could be dominant at different time periods before, during, and after a bloom (Evans, 1973). However, no species-specific differences were observed in direct bacterial counts due to overlapping cycles of bacterial growth. Buck and Pierce (1989) attempted to characterize the bacterial communities of three *K. brevis* blooms that occurred in 1982 and 1987 (one each year in Florida and one in North Carolina in 1987), but used a plate count method that missed a significant portion of the bacterial community (Buck and Cleverdon, 1960).

Research on bacterial community composition and function in a 2001 *K. brevis* bloom revealed that the associated bacterial community could be separated into distinct groups based on *K. brevis* concentrations (Jones et al., 2010). Bacterial groups present clustered separately in Principal Components Analysis (PCoA) when divided into samples with ≤ 20 *K. brevis* mL^{-1} or ≥ 500 *K. brevis* mL^{-1}. Heil et al. (2004) observed significantly higher bacterial production within a *K. brevis* bloom (^{3}H-Leucine incorporation), which suggests that microbes were active in nutrient cycling. Enumeration of bacteria surrounding the 2001 bloom revealed a range of bacterial concentrations outside and inside the bloom which corresponded with

published concentrations for oligotrophic and mesotrophic waters, respectively (Jones et al., 2010).

Viral termination of *K. brevis* blooms was investigated during a study in which researchers were able to terminate cultures of *K. brevis* with a lytic concentrate obtained from a naturally occurring bloom of *K. brevis* from the WFS (Paul et al., 2002). Viruses were not definitively identified as the cause however, as electron micrographs found no evidence of *K. brevis* lysis. Viral abundances increased over time but remained within estimated ranges normally found in coastal seawater (Wilcox and Fuhrman, 1994, Paul et al., 2002). It was hypothesized that viruses were actually affecting the bacterial community, rather than affecting the *K. brevis* directly, thereby causing a shift in bacterial community composition which caused bloom termination (Paul et al., 2002).

As part of a regional ECOHAB project focused on bloom dynamics of *K. brevis*, this study focused on microbial community dynamics (production and abundance) to examine the relationships between *K. brevis*, bacteria, and viruses during multiple blooms stages. As blooms are, by nature, highly productive, this study was undertaken as part of the ECOHAB: *Karenia* project to:

1. Enumerate bacteria and viruses across the West Florida Shelf and inside blooms of *Karenia brevis*.

2. Determine if bacterial production exhibits similar patterns as *K. brevis* production when establishing blooms.

3. Better understand bacterial community dynamics surrounding blooms of *K. brevis*.

High leucine incorporation was observed in the past (Heil et al., 2004) but alone could not provide a complete picture of bacterial dynamics. Additional measurements were chosen for this study to better elucidate microbial dynamics in an effort to determine the nature of possible interactions between *K. brevis* and co-occurring microbes.

Methods

Station sampling

Two-week research cruises were conducted aboard the R/V *Pelican* each October from 2008 to 2010. Cruises were designed so the first week (leg 1) of each cruise mapped the spatial distributions of nutrients, microbial abundance, and bacterial production along the WFS and located *K. brevis* populations. The second week (leg 2) of each cruise focused on sampling identified *K. brevis* populations for process-related research. Cruises occurred on 2-12 October, 2008; 2-12 October, 2009, and 13-23 October, 2010. Seven stations were sampled on each leg 1, with station location related to geographic proximity to nutrient sources along the WFS (Figure 3.1). Stations were: inside Tampa Bay (TB-In), outside Tampa Bay (TB-Out, not sampled in 2010), outside Sarasota Bay (SB-Out), inside Charlotte Harbor (CH-In), outside Charlotte Harbor (CH-Out), offshore (Off, seaward of the

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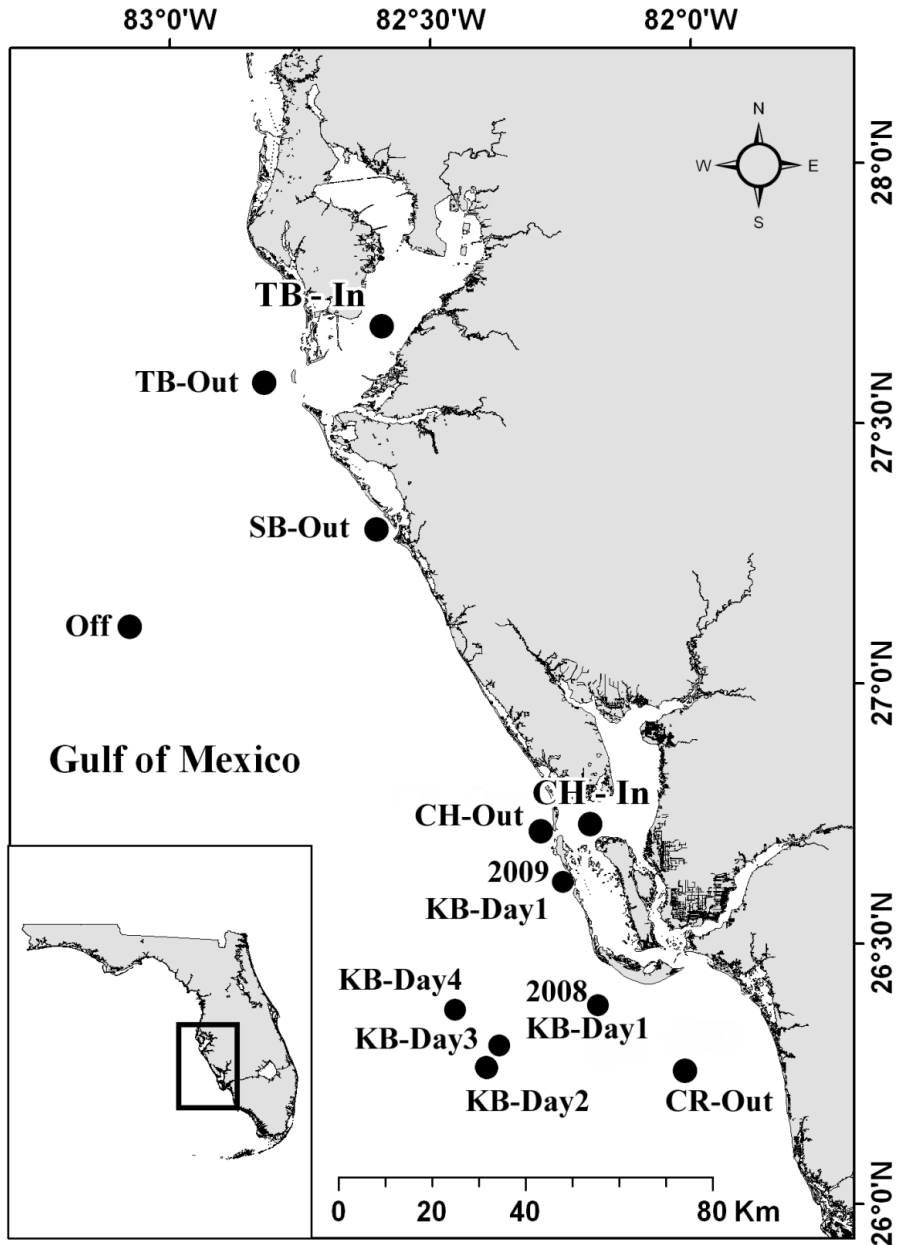
Figure 3.1: Sampling station locations for each of the three years research cruises were conducted. Insert map- Location of the study area for the ECOHAB: *Karenia* program. Bloom stations are designated by KB and the number of days spent sampling the bloom (Ex: the second day of sampling a bloom in 2009 is listed as KB-Day3).

20 m isobath), and outside the Caloosahatchee River (CR-Out, Fig. 3.1). On leg 2 sampling was conducted on consecutive days within identified blooms.

CTD profiles of the water column were taken each day within one hour of dawn at each station on both legs. Water samples for abundance and production measurements were collected concurrently using rosette-mounted 20 L Niskin bottles. Bacteria enumeration samples consisted of two 40 mL samples fixed with 2% final concentration of sterile paraformaldehyde (Troussellier et al., 1995). Two 30 mL samples for virus enumeration were fixed with 0.5% final concentration of buffered glutaraldehyde (Brussaard et al., 2010). Samples for enumeration were flash-frozen in liquid nitrogen, stored at -80 °C, and thawed in an ice bath prior to counting. Twelve 10 mL samples were taken from the same Niskin bottle for bacterial production.

In 2008 blooms were located and tracked by direct samples and cell counts of *K. brevis*. In 2009 a surface drifter with a radio beacon was deployed inside a bloom and used to track the parcel of water in which the bloom was located.

Bacteria and virus enumeration

Bacteria in samples from 2008 and virus like particles (VLPs) in samples from 2008-2010 were enumerated using SYBR® Green I nucleic acid stain and epifluorescence microscopy (Patel et al., 2007). Bacteria in 2009 and 2010 samples were enumerated using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, U. S. A.) using a combination of methods (Troussellier et al., 1995, Brussaard, 2004, Brussaard et al., 2010). SYBR® Green I and 20 µL of bead

stock (BD Trucount™ Controls in 0.02 μm Anodisc-filtered deionized water, final concentration ~2000 beads μL^{-1}) was added to each sample before analysis to increase fluorescent signature and serve as an internal reference for counts, respectively. Using CellQuest™ Pro version 4.0.2 (BD) data was collected in logarithmic mode based on side scatter (SSC, 488±5 nm) and green fluorescence (FL1, 530±15 nm), with a target even rate of 100-1000 particles s^{-1} and a target count of 20,000 total events (modified from Brussaard, 2004). Cell concentration was calculated using a modified equation from Olson et al. (1985):

(1) $Cells_{ST} = [E_{cells} \times (Beads_{ST}/E_{beads})] - Blank$

Where $Cells_{ST}$ is the concentration of total cells in the sample (cells mL^{-1}), E_{Cells} is the number of recorded events gated as bacterial cells, $Beads_{ST}$ is the number of beads added to the sample (beads in bead stock added, mL^{-1}), E_{Beads} is the number of beads recorded during acquisition of sample events, and Blank is the number of events in the gated region of a 0.02 μm filtered seawater blank (calculated from equation (1) without the variable "Blank" in the equation). The viruses to bacteria ratio (VBR) within a sample was then calculated as a measure of viral-mediated mortality (Jacquet et al., 2005). A VBR >10 is considered unbalanced growth and therefore a hostile environment for bacterial growth and a VBR < 10 is conducive for bacterial cellular division and efficient growth.

Abundance of *K. brevis* was determined by taking 1 mL subsamples from gently inverted whole water samples, fixing them with Lugol's preservative, and

allowing them to settle for at least 15 min. in individual wells of a 24-well plate before being counted using an inverted microscope. If concentrations of *K. brevis* were too high (>3000 cells mL^{-1}), samples were diluted 10-fold before being enumerated as above.

Bacterial production

Tritium (^{3}H) radiolabeled leucine (Leu) and thymidine (TdR) were added to separate whole water samples at a concentration of 10 nM ^{3}H and then incubated in on-deck flow-through incubators (Bell, 1993, Kirchman, 1993, Simon and Azam, 1989). One hour incubations were used to minimize significant changes in microbial community due to "bottle effects" (Ferguson et al., 1984, Simon and Azam, 1989). Incubations were terminated by filtration onto 0.2 μm Nucleopore filters in 2008 and 3.0 μm and 0.2 μm Nucleopore filters in 2009 and 2010 (>3.0 μm and Total size fractions, respectively). Filters were then treated with with 5% (w/vol) chilled trichloroacetic acid (TCA, Bell, 1993) for 2 min., filtered again, and triple-rinsed with with 5% TCA and single-rinsed with 80% ethanol (Kirchman, 1993). Filters were dried overnight and all samples were counted using a Packard Tri Carb 1900TR liquid scintillation counter.

Both isotopes were used because Leu incorporation is a measure of protein synthesis and TdR incorporation is a measure of nucleic acid synthesis (Franco-Vidal and Morán, 2010); the ratio of Leu to TdR incorporation provides a measure of the physiological state of the community (del Giorgio et al., 2011). A high leucine to

thymidine ratio (Leu:TdR) indicates high metabolic rates but low cellular division and therefore an environment with high cellular maintenance costs. Bacterial growth rate (BGR) was calculated by dividing isotope incorporation by cell abundance so that production on an individual-cell basis could be compared between communities to determine metabolic differences on a per-cell basis.

Statistical analysis

All statistics were done using SAS software, Version 9.2 (SAS Institute Inc., Cary, N.C., U.S.A.). All data were tested for normality; non-normal data were Log-transformed before further analysis. Data were grouped by station, year, and bloom type for analysis using a significance level of $p = 0.05$ for all tests. Bacteria and VLP abundance were compared using an ANOVA with a Tukey's post-hoc test. Incorporation rate of Leu and TdR was compared using a two-way T-Test. Correlations analyses were used to analyze all variables measured in this study with physical and environmental parameters measured during this ECOHAB regional project.

Results

Karenia brevis abundance and bloom categorization

Blooms of *K. brevis* were present during 2008 and 2009 sampling but not in 2010. Concentrations of *K. brevis* ranged from 1.18 $\times 10^5$ to 3.78×10^5 cells L^{-1} in 2008 and 1×10^3 to 5.20×10^4 cells L^{-1} in 2009 (Table 3.1). The 2008 bloom was defined as being in initiation phase. Biomass was relatively low and cell concentrations were increasing (highest abundance observed on the last day of sampling, Table 3.1) as the bloom moved from offshore to nearshore environments (physical environment aggregating the bloom, Walsh et al., 2003). Furthermore, there was no prior detection of this bloom by any regional monitoring programs. The 2009 bloom was defined as a maintenance/stationary bloom due to its offshore location (serving to disperse the bloom, Weisberg et al., in press), low to moderate concentrations of *K. brevis* present, and high numbers of lipid bodies within cells (which have been shown to indicate older cells in stationary phase, Steidinger, 1979). Abundance of *K. brevis* in the 2009 bloom remained uniform until the last day in which abundance was an order of magnitude lower (station KB-Day4, Table 3.1).

Table 3.1: Abundance of *K. brevis*, bacteria, and viruses during three bloom phases of *K. brevis* blooms along the West Florida Shelf. Abundances were determined from: light microscopy (*K. brevis*), flow cytometry (bacteria), and epifluorescence microscopy (Virus like particles and some bacteria). Virus to bacteria ratio (VBR) represents if viral abundance is high enough to cause significant mortality of the bacterial population: VBR >10 is conducive for significant viral lysis of the bacterial community (Jacquet et al., 2005). N.D.: No data available.

Station	*K. brevis* (cells L^{-1} ×10^5)	Bacteria (cells mL^{-1} ×10^6)	Viruses (mL^{-1} ×10^6)	VBR
No Bloom				
CH-Out	0	66.0 ±3.74	61.1 ±17.7	0.92±0.23
CR-Out	0	31.3 ±0.87	51.3 ±0.83	1.64±0.01
TB-In	0	70.0 ±0.0025	24.2 ±3.24	0.35±0.05
CH-In	0	88.9±0.0025	52.5 ±11.9	0.59±0.15
Initiating Bloom				
Off	0	N.D.	N.D.	N.D.
TB-Out	0	2.01 ±0.26	N.D.	N.D.
SB-Out	0	4.14 ±0.24	N.D.	N.D.
CH-Out	0	2.88 ±0.22	20.4 ±14.2	6.97±4.86
CR-Out	0	2.42 ±0.56	31.0 ±17.0	12.21±3.91
TB-In	0	2.04 ±0.092	N.D.	N.D.
CH-In	0	2.31 ±0.34	28.0 ±4.86	12.31±2.89
KB-Day1	0	2.72 ±0.35	29.9 ±7.23	10.93±2.00
KB-Day2	1.23	3.42 ±0.46	2.09 ±0.13	0.62±0.13
KB-Day3	1.18	2.71 ±0.41	2.52 ±0.26	0.95±0.21
KB-Day4	3.78	2.25 ±0.54	N.D.	N.D.
Maintenance Bloom				
Off	0	3.48	N.D.	N.D.
TB-Out	0	43.8 ±0.62	16.4	0.37±0.01
SB-Out	0	62.7 ±3.76	38.7 ±11.0	0.61±0.14
CH-Out	0.03	7.57	23.0	3.04
CR-Out	0	11.0	15.1 ±5.04	1.37±0.46
TB-In	0	71.2 ±7.29	24.7 ±7.67	0.35±0.14
CH-In	0	47.1 ±0.29	25.7	0.54±0.00
KB-Day1	0.52	10.9 ±0.66	29.1 ±16.0	2.36±1.31
KB-Day2	0.46	15.9	N.D.	N.D.
KB-Day3	0.47	74.5 ±0.54	9.12 ±1.37	1.24±0.27
KB-Day4	0.01	38.4 ±0.68	28.2 ±5.71	7.33±0.19

Bacteria and virus abundance

Bacteria and VLP abundance were grouped by station and year as combined data had high errors that overwhelmed any patterns. Bacteria abundance was not correlated to the presence or abundance of *K. brevis* in any year or bloom phase. Abundance was $2.01\text{x}10^6$-$8.89\text{x}10^7$ in the absence of *K. brevis*, and $2.71\text{x}10^6$-$1.59\text{x}10^7$ within blooms (Table 3.1). Abundance varied geographically and was positively correlated to Chl*a* ($p < 0.05$), and negatively correlated to VLP and salinity ($p < 0.05$).

VLP abundance was fairly uniform across the WFS, but higher in estuaries and estuarine outflows and negatively correlated with photosynthetically active radiation (PAR). The VBR was relatively low, < 1, for most stations, with the highest value of 12.31 inside Charlotte Harbor (Table 3.1). Daily samples from the initiating *K. brevis* bloom showed a decrease in VBR from 10.93±2.00 to 0.95±0.21. Over a similar time period within a bloom in maintenance phase, VBR increased from 2.36±1.31 to 7.33±0.19. In 2009, Bacterial abundance within the *K. brevis* bloom (maintenance phase) was significantly lower than at estuarine and nearshore stations, with station KB-Day3 and KB-Day4 having levels similar to that of station Off, which was located in oligotrophic waters. Bacterial abundance fluctuated during the four days the bloom was sampled, with the final abundance less than that of the first day (Table 3.1).

Bacterial production

Bacterial production showed different patterns for each year data were collected, prompting data to be analyzed by year and station. When no bloom was present, Leu production was highest within estuaries and decreased as distance offshore increased (Table 3.2, Fig. 3.2). TdR production was not significantly different between stations, except for CR-Out which was significantly lower ($p < 0.05$, Fig. 3.2). BGR calculated by Leucine (Leu-BGR) ranged from $4.20 \times 10^{-9} \pm 4.77 \times 10^{-10}$ to $8.21 \times 10^{-9} \pm 3.98 \times 10^{-10}$ pmol Leu cell^{-1} h^{-1} and was significantly higher within estuaries than outside ($p < 0.05$, Fig. 3.2). Leu-BGR was also negatively correlated with VPL abundance. Thymidine based BGR (TdR-BGR) ranged from $2.43 \times 10^{-10} \pm 2.25 \times 10^{-11}$ to $6.31 \times 10^{-10} \pm 1.98 \times 10^{-10}$ and followed the same pattern as production (Fig. 3.2). Both Leu- and TdR-BGR were positively correlated with RFU and PAR, and Leu-BGR was negatively correlated with VLP abundance. The Leu:TdR ratio was >10 for most stations sampled, ranging from 7.74±2.12 to 19.48±2.58.

When a *K. brevis* bloom was in initiation phase, both Leu and TdR production were significantly higher inside the bloom than in surface waters adjacent to a bloom (Fig. 3.2). The highest BGR values, for both Leu and TdR, were also samples from within the bloom (Fig. 3.2). The Leu:TdR average was 5.92±0.54 and did not show any significant difference between bloom and non-bloom stations (Table 3.2, Fig. 3.2).
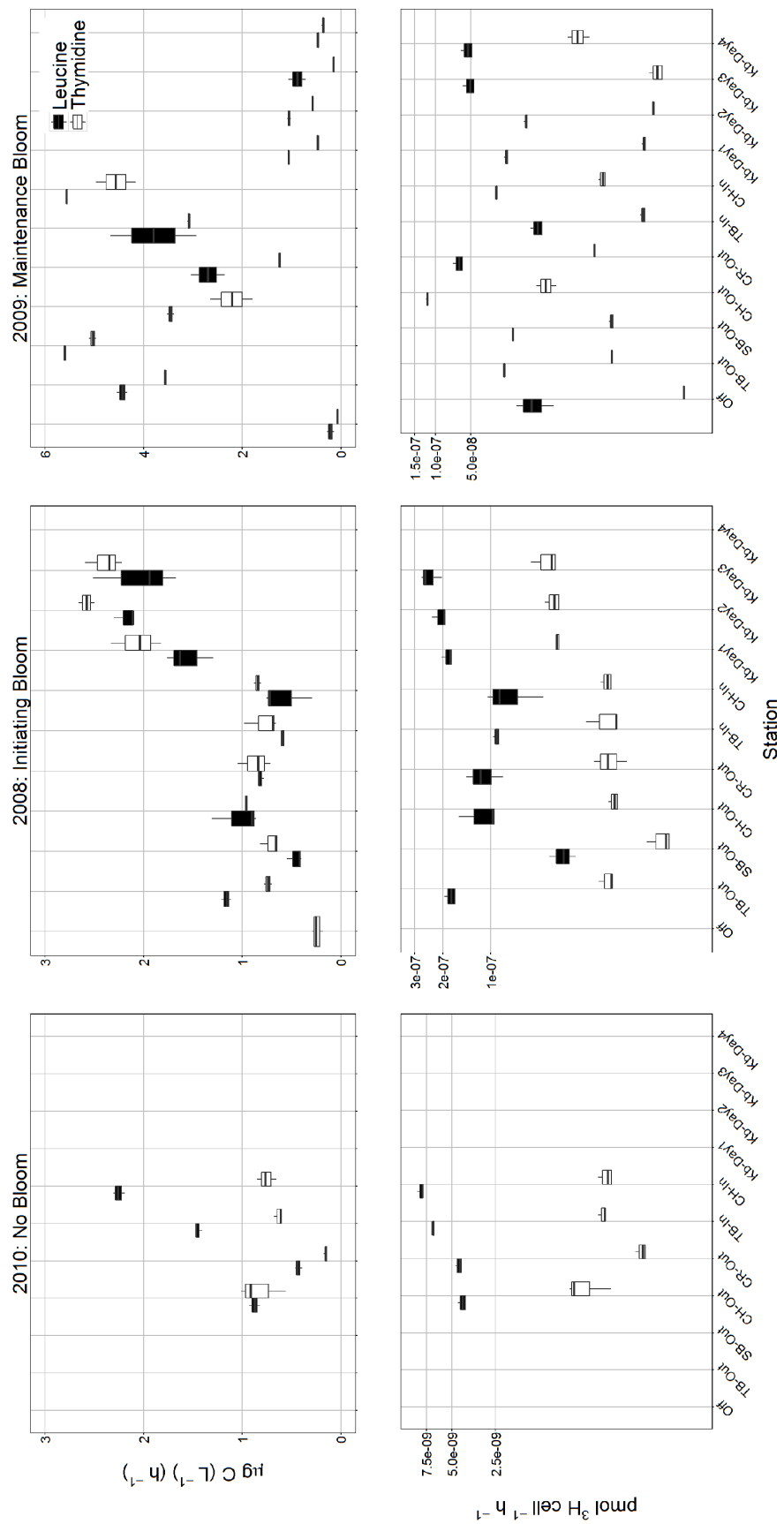
Figure 3.2: Bacterial production (μg C L^{-1} h^{-1}) determined from uptake of ^{3}H-Leucine (black boxes) and ^{3}H-Thymidine (white boxes), and bacterial growth rate (BGR, cell specific isotope uptake x10^{-8}, pmol ^{3}H cell^{-1} h^{-1}) determined from uptake of Leucine (black boxes) and Thymidine (white boxes) when no *Karenia brevis* bloom was present (left column), when a *K. brevis* bloom was initiating (center column), and when a *K. brevis* bloom was in maintenance phase (right column). The X-axes from left to right represent stations from offshore to nearshore (north to south along the Florida coast) then to estuaries and finally to within a *K. brevis* bloom. Stations with the precursor "KB-Day" represent samples taken on consecutive days (2008- 3 days, 2009-4 days) within an identified *K. brevis* bloom (bloom samples were taken in different locations each year). The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range.

Table 3.2: Production, growth, and metabolic balance of the bacterial community in three *K. brevis* bloom stages. Bacterial production was measured from incorporation of ^{3}H-Leucine (Leu) and ^{3}H-Thymidine (TdR), bacterial growth rate calculated from ^{3}H-Leucine incorporation (Leu-BGR) and ^{3}H-Thymidine (TdR-BGR), and Leucine to Thymidine incorporation ratio (Leu:TdR). Note the difference in scale for growth rate as TdR-BGR was consistently one order of magnitude lower than Leu-BGR, 10^{-9} and 10^{-8} for TdR-BGR and Leu-BGR respectively.

Station	Leu (μg C L^{-1} h^{-1})	Leu-BGR (pmol Leu cell^{-1} h^{-1} × 10^{-8})	TdR (μg C L^{-1} h^{-1})	TdR-BGR (pmol TdR cell^{-1} h^{-1} × 10^{-9})	Leu:TdR
No Bloom					
CH-Out	0.87±0.08	0.42±0.048	0.83±0.24	0.63 ±0.20	7.74±2.58
CR-Out	0.43±0.03	0.44 ±0.027	0.15±0.02	0.24±0.023	18.40±2.52
TB-In	1.45±0.04	0.67 ±0.019	0.63±0.04	0.45 ±0.032	14.87±1.03
CH-In	2.25±0.06	0.82±0.040	0.76±0.10	0.43 ±0.064	19.48±2.12
Initiating Bloom					
Off			0.24±0.06		
TB-Out	1.16±0.07	17.7 ±2.59	0.74±0.04	18.7±2.18	10.18±1.40
SB-Out	0.46±0.08	3.57 ±0.67	0.71±0.10	8.57±1.69	4.25±1.04
CH-Out	1.02±0.25	11.6 ±3.67	0.96±0.01	16.8±2.06	6.87±1.59
CR-Out	0.82±0.03	11.4±2.94	0.87±0.17	18.3±4.31	6.28±1.31
TB-In	0.59±0.03	9.17±0.70	0.78±0.18	19.2±5.17	5.64±0.45
CH-In	0.59±0.26	7.99±2.97	0.85±0.05	18.5±2.74	4.45±1.88
KB-Day1	1.56±0.24	18.5±1.56	2.06±0.26	37.9±1.44	4.91±0.61
KB-Day2	2.17±0.11	20.7±2.33	2.73±0.27	40.3±5.26	5.16±0.28
KB-Day3	2.04±0.43	24.4±3.68	2.39±0.19	45.0±9.72	5.54±1.19
KB-Day4					
Maintenance Bloom					
Off	0.21±0.10	1.92±0.95	0.07	0.98	19.51±9.66
TB-Out	4.44±0.15	3.28±0.065	3.56	4.02	8.07±0.27
SB-Out	5.60	2.77	5.04±0.10	4.02±0.32	7.30
CH-Out	3.45±0.09	14.7±0.38	2.22±0.60	14.6±3.97	10.48±3.10
CR-Out	2.79±0.49	7.91±1.42	1.25±0.01	5.64±0.049	14.04±2.63
TB-In	3.80±1.23	1.70±0.39	3.08±0.04	2.17±0.20	7.95±2.48
CH-In	5.56±0.03	3.82±0.0079	4.56±0.57	4.83±0.57	7.96±0.95
KB-Day1	1.06	3.15 ±0.21	0.47	2.14±0.13	14.67±0.08
KB-Day2	1.05±0.05	2.14±0.11	0.57	1.80±0.0076	11.88±0.56
KB-Day3	0.89±0.25	3.82±0.80	0.15±0.02	1.00±0.24	40.56±17.78
KB-Day4	0.47±0.01	3.99±0.80	0.36±0.04	4.73±1.41	8.58±0.86

Bacterial production inside a *K. brevis* bloom in maintenance phase was lower than that measured in adjacent estuarine and nearshore waters (Fig. 3.2). The lowest measurements of Leu and TdR incorporation also had the lowest calculated BGR and within the bloom both Leu and TdR incorporation decreased while corresponding BGR increased (Table 3.2). Leu and TdR incorporation measurements correlated with bacterial abundance ($p < 0.05$, Pearson's 0.83 and 0.82 for Leu and TdR respectively) but were not correlated with BGR ($p = 0.95$ and 0.29 respectively). BGR-TdR was negatively correlated with *K. brevis* abundance ($p < 0.05$, Pearson's -0.65) and positively correlated to dissolved Nitrate-N ($p < 0.05$, Pearson's 0.73) and phosphate regeneration ($p < 0.05$, Pearson's 1.0, Heil, unpublished data) possibly indicating competition for nutrients between *K. brevis* and the bacterial community. The average Leu:TdR of all stations was 13.73±5.65, with the highest value (40.56) measured within the bloom. No Leu:TdR was below 7.5 and all stations except KB-Day3 were statistically similar.

Discussion

Three consecutive years of sampling an area of the WFS known to be impacted by *K. brevis* blooms provided a fortuitous opportunity to investigate microbial dynamics in the same area when presented with no bloom, an initiating bloom, and a bloom in maintenance phase (Fig. 3.4). The oligotrophic microbial community was highly productive and efficiently growing within an initiating bloom but was metabolically stressed when the bloom was in maintenance phase. It is likely

that the microbial community structure was also changing between bloom and non-bloom states which may play an additional role in bloom dynamics (Mayali and Doucette, 2002, Roth et al., 2008a, b). Given the significant affect microbes have on biogeochemical cycles and the need to further elucidate potential nutrient sources supporting large blooms of *K. brevis*, testable hypotheses regarding bacteria-*Karenia* interactions emerge that could provide significant insight into multiple aspects of *K. brevis* bloom dynamics (Azam et al., 1983, Fuhrman, 1999, Walsh et al., 2006, Steidinger, 2009, Brand et al., 2011).

ECOHAB methods and data pooling

Bacterial production was higher than in previous studies in the same region (0.001-0.049 μg C L^{-1} h^{-1} measured by ^{3}H-Leu in 2001), which may be due in part to the smaller filter size (0.2 μm) used in this study following incubations with radioisotopes as opposed to other studies (GF/F ~0.45-1.2 μm; Heil et al., 2004), thereby increasing the capture of smaller bacteria.

When data from all years were pooled together, results were similar to a study which measured the same bacterial parameters along a transect heading from nearshore to offshore in the Pacific (del Giorgio et al., 2011). The abundance and production of bacteria decreased as distance offshore increased and VLP abundance was uniform across all stations. Comparing within-year stations highlighted differences in microbial abundance and production between bloom and non-bloom stations and comparing between-year data highlighted differences between microbial

abundance and production when *K. brevis* was in initiating phase, maintenance phases, and not present.

No bloom

The absence of a bloom of *K. brevis* in 2010 presented the opportunity to study the microbial community of the WFS without any influences of *K. brevis* during the same time of year as previously studied blooms. Bacteria and VLP abundance was highest within estuaries and decreased offshore, however, the virus to bacteria ratio (VBR) was < 2 at all stations, which may indicate that viruses were infecting and lysing bacteria but were not a significant source of mortality at the time of sampling (Jacquet et al., 2005, Table 3.1).

Bacterial production was unbalanced, Leu:TdR > 10 for all stations except CH-Out, indicating that the environment was metabolically demanding and that most fixed carbon was being metabolized rather than being used for cellular division (del Giorgio et al., 2001). Positive correlations existed between bacterial production and growth rate and nutrients which furthers the idea that a majority of the bacterial communities along the WFS were nutrient limited. Data show a preference of the bacterial community for environments with higher inorganic nutrients (correlations only between BGR and inorganic nutrients), which are typically low along the WFS (Heil et al., 2007). As bacterial production, but not abundance, is correlated to inorganic nutrients, and ammonia in particular, it is possible that the phytoplankton

community is responding to the inorganic nutrients and the heterotrophic bacteria are then utilizing organics produced by the phytoplankton (Lenes et al., 2001).

While no bloom was present in 2010, data from this year demonstrate that bacterial production along the WFS was typical of an oligotrophic system. Higher production was located in and around estuaries and decreased seaward from the shore. Bacterial production was unbalanced and would have required dramatic increases in nutrient inputs before nutrient limitation could be overcome and efficient growth achieved. The implication of this is that the natural, non-bloom, microbial community on the WFS is capable of responding quickly to new nutrient sources if given enough time to overcome initial limitations on growth and production.

Initiating bloom

Bacterial abundance was only significantly correlated with primary production and showed no geographic patterns in 2008. Leu:TdR indicates that bacteria were efficiently growing at all but one station (Table 3.2, Fig. 3.3). Despite uniformities in abundance and growth efficiency, stations with *K. brevis* present exhibited high rates of Leu and TdR production as well as Leu-BGR and TdR-BGR (Fig. 3.3). An increase in production, especially TdR production, is typically coupled with an increase in bacterial abundance yet no significant increase in abundance was observed. This indicates a highly productive bacterial community within *K. brevis* blooms that also had a high rate of mortality.
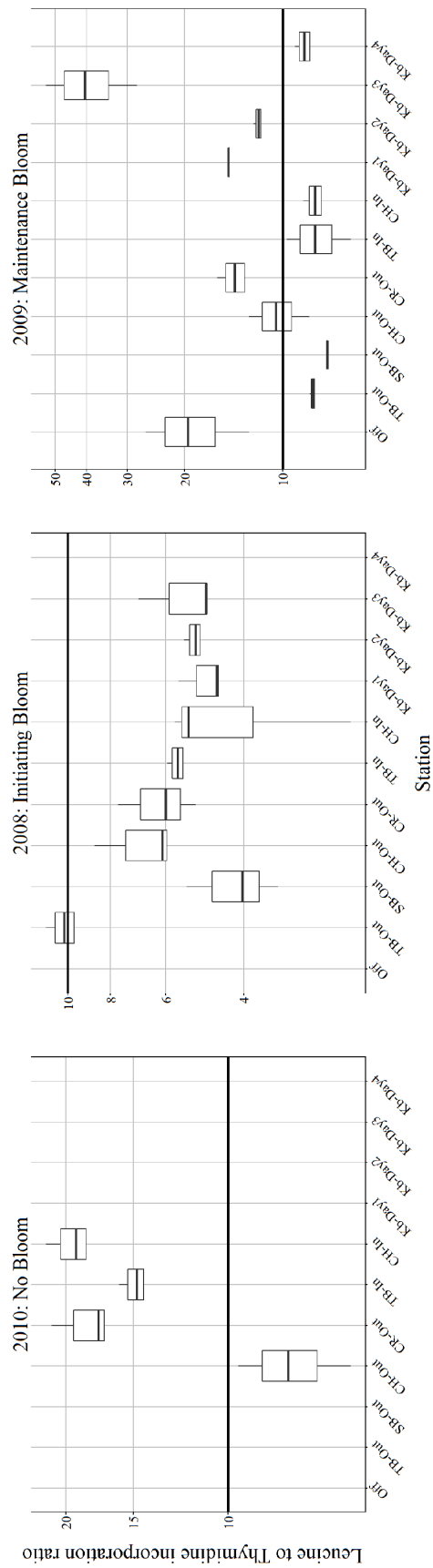
Figure 3.3: Leucine to Thymidine incorporation ratio (Leu:TdR, pmol Leu L^{-1} h^{-1}: pmol TdR L^{-1} h^{-1}) during the three *K. brevis* bloom stages sampled in this ECOHAB: *Karenia* project. As Leu is a measure of biomass production while TdR represents cell division (nucleic acid synthesis), the balance between the two represents the environmental maintenance costs of the bacteria and therefore indicate whether the growth is balanced (<10) or unbalanced (>10, Franco-Vidal and Morán, 2010, del Giorgio et al., 2011). Black bars denote a Leu:TdR ratio of 10.

One vector capable of controlling bacterial abundance is viral lysis. Jacquet et al. (2005) theorized if the ratio of viruses to bacteria ratio (VBR) is > 10 then conditions are likely conducive for virally-mediated lysis, and conversely a VBR < 10 demonstrate low levels of viral-mediated bacterial mortality. During the bloom, VBR decreased from 10.93 ± 2.00 at station KB-Day1 to 0.62 ± 0.13 at KB-Day2 and 0.95 ± 0.21 at station KB-Day3 (Table 3.2). This indicates a reduction in virally-mediated lysis as the bloom progressed (Wilcox and Fuhrman, 1994, Jacquet et al., 2005).

Studies have demonstrated that *K. brevis* is a mixotroph, capable of utilizing bacteria as a supplemental nutrient source at rates of 0.96-83.8 bacteria *K. brevis*$^{-1}$ h^{-1} (Glibert et al., 2009). Grazing rates were calculated from within the initiating bloom and found to be on the order of 1 bacteria *K. brevis*$^{-1}$ h^{-1} (L. Procise, Pers. Comm.). If the decreases in bacterial abundance observed on consecutive days within the same bloom represented total losses of bacteria to mortality, this conservative grazing rate could account for 121% and 158% of bacterial mortality between stations KB-Day2 and KB-Day3, and KB-Day3 and KB-Day4 respectively. Considering *K. brevis* grazing rate is low compared to laboratory estimates of *K. brevis* ingestion of heterotrophic bacteria (L. Procise, Pers. Comm.), bacterivory is very likely to be the cause of some, if not all, of the decrease in bacterial abundance observed. This indicates that *K. brevis* could have been using the highly productive bacterial community as an additional nutrient source to support bloom growth and eventually bloom maintenance.

Primary production had no correlation to Leu production, a positive correlation with TdR production (0.44), and a negative correlation with Leu:TdR (-0.50), indicating that bacterial abundance, and not metabolism, was responding to changes in primary production. TdR production was highest at stations where *K. brevis* was present (KB-Day1, KB-Day2, and KB-Day3) indicating that bacterial division was the most active within the bloom (Fig. 3.2). Given such, it is very likely the composition of the bacterial community was shifting throughout the course of the initiating bloom with the mortality limited to only a portion of the bacterial community (Hare et al., 2005). Unaffected bacteria would be highly productive and efficiently growing. This coincides with other hypotheses that the bacterial community composition is changing in response to the presence and abundance of *K. brevis* (Jones et al., 2010, Sipler et al., in press).

Observations from the 2008 bloom demonstrate that the microbial community (both bacteria and viruses) is very active during bloom initiation. The bacteria are growing efficiently and, coupled with the decrease in VLPs, appear to be serving as a nutrient source to supplement *K. brevis* nutritional requirements (Hare et al., 2005). This relationship between viruses, bacteria, and *K. brevis* could be shifting the bacterial community structure which may further alter the productivity and function of the microbial community.

Maintenance phase bloom

In 2009, when a bloom in maintenance phase was observed, abundance and production were highest in estuaries, lower where *K. brevis* was present, and was lowest at the most offshore station (no *K. brevis*, Table 3.2, Fig. 3.2). All stations exhibited high Leu:TdR, which indicated unbalanced growth and therefore a nutrient limited bacterial community along the WFS in 2009 (Fig. 3.3).

Using the same grazing rate as above (1 bacteria *K. brevis*$^{-1}$ h^{-1}), grazing by *K. brevis* on bacteria could account for 20% and 25% of the decrease in bacteria abundance between stations KB-Day2 and KB-Day3 and KB-Day3 and KB-Day4, respectively. As hypothesized above, bacterivory during bloom formation could significantly reduce bacterial abundance and increase *K. brevis* abundance, therefore leading to decreased competition between bacteria and *K. brevis* for available nutrients. However, bacterivory alone would not account for the entire decrease in bacterial abundance, especially considering *K. brevis* abundance was decreasing during this same time period, reducing its potential role as a source of bacterial mortality.

Bacterial production data further support virally-mediated lysis playing a large role during this bloom. Between KB-Day3 and KB-Day4 neither Leu production nor Leu-BGR changed significantly while TdR production increased by 237% and TdR-BGR increased by 475% (Fig. 3.2). This demonstrates that the bacterial community was channeling more energy into cellular division than metabolism but still decreasing in abundance by more than 50%. This magnitude of viral mediated

mortality is within ranges estimated in other studies (Bratbak et al., 1992, Bratbak et al., 1993, Suttle, 1994). Since a majority of viruses are host-specific, it is possible that high viral-mediated mortality of bacteria was also causing a shift in community composition (Fuhrman, 1999).

The microbial community in 2009 demonstrated balanced growth only in estuarine environments. In both offshore and bloom environments, bacterial production was significantly lower, the Leu:TdR was >10, and abundance was significantly lower or decreasing, which demonstrate a microbial community that was highly stressed. That this was co-occurring with a *K. brevis* bloom in maintenance phase that was also decreasing in abundance suggest there may be important interactions between bacteria and *K. brevis* that could either maintain or terminate a bloom (Paul et al., 2002, Roth et al., 2008a, b).

Bacterial production in different *K. brevis* bloom phases indicate synergistic relationships between bacteria and *K. brevis*

The difference in bacterial production between a non-bloom year, initiating *K. brevis* bloom, and bloom in maintenance phase were striking. High bacterial production during the initiating bloom in 2008 suggest that the bacterial community being physically entrained in the same water mass as *K. brevis* (Weisberg et al., in press) was significantly contributing to the carbon cycle and possibly helping to augment growth of the bloom. This is likely through the exchange of materials between *K. brevis* and associated bacteria (Amin et al., 2012). Identifying such materials and determining the rates of transfer would help to determine if bacteria

have synergistic relationships with *K. brevis*, and if so, how such interactions impact nutrient pools *K. brevis* uses to achieve high biomass in the nearshore environments were blooms occur (Vargo et al., 2008).

The relatively low bacterial production observed in a bloom in maintenance phase was a surprise as it would be expected that as *K. brevis* cells begin to die bacteria would remineralize their organic matter, increasing production. It may be that the bloom observed represented a transition in the bacterial community in which the community structure was changing from bacteria which utilize compounds produced by *K. brevis* (synergism) towards bacteria which decompose dead *K. brevis*. During this transition it is possible there would be a drop in bacterial production as the synergistic bacteria lose productivity and decomposing bacteria are still in lag phase growth. Increasing the number of measurements during a bloom as it transitions from initiation to maintenance phase, and then into termination, would help to address this hypothesis. Using multiple size fractions, as was done in this study, would determine if bacterial production shifted from smaller, 0.2-3 μm, free-living bacterial size fractions to larger, >3 μm, size fractions which indicate particle attached bacteria (bacteria attaching to dead *K. brevis*).

Bacterial communities are very dynamic and have a large impact on biogeochemical cycles, including those within blooms of marine phytoplankton (Riemann et al., 2000). The three "bloom snapshots" obtained in this study demonstrate that the bacteria associated with *K. brevis* blooms may also go through transitions that follow the state of the bloom (initiation, maintenance, termination, no bloom). More measurements are necessary to not only fill in the gaps so that a more

complete picture of bacteria dynamics throught the evolution of a *K. brevis* bloom is obtained, but also to better identify potential synergistic interactions between *K. brevis* and bacteria. Such interactions may help promote bloom formation and maintenance through increase production within the microbial loop and may also serve as a vector for bloom termination (Roth et al., 2008). Understanding these dynamics will assist in *K. brevis* modeling, management, and mitigation efforts.
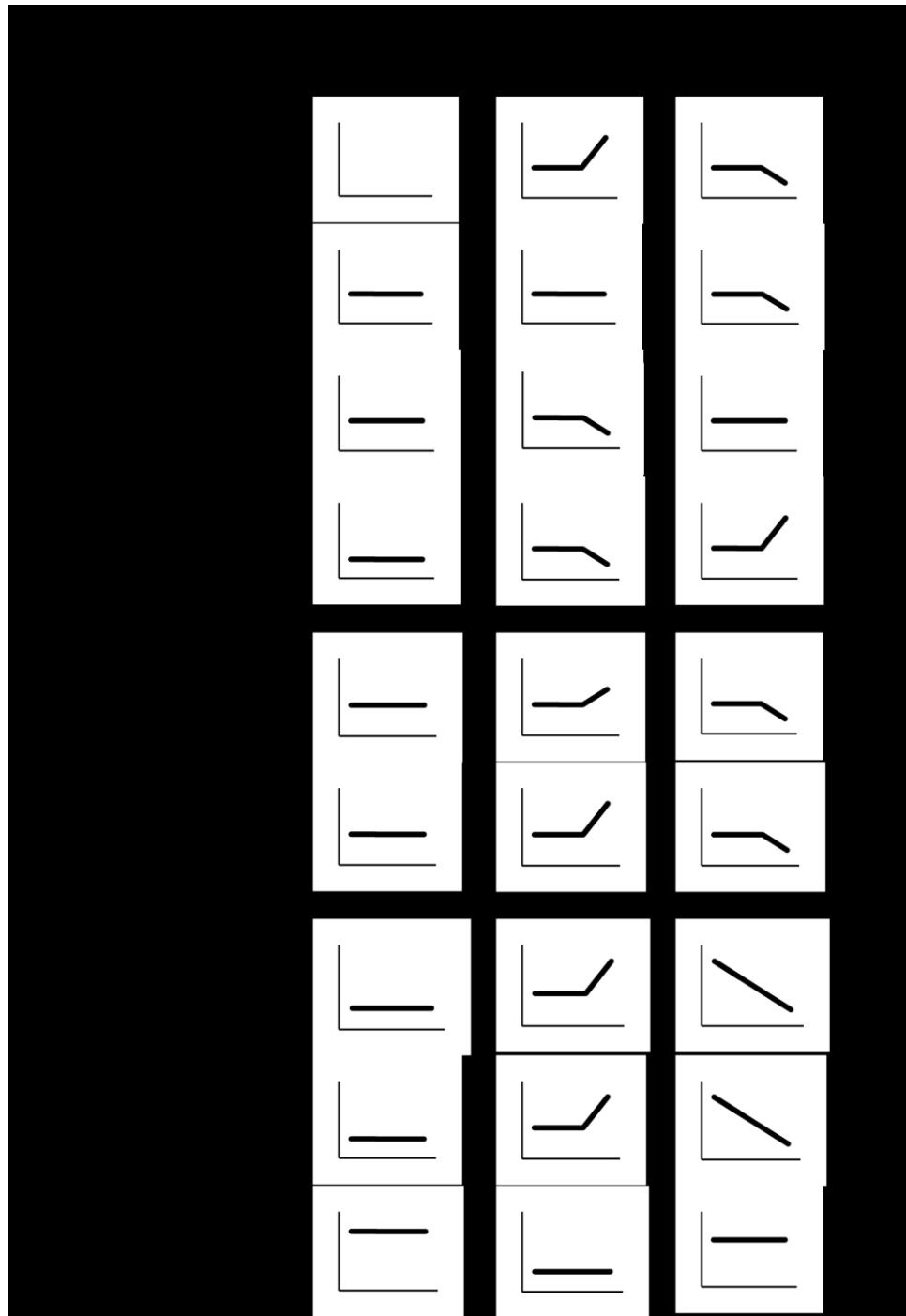
Figure 3.4: Change in measured parameters over time based on data obtained during sampling when no *K. brevis* bloom is present, during an initiating bloom, and within a bloom in maintenance phase. Units are: *K. brevis* (cells L^{-1}), bacteria (cells mL^{-1}), virus like particles (mL^{-1}), leucine/thymidine production ($\mu g\ C\ L^{-1}\ h^{-1}$), and leucine/thymidine bacterial growth rate (pmol ^3H cell^{-1} h^{-1}).

Chapter 4: Microbial community composition surrounding blooms of *Karenia brevis* on the West Florida Shelf using high-throughput sequencing

Abstract

High-throughput DNA sequencing was used to investigate the bacterial community composition in surface waters of the West Florida Shelf (WFS) inside and outside a bloom of the red tide dinoflagellate *Karenia brevis* in 2009 and when no bloom was present in 2010. All communities sampled were dominated by the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex, the cyanobacteria *Synechococcus*, and *Alphaproteobacteria* with relatively little variation between communities sampled within the same year. Sequences matching algicidal bacteria strains S03 and 41-DBG2, both in the CFB group, as well as the algicidal resistance-conferring bacteria strain D38BY were found in both bloom and non-bloom communities using BLAST. Interactions between these strains, and between *K. brevis* and these strains are not well understood, but may play a critical role in the timing and extent of bloom formation and termination. The SAR406 group was also found in all samples despite typically being more prevalent in deep waters (below the deep chlorophyll maximum to within 10m of the seafloor, Gallagher et al., 2004). The presence of SAR406 in the surface waters of the WFS supports existing *K. brevis*

bloom formation hypotheses and could potentially be used as an indicator species for conditions conducive to bloom formation and forecasting. This study highlights the need for more detailed investigations into individual components of the microbial community of the WFS and within different bloom stages of *K. brevis*. Understanding the associated microbes and potential interactions between the components of the bacterial community and *K. brevis* may improve bloom modeling, forecasting, and mitigation strategies by clarifying nutrient pathways and rates as well as better identifying the trophic structure of the ecosystem.

Introduction

In the State of Florida, there are near-annual blooms of the "Red Tide" dinoflagellate *Karenia brevis* (Davis) G. Hansen et Moestrup along the West Florida Shelf (WFS). Much research has on blooms of *K. brevis* have focused on the dinoflagellate while relatively few studies have focused on microbial communities surrounding blooms. Early studies of *K. brevis* in Florida used a variety of culture media techniques to isolate bacteria primarily by plating methods (Bein, 1954). Using plate methods chromogenic bacteria were observed to be dominant in blooms of *K. brevis* on the WFS and in unialgal cultures (Bein, 1954, Collier, 1958). These studies concluded with two hypotheses regarding potential relationships between bacteria and *K. brevis*: 1- Toxins produced by bacteria could have a role in mortality associated with blooms (Bein, 1954), and 2- A positive feedback exists between *K. brevis* and

certain bacteria in which the dinoflagellate provides organic substrate necessary for bacterial growth and bacteria provide vitamins needed by the dinoflagellate (Collier, 1958).

Neither hypothesis was tested in subsequent studies of either natural blooms or *K. brevis* cultures. The next studies investigating bacteria bloom interactions were conducted during natural blooms in the summer of 1971 and fall of 1972 which used a combination of aqueous and plate culturing methods to isolate and identify bacteria (Evans, 1973). A combination of sea water nutrient broth culturing and subsequent plating on sea water nutrient agar revealed the chromogenic bacteria from eight of ten water samples taken in 1971. Streaking of water samples followed by plate counts revealed a weak positive relationship between bacteria and *K. brevis* abundance in 1972. These data were then used to combine the two previous hypotheses into an overarching bloom formation hypothesis in which one type of bacteria (bacteria II) promoted bloom formation by synthesizing needed substrates and then a second bacteria (bacteria I) became dominant subsequent to bloom formation (Figure 4.1, Evans, 1973). According to this hypothesis prolonged blooms could occur due to overlapping cycles of bacteria (I) fueling the growth of the *Karenia* bloom by remineralizing dead fish to promote bacteria (II). The study could not accurately identify any component of either bacterial group.

Molecular techniques were applied to assess the bacterial community associated with *K. brevis* blooms as part of two different studies in 2001 and 2002. Samples were taken inside and outside identified bloom patches as part of a transect
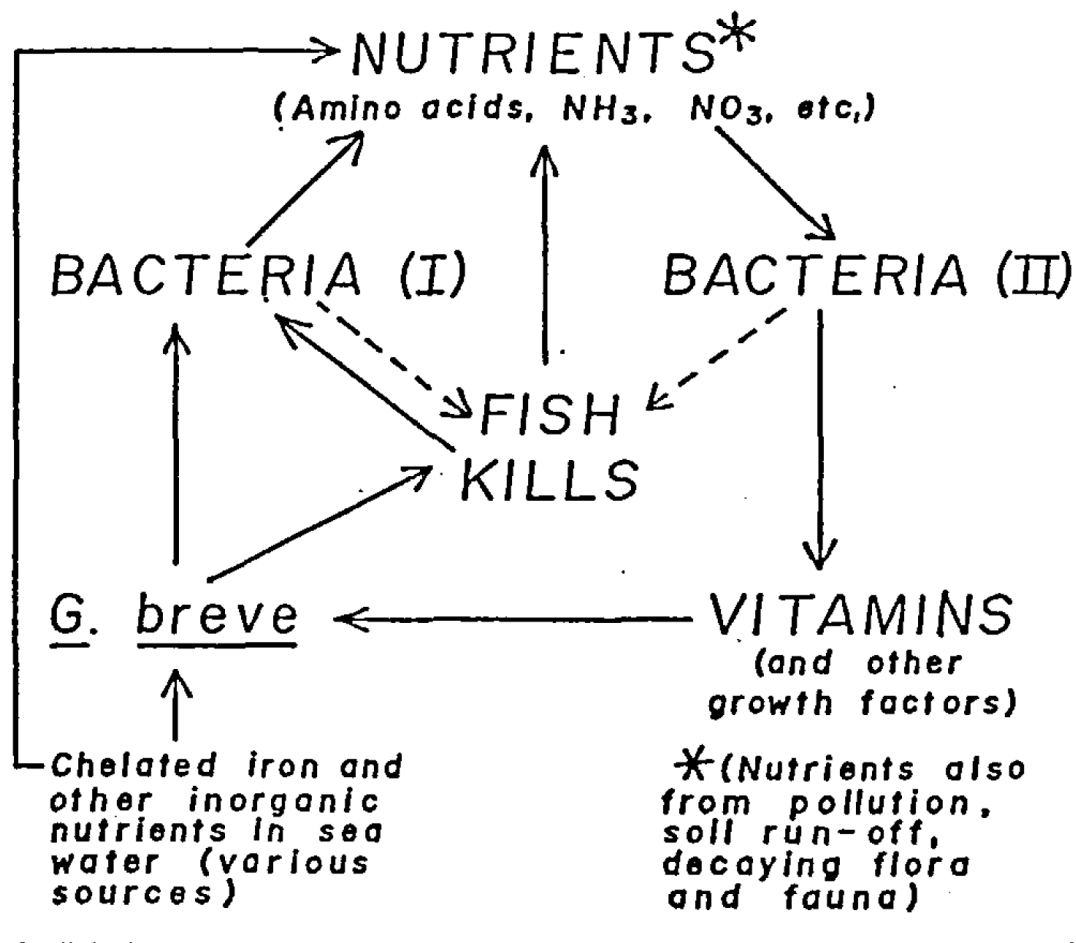
Figure 4.1: An early hypothesis regarding the relationship between *K. brevis* and bacteria in naturally occurring blooms in the Gulf of Mexico. Bacteria (II) produce compounds necessary for *Gymnodinium brevis* (*Karenia brevis*) growth, allowing blooms to form. Once a bloom becomes toxic, bacteria (I) capable of using organic matter from decaying fish become more dominant and remineralize nutrients needed to being the cycle anew. If cycles began to overlap, it would appear as large continuous bloom. From Evans, 1973.

in 2001 (Jones et al., 2010) and a Lagrangian study following a drifter in 2002 (Hewson et al., 2006). These studies used two different methods for both processing and analyses and thus achieved different results regarding the composition of microbes on the WFS, and consequently different conclusions were drawn within the context of each of the sampled conditions. The transect study used clone library plasmid sequencing for phylogenetic comparison against existing databases to identify members of the sampled community and analyzed any changes due to environmental variables measured using the Shannon diversity index (Jones et al., 2010). In the Lagrangian study automated rRNA intergenic spacer analysis (ARISA) was used which allows for easy comparison between samples taken at different locations and times, and used the Whittaker index (Hewson et al., 2006).

The transect study used a Principal Coordinate Analysis on data and found that bacteria communities were primarily separated by the concentration of *K. brevis*. Samples were then grouped for diversity comparison by a threshold value of *K. brevis* concentration: $\leq$20 *K. brevis* cells mL^{-1} or $\geq$500 *K. brevis* cells mL^{-1} (Jones et al., 2010). The Shannon diversity index did not vary between samples (mean = 3.59 for all samples), and the major differences between bacterial communities associated with the two concentrations of *K. brevis* were within bacterial orders. For example, the *Rhodobacterales* (*Alphaproteobacteria*) and *Cytophagales*/*Sphingobacteriales* (*Bacteriodetes*) were more abundant in samples with high concentrations of *K. brevis* while *Cyanobacteria* were dominant when *K. brevis* was not present or in low concentrations. This suggests that changes in the bacterial community are

metabolically driven by potential synergistic relationships that would also benefit *K. brevis* and the proliferation of blooms.

The Lagrangian study was a multi-year effort in the offshore oligotrophic waters of the WFS that captured blooms of both *K. brevis* (2002) and the diazotrophic cyanobacteria *Trichodesmium* (2001 and 2003, Hewson et al., 2006). This study found lower variability between bacterial fingerprints from within the *K. brevis* bloom, but no significant differences between Shannon index values (mean = 3.37 for all samples). Bacterial assemblage variability was highest in 2002, when a *K. brevis* bloom was sampled; the daily change in whole bacterial assemblages observed in this study suggest a high temporal variation in the bacterial community on the WFS (Hewson et al., 2006). The dynamic nature of the bacterial community observed in this study inside and outside observed blooms suggested an ecosystem in which such shifts are a common occurrence; whether bacterial diversity is influenced by bloom presence or stage needs further research.

Given that bacterial diversity is often linked to ecosystem function several studies investigated potential relationships between *K. brevis* and associated bacteria. One avenue of study was to determine if bacteria could be a vector for bloom prevention or termination. This was prompted when several bacteria were isolated from bloom and nonbloom waters of the WFS (Doucette et al., 1999, Roth et al., 2008b) and screened for algicidal activity (lysis of algal cells within 1-2 days of introduction of bacteria or bacterial compounds, Mayali and Doucette, 2002). Two such bacteria were found to be algicidal to *K. brevis*, *Cytophaga*/*Flavobacterium*/*Bacteroidetes* (CFB)-bacterium strain S03 and CFB-

bacterium strain 41-DBG2 (Roth et al., 2008b). Strain S03 required direct contact with the algal culture whereas strain 41-DBG2 was capable of indirect algicidal activity. A third bacteria classified as *Tenacibaculum* strain D38BY provided resistance to algicidal attack from strain S03 while cultures which were bacteria-free did not have any resistance to algicidal effects (Roth et al., 2008a). The dynamics of both algicidal and algicidal-resistance bacteria demonstrate that not all bacteria are influencing *K. brevis*, and *K. brevis* blooms, via metabolism and metabolic requirements/products. These studies emphasize the multiple potential interactions between bacteria and *K. brevis* in both naturally occurring blooms and cultures which can change based on the composition of the bacterial community.

There are numerous interactions between algae and both planktonic and attached bacteria. These interactions become more critical in the case of algal blooms as they can either prolong or terminate the bloom depending on the relationship. Such interactions can change over the course of the bloom as physiological and molecular changes take place, and nutrient pools can lead to a succession in the bacterial community which can lead to portions of the bacterial community becoming dominant or the bacterial community remaining mixed (Doucette, 1995). Previous studies of bacteria associated with naturally occurring *K. brevis* blooms demonstrate high temporal variability within communities with the potential for certain bacterial groups to become dominant. This highlights the need to further study bacterial communities within naturally occurring blooms to assess whether:

1. Certain bacterial communities are dominant within blooms of *Karenia brevis*.

2. Different bacterial communities are dominant within different bloom stages of *K. brevis*.

3. Previously identified algicidal bacteria from the Gulf of Mexico (strain S03 or 41-BDG2) are present within any given bloom, and individual bloom stages.

4. Bacteria capable of conferring algicidal resistance, such as strain D38BY, are present within any given bloom or specific bloom stage of *K. brevis*.

This study was designed to investigate microbial community composition inside and outside naturally occurring blooms of *K. brevis* along the WFS using high-throughput sequencing technologies. The primary focus was to identify the bacterial community inside and outside of *K. brevis* blooms, and to measure metrics of community diversity. A secondary objective was to search for identified algicidal and algicidal resistance-conferring bacteria within sampled communities from the WFS and naturally occurring blooms to determine if such bacteria were present. This study will provide information regarding bacterial community composition surrounding naturally occurring blooms of *K. brevis* on the WFS using state-of-the-art DNA sequencing technologies and the most up-to-date bacterial genomic databases. Emphasis was placed on identifying the primary components of the bacterial community, characterizing community diversity inside and outside blooms, and searching for identified bacteria of interest (algicidal and algicidal-resistance conferring bacteria).

Methods

Sampling the microbial community on the West Florida Shelf

Two-week research cruises were conducted aboard the R/V *Pelican* in October 2009 and 2010. The first week of each cruise mapped the spatial distributions of nutrients, microbial abundance, and bacterial production along the WFS while also searching for *K. brevis* populations. The second week of each cruise focused on sampling identified *K. brevis* populations and other targeted studies. Cruises occurred on 2-12 October, 2009 and 13-23 October, 2010. Stations were sampled along the WFS and named according to geographic proximity to nutrient sources. Only a few stations had successful DNA extractions and amplifications. In 2009 those stations were: outside Tampa Bay (TB-Out), within an identified *K. brevis* bloom on consecutive days (KB-Day1, Day2, Day3), and adjacent to the bloom in a coastal environment (KB-adjacent, Fig. 4.2). In 2010 extractions were done on two samples, one each from outside Charlotte Harbor (CH-Out) and nearshore to Bonita Springs and Naples, Florida (Coastal, Fig. 4.2). The limited number of samples from 2010 was due to the fact that no *K. brevis* blooms were found during the second week of the cruise (Weisberg et al., in press).

Profiles of the water column were taken each day using a CTD within one hour of dawn at each station on both legs. Surface samples were collected using rosette-mounted 20 L Niskin bottles. Water was transferred to acid-rinsed

polycarbonate containers for DNA collection (Crump et al., 2003). Using a 60-mL syringe with a Sterivex-GP™ 0.22 μm pressure driven filter attached, approximately 500 mL of surface water was passed through each filter. The syringe was then used to remove all water from the Sterivex™ before adding ~1 mL of DNA extraction buffer (DEB) to the filter (Crump et al., 2003). Sterivex™ filters were then sealed and flash-frozen in liquid nitrogen until analysis.

DNA Extraction

Frozen Sterivex™ filters were processed for DNA extraction (Crump, 2007). Filters were manually opened under sterile conditions and the DEB within the filter cartridge was distributed between two sterile 2 mL microcentrifuge tubes (Corning Incorporated, Corning, New York, U.S.A.). The filters were cut into ~12 pieces and also divided between the two microcentrifuge tubes. All tubes then had 20 μL of proteinase-K (10 mg mL^{-1}) and 20 μL of lysozyme (100 mg mL^{-1}) added before undergoing three rounds of a freeze-thaw cycle consisting of 15 minutes at -80 °C and 5 minutes at 37 °C. Following the freeze-thaw cycle, all samples were incubated in a 37 °C water bath for 30 minutes, 50 μL of filter-sterilized sodium dodecyl sulfate (SDS) was added, and then incubated for 120 minute in a 65 °C water bath.
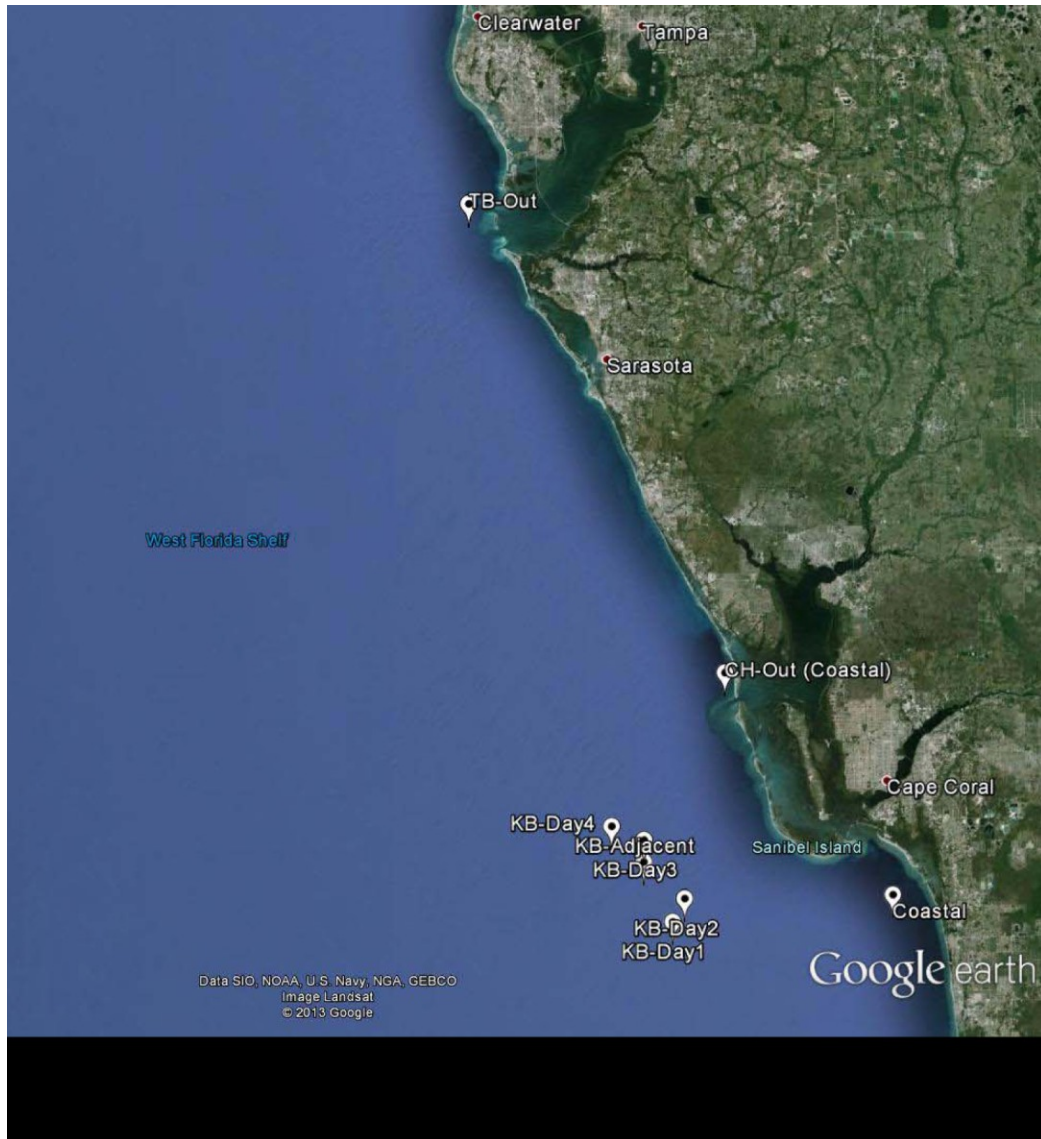
Figure 4.2: Stations on the West Florida Shelf during the 2009 and 2010 ECOHAB: *Karenia* project process cruise from which DNA was amplified. Stations sampled in 2009 were outside Tampa Bay (TB-Out), within a bloom sampled on consecutive days (KB-Day1, Day2, Day3), and adjacent to the bloom (KB-Adjacent). In 2010 samples were taken outside Charlotte Harbor (CH-Out) and nearshore to Naples and Bonita Springs Florida. Map from Google, 2013.

Upon removal from the water bath, the DNA/filter/DEB slurry was washed three times with phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). Room temperature isopropanol (100%) alcohol was added to the slurry (0.6 volumes of the slurry) and incubated at room temperature of ~120 minutes. The DNA was then spun down into a pellet at high speed (1300 rpm centrifugation) for 30 min. before rinsing the pellet three times with 70% ethanol. The pellet was then dried in a roto-evaporator before being re-suspended in 250 µL autoclaved-UV sterile ultra-pure water. One of the two aliquots was archived in a -80 °C freezer and the other used for amplification and subsequent sequencing (Crump, 2007).

Amplification of genetic material

Extracted DNA was amplified in triplicate using a modified Polymerase Chain Reaction (PCR, Crump et al., 1999) with primers designed to amplify the V2, 27F hypervariable region of the 16S ribosomal RNA genes while simultaneously adding unique barcoded reverse primers (Hamady et al., 2008). Primers for the 454 pyrosequencing samples were bacteria-specific with 454B FLX linker (GCCTTGCCAGCCCGCTCAG TC AGRGTTTGATYMTGGCTCAG) and 338R with 454A linker containing a unique 8 base pair barcode, denoted by N in primer sequence (Modified from Hamady et al., 2008): GCCTCCCTCGCGCCATCAG NNNNNNN CA TGCWGCCWCCCGTAGGWGT. Primers for the Illumina samples were bacteria-specific 515F Earth Microbiome Project (EMP) primers with 806 reverse primers, Golay barcodes (denoted by N), reverse primer pad, reverse primer linker, and reverse primer (806r): CAAGCAGAAGACGGCATACGAGAT

NNNNNNNNNNNN AGTCAGTCAG CC CCGGACTACHVGGGTWTCTAAT

(Caporaso et al., 2012).

Cycling conditions were: initial denaturing at 94 °C for 3 min; 30 cycles of 30

s at 94 °C, 45 s at 57 °C, and 60 s at 72 °C; with a final extension step of 2 min at 72

°C (Crump et al., 1999, Sogin et al., 2006). Amplification was confirmed with gel

electrophoresis using a 2.5% agarose gel (Agarose 3:1, Thermo Fisher Scientific)

with TAE (0.04 M Tris-acetate, 1mM EDTA) and SYBR® Green I nucleic acid stain

(Invitrogen) added. Triplicate amplifications were combined and quantified with a

Quant-iT™ PicoGreen® double-stranded DNA Assay Kit according to the

manufacturer's instructions (Life Technologies Incorporation) and then purified with

a S.N.A.P. UV-Free Gel Purification Kit (Invitrogen, Carlsbad, CA), by gel isolation

from a 0.8% agarose gel according to manufacturer's instructions.

Sequencing and Community Assessment

Purified samples from 2009 were sent for pyrosequencing on a Roche-454

FLX Pyrosequencer at Engencore at the University of South Carolina using titanium

chemistry (http://engencore.sc.edu/). Samples from 2010 were sent for sequencing on

the Illumina-MiSeq (Illumina, Inc.) platform at the University of Maryland Genomics

Resource Center (http://www.igs.umaryland.edu/grc). Sequences were demultiplexed

based on the TAG barcodes and primer sequences removed using the Quantitative

Insights Into Microbial Ecology (QIIME, Caporaso et al., 2010) pipeline. Additional

quality control, alignment, and operational taxonomic unit (OTU) assignment was

done with QIIME, while taxonomy assignment was done in the programs QIIME (Illumina sequences) and Mothur (454 sequences, Schloss et al., 2009).

Pyrosequencing demultiplexed flowgrams had distances calculated and were clustered using Complete Linkage Hierarchical Clustering. Single nucleotide polymorphisms brought about as an artifact of the pyrosequencing process and distances errors brought about by PCR were then checked for and corrected using the PyroNoise and SeqDistT programs (Quince et al., 2011). The SeqNoise and Perseus programs were then used to removed Single-base errors and PCR chimeras, respectively (Gaspar and Thomas, 2013). Sequences were then assigned to operational taxonomic units (OTUs) with the uclust method in QIIME, which generates clusters of sequences based on percent identity of sequences (Edgar, 2010). Taxonomic assignments were then generated for representative sequences using the program Mothur, with classifications based on the GreenGenes May, 2013 database (Schloss et al., 2009).

The QIIME pipeline for Illumina sequences first requires sequence reads to be paired together (Sequences are generated separately for the 5' and 3' strands of DNA) and demultiplexed; joining overlapping 5' and 3' strands together and separating them by sample using barcoded primers (D. Smith, Argonne National Laboratory, 2012). Demultiplex sequences were assigned OTUs using the usearch method; similar to the uclust method with the additional steps of filtering low abundance clusters and chimera detection (Edgar, 2010). Sequences were aligned using Python Nearest Alignments Space Termination Tool (PyNAST, Caporaso et al., 2010) and the Greengenes May 2013 taxonomic database as a template (McDonald et al., 2012).

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Taxonomy was assigned with the Ribosomal Database Project (RDP) Classifier which uses a naïve Bayesian classifier which allows for a high level of taxonomic assignments even with partial sequences (Wang et al., 2007).

Diversity analysis

All sequences were rarefied before diversity analyses: a process to maximize the number of samples and unique sequences per sample, in this case 12,000 sequences per sample (Sogin et al., 2006). Alpha diversity, a measure of species richness within an individual sample, was measured using the default QIIME workflow which used Faith's Phylogenetic Diversity metric (Faith, 1992). Beta diversity, a measure of taxonomic diversity between samples, was calculated with weighted and unweighted UniFrac analysis (Lozupone and Knight, 2005, Lozupone et al., 2007). The UniFrac distance metrics were then used in a Principal Coordinates Analysis (PCoA) within QIIME, which also provided visualizations of both the quantitative (weighted UniFrac) and qualitative (unweighted UniFrac) measurements provided. Qualitative measures compare communities through a presence or absence of data whereas quantitative measures account for the relative abundance of each type of organism. These measures can provide insight into different drivers of community diversity between samples. By using both indices a better overall picture of both diversity and driving factors is obtained (Lozupone et al., 2007).

Following multivariate analyses a Dufrene-Legendre Indicator Species Analysis (R package {labdsv}, Dufrene and Legendre, 1997) was run on OTU tables using an "around medoids" partitioning of Bray-Curtis distances (R package

{cluster}, Reynolds et al., 1992). Significant indicator species were further tested for correlations with culture abundance and physical conditions using a Spearman's *rho* product moment correlation coefficient.

Search for algicidal and resistance-conferring bacteria

Representative Illumina OTUs generated by the usearch method (pick_rep_set.py, which chooses the most abundant sequence showing up in each OTU) were compared to sequences uploaded to the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) megablast algorithm (Altschul et al., 1997). The sequences used in the comparision were GenBank sequences for algicidal bacteria strains S03 (accession number EU021292) and 41-DBG2 (accession no. AF427479, Roth et al., 2008b) and the bacteria strain D38BY (accession no. EU021293) which is antagonistic towards strain S03 (Roth et al., 2008a). An identity % of 90 was used to find matches between the usearch OTUs and the GenBank sequences.

Results

In two years of sampling two different physical and biological environments were observed. In 2010 there was no bloom of *K. brevis* on the WFS. This year was characterized by continual upwelling along the WFS which dispersed any *K. brevis* cells from the nearshore environment, preventing bloom aggregation and formation (Weisberg et al., in press). In 2009 the *K. brevis* bloom was classified to be in

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maintenance phase. This bloom was moving from nearshore to offshore (dispersal), had low to moderate concentrations of *K.brevis*, and cell had a high amount of lipid bodies (an indicator of aged cells, Steidinger, 1979).

Microbial community composition

The sequencing platforms used, 454 and Illumina, produced both different amounts of data and different community composition results in terms of identified OTUs. There were 680 total OTUs identified from Illumina sequences with an average of 33073 sequences per sample (max 51972, min 9489, data not shown); whereas for the 454 pyrosequencing data there were 402 total OTUs with an average of 2160 sequences per sample (max 3160, min 1670).

The proportion of OTUs in each sample was used to look at community composition between the two sequencing platforms to account for Illumina samples having significantly more sequences and OTUs. The 454 sequences had a higher proportion of *Alphaproteobacteria* classified as SAR11 (22-29% of communities) than Illumina sequences (2.13-3.71% of communities, Fig. 4.3). It is possible that some SAR11 were classified as *Alphaproteobacteria sp.* in the Illumina dataset, however even if all *Alphaproteobacteria sp.* were SAR11 the proportion of SAR11 would still be lower than that in the 454 data. Additionally, 1.33% of the OTUs were identified as SAR406 in Illumina sequences from outside Tampa Bay but no SAR406 OTUs were identified in the 454 data from the same station. Within the Illumina dataset, there were higher proportions of the *Flavobacteriales, Planctomycetes*, *Rhodobacterales*, *Oceanospirillales*, and *Gammaproteobacteria* groups. However,

Bacteria community composition in surface waters of the Gulf of Mexico sampled during ECOHAB: Karenia 2009 and 2010 process cruises

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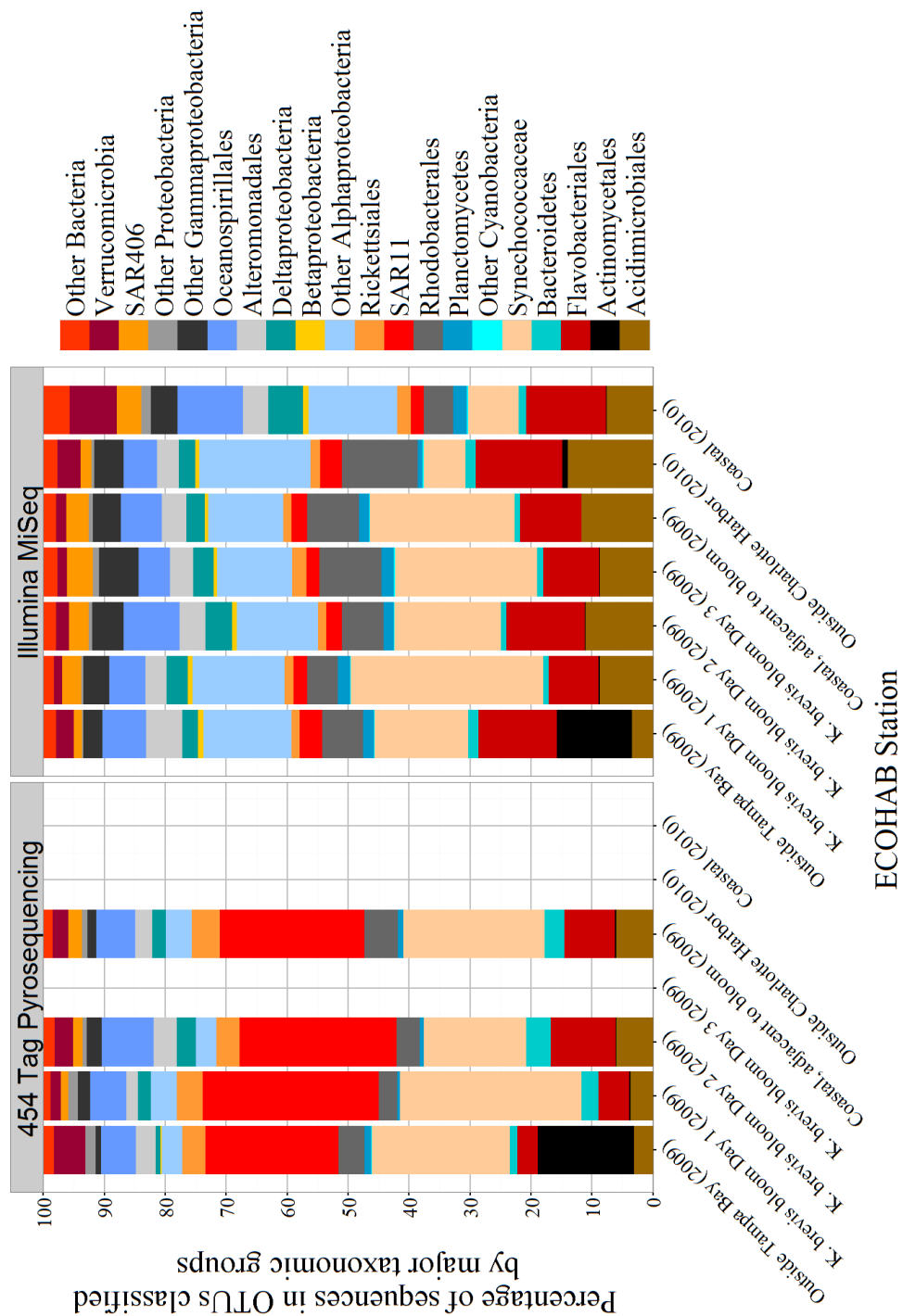
Figure 4.3: Bacterial community composition along the West Florida Shelf depicted as the percentage of OTUs classified as major taxonomic groups. Samples collected during the 2009 ECOHAB: *Karenia* process cruise and represent five different stations: Outside Tampa Bay (TB-Out), three consecutive days of *K. brevis* bloom sampling (KB-Day1-3), and adjacent to the bloom sample on day 2 (St. 9A). Samples in 2010 were from outside Charlotte Harbor (CH-Out) and south of Sanibel Island, FL (Coastal). A total of 10,800 sequences were generated by 454 tag-pyrosequencing and classified into 402 OTUs (2009 samples only). The Illumina® MiSeq platform generated 215,365 sequences which were classified into 680 OTUs (2009 and 2010 samples). Classifications were generated using Mothur (Schloss et al., 2009) and the Greengenes taxonomic database (McDonald et al., 2012).

the lack of replicate treatments limited statistical comparisons to determine if such differences between the two sequencing platforms were significant.

In the estuarine outflows of Tampa Bay and Charlotte Harbor there were higher percentages of *Actinomycetales* OTUs than both bloom and coastal samples (>10% vs. < 1%, Fig. 4.3). Non-bloom stations had a higher proportion of *Verrucomicrobia* than bloom and near-bloom stations; the non-bloom proportion of *Verrucomicrobia* OTUs averaged 4.81% and 5.17% and bloom and near-bloom stations averaged 1.68% and 2.13% for Illumina and 454 sequencing, respectively. The coastal environments had higher proportions of *Deltaproteobacteria*, *Gammaproteobacteria*, and SAR406 than in estuarine outflows (Fig. 4.3). The proportion of these groups was higher in Illumina sequences but both platforms exhibited 1-2% more of the OTUs in coastal environments. There were 1.40% and 1.70% *Deltaproteobacteria*, 1.01% and 1.27% more *Gammaproteobacteria*, and 2.11% and 1.59% more SAR406 (Illumina and 454, respectively) in the coastal samples.

BLAST search for algicidal or algicidal resistance-conferring bacteria

The algicidal *Flavobacterium* sp. strain S03 had the most hits in the Illumina representative sequences database, with 60 sequences having a % identity ranging from 89.6-97.63% (data not shown). Sequences had alignment lengths of 65-254 nucleotides and 4-26 mismatches. Each station sampled had at least one BLAST match to strain S03. Station KB-Day2 had the most hits (15) with an average % identity of 92.64%, average length of 236.80, and the fewest average mismatches

(17.13 per hit). Stations Outside Tampa Bay and KB-Day1 also had high abundances of strain S03 (13 and 12 hits, respectively) with similar % identities (~92%) and mismatches (~19). The final day of bloom sampling, KB-Day3 only had 1 BLAST hit with >90% identity (91.70%) but also had the highest number of mismatches (21). Adjacent to the bloom, there were 5 BLAST hits which averaged 92.45% identity. In 2010, when no bloom was present, 5 hits to strain S03 were found near Charlotte harbor and 9 hits were found southeast of Sanibel Island, FL.

There were 15 hits for the *Tenacibaculum* sp. strain D38BY, which is antagonistic towards strain S03, with % identities ranging from 89.8-99.21%. Alignment lengths were between 251 and 255 with 2-22 mismatches. The only sample from within the 2009 bloom with hits to D38BY was KB-Day2, which had 4 hits (95.25%, 252.75, 11, average identity, length, and mismatches, respectively). The other samples from within the bloom, KB-Day1 and KB-Day3, had hits, but all had <90% identity. There were 5 hits Outside Tampa bay and 1 hit adjacent to the bloom. In 2010 there were 5 hits total, 2 Outside Charlotte Harbor and 3 in the coastal environment south of Sanibel Island.

The second algicidal bacteria, *Cytophaga* sp. strain 41-DBG2 had the fewest BLAST hits, 10, with similar alignment lengths (52-253) and number of mismatches (2-26) as the other two strains. There were 4 hits each in the stations Outside Tampa Bay and KB-Day2 in 2009 and 2 hits Outside Charlotte Harbor in 2010. The highest % identities were in the hits from the bloom station though these alignments were also the shortest in average length (152.25 compared to >200 for other stations). All stations had BLAST hits to strain 41-BBG2, but for stations KB-Day1, KB-Day3,

Coastal, adjacent to bloom, and Coastal (2010) no hits had a % identity greater than 90%.

Indicator analysis of bacterial OTUs

The indicator analysis of OTU datasets was more useful for Illumina data as 27% of OTUs in the 454 dataset were considered significant ($p < 0.05$) compared to only 7.5% of the Illumina sequences. In both datasets SAR406 was a significant indicator species, but only two of the eight SAR406 OTUs were significant. In the Illumina dataset these two OTUs comprised 58-88% and 34-58% of SAR406 OTUs in 2009 and 2010, respectively. In the 454 dataset the significant OTUs are 18-36% of the total identified SAR406 OTUs.

Clustering and statistical analysis of bacterial communities

Clustering of communities using Bray-Curtis dissimilarities demonstratedthat bloom and near-bloom communities cluster more closely with one another than with non-bloom communities (Fig. 4.4). The major difference in clustering between the two sequencing methods is that the 454 data clusters the community from adjacent to the bloom more closely with the early bloom community (KB-Day1) than with later bloom samples (Fig. 4.4, top). The Illumina data clusters the bloom stations KB-Day1 and KB-Day2 together, near-bloom and bloom station KB-Day3, and then the three

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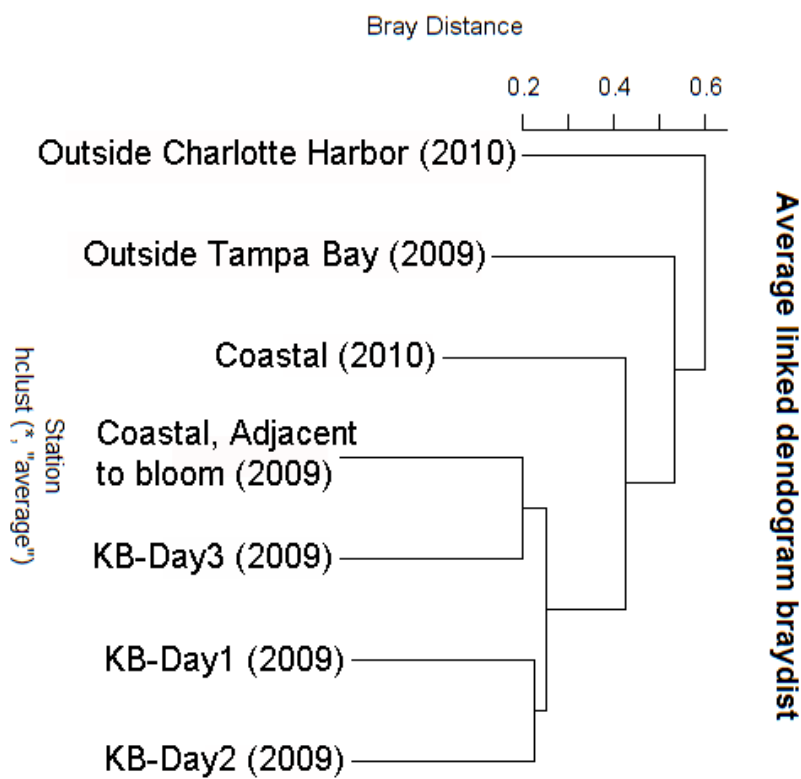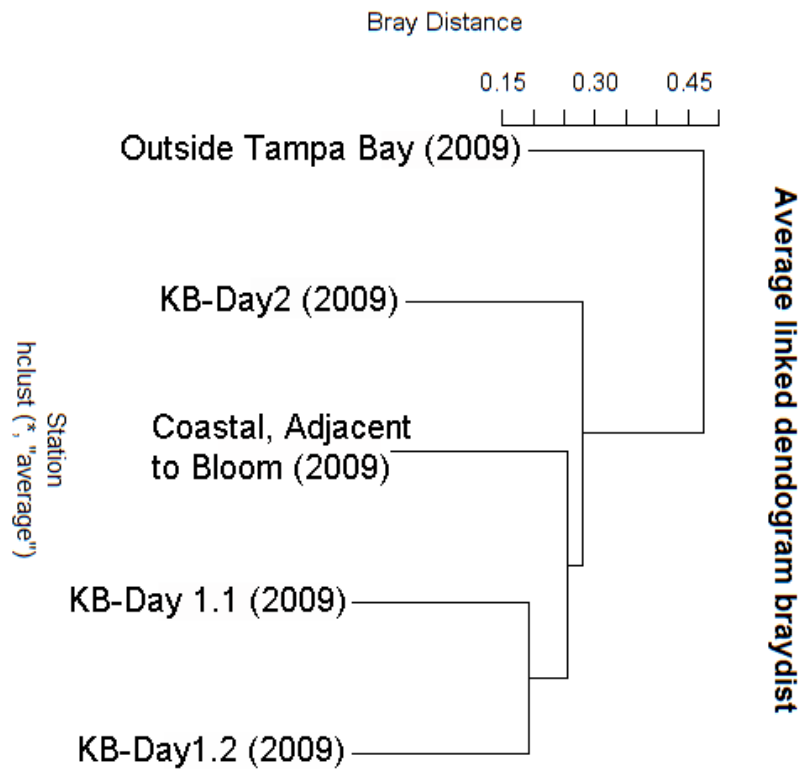
Figure 4.4: Average linked dendrogram of bacterial communities in *K. brevis* blooms sequenced by 454 Tag Pyrosequencing (top) and Illumina MiSeq (bottom) platforms. Calculations are based on weighted UniFrac distances provided by QIIME and analyzed using a hierarchical clustering algorithm within the {stats} package in R (both version 3.0.1). Labels denote the Station location and year in which the samples were taken.

non-bloom stations (Outside Tampa Bay in 2009 and outside Charlotte Harbor and Coastal from 2013). A NMDS of data revealed that 454 sequences separated primarily by geographic location, outside Tampa Bay vs. coastal, along the first axis (MDS1, Fig. 4.5). The Illumina data also separated along the first axis, but there appears to be influence by Charlotte Harbor and the Caloosahatchee River (Coastal station) on the 2010 samples (Fig. 4.6).

Principal components analyses (PCoA) were done on both datasets twice, with and without bacterial production data. For the 454 data, abundance of *K. brevis*, abundance of bacteria, and dissolved total nitrogen (DTN) were significant variables in the model (explaining 89.23% of the variation, CCA). Using only count and environmental data, bloom and non-bloom stations were separated along the first axis which accounted for 84.24% of the variation (PC1, Fig. 4.7). The addition of production data did not change the clustering, but did support the observation that production was significantly higher outside the bloom (Fig. 4.8). The first and second principal components encompassed all of the variation in the model (PC1 78.80%, PC2 21.20%). Illumina data were explained primarily by the first two axes (97.64% of variation, CCA) which were most heavily influenced by *K. brevis* abundance and bacteria abundance (axis 1) as well as bacterial production measurements (axis 2, Fig. 4.9). Removing production data from the PCoA also showed the separation of communities from Outside Tampa Bay from other stations as well as bloom from non-bloom stations (Fig. 4.10).
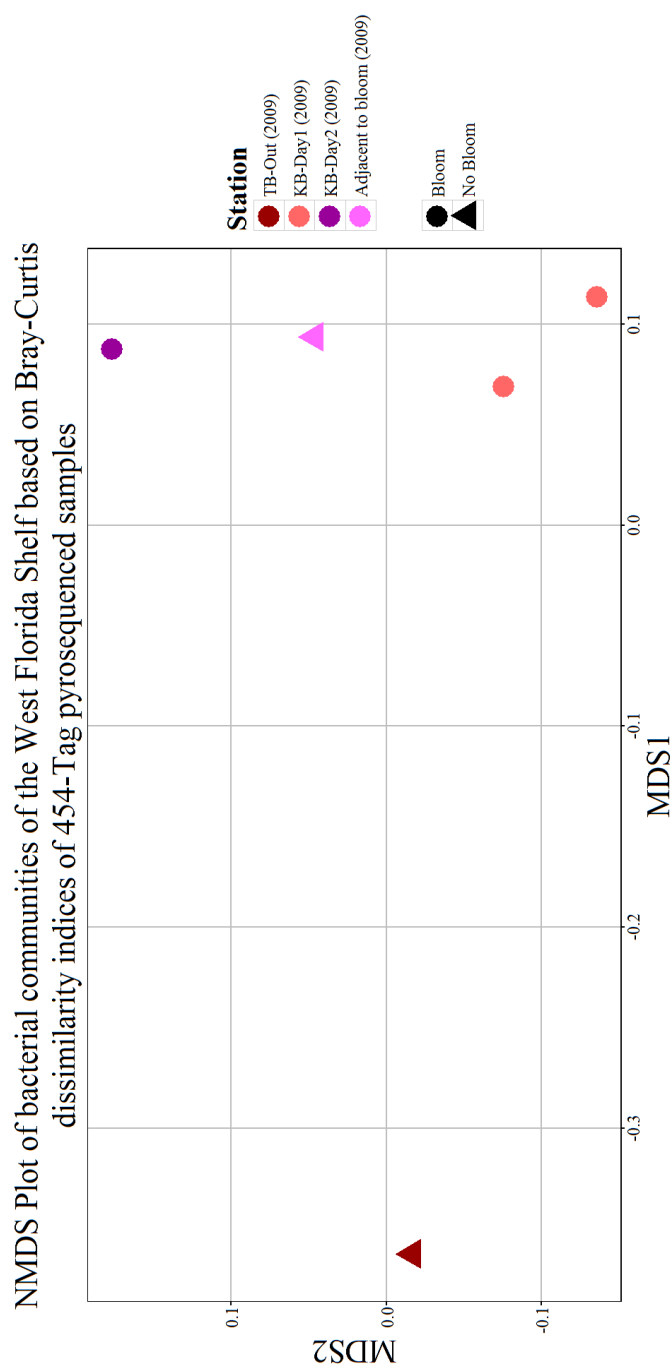
Figure 4.5: Clustering of bacterial OTUs in samples inside (circles) and outside (triangles) blooms of *Karenia brevis* on the West Florida Shelf. Sequences were generated with 454-Tag pyrosequencing and OTUs assigned using Mothur (Schloss et al., 2009). Bray-Curtis dissimilarity indices were calculated with the {vegan} package using R. Stress of the plot was near zero.
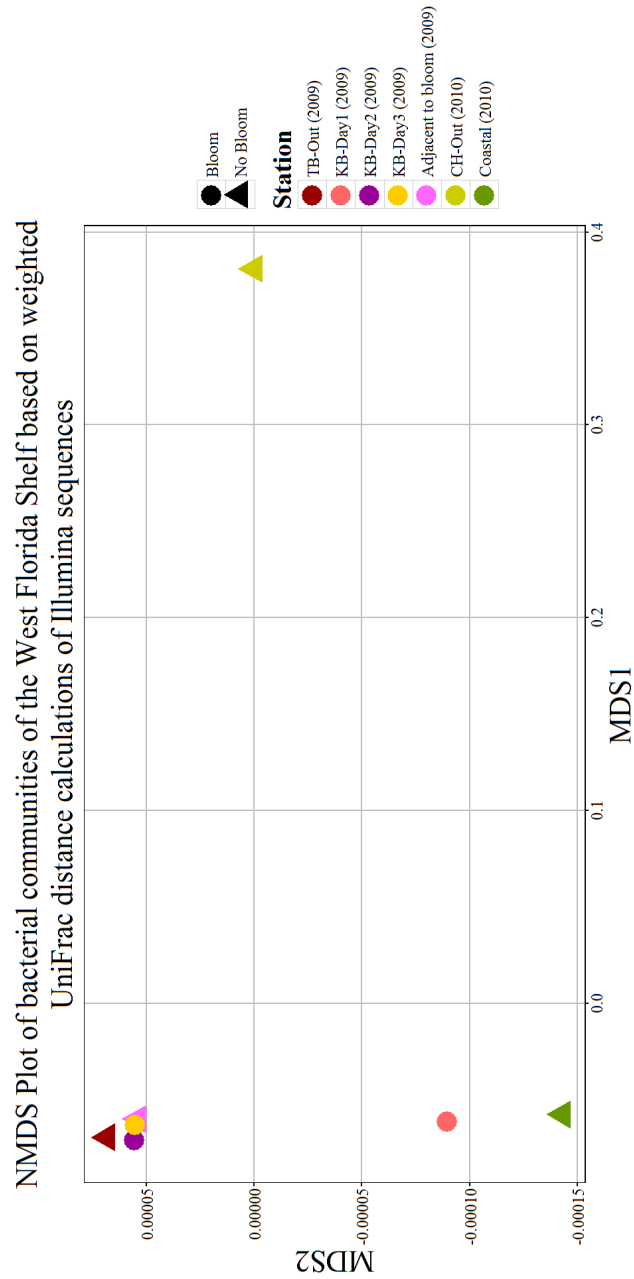
Figure 4.6: Samples from surface waters of the WFS when a bloom was present (2009) and no blooms were identified (2010). Samples were taken from inside (circles) and outside (triangles) blooms of *Karenia brevis*. Samples were sequenced on the Illumina® MiSeq platform. The GreenGenes May 2013 taxonomic database (McDonald et al., 2012) was used as a template to identify OTUs using QIIME (Caporaso et al., 2012), which also calculated UniFrac distances. Clustering was done R using the {vegan} package.
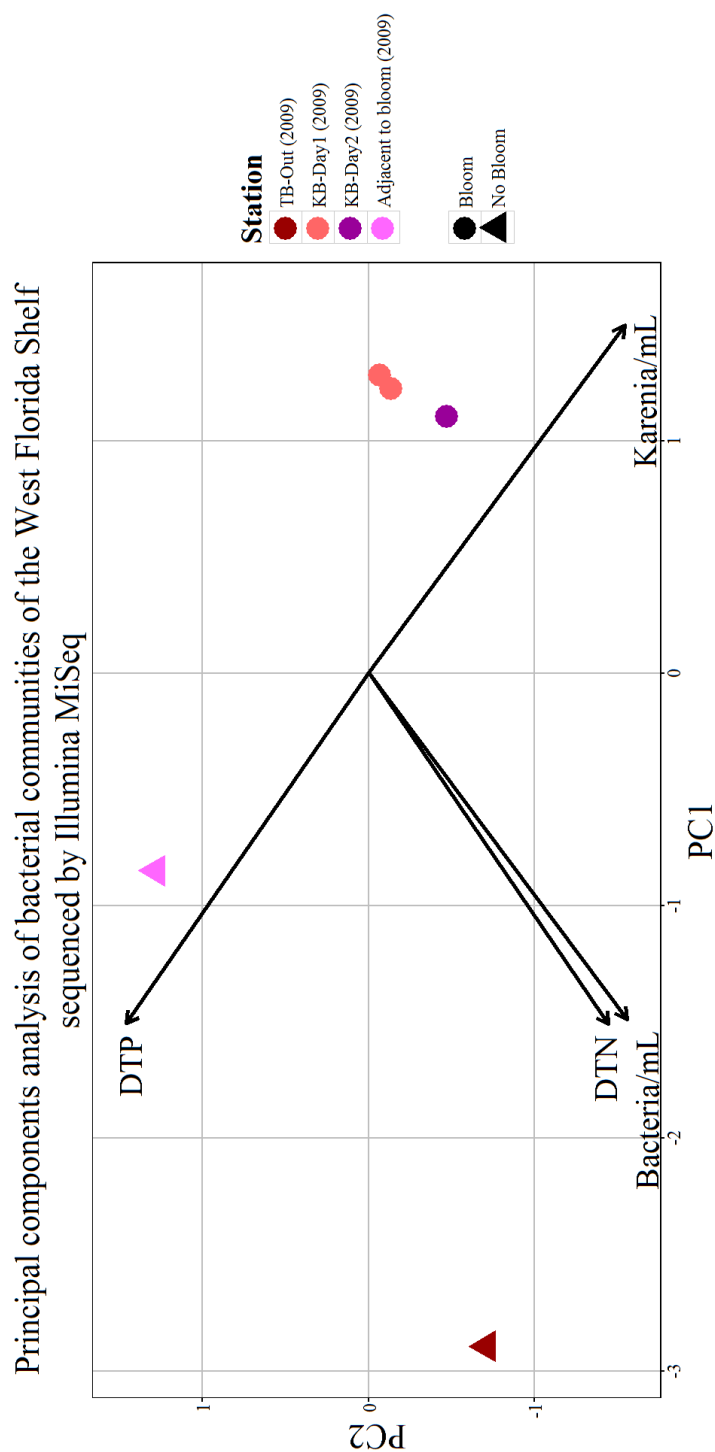
Figure 4.7: Influence of environmental and count data on bacterial communities sequenced by 454 tag pyrosequencing. The displayed axes explain 99.74% of the variance (PC1 84.24%, PC2 15.50%). Stations are displayed by both location (color) and bloom status (shape).

Figure 4.8: Stations from which genetic sequences (454 tag pyrosequencing), environmental data, and production data were available from the 2009 ECHOAB: *Karenia* cruise. All of the variation was explained by this model (PC1 78.80%, PC2 21.20%).
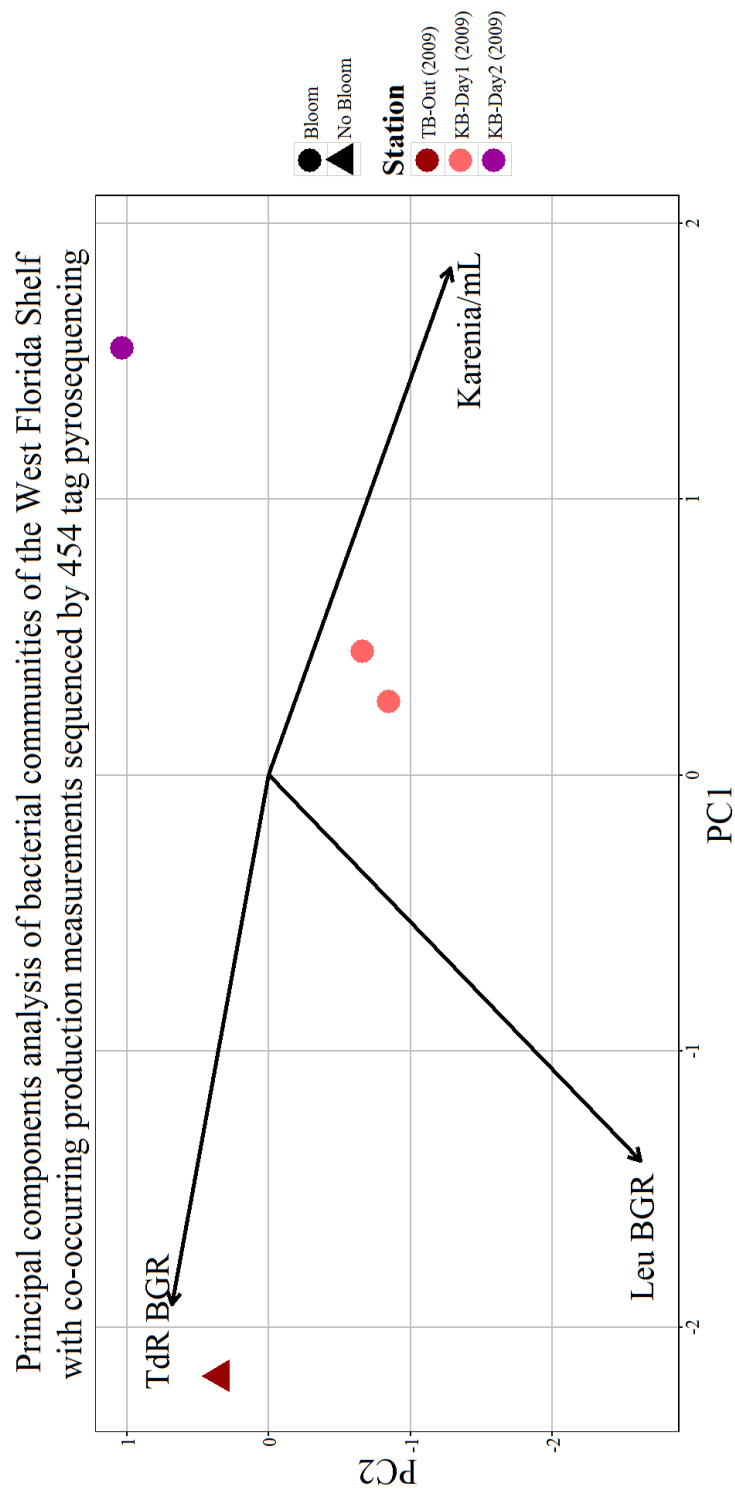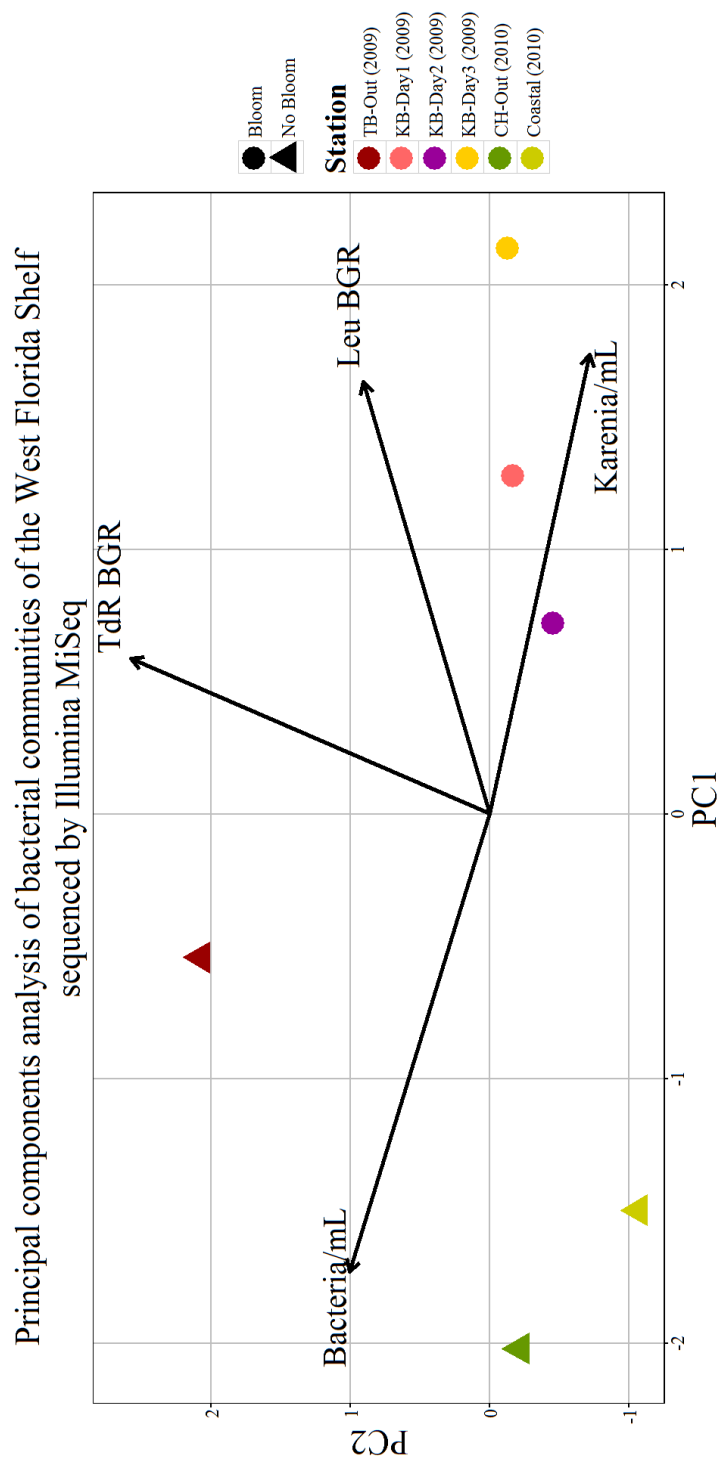
Figure 4.9: Distribution of bacterial communities based upon count, environmental, and production data in 2009 when a bloom was present and 2010 when no bloom was observed. The first and second principal components explained 91.04% of the variation (PC1 62.18%, PC2 28.85%).
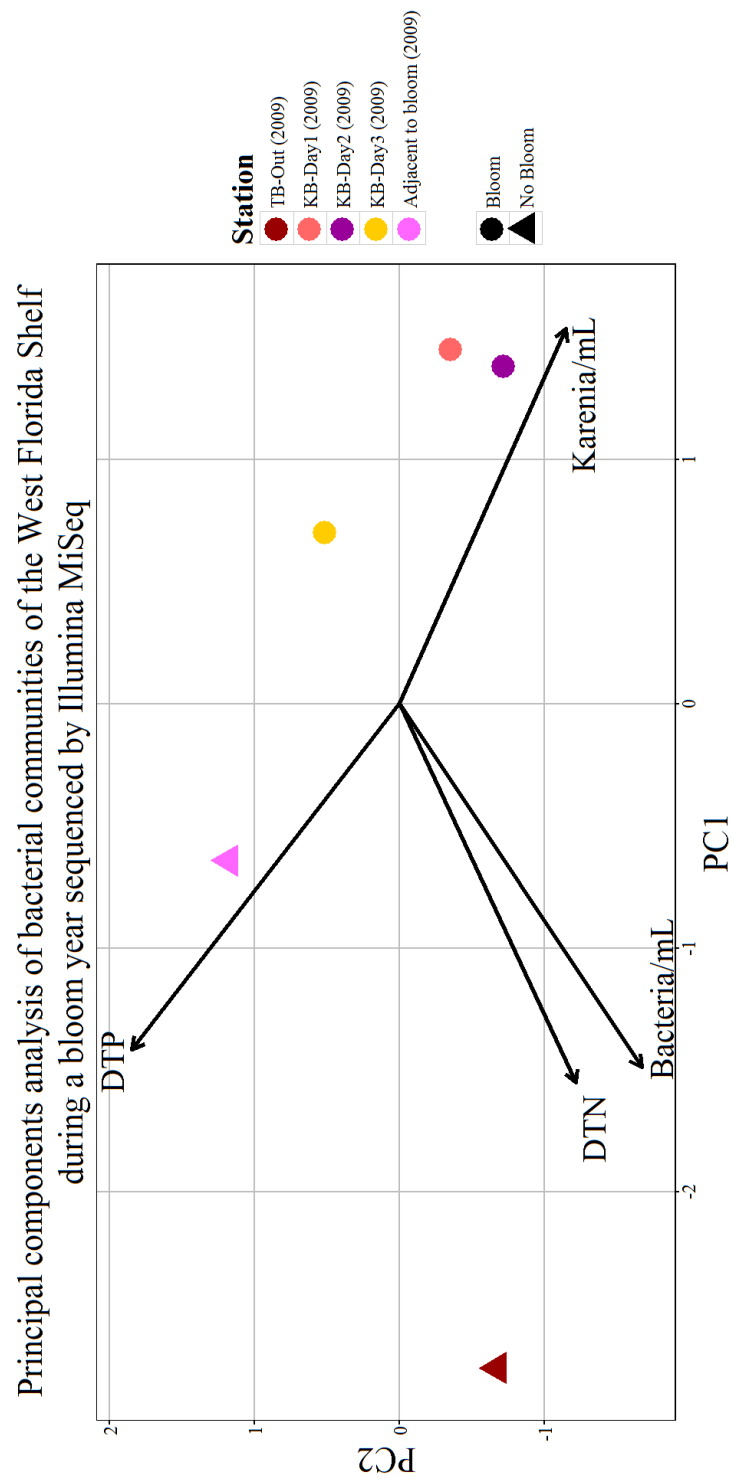
Figure 4.10: Incorporation of nutrient data into a PCoA of data from the 2009 bloom of *K. brevis*. No bacteria production data were available for the station adjacent to the bloom so such factors were excluded from this model. The first two components explain 95.01% of the variation in the model (PC1 77.84%, PC2 17.17%).

An Adonis (analysis of variance using distance matrices, AKA PERMANOVA, permutational multivariate analysis of variance using distance matrices) of the Illumina data (excluding the station Adjacent to bloom) only found a significant difference in bacterial abundance between samples (data not shown). Given that only one sample was a distance away from the bloom, rather than inside or adjacent (Outside Tampa Bay), this may have had a disproportionate effect on the variance in the analysis.

Discussion

The results of this study showed that bacterial communities on the WFS were similar within any given year regardless of the presence of a *K. brevis* bloom. All communities sampled were dominated by the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex, the cyanobacteria *Synechococcus*, and *Alphaproteobacteria*. Sequences matched those of previously identified strains of bacteria that exhibit algicidal activity and resistance to algicidal activity were also found inside and outside blooms and may play a role in the timing of bloom formation and termination. The SAR406 group, typically found below the deep chlorophyll maximum, was also found in surface samples. This group was a significant indicator species and supports an existing bloom formation hypothesis involving upwelling. Further investigations are needed to more closely study the CFB complex as well as *Alphaproteobacteria* to determine if there are interactions between *K. brevis* and specific bacteria which may play a role in bloom dynamics.

Methodological considerations

The primary difference between the two sequencing platforms is that 454 pyrosequencing generates DNA reads ~250 base pairs (bp) in length and Illumina MiSeq generates shorter reads with lengths of 75-150 bp (Quince et al., 2009, Werner et al., 2012). Pairing the 5' and 3' ends of Illumina reads, as was done in this study, can double the bp of each read but does not necessarily increase the resolution of OTU assignment or diversity results (Werner et al., 2012). The marginally better results produced by paired-end (PE) data as well as the pairing allowing for an additional quality control step prompted the use of PE data instead of a single read (Bartram et al., 2011).

Tag pyrosequencing has been found to provide conservative taxonomic resolution of the V6 variable region of 16S rRNA, with taxonomic resolution extending to the genus level (Thompson et al., 2010). Illumina sequencing has been found to have taxonomic resolution linked to sequence length as well as repeatability between samples (Bartram et al., 2011). In this study, data from both platforms had limited taxonomic resolution past the Order level; in the Illumina dataset 56% of all OTUs were classified down to Order (63% when just looking at OTUs which classified beyond Kingdom: Bacteria) and in the 454 dataset 77% were classified to Order (82% for OTUs beyond Kingdom). Differences in taxonomic results most likely resulted from the use of different primers during amplification of DNA prior to sequencing; primer choice did not have a large affect on either community clustering or indicator analysis (Liu et al., 2007).

Samples were amplified from a single bloom in 2009 which was in maintenance phase (K. Steidinger, pers. comm.) as well as when no bloom was present. This did not allow for comparison of bacterial communities between bloom stages, but instead between a maintenance-phase bloom and a non-bloom community. A similar comparison was done during a 2001 *K. brevis* bloom on the WFS but the growth phase of the bloom was not established (Jones et al., 2010); this study therefore adds to the available data on bacterial communities inside and outside *K. brevis* blooms by specifying growth stage of the bloom. This is an important classifier as bacteria-algal interactions can be linked to the growth and physiology of the algae, but more studies focused on bacterial community composition within different blooms stages are needed (Amin et al., 2012).

Bacterial communities were similar inside and outside blooms of *Karenia brevis*

Most bacteria on the WFS are free-living and account for a majority of bacterial production (Chapter 3, data not shown). The community in all samples was primarily represented by members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex, the cyanobacteria *Synechococcus*, and *Alphaproteobacteria*. Both CFB bacteria and *Alphaproteobacteria* have been found to be significant components of communities associated with toxic and non-toxic dinoflagellates (Hold et al., 2001), including *K. brevis* (Jones et al., 2010).

The MDS of the 454 data showed separation between all communities, including subsequent samples with the *K. brevis* bloom. However, when samples

from a non-bloom year were included (Illumina data) the samples KB-Day2 and KB-Day 3 (within the bloom) were more similar to non-bloom samples from the same year. Given that the 2009 bloom was in maintenance phase could explain the separation between the first day of bloom sampling and other 2009 samples: physical and/or physiological changes in the bloom environment would promote bacterial succession (Massana et al., 2001). The longer the bloom stays in maintenance phase the more potential that physiological or molecular changes may lead to bacterial succession and the more likely communities become more similar (Doucette, 1995, Fandino et al., 2001).

Given physical conditions on the WFS during the bloom, onshore winds would be expected to entrain blooms and bloom-communities in nearshore enviornments and lead to natural shifts in bacterial communities; in this case, less diversity as the bloom progressed (Walsh et al., 2003). Alternatively, offshore winds could lead to bloom dispersal, and possibly bloom termination, and could lead to a more diverse bacterial ecosystem with similarities to the central Gulf of Mexico but localized community differences centered on nutrient sources (Zinger et al., 2011).

Algicidal and antagonistic bacteria were present in bloom and non-bloom environments

Closely related species to previously identified bacteria that are capable of lysing significant portions of *K. brevis* cultures were found in both bloom and non-bloom communities on the WFS. All were found at the stations Outside Tampa Bay and during the second day of bloom sampling (KB-Day2) which demonstrate these

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bacteria are ubiquitous throughout the area in which blooms typically occur (Roth et al., 2008b, Steidinger, 2009).

The strain with the most BLAST hits was *Flavobacterium* sp. strain S03, which exhibits algicidal activity towards *K. brevis* (Roth et al., 2008b). This strain requires direct contact with algal cells for algicidal activity but can almost immediately retard algal growth and cause lysis within a few days. The 2009 *K. brevis* blooms was categorized as being in maintenance phase (K. Steidinger, pers. comm.) and therefore had likely been experiencing bloom concentrations long enough to experience increased cell lysis. Direct contact with cells could provide a competitive advantage to strain S03 as the bacteria would still be near individual *K. brevis* cells at the time of lysis, and lysis of *K. brevis* would create microenvironments of cellular components like protein and cellulose that would further promote the growth of Strain S03 (Cottrell and Kirchman, 2000, Fandino et al., 2001, Konopka, 2009).

It was surprising to find fewer BLAST hits to 41-DBG2 as this strain has more potent algicidal activity against *K. brevis* and does not have a described interaction with the antagonistic bacteria strain B38BY (Roth et al., 2008 a,b). Strain 41-DBG2 has an indirect method (extracellular compounds) of algicidal activity. This method would require more time for such activity to take place as it depends on diffusion; a maintenance phase bloom in a frontal zone would provide an ideal environment and time frame for the algicidal activity of strain 41-DBG2 to initiate cell lysis and benefit from released DOM. However, undescribed bacteria capable of limiting and/or inhibiting growth of 41-DBG2 have been observed in culture; if such

bacteria were present during this study they could have contributed to the low abundance of hits to 41-DBG2 (Mayali and Doucette, 2002). This suggests that 41-DBG2 was not a large component of the microbial community, even within the bloom, and therefore was probably not a significant source of *K. brevis* mortality during the 2009 bloom.

The *Tenacibaculum* sp. strain D38BY had the second most BLAST hits; given this strain has been shown to have a specialized antagonistic interaction towards algicidal strain S03 within cultures of *K. brevis* it appears this relationship also occurs within natural blooms (Roth et al., 2008a). The variation in D38BY hits both inside and outside of *K. brevis* blooms demonstrate that relatives of this microbe are found across the WFS in both nearshore and coastal environments but flourishes when strain S03 is also present. Stations with the most hits to D38BY also had a large number of hits for strain S03. The antagonistic relationship requires direct contact between S03 and D38BY so a large abundance of S03 would be conducive for such contact with D38BY, especially in frontal zones which develop on the WFS (Roth et al., 2008a, Vargo et al., 2002).

The ability of strain D38BY to provide protection from the algicidal effects of strain S03 was found to act in a concentration dependent manner which prolonged the growth period of cultures rather than providing true immunity to the co-occurring dinoflagellate (Roth et al., 2008a). The nature of the relationship between S03 and D38BY needs to be further explored to determine how D38BY provides resistance to the algicidal effects of S03 and if this resistance also promotes the growth of D38BY.

In addition to being found within the bloom, all strains of interest were found in samples from the two stations with estuarine influences; Outside Tampa Bay and Charlotte Harbor. When first characterized it was hypothesized that the algicidal bacteria require high amounts of dissolved organic matter (DOM, Mayali and Doucette, 2002). When present within a *K. brevis* bloom, the bloom itself may serve as a DOM source (either from direct excretion of compounds by *K. brevis* or through lysis of *K. brevis* due to algicidal activity). Outside of blooms in the estuarine outflows of Tampa Bay and Charlotte Harbor it appears these bacteria utilize natural and anthropogenically derived nutrients from these bodies of water (Vargo et al., 2004). Furthermore, the presence of strain S03 appears to promote stain D38BY, so the BLAST hits of sequences similar to D38BY in the estuarine outflows were more likely a result of estuarine DOM supporting growth of S03 and subsequently supporting D38BY in the same environments.

Unfortunately the nature of the interaction between these strains has not been discerned and this study was not designed to make any strides towards that end. What is evident, however, is that all of these bacteria are ubiquitous on the WFS and can respond to natural stimuli other than *K. brevis* blooms. More research needs to be conducted to determine how these microbes interact with one another as the current knowledge highlights just how complex such interactions are between microbes and *Karenia* as well as between the microbes themselves. Additionally, at least one strain exists which is antagonistic towards 41-DBG2, but this strain has yet to be isolated and identified (Mayali and Doucette, 2002). The duration and impact of *K. brevis* blooms could be significantly altered by the interaction between algicidal and

antagonistic bacteria interactions, and could also lead to new mitigation strategies as well as more accurate predictions of bloom duration by models.

The presence of SAR406 supports bloom-formation hypotheses

All indicator species, as determined by indicator analysis, were either single OTUs or a subset of OTUs grouped at a higher taxonomic level due to low abundance (<1% of all OTUs in every sample). One of the most interesting indicator species were contained within the Phylum SAR406. This Phylum contains free-living bacteria that have been identified in both the Atlantic and Pacific oceans throughout the water column, including deep ocean and near-bottom (10m above the sea floor) environments (Gordon and Giovannoni, 1996, Gallagher et al., 2004). In the Sargasso Sea the proportion of SAR406 ribosomal DNA at 200m was four to five times higher than that observed in surface waters with a maximum abundance below the deep chlorophyll maximum. Increases in SAR406 in surface waters of the Sargasso Sea were positively correlated with chlorophyll *a* abundance which in turn is brought about by spring phytoplankton blooms resulting from stratification following mixing events (Gordon and Giovannoni, 1996). This suggests the SAR406 found in the surface waters of the WFS is linked to a mixing event and possibly the presence of *K. brevis*.

A primary hypothesis for formation of *K. brevis* blooms on the WFS involves season upwelling of near-bottom water of the middle to outer WFS (Walsh et al., 2006). When upwelling is localized and relatively weak *K. brevis* can become aggregated within frontal zones utilizing vertical migration to take advantage of

estuarine and benthic nutrients and colored dissolved organic matter for ultraviolet protection (Walsh et al., 2003). Indeed, several blooms were observed surrounding nearshore salinity or thermal fronts which developed in conjunction with upwelling favorable winds (Vargo et al., 2002). The proportion of SAR406 in the surface samples of this study provides confirmation of near-bottom offshore water intrusion into surface waters of the WFS. This supports the offshore-transport hypothesis of *K. brevis* seed stocks to nearshore waters. This does not mean SAR406 will indicate a *K. brevis* bloom, or help identify seed stocks of *Karenia* in deep offshore waters (Brand, 2012), but rather provides another way to monitor for conditions which may preface *K. brevis* bloom formation. If physical conditions lead to the formation of a *K. brevis* bloom, SAR406 would be expected to be part of the associated bacterial community.

Bacteria-*Karenia* interactions are important to bloom dynamics

This study found bacterial communities inside and outside blooms of *K. brevis* to be more related than previous studies (Jones et al., 2010), stable over multiple days of sampling, and containing several groups that warrant further study (Figs. 4.3, 4.4, 4.6). It also highlights the importance of understanding the composition of bacterial community and potential bacteria-bacteria and bacteria-*Karenia* interactions.

Closely related species to previously identified strains of bacteria that are algicidal towards *K. brevis*, as well as bacteria that can confer algicidal resistance, were found within a 2009 bloom and across the WFS. The presence of these bacteria in the estuarine outflows of Tampa Bay and Charlotte Harbor demonstrate that *K.*

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brevis blooms are not the only stimulus to which these bacteria respond. This also suggests if algicidal bacteria are abundant before a bloom initiates, due to non-bloom stimuli, a bloom may be prevented or minimized in both extent and duration.

The SAR406 group was determined to be an ideal indicator species of a *K. brevis*-associated microbial community. Normally found at depths greater than the deep chlorophyll maximum, the presence of SAR406 in nearshore surface waters suggest upwelling events that support existing hypotheses regarding formation of *K. brevis* blooms. The use of probes specific to SAR406 should be investigated as a potential bloom forecasting tool. While the presence of SAR406 is not always followed by a *K. brevis* bloom, it does indicate that several events leading to previous blooms have occurred and that the chances of a bloom are increased.

Microbial communities in different bloom stages differ in their production and total abundance (Chapter 3) however, more coupled production-genetic data are needed to determine if community production does indeed change within different bloom stages on the WFS without a similar change in community composition. This study was only able to compare bacterial community composition between a bloom in maintenance phase and an environment with no bloom. A complete picture can only be achieved when the microbial communities of initiating and terminating blooms are also investigated. Doing so would determine if SAR406 presence matches physical models and measurements of upwelling along the WFS and if algicidal and antagonistic bacteria occur within deep waters. This may help determine the earliest point at which a bloom may occur within a given physical event (there may be a time lag between the onset of upwelling and the arrival of near-bottom offshore

communities) and if algicidal resistance-conferring bacteria are associated with specific *K. brevis* communities or are mixed with upwelling communities when they reach nearshore surface waters.

Bacteria-algae interactions are being better elucidated in many ecosystems, but in the case of a harmful algal bloom species such interactions can have wide ranging impacts on the ecosystem and economy surrounding blooms of such species. While members of CFB-group, *Alphaproteobacteria*, and *Synechococcus* are dominant in bacterial communities inside and outside blooms of *K. brevis*, more focus should be directed towards the CFB and SAR406 groups. The functionality of these groups suggests potentially large roles in bloom dynamics that may lead to improved bloom models, forecasting, and mitigation strategies. Existing methods and technology will allow for hypothesis testing but care needs to be taken to ensure that the bloom stage is considered so that these dynamics are characterized from the early stages of bloom initiation through termination of blooms.

Chapter 5: Response of *Karenia brevis* and associated microbial communities to viruses concentrated from the Gulf of Mexico.

Abstract

Previous research found that cultures of the "red tide" dinoflagellate *Karenia brevis* were susceptible to a filterable lytic agent found in the Gulf of Mexico where *K. brevis* frequently forms large blooms (Paul et al., 2002). It was not determined if the lytic agent was acting upon *K. brevis* directly or upon the associated bacterial community. This study sought to test whether bacteria found in cultures of *K. brevis* were being affected by viruses and if mortality of *K. brevis* followed thereafter. Viruses were concentrated from the West Florida Shelf (WFS), where *K. brevis* frequently blooms, and added to unaltered cultures (*K. brevis* and associated bacteria) and cultures pre-filtered through at GF/F filter to remove *K. brevis* (associated bacteria only). Abundance, production, growth, and bacterial community composition were examined at the beginning and end of a 24 h incubation. Virus abundance did not increase significantly and bacterial communities were not significantly altered by the addition of a viral concentrate. In unaltered cultures *Flavobacteriales* were > 45% of identified OTUs in all treatments, but <10% in filtered cultures. Abundance of *K. brevis* declined the most in treatments with addition of the viral concentrate; interestingly, these treatments also had the highest rate of nucleic acid synthesis. This

suggests that bacteria exposed to a viral concentrate were channeling carbon uptake into cellular division. This shift in growth may have disrupted a *K. brevis*-bacteria interaction which warrants further investigation to determine the nature and extent of this relationship. Differences in bacterial production and growth indicate that viruses were acting upon the bacterial community and not the dinoflagellate. More study is needed however to determine if viral infection is limited to key bacterial groups or the entire community.

Introduction

Viruses are the most abundant biological entity on the planet, existing everywhere there is life (Suttle, 2005). As more knowledge is gained concerning viruses their importance in global processes is being brought to the forefront. Viruses represent a massive genetic reservoir capable of driving changes in community composition, biogeochemical cycles, and host metabolic function. Oceanic viruses can terminate massive algal blooms, sometimes in a matter of days (Frada et al., 2008). Termination of phytoplankton blooms can take place when nutrients are non-limiting, allowing previously out-competed phytoplankton to grow or even form a second bloom (Bratbak et al., 1993).

A common bloom-forming species in the Gulf of Mexico is the toxic dinoflagellate *Karenia brevis* (Davis) G. Hansen et Moestrup. Blooms typically form on the West Florida Shelf (WFS), often along the Gulf Coast of Florida right up to the

beach (Steidinger, 2009 and Derby et al., 2003). These blooms have been documented to exceed 10^6 cells L^{-1}, cover an area greater than 2000 km^2, and persist for months (Heil and Steidinger, 2008). Blooms of this nature represent an ideal situation for viral infection to play a significant role in several bloom dynamics, as viral infection is density dependent (Fuhrman, 1999).

A study using cultures of *K. brevis* found that seawater from the Gulf of Mexico filtered through a 0.2 µm filter resulted in lysis of the cultures (Paul et al., 2002). Filtrate from the lysed cultures caused further lysis when added to other cultures and transmission electron microscopy (TEM) analysis of the lytic filtrate found siphoviridae to be the dominant phage. Lytic activity was lost when the lytic filtrate was treated with heat (50 °C for 50 min.), 0.05 µm filtration, addition of RNase, acidification (pH 5.0), UV treatment, or protease digestion which suggested a viral lysate. Analysis of intact and lysing *K. brevis* cells from cultures with TEM did not show any phages within or surrounding *K. brevis*. This finding led to the hypothesis that phages were lysing bacteria within the cultures: bacteria either had a positive relationship with *K. brevis* or released a lytic agent or enzyme when lysed as a result of viral infection (Paul et al., 2002). No aspect of this hypothesis was tested and a follow up experiment was unsuccessful at terminating cultures of *K. brevis* (I. Hewson, pers. comm.).

While the original study did provide evidence that viruses were capable of affecting *K. brevis* and possibly terminating blooms it did not test any aspect of bacterial dynamics. In other studies, a majority of detected viral genomes in pelagic waters of the eastern Pacific were of a size typical of bacteria and not algae (Steward

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et al., 2000) and bacteria and viruses have been observed to co-vary on the WFS, suggesting a close link between virus communities and their most abundant hosts, bacteria (Suttle, 1994, Hewson et al., 2006).

In coastal Pacific waters changes in the viral assemblage were observed to take place over a short time scale ($\leq$2 days) within a dinoflagellate bloom (Steward et al., 2000). On the WFS the virus-to-bacteria ratio (VBR), an indicator of viral-mediated bacterial lysis (Jacquet et al., 2005), was lowest in samples taken during a *K. brevis* bloom on the WFS (Hewson et al., 2006). Given that bacteria are known to produce nucleases which can also degrade viral particles, high concentrations of nucleases produced by bacteria within a bloom could create a refuge from viral infection (Ammerman and Azam, 1991, Noble and Fuhrman, 1997)

Viral-mediated termination of *K. brevis* blooms needs to be revisited for several reasons. The near-annual occurrence of blooms along the WFS causes economic losses to the local economy, health risks from toxin exposure, and costly dead-fish removal efforts (Backer et al., 2009, Morgan et al., 2009). Sarasota County, FL alone spends $500,000 to $4 million annually in bloom mitigation and cleanup efforts depending on the severity of the bloom (Hoagland et al., 2009). Rapid lysis of a bloom due to viral infection could make a bloom more toxic depending on bloom stage. Higher concentrations of toxins are found within cells in stationary growth, so if blooms reach stationary growth before being termianted due to viral lysis more toxins would be released into the surrounding water (Maier Brown et al., 2006). Viral termination of a *K. brevis* bloom could increase the health risk to humans in the bloom area, require expanded commercial fisheries restrictions, potentially kill more

fish and marine mammals, and potentially reduce local tourism and beach-related business (Morgan et al., 2009).

A better understanding of viral dynamics within blooms could yield a potential means of bloom control. If viral concentrates could be applied to initiating blooms to prevent large blooms from becoming established the impacts of nearshore blooms would be significantly reduced. Large-scale applications of such techniques require a thorough understanding of the viral-host relationship, timeline of infection, and an understanding of any effects viral-mediated termination would have on the toxicity of *K. brevis* blooms.

This study was conducted to determine the role of viruses within *K. brevis* bloom dynamics. The primary hypothesis was that viruses were acting upon the bacterial community (bacteriophages) and not directly upon the dinoflagellate. Lysis of a portion of the bacterial community would alter the bacterial community enough that *K. brevis* growth would be negatively affected; either through the loss of beneficial bacteria, or growth of antagonistic bacteria. The secondary hypothesis was that the bacterial community composition would be significantly different in treatments in which a viral concentrate was added. Viruses were concentrated from the Gulf of Mexico along the WFS and used to inoculate cultures of *K. brevis*, the co-occurring bacteria found in the *K. brevis* cultures, and the bacteria isolated from cultures (without *K. brevis* present). The goal was to help provide insight into viral and bacterial dynamics within the Gulf of Mexico and surrounding a bloom of a toxic dinoflagellate. If better understood, viral dynamics should be considered in *K. brevis*

bloom modeling and forecasting, bloom mitigation strategies, and possibly bloom management.

Methods

Concentrating viruses from the Gulf of Mexico

Surface water was collected by Niskin bottle from the Gulf of Mexico on 19 October 2010 approximately 21.5 km south of Sanibel Island, Florida. There was a high concentration of *Trichodesmium sp.* on the surface of the water, however Niskin bottles were deployed below the surface layer and no colonies of *Trichodesmium sp.* were observed in water samples. Using tangential-flow filtration (TFF) ~20 L of pre-filtered seawater (64 μm mesh followed by a 0.2 μm cellulose acetate filter) was concentrated to ~20 mL of viral concentrate (Figure 5.1, Wommack et al., 2010). Half of the viral concentrate was placed under a UV lamp for ~30 min. to serve as a negative control (deactivated viral particles, Paul et al., 2002).

Cultures of *Karenia brevis*

Florida Fish and Wildlife Research Institute (FWRI) provided batch cultures of *K. brevis* strain Sarasota B3 (FWRI Culture Identifier CCFWC 254). Cultures were amended with GP media at the start of culturing and growth at a salinity of 35 (M. Garrett, pers. comm.). Cultures were grown in environmental chambers at 25 °C with
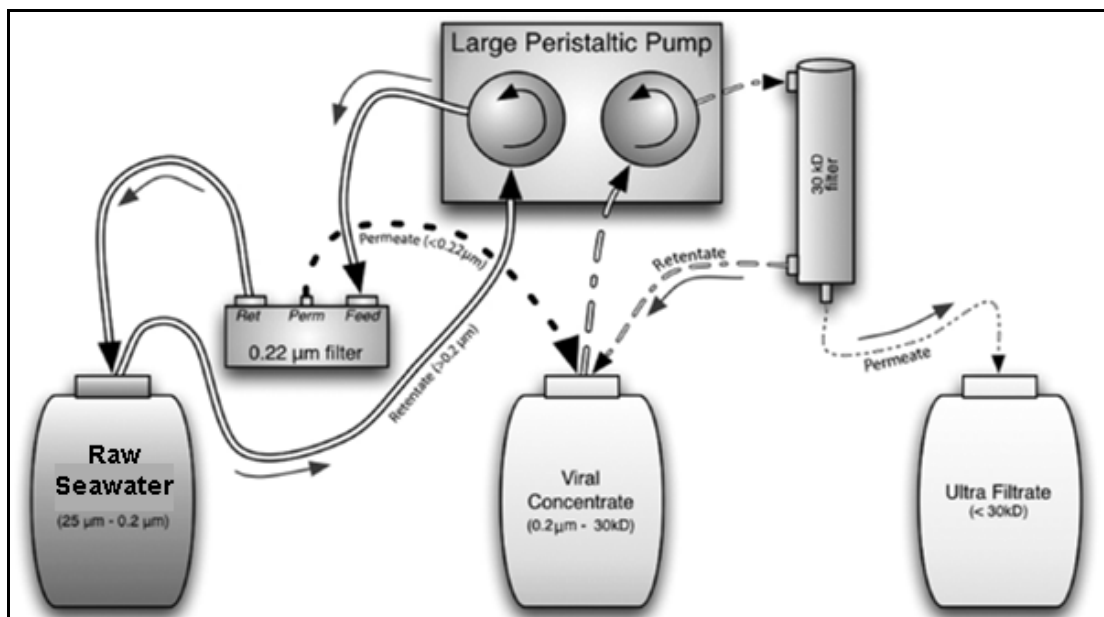
Figure 5.1: Schematic for obtaining a viral concentrate. A large volume of raw seawater is pre-filtered through at 64 µm mesh and then a 0.22 µm cellulose acetate filter to remove all large particles so that the Tangential Flow Filter (TFF, 30 kD filter) does not clog. The viral concentrate is obtained through cycling the pre-filtered seawater through a 30 kD TFF. Particles less <30 kD pass through the TFF and exit as ultra filtrate (discarded) while particles between 0.22 µm and 30 kD will be recycled (retained) in the ever-diminishing volume of viral concentrate. Modified from Wommack et al. (2010).

an irradiance of 60 μE m^{-2} s^{-1} provided by cool white fluorescent light bulbs on a

12:12 light: dark cycle. Cultures were then transferred to on-deck flow-through

incubators aboard the R/V *Pelican* covered with 2 layers of neutral density screening

to replicate surface waters of the WFS. All cultures were used to inoculate

experimental treatments within 3 days after being brought aboard.

Experimental Treatments and Incubations

Five treatments were used to determine if viral concentrates had an effect on *K.*

brevis cultures or its associated bacteria (Table 5.1). Cultures were divided in half

with one half left unchanged and the second half gently filtered (~20 mmHg) through

a GF/F filter (Whatman Ltd.) using a vacuum pump to separate *K. brevis* from the

associated microbial community. Treatments were comprised of 750 mL 0.2 μm

filtered seawater plus 250 mL of whole *K. brevis* cultures water (*K. brevis* plus any

associated bacteria, "whole") or 250 mL GF/F filtered culture water (associated

bacteria only, "bacteria"). All treatments were carried out in triplicate and stored in 1

L cubitainers with all air removed before sealing. Individual cuibitainers were then

sacrificed at the time of sampling.

Whole *K. brevis* culture treatments consisted of: *K. brevis* with no additions

(control), *K. brevis* and 1 mL viral concentrate, and *K. brevis* and 1 mL UV-treated

viral concentrate (negative control). The control treatment was duplicated (two sets of

three) with one set sacrificed for time-zero sampling and the second set incubated

along with other treatments. There were two bacterial treatments which consisted of

Table 5.1: Experimental treatments used to determine the effect of concentrated viruses from the Gulf of Mexico on *Karenia brevis* or associated bacterial growth in culture. Whole culture treatments consisted of a control (250 mL of *K. brevis* culture in 750 mL 0.2 µm filtered seawater), a live virus treatment (addition of 1 mL virus concentrate, [Virus]), and a killed virus treatment (addition of 1 mL UV treated viral concentrate [UV Virus]). Bacteria-only treatments were made by gently filtering (< 20 mmHg) cultures of K. brevis with a GF/F filter (nominal pore size 0.7µm) using a vacuum pump before adding 250 mL GF/F filtrate to 750 mL 0.2 µm filtered seawater. There were only live and killed virus treatments for the bacteria-only samples. Time-zero samples were taken of separate whole culture and bacteria-only treatments.

Treatment	Whole culture	GF/F filtered culture	0.2 µm filtered seawater	[Virus]	[UV Virus]
Karenia: Time zero	250 mL		750 mL		
Karenia: Final	250 mL		750 mL		
Karenia + [Virus]	250 mL		750 mL	1 mL	
Karenia + [UV Virus]	250 mL		750 mL		1 mL
Bacteria initial		250 mL	750 mL		
Bacteria + [Virus]		250 mL	750 mL	1 mL	
Bacteria + [UV Virus]		250 mL	750 mL		1 mL

GF/F filtered culture with the addition of 1mL of either viral concentrate (live viruses) or 1 mL UV-treated viral concentrate (negative control). A third bacterial treatment consisted of GF/F filtered culture and no additions which was immediately sampled (time-zero).

All treatments were incubated aboard the R/V *Pelican* in on-deck flow-through incubators covered in two layers of neutral density mesh screening for ~40 hours. All treatments were sampled for bacteria, virus, and *K. brevis* enumeration, bacterial community composition, and community production and growth.

Cell enumeration

Abundance of *K. brevis* was determined by fixing 1 mL subsamples of gently inverted cubitainers with Lugol's preservative and allowing them to settle for at least 15 min in individual wells of a 24-well plate before being counted using an inverted microscope. If concentrations of *K. brevis* were too high (>3000 cells mL^{-1}), samples were diluted 10-fold before being enumerated as above.

Bacteria were enumerated using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, U. S. A.) using a combination of methods (Troussellier et al., 1995, Brussaard, 2004, Brussaard et al., 2010). The fluorescent dye SYBR® Green I and 20 μL of bead stock (BD Trucount™ Controls in 0.02 μm Anodisc-filtered deionized water, final concentration ~2000 beads μL^{-1}) was added to each sample before analysis to increase fluorescent signature and serve as an internal reference for counts, respectively. Using CellQuest™ Pro version 4.0.2 (BD) data was collected in logarithmic mode based on side scatter (SSC, 488±5 nm) and green

fluorescence (FL1, 530±15 nm), with a target even rate of 100-1000 particles s^{-1} and a

target count of 20,000 total events (modified from Brussaard, 2004). Cell

concentration was calculated using a modified equation from Olson et al. (1985):

(1) Cells$_{ST}$ = [E$_{cells}$ x (Beads$_{ST}$/E$_{beads}$)] - Blank

Where Cells$_{ST}$ is the concentration of total cells in the sample (cells mL^{-1}), E$_{Cells}$ is the

number of recorded events gated as bacterial cells, Beads$_{ST}$ is the number of beads

added to the sample (beads in bead stock added, mL^{-1}), E$_{Beads}$ is the number of beads

recorded during acquisition of sample events, and Blank is the number of events in

the gated region of a 0.02 μm filtered seawater blank (calculated from equation (1)

without the variable "Blank" in the equation).

The viruses to bacteria ratio (VBR) within a sample was then calculated as a

measure of viral-mediated mortality (Jacquet et al., 2005). A VBR >10 is considered

unbalanced growth and therefore a hostile environment for bacteria and a VBR < 10

is conducive for bacterial cellular division and efficient growth.

Epifluorescence microscopy was used to enumerate virus-like particles

(VLPs). Samples were thawed or stored as above. Thawed samples were then filtered

onto a 0.02 μm Anodisc Al$_2$O$_3$ filter (Whatman Inc., Pescataway, NJ), stained with a

1:400 dilution of SYBR® Green I nucleic acid stain, and then slide mounted with a

0.1% (vol/vol) *p*-phenylenediamine anti-fade mounting medium (Patel et al., 2007).

Slides were counted using an epifluorescence microscope, and VLP

abundance was calculated using the equation:

(2) $VLP_{ST} = (\{RSF \times A_{Cells} \times [(100/n)/V]\} \times DF) - Blank$

VLP_{ST} is the number of total VLPs in the sample (VLPs mL^{-1}), RSF is the grid-reticule scaling factor, A_{Cells} is the average number of viral particles per field, n is the number of boxes counted within the grid per field of view, V is the volume of sample filtered, and Blank is the $Cells_{ST}$ of a 0.02 μm filtered seawater blank calculated from equation (2) without subtraction of the variable Blank (Patel et al., 2007).

Production measurements

Tritium (^{3}H) radiolabeled leucine (Leu) and thymidine (TdR) were added to separate whole water samples at a concentration of 10 nM ^{3}H and then incubated in on-deck flow-through incubators (Bell, 1993, Kirchman, 1993, Simon and Azam, 1989). One h incubations were used to minimize significant changes in microbial community due to "bottle effects" but also to allow time for *K. brevis* to incorporate the isotope (Ferguson et al., 1984, Simon and Azam, 1989). Incubations were terminated by filtration onto 3.0 μm and 0.2 μm Nucleopore filters (>3.0 μm and Total size fractions, respectively). Filters were then treated with 5% (w/vol) chilled trichloroacetic acid (TCA, Bell, 1993) for 2 min, filtered again, and triple-rinsed with 5% TCA and single-rinsed with 80% ethanol (Kirchman, 1993). Filters were dried

overnight and all samples were counted using a Packard Tri Carb 1900TR liquid

scintillation counter.

The ratio of Leu to TdR incorporation provides a measure of the physiological

state of the community (del Giorgio et al., 2011) as Leu incorporation is a measure of

protein synthesis and TdR incorporation is a measure of nucleic acid synthesis

(Franco-Vidal and Morán, 2010). A high leucine to thymidine ratio (Leu:TdR)

indicates high metabolic rates but low cellular division and therefore an environment

with high cellular maintenance costs. Bacterial growth rate (BGR) was calculated by

dividing isotope incorporation by cell abundance so that production on an individual-

cell basis could be compared between communities to determine metabolic

differences on a per-cell basis. For the "whole" treatments the abundance of *K. brevis*

was used, and for the "bacteria" treatments, the abundance of bacteria was used.

DNA Extraction

A 60-mL syringe with a Sterivex-GP™ 0.22 µm pressure driven filter

attached was used to filter 500 mL of each treatment. The syringe was then used to

remove all water from the Sterivex™ before adding ~1 mL of DNA extraction buffer

to the filter (Crump et al., 2003). Sterivex™ filters were then sealed and flash-frozen

in liquid nitrogen until analysis.

Frozen Sterivex™ filters were processed for DNA extraction (Crump et al.,

2003). Filters were manually opened under sterile conditions and the DEB distributed

between two sterile 2 mL microcentrifuge tubes (Corning Incorporated, Corning,

New York, U.S.A.). The filter was cut into ~12 pieces and also divided between the

two microcentrifuge tubes. All tubes then had 20 µL of proteinase-K (10 mg mL^{-1}) and 20 µL of lysozyme (100 mg mL^{-1}) added before undergoing three rounds of a freeze-thaw cycle consisting of 15 minutes at -80 °C and 5 minutes at 37 °C. Following the freeze-thaw cycle, all samples were incubated in a 37 °C water bath for 30 minutes, 50 µL of filter-sterilized Sodium dodecyl sulfate (SDS) was added, and then incubated for 120 minute in a 65 °C water bath.

Upon removal from the water bath, the DNA/filter/DEB slurry was washed three times with phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). The slurry then had 0.6 volumes of room temperature 100% Isopropanol added followed by an incubation at room temperature for ~120 minutes. The DNA was then spun down into a pellet at high speed (1300 rpm centrifugation) for 30 min before rinsing the pellet three times with 70% ethanol. The pellet was then dried in a roto-evaporator before being re-suspended in 250 µL autoclaved-UV sterile ultra-pure water. One of the two duplicates was archived in a -80 °C freezer and the other used for amplification and subsequent sequencing (Crump, 2007).

Amplification of genetic material

Extracted DNA was amplified in triplicate using modified Polymerase Chain Reaction (PCR, Crump et al., 1999) with bacteria-specific primers from the Earth Microbiome Project (EMP). The 806 reverse primers (CCGGACTACHVGGGTWTCTAAT) had attached Golay barcodes, denoted by N in the primer sequence (Caporaso et al., 2012).

Cycling conditions were: initial denaturing at 94 °C for 3 min; 30 cycles of 30 s at 94 °C, 45 s at 57 °C, and 60 s at 72 °C; with a final extension step of 2 min at 72 °C (Crump et al., 1999, Sogin et al., 2006). Amplification was confirmed with gel electrophoresis using a 2.5% agarose gel (Agarose 3:1, Thermo Fisher Scientific) with TAE (0.04 M Tris-acetate, 1 mM EDTA) and SYBR® Green I nucleic acid stain (Invitrogen) added. Triplicate amplifications were combined and quantified a Quant-iT™ PicoGreen® double-stranded DNA Assay Kit according to the manufacturer's instructions (Life Technologies Incorporation).

Sequencing and Community Assessment

Samples were sent for sequencing on the Illumina-MiSeq platform (Illumina, Inc.) at the University of Maryland Genomics Resource Center (http://www.igs.umaryland.edu/grc). Sequences were demultiplexed based on the Golay barcodes and primer sequences removed the Quantitative Insights Into Microbial Ecology (QIIME, Caporaso et al., 2010) Illumina pipeline. Additional quality control, alignment, and operational taxonomic unit (OTU) assignment was done with QIIME, while taxonomy assignment was done in QIIME (Illumina sequences) and mothur (454 sequences, Schloss et al., 2009).

The QIIME pipeline for Illumina sequences first requires sequence reads to be paired together (Sequences are generated separately for the 5' and 3' strands of DNA) and demultiplexed; joining overlapping 5' and 3' strands together and separating them by sample using barcoded primers (D. Smith, Argonne National Laboratory,

2012). Demultiplex sequences were assigned OTUs using the usearch method; similar to the uclust method with the additional steps of filtering low abundance clusters and chimera detection (Edgar, 2010). Sequences were aligned using Python Nearest Alignments Space Termination Tool (PyNAST, Caporaso et al., 2010) and the Greengenes May 2013 taxonomic database as a template (McDonald et al., 2012). Taxonomy was assigned with the Ribosomal Database Project (RDP) Classifier which uses a naïve Bayesian classifier which allows for a high level of taxonomic assignments even with partial sequences (Wang et al., 2007).

Statistical analysis

Data analyses were run using Statistical Analysis Software, Version 9.2 (SAS Institute Inc., Cary, N.C., U.S.A.). Data were tested for normality and Log-transformed when not normally distributed.

Dinoflagellate abundance, bacterial production, and bacterial abundance data were tested with an ANOVA that investigated interactions between the lab or culture origin, strain (or species) of dinoflagellate, and growth phase. A significant interaction was found between strain and growth phase ($p < 0.05$) so comparisons were made between all strains in each growth phase as well as all growth phases of each strain using a Tukey's post-hoc test.

Bacterial community alpha and beta diversity were both analyzed using the default programs in QIIME. Alpha diversity, a measure of species richness within an individual sample, was measured using Faith's Phylogenetic Diversity metric (Faith, 1992). Beta diversity, a measure of taxonomic diversity between samples, was calculated with weighted and unweighted UniFrac analysis (Lozupone and Knight,

2005, Lozupone et al., 2007). The UniFrac distance metrics were then used in a Principal Coordinates Analysis (PCoA) within QIIME, which also provided visualizations of both the quantitative (weighted UniFrac) and qualitative (unweighted UniFrac) measurements provided. Qualitative measures compare communities through a presence or absence of data whereas quantitative measures account for the relative abundance of each type of organism. These measures can provide insight into different drivers of community diversity between samples so by using both a better overall picture of both diversity and driving factors (Lozupone et al., 2007).

Results

Dinoflagellate and microbial abundance

When the experiment was initiated, the abundance of *K. brevis* was 4,709 cells mL^{-1} (Fig. 5.2) which equates to an addition of ~1,177,250 *K. brevis* in each cubitainer. At the end of the incubation, this number had significantly decreased ($p <$ 0.05) in all "whole" treatments. The control and negative control treatments decreased to abundances of 2541±93 and 2474±41 cells mL^{-1} (± standard deviation), respectively, and were not significantly different from one another (Fig. 5.2). The viral concentrate treatment had a *K. brevis* abundance of 1356±114 cells mL^{-1} which
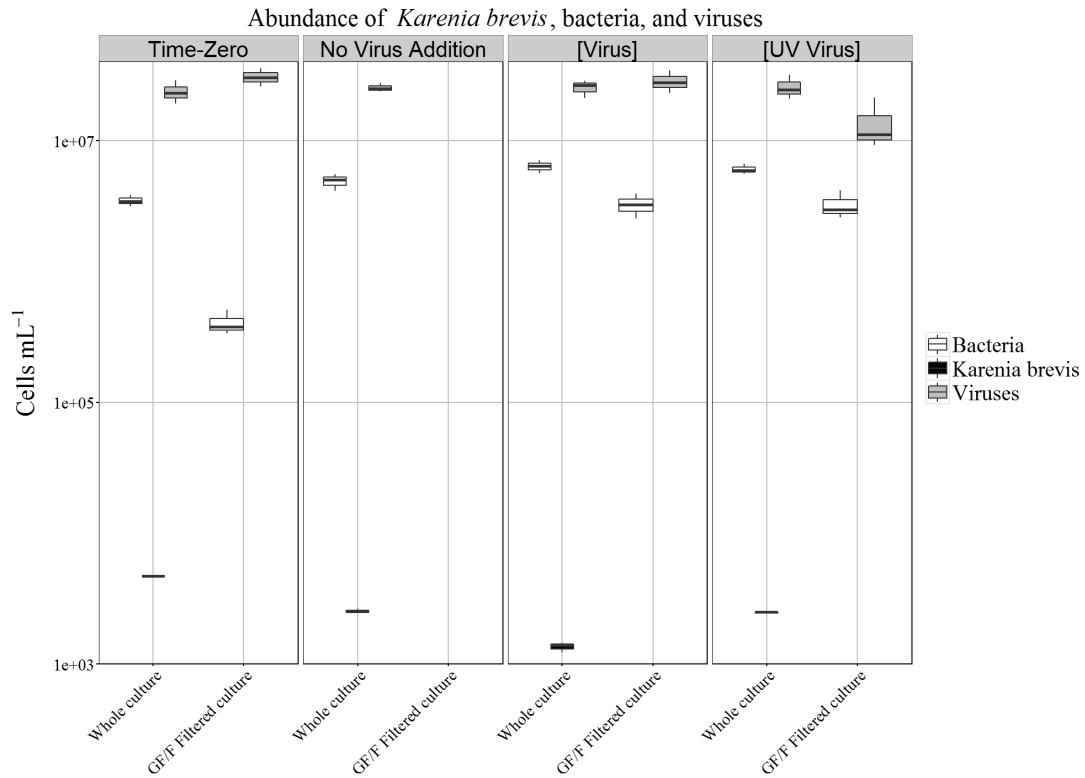
Figure 5.2: Abundance of bacteria (white boxes), *Karenia brevis* strain Sarasota B3 (black boxes) and virus-like particles (gray boxes) at the start (time-zero) and after ~40 hours of incubation with additions no viruses, viral concentrate [Virus], and UV-treated viral concentrate [UV Virus]. Co-occurring bacteria were separated from *K. brevis* by gentle filtration using a GF/F filter. The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extending to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range.

was the largest decrease in *K. brevis* abundance and a significantly lower abundance

than both controls (Fig. 5.2). Filtration of the whole culture through GF/F filter to

separate bacteria from *K. brevis* removed approximately 88% of the bacterial

community (Fig. 5.2, $p < 0.05$). Due to this artifact, abundance in all non-*Karenia*

treatments was significantly lower than whole culture treatments ($p < 0.05$) with an

average of 3.24×10^6 cells mL^{-1}. Within "whole" treatments (*K. brevis* present)

abundances increased significantly over the course of the incubation, from 3.47×10^6

cells mL^{-1} to an average of 5.77×10^6 cells mL^{-1}. Abundance in the control treatment

was similar to the negative control ($p = 0.05$) but lower than the virus treatment ($p <$

0.05) and abundances between the two viral concentrate treatments was similar ($p =$

0.57). In "bacteria" treatments abundances significantly increased between the start

and end of the experiment (Fig. 5.2, $p < 0.05$).

      VLP were all statistically similar with the exception of the "bacteria" negative

control treatment, which had a reduced abundance when compared to other "bacteria"

treatments and the "whole" negative control (Fig. 5.2, $p < 0.05$). The average for all

"whole" treatments was 2.51×10^7 VLP mL^{-1} for "bacteria" treatments which were not

significantly different 2.96×10^7 VLP mL^{-1}, and for the "bacteria" negative control

1.39×10^7 VLP mL^{-1}.
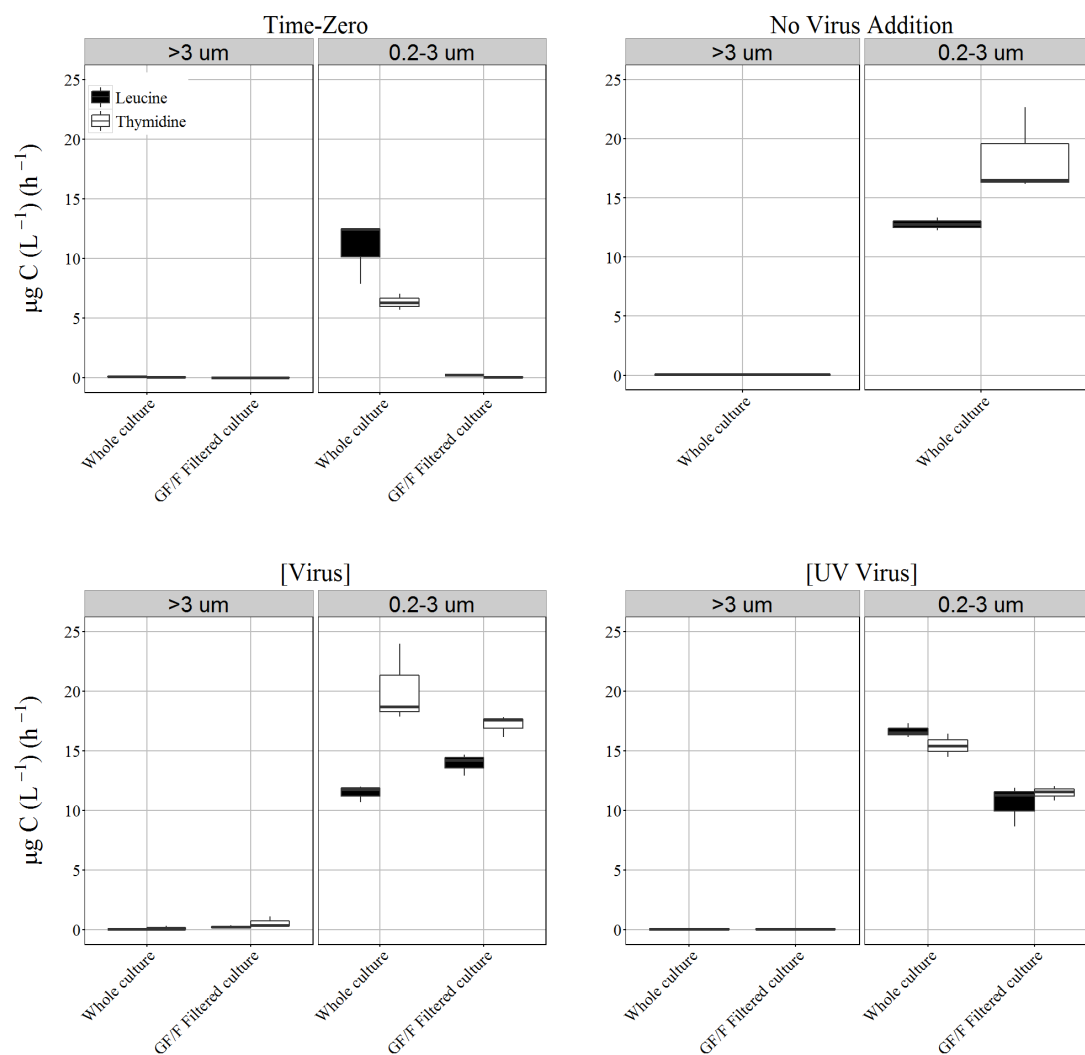
Figure 5.3: Bacterial production measured using ^{3}H-Leucine (black boxes) and ^{3}H-Thymidine (white boxes) in size fractions to separate the dinoflagellate *Karenia brevis* (>3 μm) from bacteria (0.2-3 μm). All treatments were incubated for ~1 hour after addition of isotope and production was normalized to the exact incubation time. Both size fractions were used in all treatments. The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extending to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range.

Production measurements

      Leucine production showed different results for the "whole" and "bacteria" treatments as well as between the >3 μm and 0.2-3 μm size fractions (Fig. 5.3). In the >3 μm size fraction production was similar in all "whole" treatments; within "bacteria" treatments production was higher and more variable, with the highest production in the viral concentrate treatments (0.24 ± 0.05 μg C L^{-1} h^{-1},± standard error, $p < 0.05$). Comparing like treatments of the "whole" and "bacteria" incubations found that production was higher in the "whole" time-zero treatments, higher in the "bacteria" virus concentrated treatments, and similar in the negative controls ($p = 0.48$, Fig. 5.3). In the "whole" treatments Leucine production was highest in the negative control (16.65 ± 0.34 μg C L^{-1} h^{-1}) while no other treatments were significantly different ($p=0.11$, 0.63, and 0.24 for T_0-control, T_0-concentrate, and control-concentrate comparisons, respectively). In the "bacteria" treatments, production was highest in the viral concentrate treatment (13.91 ± 0.53 μg C L^{-1} h^{-1}), significantly lower in the negative control (10.60 ± 0.99 μg C L^{-1} h^{-1}), and lower still in the time-zero treatments (0.21 ± 0.02 μg C L^{-1} h^{-1}).

      Thymidine production had similar trends in both size fractions. In the >3 μm size fraction the only significant difference was that the "bacteria" virus concentrate treatments was higher than the Time-zero, negative control, and "whole" virus concentrate treatments ($p < 0.05$, Fig. 5.3). In the 0.2-3 μm size fraction, the virus concentrate treatment had the highest production in both "whole" and "bacteria" treatments (20.17 ± 1.92 and 17.17 ± 0.52 μg C L^{-1} h^{-1} for "whole" and "bacteria," respectively $p = 0.08$). Comparing within size fractions found that the "whole"

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control treatment was not significantly different from either the viral concentrate or

negative control (p = 0.30 and 0.08, respectively); all had higher production than at

Time-zero (Fig. 5.3). As with Leucine, in the "bacteria" treatments production was

highest in the viral concentrate treatment and lowest in the Time-zero (17.17±0.52,

11.48±0.36, and 0.04±0.003 for concentrate, negative control, and time-zero,

respectively, Fig. 5.3).

There were no significant differences in bacterial growth rate (BGR)

calculated from leucine or thymidine uptake in the >3 µm size fraction ("whole"

treatments only); BGR averaged 7.12×10^{-6} pmol ^{3}H cell^{-1} h^{-1} for leucine and 1.72×10^{-6}

pmol ^{3}H cell^{-1} h^{-1} for thymidine (Fig. 5.4). In the 0.2-3 µm size fraction Leucine-BGR

was significantly higher than Thymidine-BGR in all time-zero and post-incubation

measurements. The highest BGR was measured with leucine in the viral concentrate

"bacteria" treatment (1.43×10^{-3}). In the "whole" treatments Leucine-BGR decreased

during the incubation while the Thymidine-BGR increased: BGR in all incubated

treatments was statistically similar so while growth did change during the incubation

period, it was not due to the presence or lack of a viral concentrate. In the "bacteria"

treatments leucine and thymidine BGR followed the same pattern; highest BGR in the

viral concentrate followed by the negative control and time-zero, respectively (all

significantly different).

There were no significant differences in the Leu:TdR between any treatments

in the >3 µm size fraction. Most Leu:TdR encompasses the cutoff for balanced

growth (≤10) with only the "bacteria" viral concentrate treatment completely below

the cutoff (Fig. 5.5). In the 0.2-3 µm size fraction Leu:TdR dropped significantly

between the time-zero and post-incubation measurements for all both "whole" and "bacteria" treatments, treatments were not significantly different from one another.

Microbial community composition

Samples had an average of 52,340 OTUs with one exception which had only 17,622 OTUs; this sample was removed from further analyses. Without this sample, there were a total of 1,046,797 OTUs. Nonmetric Multidimensional Scaling (NMDS) of weighted UniFrac distances demonstrate that the "whole" and "bacteria" treatments did not clustered with one another (Fig. 5.6). The "Whole" samples retained clustering across treatments, with the time-zero samples having the tightest clustering (Fig. 5.7). The "bacteria" samples formed two clusters both removed from the "whole" samples: time-zero samples clustered separately from samples incubated with live or UV-treated viral concentrates.

The Principal Components Analysis (PCoA) of bacterial communities only had one significant axis; the first axis (PC1) accounted for 64.18% of the variance (Fig. 5.8). The second axis (PC2) was significant when a Kaiser's criterion was used and explained 15.19% of the variation in the PCoA. Production and abundance data captured 93.43% of this variation (CCA, data not shown), of which almost 98% was accounted for in the first two constraints (CCA1, 82.45% and CCA2, 15.19%). Both constraints were significant (ANOVA, p=0.05) with the main factors influencing them being the abundance of *K. brevis* and bacterial abundance (CCA1) or the VBR and bacterial production (CCA2). An ANOVA of these factors found *K. brevis*,
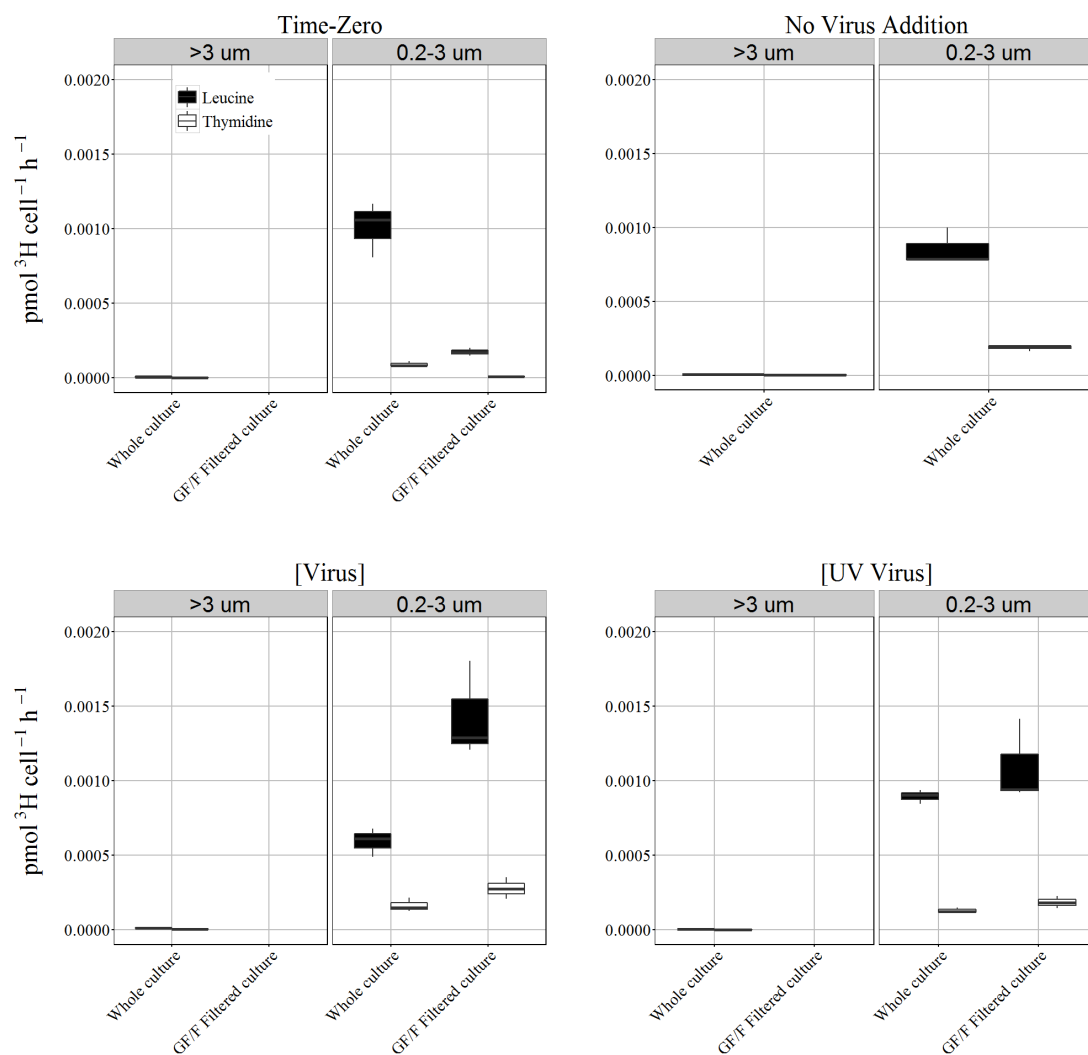
Figure 5.4: Cell-specific incorporation of ^{3}H-Leucine (black boxes) and ^{3}H-Thymidine (white boxes) in the dinoflagellate (>3 µm) and bacteria (0.2-3 µm) size fractions. Incorporation was normalized by treatment to the number of intact *Karenia brevis* cells (>3 µm size fraction) or the number of bacterial cells (0.2-3 µm size fraction) in that treatment.
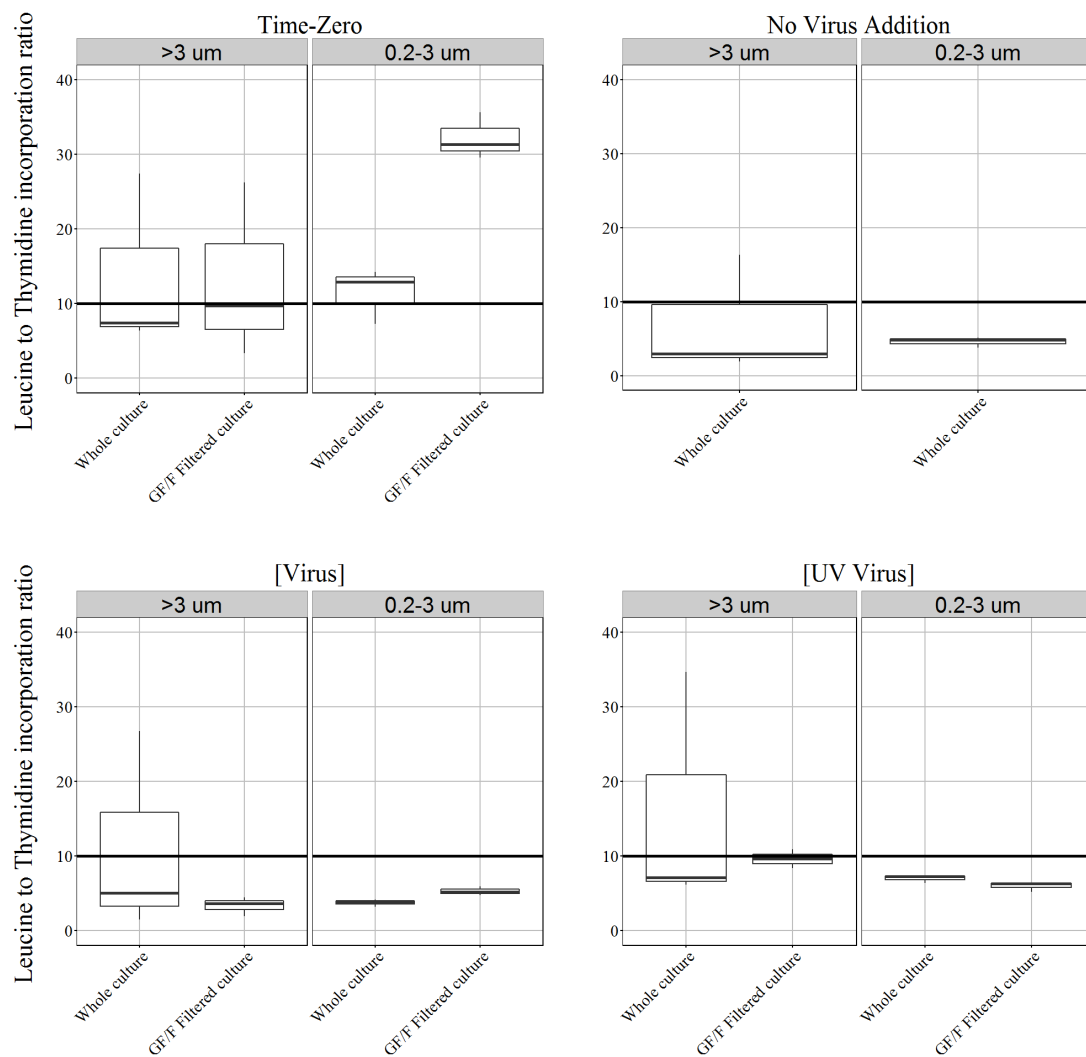
Figure 5.5: The leucine to thymidine ratio of *Karenia brevis* strain Sarasota B3 treated with live or UV-treated viral concentrate, and controls. The black line represents the threshold for balanced (above) or unbalanced (below) growth (del Giorgio et al., 2011).
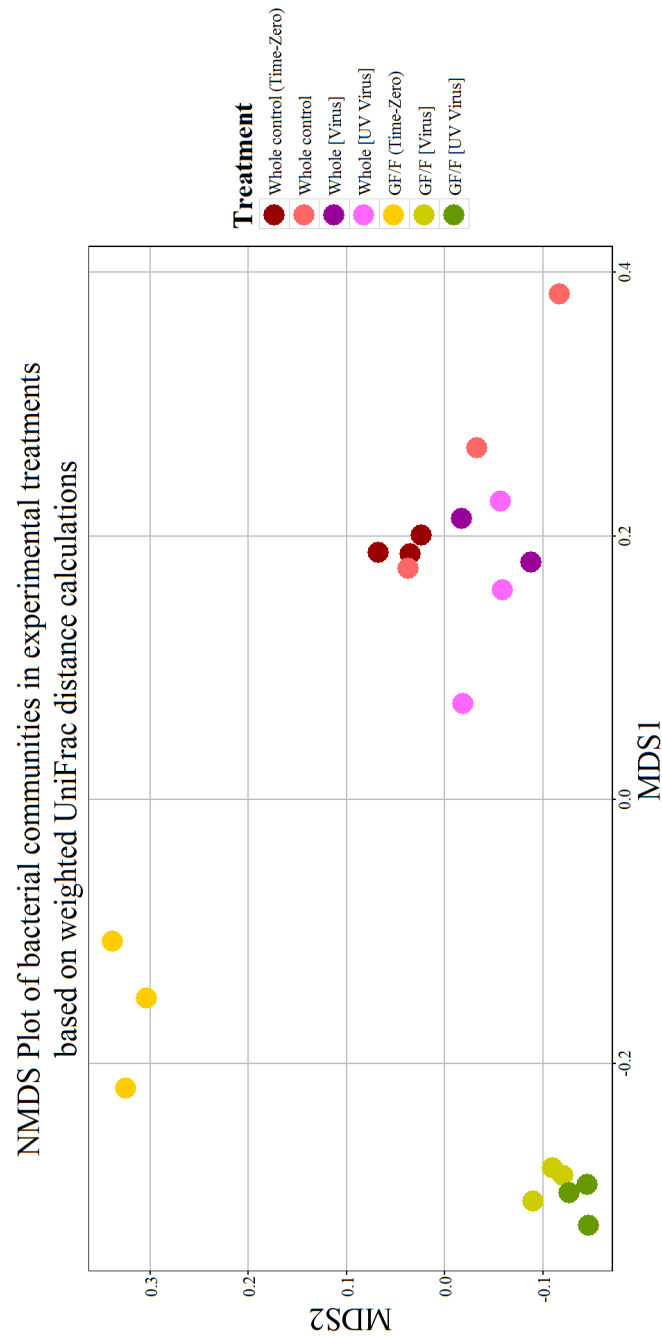
Figure 5.6: Clustering of bacterial OTUs in a *K. brevis* culture before and after exposure to live and UV-treated viral concentrates. Bacterial OTUs were classified according to the Greengenes May 2013 taxonomic database (McDonald et al., 2012) and UniFrac distance calculations were done using QIIME (Caporaso et al., 2010). "Whole" represents treatments of raw culture and "GF/F" represents culture pre-filtered through a GF/F filter (nominal pore size 0.7 μm) to isolate the bacterial community from the dinoflagellate, further amended as described in the methods. Stress of the plot was 0.059.
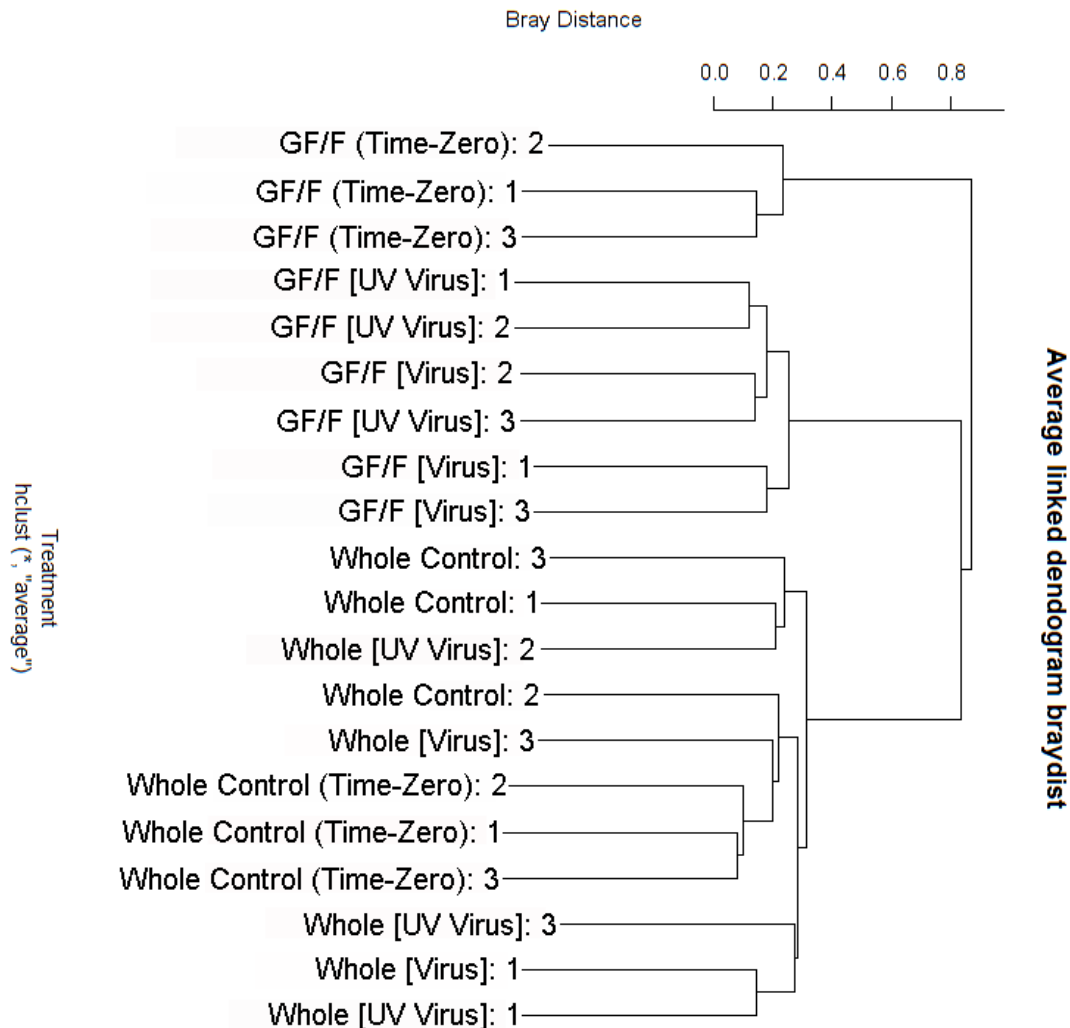
Figure 5.7: Average linked dendrogram of bacterial communities in *K. brevis* cultures unaltered and pre-filtered to remove the dinoflagellate. Calculations are based on weighted UniFrac distances provided by QIIME and analyzed using a hierarchical clustering algorithm within the {stats} package in R (both version 3.0.1). Labels denote the size fraction, treatment, and replicate.
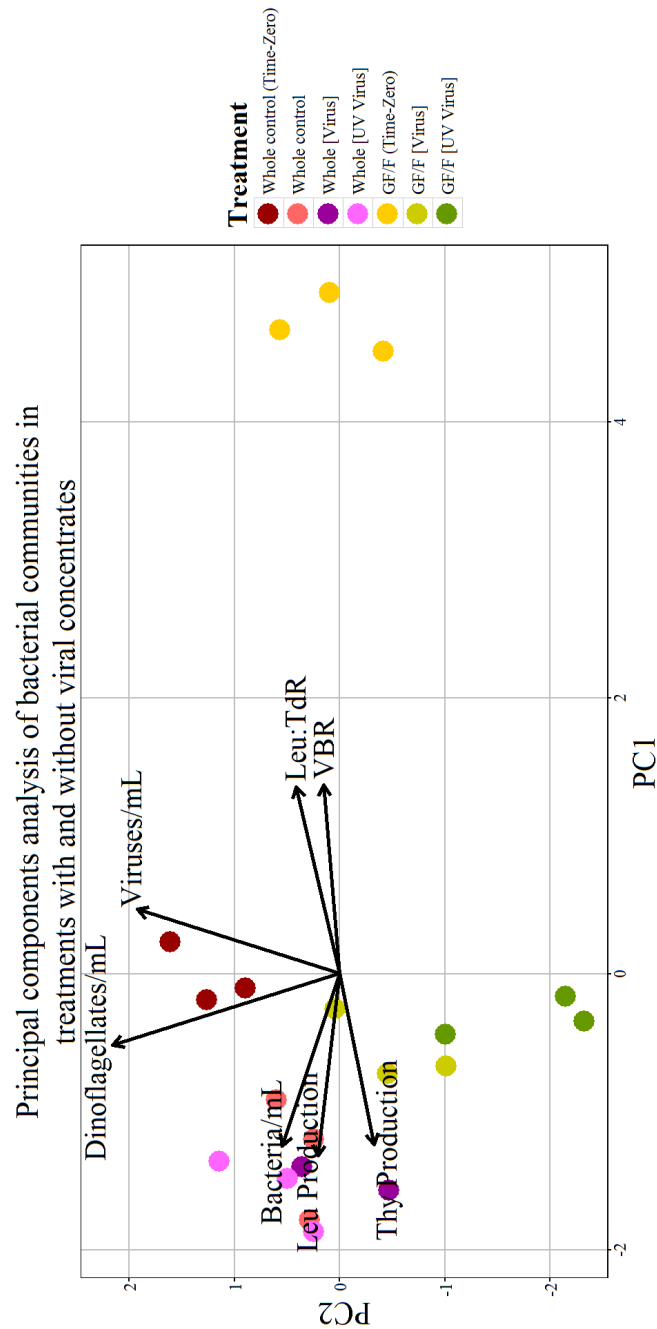
Figure 5.8: Bacterial communities in cultures of *K. brevis* clustered using Principal Components Analysis (PCoA). Communities were left unaltered (whole) and pre-filtered through a GF/F filter to isolate the bacterial community from the dinoflagellate (nominal pore size 0.7 μm) before addition of live or UV-treated viral concentrates. Communities separated significantly along the first principal component axis (PC1) according to a broken stick analysis and along PC1 and PC2 according to Kaiser's criterion. These axes explained 79.37% of the variation within the analysis.

bacteria, and virus abundance, the VBR, and TdR-production all significant (Data not shown).

Dissimilarity between samples was best explained by partitioning samples by individual treatment (ADONIS, p=0.001, R^2=0.9361). Partitioning samples by the type of viral concentrate addition (live, UV-treated, or none) was also significant but had a very low coefficient of determination (p=0.04, R^2=0.2281).

The most striking difference between bacterial communities in the "whole" and "bacteria" treatments is the difference in the proportion of *Flavobacteriales* and *Rhodobacteraceae* (Fig. 5.9). In all whole treatments the *Flavobacteriales* make up more than 45% of the community, and in the control and live virus treatments >60%. On the other hand, *Rhodobacteraceae* were ~12% of the time-zero "whole" treatments and decreased to ~4, 9, and 6% in control, virus, and negative control incubations, respectively. In "bacteria" treatments, *Flavobacteriales* were only 12% of the community at the beginning of the incubation and less than 6% after 24 hours. In "bacteria" time-zero samples *Rhodobacteraceae* were less than a percent, but increased to ~32% in live virus treatments and ~26% in UV-treated viral concentrate incubations (Fig. 5.9).

Both *Flavobacteriales* and *Rhodobacteraceae* were significant indicator taxa. The abundance of *K. brevis* and bacteria were both positively correlated with *Flavobacteriales* (p = 0.001 and 0.022, respectively) with correlation coefficients of 0.74 for *K. brevis* and 0.50 for bacteria. Bacterial growth rate was correlated with the presence of *Rhodobacteraceae*; both Thymidine-BGR (p = 0.0002, R^2 = 0.75) and Leucine-BGR (p = 0.009, R^2 = 0.56) had positive interactions with this group.

Bacterial community composition before and and after exposure to live and UV-treated viral concentrates in whole and pre-filtered cultures of *Karenia brevis*

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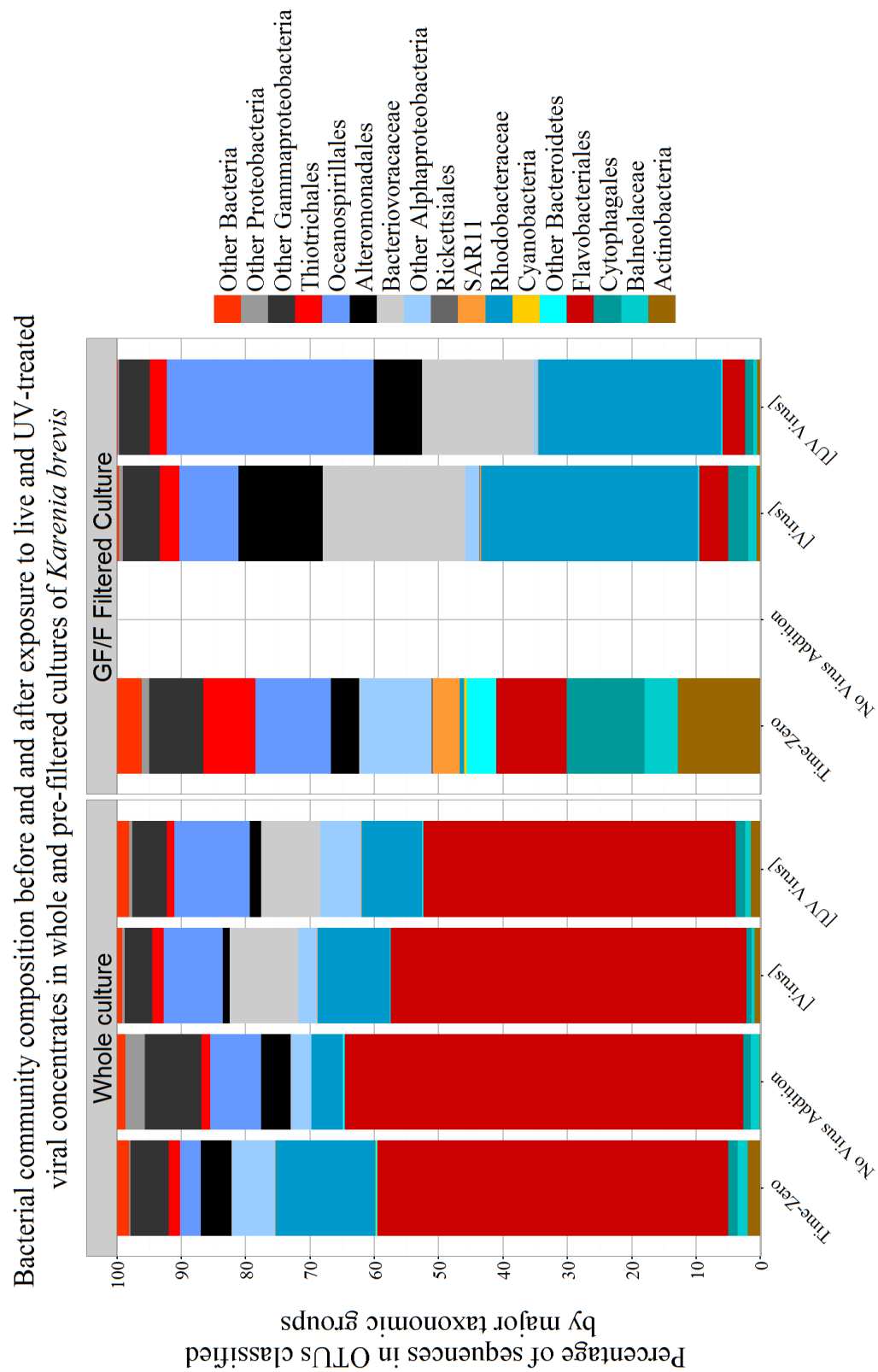
Figure 5.9: Bacterial community composition in whole and bacterial size fractions of *Karenia brevis* cultures before and after exposure to live and UV-treated viral concentrates. Composition is depicted at the percentage of OTUs classified by major taxonomic groups as determined by Mothur using the Greengenes database (Schloss et al., 2009, McDonald et al., 2012). There were a total of 809,664 sequences divided into 679 OTUs.

There was also an increase in *Bacteriovoracaceae* in all treatments that had a viral concentrate added (both live and UV-treated). In time-zero and control treatments *Bacteriovoracaceae* were < 0.2% of identified OTUs but were 7-10% and ~19% of OTUs in "whole" and "bacteria" treatments with viral concentrate additions, respectively. According to the indicator analysis, *Bacteriovoracaceae* constituted an indicator species and linear analysis indicated a negative correlation with *K. brevis* abundance ($p = 0.0038$, $R^2 = -0.60$) and positive correlations with Thymidine-BGR and Leucine-BGR ($p = 0.0023$ and 0.026, $R^2 = 0.63$ and 0.49, respectively).

Both *Altermonodales* and *Oceanospirillales* were significant indicator taxa. The proportion of *Altermonodales* OTUs was negatively correlated with bacteria abundance ($p = 0.003$, $R^2 = -0.62$) and positively correlated with Leucine-BGR ($p = 0.003$, $R^2 = 0.63$). The *Oceanospirillales* were negatively correlated with the abundance of *K. brevis* ($p = 0.002$, $R^2 = -0.64$). In "whole" treatments, the proportion *Altermonadales* decreased during the incubation and the proportion of *Oceanospirillales* increased; these differences were less pronounced in samples amended with the viral concentrate. In "bacteria" treatments both groups increased during the incubation. The *Altermonadales* increased threefold in samples amended with the viral concentrate and twofold in those with the UV-treated concentrated. The opposite was seen for *Oceanospirillales*, which increased only twofold after addition of the live virus concentrate and threefold after addition of the UV-treated concentrate.

Discussion

While results were inconclusive as to whether the viral concentration procedure increased the abundance of viruses at the beginning on treatment incubations, other data support previous hypotheses that phages lyse bacteria which co-occur with *K. brevis*, and not the dinoflagellate directly. A large proportion of the bacterial communities in treatments that contained *K. brevis* were *Flavobacteriales*, which have been found associated with both toxic and non-toxic dinoflagellates (Hold et al., 2001). In treatments where *K. brevis* was first removed, the bacterial communities were dominated by *Alphaproteobacteria, Deltaproteobacteria*, and *Gammaproteobacteria* which are commonly observed in decaying algal blooms (Pinhassi et al., 2004). This suggests a synergistic interaction exists between *K. brevis* and members of the *Flavobacteriales*. In treatments where the live virus concentration was added there were high levels of bacterial production and growth but no significant increases in bacterial abundance which indicate lytic viral attack. If viruses were infecting and lysing members of the *Flavobacteriales*, a potential synergitic interaction with *K. brevis* could have been disrupted, leading to termination of the culture. These relationships need to be further explored. However, this study provides observations of bacterial abundance, physiology, and community composition that support previous work and also conclude that viruses are capable of terminating blooms of *K. brevis* through lysing co-occurring bacteria.

Experimental design

This experiment was designed to explore the hypotheses posed by Paul et al. (2002) that viruses were altering relationships between bacteria and *K. brevis* to the detriment of the dinoflagellate. It was not designed to determine if a viral concentrate would reduce or terminate *K. brevis* directly, as this has been previously investigated. Tangential-flow filtration, one of the most common methods for viral concentration requires close monitoring of flow rate and back pressure (Wommack et al., 2010). Prefiltration of seawater before concentration can reduce the overall viral abundance, but prolongs the integrity of the TFF. This experiment used a prefiltration step using 0.2 μm pore size cellulose acetate filters which have been shown effective at removing both particulate matter and bacteria (Gasol et al., 1998), but did not account for the potential removal of up to two-thirds of the viral particles prior to concentration (Boehme et al., 1993). This may have contributed to a lack of variation in the total virus abundance between treatments.

Use of a GF/F filter (effective pore size 0.7 μm) to separate *K. brevis* from the associated microbial community co-occurring in culture was not effective as intended. Almost 90% of the bacteria were removed during filtration and the community that remained was different from that found in the unmodified culture. These filters were chosen because *K. brevis* is capable of passing through larger pore-sizes (C. Heil, pers. comm.). Efforts were made to replace filters before they became clogged, but this was not an efficient way to separate the bacteria from the dinoflagellate. Given the significant difference in production between the >0.3 μm and 0.2-3 μm size classes it appears that most bacteria within cultures were free-

living (Eloe et al., 2011), and filtration removed the most productive components of the community. Furthermore, of the communities that passed through the filters, only one (Leucine-BGR, virus treatment) had a higher growth rate and none had higher bacterial abundance, as has been previously observed (Ferguson et al., 1984). More consideration needs to be placed on effective filtration methods that do not also alter the community composition but are still capable of separating *K. brevis* from associated free-living bacteria.

The length of incubations to obtain production measurements was deliberately kept short so that even in the larger size fraction the measurement would be limited to the bacterial community (del Giorgio et al., 2011). The difference in production between the algal (>3 µm) and bacterial (0.2-3 µm) size fractions indicate the 1h incubations were able to measure bacterial uptake of the isotope without significant uptake by *K. brevis* occurring (Wholers-Zöllner et al., 2011). Uptake was measured in the algal size fraction, but it was minimal; even if all production in that size fraction were attributed to *K. brevis*, algal growth rate (calculated the same way as BGR) was less than that of the bacterial size fraction for the same treatment.

There is a possibility that the ^{3}H-thymidine (^{3}H-TdR) used to measure bacterial production was also incorporated into phage genomes during lytic replication of viruses within host cells (Steward et al., 1992). This method focuses on measuring incorporation of ^{3}H-TdR into the viral size fraction (0.02-0.2 µm), which was not retained in this study, and has been found to be minimal in oligotrophic waters (at or near background concentrations, Noble and Fuhrman, 2000). Given this information it is likely that measured incorporation of ^{3}H-TdR was due to synthesis of

bacterial nucleic acids. It is possible that some incorporation of ^{3}H-TdR went to synthesis of phages, but this requires the molar content of viruses to be known or direct measurement of label incorporation in the viral size fraction (Steward et al., 1992). Future studies may want to also filter the 0.2 μm filtrate across 0.02 μm filters to determine incorporation of ^{3}H-TdR into phage genomes in an attempt to correct bacterial uptake rates and determine the portion of ^{3}H-TdR incorporation being utilized for bacteria vs. phage nucleic acid synthesis.

Viral lysis was not a major factor of bacterial mortality

Viral abundances within samples, including those with viral concentrates added, were typical of those found in Tampa Bay during the fall months (Williamson et al., 2002). The ratio of VLPs to bacteria in all treatments was also <10, which typifies communities with a low incidence of viral-mediated bacterial mortality (Jacquet et al., 2005). Higher virus counts would be expected in incubated treatments given either the addition of a viral concentrate or a high viral production rate (Steward et al., 1992).

The environment exerts selective pressure on the viral assemblage and given that the bacterial assemblage was cultured and the viral assemblage was not, the difference in environments could have led to a mismatch between the viral community and available hosts (Angly et al., 2006). The most available host in "whole" treatments were *Flavobacteriales* and while it is likely there were *Flavobacteriales*-specific phages present they likely comprised less than 3% of the viral community given the high diversity observed in viral communities (Breitbart et

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al., 2002). If this were the case there would be enough *Flavobacteriales*-specific phages to infect ~24% of that group in the "whole" treatments. Using an average burst size of 27.6 VLPs per cell (Williamson et al., 2002) and a viral decay rate of 7.2% h^{-1} (Noble and Fuhrman, 1997) a 24 hour period with only one lysis event would yield ~1.86x10^6 VLPs mL^{-1} which is an order of magnitude lower than observed. Two lysis events would produce VLP abundance on the order of 10^7 mL^{-1} but also require lysis of more than 100% of the *Flavobacteriales* population. A diverse viral community would lead to infection and lysis of some of the bacteria present, but the differences in the cultured and naturally occurring bacterial communities would require an extended incubation before community shifts would be observed (Frada et al., 2008). It is possible that the one day length in this study did not allow ample time for lytic cycles to fully complete and therefore new viruses may have been within intact hosts (Wilcox and Fuhrman, 1994). The stable VLP abundance suggests that viral production was occurring but at the same rate of removal of particles and therefore not indicative of increased lysis following the addition of the viral concentrate (Noble and Fuhrman, 2000).

Bacterial production data suggests that there were physiological differences in the communities exposed to live vs. UV-treated viral concentrates. The viral concentrate communities had significantly higher thymidine production than those exposed to the negative control. As such communities exposed to the live virus were undergoing more cell division than biomass production, whereas the negative control communities had equal levels of each (Franco-Vidal and Morán, 2010). The high rate of thymidine production would allow for bacterial division to account for mortality

due to lysis as well as the increase in bacteria abundance within the virus treatments (Suttle, 1994). The Leu:TdR ratio of these treatments also indicates that the bacterial communities were efficiently growing, and therefore in an environment conducive for both catabolic and anabolic processes (del Giorgio et al., 2011). Nutrient pools may have been amended by decay of *K. brevis*, allowing for balanced bacterial growth (Schäfer et al., 2002).

Production in negative controls was balanced between both biomass production (Leucine) and cell division (Thymidine) but yielded similar bacterial abundance as live virus treatments. This indicates less cell division was needed for the same increase in total abundance and therefore reduced bacterial mortality within these treatments. As cultures were maintained without zooplankton or protist grazers (M. Garrett, pers. comm.) two possible sources of bacterial mortality were bacteriophages (Fuhrman, 1999) and *K. brevis* mixotrophy (Glibert et al., 2009), yet neither appear to have been an important component of bacterial mortality within this experiment.

Since the viral concentrate added was first subjected to UV irradiation the only active phages were ones already within the culture (Noble and Fuhrman, 1997). If existing phages had a high rate of infection and lysis the bacterial populations within cultures would have crashed before the experiment began or an increase in viral particles would have been observed (Wilcox and Fuhrman, 1994). Both bacteria and virus abundances were statistically similar between live and UV-treated viral treatments suggesting there was not significant viral-induced mortality of bacteria within the negative controls.

Mixotrophy is a strategy typically employed in nutrient-poor habitats as a means to augment necessary nutrients for growth (Burkholder et al., 2008). As the initial cultures were grown in batch for several days before being further diluted using oligotrophic water from the Gulf of Mexico, it is possible experimental nutrient conditions favored mixotrophy instead of autotrophy. Mixotrophy requires fewer cellular resources to maintain photosynthetic apparatus so more efficient, and possibly increased, growth of *K. brevis* would be expected if mixotrophy were being employed (Stoecker et al., 2006b). In all treatments *K. brevis* abundance decreased; if mixotrophy was occurring it was likely not an important component of *K. brevis* growth or bacterial mortality.

No cultures exhibited signs of large scale lysis events brought about by viral infection. A study of bloom termination of the coccolithophore *Emiliania huxleyi* was accompanied by an increase in VLPs at the same time, and following, significant decreases in abundance of *E. huxleyi* (Bratbak et al., 1993). No such shifts were observed in either the bacterial community composition or *K. brevis* abundance despite there being large numbers of potential hosts (*Flavobacteriales* and *K. brevis*, respectively) for viral infection (Fuhrman, 1999). Despite bacterial production in some treatments indicating high rates of cell division, a hallmark of viral-mediated lysis, no increases in viral abundance were observed. Instead, balanced bacterial growth in all treatments suggests that bacterial mortality was minimal and communities were not energetically limited. Perhaps the most telling of all was the similarity between bacteria community composition in live virus and negative controls. In "whole" treatments community composition was stable throughout the

experiment and "bacteria" treatments appeared to be reaching a new steady-state community. The difference between the communities in "whole" and "bacteria" treatments was most likely an artifact of filtration prior to the start of the experiment rather than any additions of viral concentrate.

Interactions between *K. brevis* and bacteria play a vital role in bloom dynamics

Data indicate the viral concentrate was either not successful or the experiment itself was not of sufficient length for lytic cycles to affect either *K. brevis* or bacteria. The secondary hypothesis, therefore, could not be addressed with data produced by this experiment. Bacterial communities showed no differences between treatments amended with filtered seawater, live virus concentrates, or UV-treated virus concentrates. Communities clustered according to experimental design indicating a primary influence on communities prior to the start of the experiment and not as a result of any treatment.

Building upon a previous hypothesis, production and community data strengthen the case that interactions between *K. brevis* and bacteria have an impact on the growth and survival of both and that viruses are acting upon the bacteria. In unaltered cultures bacterial communities were dominated by the *Flavobacteriales* which are dominant in cultures of both toxic and non-toxic dinoflagellates (Hold et al., 2001) as well as diatoms (Pinhassi et al., 2004). When the cultures were filtered to remove *K. brevis* the community was initially diverse, with many groups in similar proportions, but then quickly became dominated by *Alphaproteobacteria,*

Deltaproteobacteria, and *Gammaproteobacteria*. These groups have been observed to increase in abundance when algal blooms are decaying (*alpha-* and *gammaproteobacteria*, Pinhassi et al., 2004) or when *K. brevis* is in low abundance (Jones et al., 2010). The high proportion of *Flavobacteriales* in "whole" treatments and *Alphaproteobacteria, Deltaproteobacteria*, and *Gammaproteobacteria* in "bacteria" treatments suggests a synergistic link between *K. brevis* and *Flavobacteriales*. When this link is disrupted (as was done in this case by removal of *K. brevis*), then other groups common on the WFS when *K. brevis* abundance is low are able to thrive.

Bacterial production and growth within the "whole" samples treated with the live virus concentrate suggest the synergistic interaction between *K. brevis* and *Flavobacteriales* was disrupted. Synergistic interactions are typically driven by exchange of vitamins, trace elements, and dissolved carbon and nitrogen compounds (Amin et al., 2012). Production measurement show higher rates of nucleic acid synthesis and balanced growth within live virus treatments, which indicates bacteria in these treatments were putting more resources into cellular division. While viral infection did not completely collapse any community, the increase in heterotrophic production within the bacterial community would have strained or eliminated availability of compounds for use by *K. brevis* and could have been a driving factor in the significant decrease of *K. brevis* within these treatments (Fuhrman, 1992).

Therefore, relationships between *K. brevis* and associated bacteria need to be further explored to determine the rate, currency, and dominant bacteria of any such interactions. Once these are better characterized, longer experiments utilizing viral

concentrates will be able to focus on these interactions to determine if disruption of this relationship by lytic infection contributes to termination of *K. brevis* blooms. The primary hypothesis of this study remains but can be modified to narrow the scope. A new working hypothesis is proposed: A synergistic relationship between *K. brevis* and *Flavobacteriales* helps to maintain and promote blooms of *K. brevis*; lytic infection of the *Flavobacteriales* by viruses (likely given the dominance of this group) disrupts the bacteria-dinoflagellate interaction leading to termination of the dinoflagellate and a shift in the bacterial community. Testing this hypothesis would provide valuable insight into the viral dynamics as well as the interactions between bacteria and the dinoflagellate within blooms of a toxic dinoflagellate. Potential bacteria-dinoflagellate interactions will also help in the determination of the nutrient requirements of *K. brevis* blooms in multiple growth stages and how the bacterial community may alter the timeframe of said growth stages (Amin et al., 2012). This could provide new tools for managers to better monitor naturally occurring blooms and for modelers to better predict the occurrence and duration of blooms so that bloom mitigation strategies can begin to shift from reactive to proactive (Heil and Steidinger, 2009).

Chapter 6: Microbial community composition and production within different cultured isolates of Karenia brevis *and related species.*

Abstract

Multiple strains of the red tide dinoflagellate *Karenia brevis* are maintained in cultures that also contain associated bacterial communities. This study used a combination of enumeration, uptake of ^{3}H-Leucine and ^{3}H-Thymidine, and genetic sequencing to investigate the composition and function of bacterial communities associated with cultures grown at Florida Fish and Wildlife Research Institute (FWRI) and MOTE Marine Laboratory (MOTE). Bacterial communities from cultures maintained for varying numbers of years were dominated by the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex and *Alphaproteobacteria* and were not reflective of communities found occurring naturally on the West Florida Shelf or within blooms of *K. brevis*. The primary bacteria within of each group could further identify strains grown at FWRI vs. MOTE indicating culturing conditions can further alter the community. The oldest cultured strain of *K. brevis*, the Wilson clone, had a unique microbial community which clustered separately from cultures more recently isolated. A "low toxin" variety of the Wilson clone maintained at MOTE (originally obtained with normal toxin production from FWRI), had a higher proportion of *Alphaproteobacteria* and lower proportion of CFB bacteria than the standard Wilson clone. Natural communities of *Alphaproteobacteria* dominate consumption of amino acids, which are also utilized by *K. brevis* in toxin synthesis pathways. Given the

dominance of *Alphaproteobacteria* in the Wilson "Low Toxin" cultures, it is possible that there is a competitive interaction between the bacteria and dinoflagellate which limits toxin production. While bacterial community composition in cultures is different from natural blooms, specific bacteria-dinoflagellate interactions may be preserved in cultures which mimic ecological relationships. A better understanding of these relationships is needed to further understand *Karenia*-bacteria interactions and determine how well cultures represent naturally occurring processes.

Introduction

Obtaining accurate physiological measurements of marine phytoplankton can be difficult to accomplish in the field. Therefore as a proxy, cultures have been isolated and grown in laboratories for controlled experiments (Wood and Leatham, 1992). Use of cultures can provide relevant information about naturally occurring populations but any such conclusions are based on critical assumptions regarding both the nature of the culture, and its response to experimental conditions (Brand et al., 1981). The primary assumption is that a single-isolate culture, often called a "strain" or "isolate," accurately represents all cells of that particular species in the field (Schaeffer et al., 2007). This is based on a hypothesis that genetic variability is limited enough to minimize physiological (phenotypic) variability. The assumption is considered valid as a compromise in experimental design; experiments that study all strains of a given population can identify a range of observations which can allow for

a single-strain experiment to provide a representation of the species as a whole (Brand et al., 1981). The studies that developed the above assumption did not account for two other components which also lead to unacknowledged assumptions. The first is the time spent in culture and the second is whether the culture was grown axenically (bacteria-free) or non-axenically (containing a bacterial community subject to the same culturing conditions as the phytoplankton).

The dinoflagellate *Karenia brevis* (Davis) G. Hansen et Moestrup is a prime organism for re-evaluating the assumption that a single-isolate culture provides an accurate representation of naturally-occurring cells. Due to regularly occurring blooms of *K. brevis* on the West Florida Shelf (WFS) near the Florida Coast that caused fish kills and human health issues, *K. brevis* was isolated in laboratory culture for further study in 1953. This first isolated is now referred to as the Wilson clone (Wilson and Collier, 1955), which has been the primary culture for *K. brevis* research until the turn of the century (Lidie et al., 2005).

As more isolates of *K. brevis* became available, several studies investigated the physiological ranges of *K. brevis* across multiple strains. Studies determined the electron transport efficiency and photoresponse of ten *K. brevis* isolates were not significantly different (Schaeffer et al., 2007) but uptake and growth efficiency when grown on urea, nitrate, or ammonium were significantly different (Sinclair et al., 2009). This highlights that the parameter being measured is an important consideration when choosing between one or multiple isolates for a culture experiment. The experimental design of these studies failed to address the fact that *K.*

brevis is difficult to culture under axenic (bacteria free) conditions, and therefore is typically studied with bacteria present (Schaeffer et al., 2007).

A genetic analysis of 40 cultures of *K. brevis* determined that a genetic divergence exists between *K. brevis* isolated in Texas and Florida waters (Henrichs et al., 2013). Despite these divergences between isolates of *K. brevis* a majority of the genetic diversity within naturally occurring populations is contained within cultured isolates. Other populations of dinoflagellates have shown genetic divergences over geographic ranges (Nagai et al., 2009) which may have been influenced by human-ocean interactions (Nagai et al., 2007). These studies did not study co-occurring bacteria, highlighting the importance of determining whether there are differences in bacterial community composition across similar geographic distances which may be exerting selective pressures on the genetic makeup of dinoflagellate communities.

Studies of bacteria co-occurring in phytoplankton cultures have found multiple interactions that can confound both physiological and molecular results. Bacteria have diverse metabolic capabilities that can alter culture growth on a variety of media (López-Lozano et al., 2002) and can also enhance toxin production in some cases (Bates et al., 1995). Polyketide synthase genes, necessary for the formation of many harmful algae associated toxins, have been found in both dinoflagellates and associated bacteria (Snyder et al., 2005, Perez et al., 2008). In other cases, researchers have had to ensure co-occurring bacteria are eliminated so that an accurate genotype of the cultured phytoplankton is achieved; this is also an important consideration in probe design (Laloui et al., 2002). Not accounting for the physiology and biogeochemistry of bacteria within experimental cultures can lead to inaccurate

estimation of parameters, false inferences about naturally occurring populations, and a lack of recognition of the potential relationships between *K. brevis* and bacteria.

In field studies, bacterial communities had significantly higher production within blooms of *K. brevis* (Heil et al., 2004), higher production in blooms in initiation phase, and lower production within blooms in maintenance phase (Chapter 4). Bacterial abundance surrounding blooms is variable (Jones et al., 2010) which could be due to mixotrophic capabilities of *K. brevis* (Glibert et al., 2009), brevetoxins produced by *K. brevis* affecting community composition (Sipler, 2009), or allelopathy-induced trophic cascades (Weissbach et al., 2010). Several bacteria isolated from the Gulf of Mexico have been found to be algicidal towards *K. brevis* and to protect *K. brevis* from algicidal bacteria (Mayali and Doucette, 2002, Kodama et al., 2006, Roth et al., 2008a and b).

Given the wide array of potential bacteria-phytoplankton interactions observed in both field and laboratory studies of *K. brevis* and other toxic and non-toxic dinoflagellates and diatoms (Amin et al., 2012), gaining a better understanding of the bacterial communities in the many different cultures of *K. brevis* in use is essential. Cultures were chosen from Florida Fish and Wildlife Research Institute (FWRI) and MOTE Marine Laboratory. Both of these laboratories have a history of studying and culturing *K. brevis* along the WFS and continue to maintain non-axenic cultures for research purposes (Haywood et al., 2007, Sinclair et al., 2009). This study was undertaken to determine:

1. Whether the laboratory in which cultures were grown impacts the abundance and composition of bacterial communities within cultures;

2. Whether each cultured strain of *K. brevis* has unique bacterial communities and if these communities change in association with the growth phase of the *K. brevis* culture;

3. Whether bacterial communities' productivity changes between strains, laboratories, and growth phases of *K. brevis* cultures.

Data from this study provides information useful beyond the context of dinoflagellate-bacteria interactions. The targeted study of both bacteria and *K. brevis* in cultures helps determine abundances and rates that may be useful to future researchers when deciding to use one or multiple strains of *K. brevis* to accurately represent natural populations in a laboratory setting.

Methods

Phytoplankton cultures

The Florida Fish and Wildlife Research Institute (FWRI), St. Petersburg, FL provided four *Karenia brevis* isolates, one *K. mikimotoi* isolate, one *Prorocentrum minimum* isolate, and one *Karlodinium veneficum* isolate. Isolates of *K. brevis* were: Wilson (CCFWC268, 1953 isolate from Johns Pass, Madeira Beach, FL, M. Garrett, K. Steidinger, L. Campbell, pers. comm.), Sarasota (B3), Charlotte Harbor (C2), and Jacksonville (C4). All *Karenia* species were grown in three treatments so at the time of sampling triplicates of cultures were available which were: 1- in logarithmic

growth, 2- stressed, and 3- grown in no light (to stimulate termination of blooms). The dinoflagellates *P. minimum* and *K. veneficum* were only available in logarithmic growth treatments (Table 6.1). All cultures were at a salinity of 35 and grown at 25 °C at an irradiance of 60 μE m^{-2} s^{-1} provided by cool white fluorescent light bulbs on a 12:12 light: dark cycle. Cultures in logarithmic growth treatments (GR) were inocula of cultures with GP media added at two time points to increase the overall volume of culture (M. Garrett, pers. comm.). Stressed cultures (ST) were inoculations of culture with GP media followed by addition of filtered and autoclaved seawater to increase culture volume without adding additional nutrients. No Light treatment cultures (NL) were inocula of cultures with filtered autoclaved seawater to the desired volume followed by covering the culture flasks with aluminum foil two days prior to sampling. Each culture was grown in a single flask.

MOTE Marine Laboratory, Sarasota, Florida, provided four *K. brevis* isolates, one *K. mikimotoi* isolate, one *P. rhathymum* isolate, and one *Gyrodinium dorsum* isolate. Isolates of *K. brevis* were: Wilson (CCMP718), New Pass (CCMP2228), Manasota Key (CCMP2229), and Wilson "Low Toxin." The Wilson "Low Toxin" culture typically has toxin measurements an order of magnitude lower than other *K. brevis* cultures at MOTE (V. Lovko, pers. comm.). Strains New Pass, Manasota Key, and Wilson "Low Toxin" were sampled in GR and ST cultures. Two ST New Pass cultures were available; the first was a recent culture (2011) and the second an older culture (2008) which was called New Pass "Maintained" (ST*). Cultures of *K. brevis* Wilson, *K. mikimotoi*, *P. rhathymum*, and *G. dorsum* were only in GR treatments (Table 6.1). All cultures were grown in modified L1 medium (Guillard and

Hargraves, 1993) with salinity around 34 at 26-27 °C under 70-80 $\mu E\ m^{-2}\ s^{-1}$

irradiance provided by cool white fluorescent light bulbs on a 12:12 light: dark cycle.

Each culture was grown in a single flask.

Culture sampling

Cultures were sampled at their respective culturing facilities to maintain

conditions and prevent damage or stress from handling and transport. FWRI cultures

were sampled from 14-15 February 2012 and MOTE cultures were sampled from 16-

17 February 2012. All cultures in the same growth treatments were sampled on the

same day.

Cultures were gently inverted several times to ensure homogeneity before

sampling. Dinoflagellate abundance samples were taken first and consisted of

triplicate 1 mL samples which were placed in individual wells of a Falcon® 24-well

plate (Becton Dickinson, Franklin Lakes, NJ) and fixed with Lugol's iodine.

Triplicate samples were also taken for bacterial and viral abundance and consisted of

1.5 mL fixed with 2% sterile paraformaldehyde and 1 mL fixed with 0.5%

glutaraldehyde (25% electron microscopy grade glutaraldehyde stock solution) final

concentration, respectively (Brussaard et al., 2010, Troussellier et al., 1995). Samples

were placed in 2.0 mL cryogenic vials (Internally threaded with silicone washer,

Corning®, Corning, NY) and allowed to sit for 30 min. at 4 °C before being wrapped

in Nunc CryoFlex Tube Wrap (Thermo Scientific®, Waltham, MA) and flash-frozen

in liquid nitrogen for storage and transport.

Table 6.1: Specifics of the culture history and culturing conditions of phytoplankton sampled in this study (λ = light intensity, μmol photons m^{-2} s^{-1}, BP = cultures in which bacterial production was measured). All cultures were grown in batch. Enumeration and DNA samples were taken from all cultures to enumerate bacteria and viruses and to assess the bacterial community composition. Growth treatmentss consisted of logarithmic growth (GR), stressed growth under limited nutrient additions (ST), and no light treatments (NL). Bacterial production samples were shipped overnight to Horn Point Laboratory before incubations with ^{3}H-Leucine (Leu) and ^{3}H-Thymidine (TdR). The *K. brevis* Wilson (CCMP 1953*) strain sampled at FWRI has unconfirmed lineage but is believed to be CCMP 1953 (M. Garrett, pers. comm.).

Species	Strain	Lab	Database Number	First Isolated	Toxin	Growth Treatment	PSU	°C	λ	Growth Media	BP
Karenia brevis	Wilson	FWRI, MOTE	CCMP 1953*	1960	Brevetoxins	FWRI: GR, ST, NL MOTE: GR	35/34	25/26.5	60/70-80	GP	FWRI Strain
	Wilson "Low Toxin"	MOTE		2001	Brevetoxins	GR, ST	34	26.5	70-80		Yes
	New Pass	MOTE	CCMP 2228	2001	Brevetoxins	GR, ST	34	26.5	70-80		Yes
	Manasota Key	MOTE	CCMP 2229		Brevetoxins	GR, ST	34	26.5	70-80		No
	Sarasota	FWRI	B3		Brevetoxins	GR, ST, NL	35	25	60	GP	No
	Charlotte Harbor	FWRI	C2		Brevetoxins	GR, ST, NL	35	25	60	GP	No
	Jacksonville	FWRI	C4		Brevetoxins	GR, ST, NL	35	25	60	GP	Yes
Kareenia mikimotoi		FWRI, MOTE			Cytotoxins	FWRI: GR, ST, NL MOTE: GR	35/34	25/26.5	60/70-80	GP	No
Prorocentrum rhathymum		MOTE			Hemolytic toxin	GR	34	26.5	70-80		No
Prorocentrum minimum		FWRI			??	GR	35	25	60	GP	No
Gyrodinium dorsum		MOTE			None	GR	34	26.5	70-80		No
Karlodinium veneficum		FWRI			Karlotoxins	GR	35	25	60	GP	No

Duplicate bacterial DNA samples were filtered through duplicate Sterivex-GP

0.22 μm pressure driven filter units (Millipore, Billerica, MA) using a peristaltic

pump (Crump et al., 2003): 400-1000 mL, depending on the culture, with the volume

of each culture filter being recorded. The outflow port of each filter was sealed with

HemataSEAL® tube sealant (Thermo Fisher Scientific, Pittsburg, PA) and 1mL of

DNA Extraction Buffer (DEB) was injected into the filter cartridge before sealing the

inport with a male luer plug with locking nut (Ark-Plas Products, Inc., Flippin, AR,

Crump et al., 2003). Filters were then flash-frozen in liquid nitrogen for storage and

transport.

Any remaining culture volume was then transferred into acid-rinsed

polycarbonate bottles and shipped overnight back to Horn Point Laboratory (HPL) for

bacterial production incubations.

Enumeration

Dinoflagellate samples fixed in Lugol's iodine were allowed to settle for at

least 15 min before counting on an inverted microscope. Counts consisted of all cells

with intact cell membranes. This included rounded cells identified as stressed or

senescing individuals that are still biologically active within the culture (Walker,

1982).

Bacteria were enumerated using a FACSCalibur flow cytometer (Becton

Dickinson, Franklin Lakes, New Jersey, U.S.A.). Two of the samples were thawed

slowly in an ice bath placed in the dark (Troussellier et al., 1995) and the third was

stored in a -80 °C freezer. Thawed samples were then stained with SYBR Green I

(5×10^{-5} dilution of commercial stock) for 24 h at 5 °C in the dark (Brussaard et al.,

2010). Stained samples had 20 μL of bead stock (known concentration of BD

Trucount™ Controls in 0.02 μm filtered deionized water) added and were run through

the flow cytometer with a target event rate of 100-1000 particles s^{-1} (Brussaard,

2004).

Data was collected in logarithmic mode with a target count of 20,000 total

events (modified from Brussaard, 2004), and analyzed using CellQuest™ Pro version

6.0.4 (BD). Total bacterial concentration was calculated using a modified equation

from Olson et al. (1985):

(1) $Cells_{ST} = \{[(E_{cells}/Beads_{ST}) \times E_{beads}] \times DF\}$ - Blank

$Cells_{ST}$ is the number of total cells in the sample (cells mL^{-1}), E_{Cells} is the

number of recorded events gated as bacterial cells, $Beads_{ST}$ is the number of beads

added to the sample (beads in bead stock added), E_{Beads} is the number of beads

recorded during acquisition of sample events, DF is the dilution factor if the sample

was diluted before running through the flow cytometer, and Blank is the number of

events in a 0.02 μm filtered seawater blank (calculated from equation (1) without the

variable Blank in the equation).

Epifluorescence microscopy was used to enumerate viruses. Samples were

thawed or stored as above. Thawed samples were then filtered onto a 0.02 μm

Anodisc Al_2O_3 filter (Whatman Inc., Pescataway, NJ), stained with a 1:400 dilution

195

of SYBR® Green I nucleic acid stain, and then slide mounted with a 0.1% (vol/vol)

p-phenylenediamine anti-fade mounting medium (Patel et al., 2007).

Slides were counted using an epifluorescence microscope, and viral

abundance was calculated using the equation:

(2) $Cells_{ST} = (\{RSF \times A_{Cells} \times [(100/n)/V]\} \times DF) - Blank$

$Cells_{ST}$ is the number of total cells in the sample (cells mL^{-1}), RSF is the grid-

reticle scaling factor, A_{Cells} is the average number of viral particles per field, n is the

number of boxes counted within the grid per field of view, V is the volume of sample

filtered, and Blank is the $Cells_{ST}$ of a 0.02 μm filtered seawater blank calculated from

equation (2) without the variable Blank (Patel et al., 2007).

Viral abundance and total bacterial abundance were used to calculate the

Virus to Bacteria Ratio (VBR), an indicator of whether conditions are ripe for virally-

mediated lysis. A VBR >10 indicates higher chances of virus-host encounters, and

therefore conditions ripe for bacterial analysis. A VBR <10 suggests low viral-

mediated bacterial mortality due to low encounter rate between viruses and potential

hosts (Jacquet et al., 2005).

Table 6.2: Microbial parameters measured from cultures, the parameters used to calculate each variable, and assumptions of each method. Bacterial production, protein to nucleic acid synthesis (Leu:TdR), and bacterial growth rate (BGR) were only measured for *K. brevis* FWRI cultures Wilson and Jacksonville (3 growth treatments), and MOTE cultures Wilson "Low Toxin," New Pass, and Manasota Key (2 growth treatments). Modified from del Giorgio et al., 2011.

Variable	Method	Assumptions
Bacterial abundance	1. Epifluorescence microscopy 2. Flow Cytometry	Minimal loss of cells after freeze-thaw cycle
Viral abundance	Epifluorescence microscopy	Same as bacterial abundance
VBR	Virus:Bacteria abundance	Same as abundance
Bacterial production	1. Incorporation of ^{3}H-Leucine (pmol Leu L^{-1} h^{-1}) 2. Incorporation of ^{3}H-Thymidine (pmol TdR L^{-1} h^{-1}) 3. Conversion to C production (µg C L^{-1} h^{-1})	Leu: 0.073% of Leucine in protein, 0.86 C to protein ratio, and isotope dilution factor of 2 (Kirchman, 1993 and Simon and Azam, 1989) TdR: Thymidine conversion factor of 2×10^8 cells mole^{-1}, and carbon conversion factor of 10fg C cell^{-1} (Bell, 1993)
Protein to nucleic acid synthesis	Leucine:Thymidine incorporation ratio	
Bacterial growth rate (cell specific isotope incorporation)	1. Leucine incorporation divided by abundance (pmol Leu cell^{-1} h^{-1}) 2. Thymidine incorporation divided by abundance (pmol TdR cell^{-1} h^{-1})	Equal uptake of isotope by all cells counted

Bacterial production

Multiple parameters were measured to characterize the microbial community (Table 6.2) of certain *K. brevis* cultures. FWRI strains Wilson and Jacksonville were measured in GR, ST, and NL treatments and MOTE cultures Wilson "LT," New Pass, and Manasota Key were measured in GR and ST treatments.

Tritium (^{3}H) radiolabeled leucine (Leu) and thymidine (TdR) were used to measure production of protein and DNA, respectively (Franco-Vidal and Morán, 2010). Identical procedures were used for each isotope. Twelve 10mL water samples were taken from each culture, and half were fixed with formalin (0.1% final concentration) to serve as controls by measuring non-biological uptake of isotope (absorption by particle or filter, modified from Bell, 1993). The isotope was added at a concentration of 10nM ^{3}H, and tubes were gently inverted before being incubated for ~1 h (Bell, 1993, Kirchman, 1993, Simon and Azam, 1989).

After incubation, six samples (3 live, 3 fixed) were filtered onto 0.2 μm and 3.0 μm filters to isolate the bacterial production (0.2-3 μm) from dinoflagellate production (>3.0 μm). Filters were then treated with with 5% (w/vol) chilled trichloroacetic acid (TCA, Bell, 1993) for 2 min., filtered again, and triple-rinsed with with 5% TCA and single-rinsed with 80% ethanol (Kirchman, 1993). Filters were allowed to dry overnight before the addition of scintillation cocktail. All samples were counted on a Packard 2200CA Tri-Carb liquid scintillation counter.

Scintillation counts were converted to carbon production equivalents (μg C L^{-1} h^{-1}) using the methods of Kirchman (1993) and Bell (1993). Bacterial growth rate (BGR, pmol isotope cell^{-1} h^{-1}) was calculated using FCM counts and isotope

incorporation (del Giorgio et al., 2011). Lastly, balance between protein and nucleic acid synthesis within cells was calculated using the ratio between Leu and TdR incorporation (del Giorgio et al., 2011, Table 6.1).

DNA extraction and PCR

Frozen Sterivex™ filters were thawed and physically cracked open in a manner to prevent damage to the filter membrane or loss of DNA Extraction Buffer (DEB, Crump, 2007). The filter was sliced into small ribbons before being placed into a microcentrifuge tube with the DEB as well as 20 µL of proteinase-K (10mg mL^{-1}) and 20 µL of lysozyme (100 mg mL^{-1}). Samples were then subject to three rounds of a freeze-thaw cycle (15 min @ -80 °C, 5 min @ 37 °C) followed by a 30 min incubation in a 37 °C water bath. This was followed by the addition of 50 µL Sodium dodecyl sulfate (SDS) and another incubation of 120 min in a 65 °C water bath (Crump, 2007). Phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) was then used to extract the DNA and 100% isopropanol was used to precipitate the DNA. Precipitated DNA was washed twice with 70% ethanol and condensed in a microcentrifuge at 13000 rpm before being dried in a roto-evaporator. The dried pellet was then resuspended in 250 µL autoclaved-UV sterile ultra-pure water. Of the 250 µL of DNA, 150 µL was archived and 100 µL was amplified for sequencing (Crump, 2007).

Extracted DNA was amplified in triplicate using modified Polymerase Chain Reaction (PCR, Crump et al., 1999) with bacteria-specific primers from the Earth

Microbiome Project (EMP, Caporaso et al., 2012). Cycling conditions were: initial

denaturing at 94 °C for 3 min; 30 cycles of 30 s at 94 °C, 45 s at 57 °C, and 60 s at 72

°C; with a final extension step of 2 min at 72 °C (Crump et al., 1999, Sogin et al.,

2006). Amplification was confirmed with gel electrophoresis using a 2.5% agarose

gel (Agarose 3:1, Thermo Fisher Scientific) with TAE (0.04 M Tris-acetate, 1 mM

EDTA) and SYBR® Green I nucleic acid stain (Invitrogen) added. Triplicate

amplifications were combined and quantified a Quant-iT™ PicoGreen® double-

stranded DNA Assay Kit according to the manufacturer's instructions (Life

Technologies Incorporation).

DNA sequencing and bioinformatics

Samples were sent for sequencing on the Illumina-MiSeq platform (Illumina,

Inc.) at the University of Maryland Genomics Resource Center

(http://www.igs.umaryland.edu/grc). Sequences were demultiplexed based on the

Golay barcodes and primer sequences removed the Quantitative Insights Into

Microbial Ecology (QIIME, Caporaso et al., 2010) Illumina pipeline. Additional

quality control, alignment, operational taxonomic unit (OTU) assignment, and

taxonomy assignment were done in QIIME.

The QIIME pipeline for Illumina sequences first requires sequence reads to be

paired together (Sequences are generated separately for the 5' and 3' strands of DNA)

and demultiplexed; joining overlapping 5' and 3' strands together and separating

them by sample using barcoded primers (D. Smith, Argonne National Laboratory,

2012). Demultiplexed sequences were assigned OTUs using the usearch method; similar to the uclust method with the additional steps of filtering low abundance clusters and chimera detection (Edgar, 2010). Sequences were aligned using Python Nearest Alignments Space Termination Tool (PyNAST, Caporaso et al., 2010) and the Greengenes May 2013 taxonomic database as a template (McDonald et al., 2012). Taxonomy was assigned with the Ribosomal Database Project (RDP) Classifier which uses a naïve Bayesian classifier which allows for a high level of taxonomic assignments even with partial sequences (Wang et al., 2007).

Statistical analysis

Dinoflagellate abundance, bacterial production, and bacterial abundance data were tested with an analysis of variance (ANOVA) using Statistical Analysis Software, Version 9.2 (SAS Institute Inc., Cary, N.C., U.S.A.). Data were tested for normality and Log-transformed when not normally distributed. The ANOVA tests investigated interactions between the lab or culture origin, strain (or species) of dinoflagellate, and growth treatment. A significant interaction was found between strain and growth treatment ($p < 0.05$) so comparisons were made between all strains in each growth treatment as well as all growth treatments of each strain using a Tukey's post-hoc test.

Bacterial community beta diversity was analyzed using default programs in QIIME followed by statistical analysis using the {stats} (3.0.1) and {vegan} (2.0-8, Oksanen et al., 2013) packages as well as the biostats.R functions (McGarigal, 2013)

in RStudio (RStudio, 2012, version 0.97.449; R Core Team, 2013, version 3.0.1). Distance measurements between samples was calculated with weighted UniFrac measurement within QIIME (Lozupone and Knight, 2005 and Lozupone et al., 2007) and then distance matrices were read into R.

Hierarchical clustering was used to produce dendrogram and Non-metric Multidimensional Scaling plots; clusters were tested using Analysis of Similarities (ANOSIM, {vegan} package) with a Morisita-Horn index of distance. Bacterial communities were compared between laboratories, strain, and dinoflagellate (combined strains). Samples were split into two groups for further analysis using culture metadata; one group included strains with bacterial production and growth rates and the second group was all strains without production and growth rates. Metadata were analyzed with principal coordinates analyses (PCoA) in the {stats} package and canonical correspondence analyses (CCA) in the {vegan} package, with the significance of metadata variables tested using ANOVA ({stats} package).

Following multivariate analyses a Dufrene-Legendre Indicator Species Analysis (R package {labdsv}, Dufrene and Legendre, 1997) was run on OTU tables using an "around medoids" partitioning of Bray-Curtis distances (R package {cluster}, Reynolds et al., 1992). Significant indicator species were further tested for correlations with culture abundance and physical conditions using a Spearman's *rho* product moment correlation coefficient.

Results

Dinoflagellate abundance

Dinoflagellate abundance in cultures displayed significant differences between labs, species, strains, and growth treatments. FWRI non-*Karenia* species had the highest abundances: *P. minimum* had a concentration of 1.74×10^8 cells L^{-1} and *K. veneficum* 8.38×10^7 (Table 6.3, Figure 6.1). Three of the four *K. brevis* strains surpassed 10^6 cells L^{-1} in at least one growth treatment, and the Jacksonville strain surpassed 10^6 cells L^{-1} for all three growth treatments. Concentrations of *K. brevis* $>10^6$ cells L^{-1} are classified as "High" by the FWRI Red Tide Status Report. The Wilson strain was the only strain in the study that showed a significant decline in cell abundance between growth treatments; from 9.63×10^6 in GR cultures to 2.41×10^5 in NL treatments (Table 6.3, Fig. 6.1). The MOTE *K. brevis* Wilson "LT" culture had the highest abundance of any species or strain from either lab and was the only strain with a significant difference in abundance between growth treatments (Fig. 6.1). Abundance in the ST* culture of *K. brevis* New Pass was significantly lower than younger New Pass cultures (Table 6.3). Significant differences in dinoflagellate abundance existed under similar growing conditions.

Bacterial abundance

Total bacterial abundance was highest in the MOTE *K. brevis* New Pass culture in ST treatments ($3.33 \pm 0.44 \times 10^9$ cells mL^{-1}) and lowest in the FWRI *K. brevis* Wilson culture in ST treatments ($57.45 \pm 6.61 \times 10^6$ cells mL^{-1}, $p < 0.05$, Table

6.3, Fig. 6.2). Every culture sampled in multiple growth treatments had abundances that were significantly different between at least two growth treatments ($p < 0.05$, Table 6.3). In GR cultures, total abundance was highest in MOTE cultures of *K. brevis* New Pass ($1.29\pm0.11 \times 10^9$ cells mL^{-1}) and *K. mikimotoi* ($9.78\pm1.04 \times 10^8$ cells mL^{-1}) and lowest in FWRI *K. brevis* Wilson ($1.01\pm0.02 \times 10^8$ cells mL^{-1}, Fig. 6.2). In ST treatments *K. brevis* New Pass (MOTE), Manasota Key (MOTE), Wilson "LT", and Charlotte Harbor (FWRI), had the highest abundances ($p < 0.05$, Table 6.3). The FWRI *K. brevis* strains Wilson, Jacksonville, and Sarasota had the fewest bacteria in ST treatments. Only FWRI provided NL treatements of cultures, of which *K. brevis* Sarasota and Charlotte Harbor had the highest abundances ($5.52\pm0.04 \times 10^8$ cells mL^{-1}) which were not significantly different from GR cultures (Table 6.3). Only FWRI *K. mikimotoi* had a significant downward trend in total bacterial abundance (Table 6.3, Fig. 6.3).

Total abundance in *K. veneficum* cultures was similar to *K. mikimotoi* in GR growth treatments (FWRI) as well as *P. rhathymum* and *G. dorsum* cultured at MOTE (Fig. 6.3). The total concentration of bacteria in the *P. minimum* culture was higher than any other culture sampled at FWRI but lower than the abundance found in *K. mikimotoi* (MOTE, Table 6.3, Fig. 6.3).

Table 6.3: Cell counts for dinoflagellates, bacteria, and viruses in cultures sampled in this study. The virus to bacteria ratio (VBR) represents how likely virally-mediated lysis is playing a role in bacterial mortality: VBR < 10 demonstrate low levels of viral-mediated bacterial mortality (Jacquet et al., 2005). GR: logarithmic growth, ST stressed growth, NL: no light treatments, ST*: "Maintained" cultures. GR treatments were maintained by the addition of GP media at two different time points. ST treatments had only one amendment with GP media at the start of culturing. NL treatments were covered in foil two days prior to sampling. The ST* culture had been left alone (no nutrient additions) for approximately four months.

Species	Strain	Lab	Treatment	Dinoflagellate	Bacteria cells	Viruses mL^{-1}	VBR
K. brevis	Wilson	FWRI	GR	9.63±1.63	100.99±2.09	23.30±8.99	0.23±0.09
			ST	1.45±0.30	57.45±6.61	11.93±2.89	0.21±0.03
			NL	0.24±0.04	102.16±1.50	16.34±7.10	0.16±0.07
		MOTE	GR	2.30±0.26	179.47±2.44	9.37	0.05
	Wilson "LT"	MOTE	GR	19.90±0.61	179.65±15.93	28.26±3.69	0.16±0.01
			ST	27.53±1.38	684.29±150.51	25.69±0.66	0.04±0.01
	New Pass	MOTE	GR	10.07±1.62	1290.77±109.64	23.68±4.88	0.02±0.01
			ST	9.83±1.13	3332.33±440.25	19.47±0.39	0.01±0.00
			ST*	5.43±0.25	1162.08±17.93	37.59±5.59	0.03±0.01
	Manasota Key	MOTE	GR	10.10±2.44	240.54±54.80	11.02±0.84	0.05±0.01
			ST	9.09±1.15	2308.58±148.49	25.94	0.14
	Sarasota	FWRI	GR	2.07±0.13	539.89±31.58	12.97	0.43
			ST	0.09±0.02	353.01±36.84	8.31±0.26	0.02±0.00
			NL	0.27±0.05	552.48±4.44	9.62	0.07
	Charlotte Harbor	FWRI	GR	0.02±0.01	350.29±28.88	3.51±0.78	0.01±0.00
			ST	0.01±0.001	549.99±12.47		
			NL	0.01±0.003	475.55±53.98	6.12±0.11	0.01±0.00
	Jacksonville	FWRI	GR	1.51±0.41	496.13±39.65	16.94±7.92	0.03±0.02
			ST	8.58±0.78	165.93±16.83	3.52±0.03	0.02±0.002
			NL	2.49±0.39	265.98±6.55	8.54±0.39	0.03±0.002
K. mikimotoi		FWRI	GR	4.84±1.58	186.67±0.43	8.79±2.22	0.05±0.01
			ST	4.45±0.07	131.30±2.31	13.31±5.21	0.10±0.04
			NL	1.81±0.33	86.65±3.88	13.97±2.66	0.16±0.04
		MOTE	GR	5.82±1.02	978.90±104.11	14.06±4.87	0.01±0.00
P. rhathymum		MOTE	GR	7.79±1.19	218.93±11.09	12.61±7.67	0.06±0.04
P. minimum		FWRI	GR	174.07±4.37	607.49±61.93	31.52±5.92	0.05±0.00
G. dorsum		MOTE	GR	1.90±0.18	243.64±12.64	13.95±0.89	0.06±0.00
K. veneficum		FWRI	GR	83.79±2.30	192.98±41.57	8.50±3.37	0.04±0.01
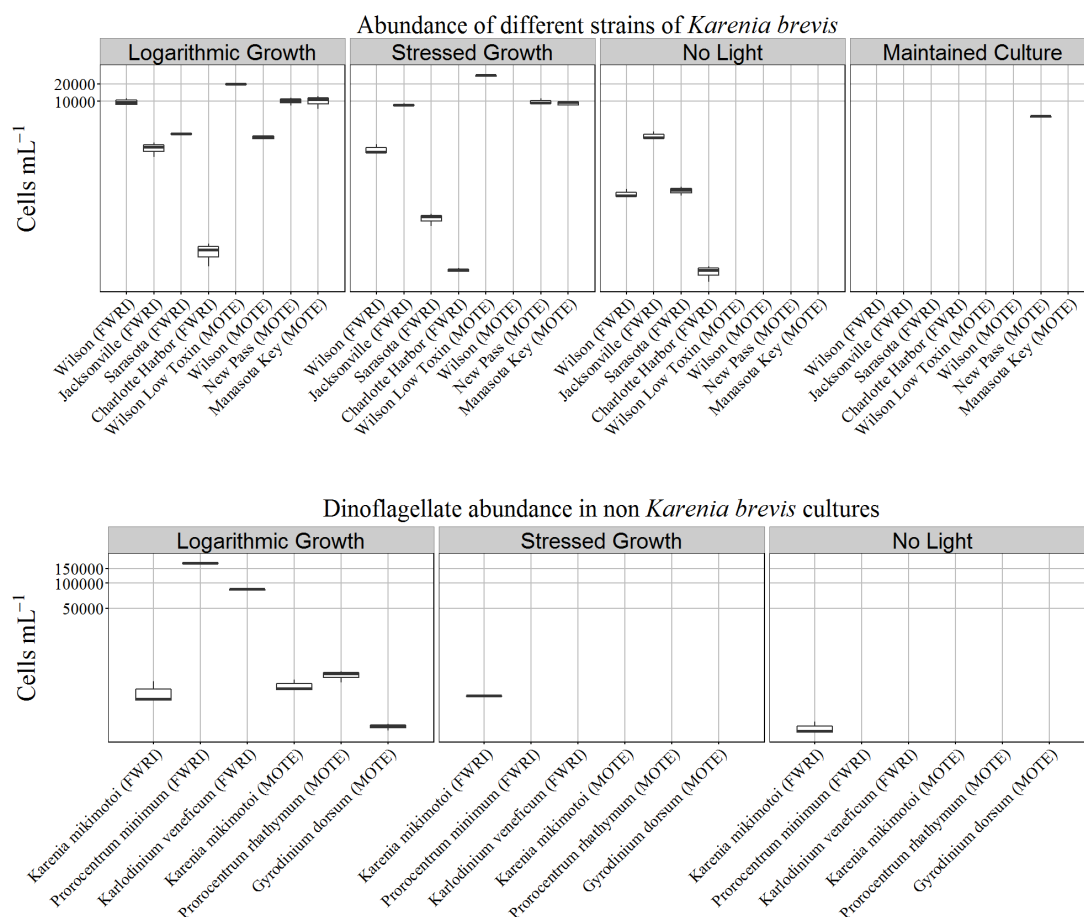
Figure 6.1: Abundance of *Karenia brevis* strains (Top, cells mL^{-1}) and multiple other dinoflagellates (Bottom, cells mL^{-1}) cultured in multiple growth treatments. The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extending to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Cultures were grown in batch.
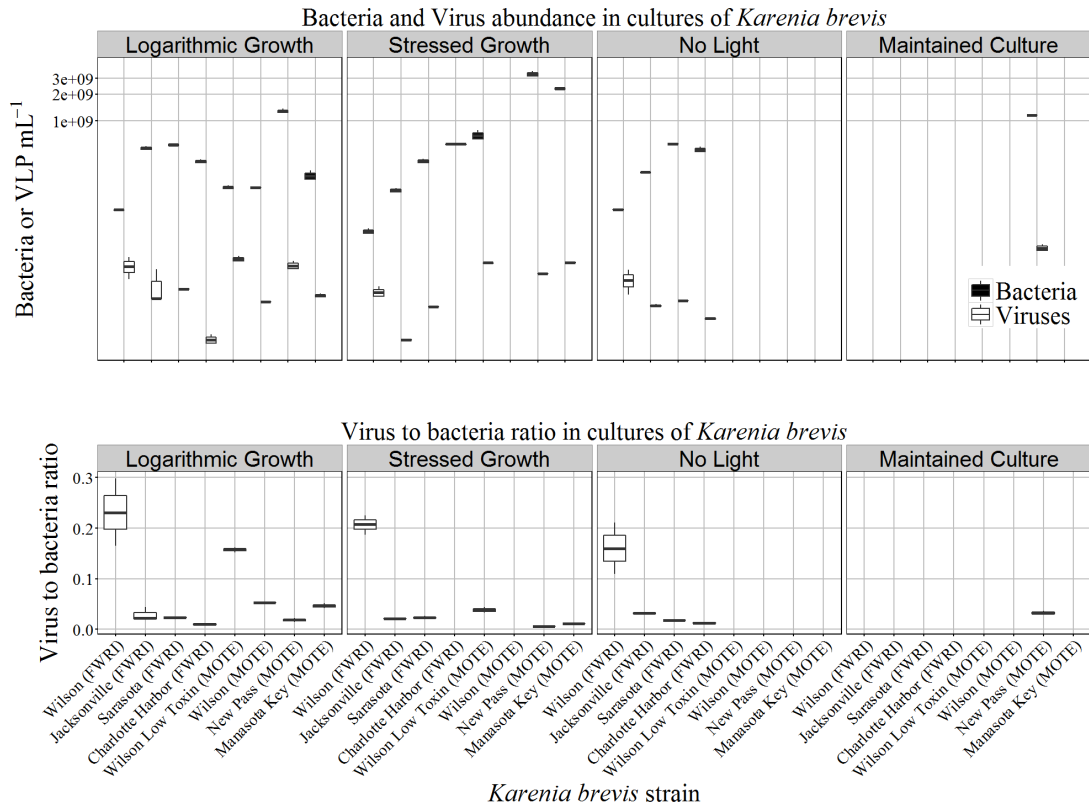
Figure 6.2: Abundance of bacteria (Black boxes) and virus-like particles (VLPs, white boxes) and the virus to bacteria ratio in different strains of *Karenia brevis* cultured at Florida Fish and Wildlife Research Institute (FWRI) and MOTE Marine Laboratory (MOTE). The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extending to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Cultures were grown in batch.
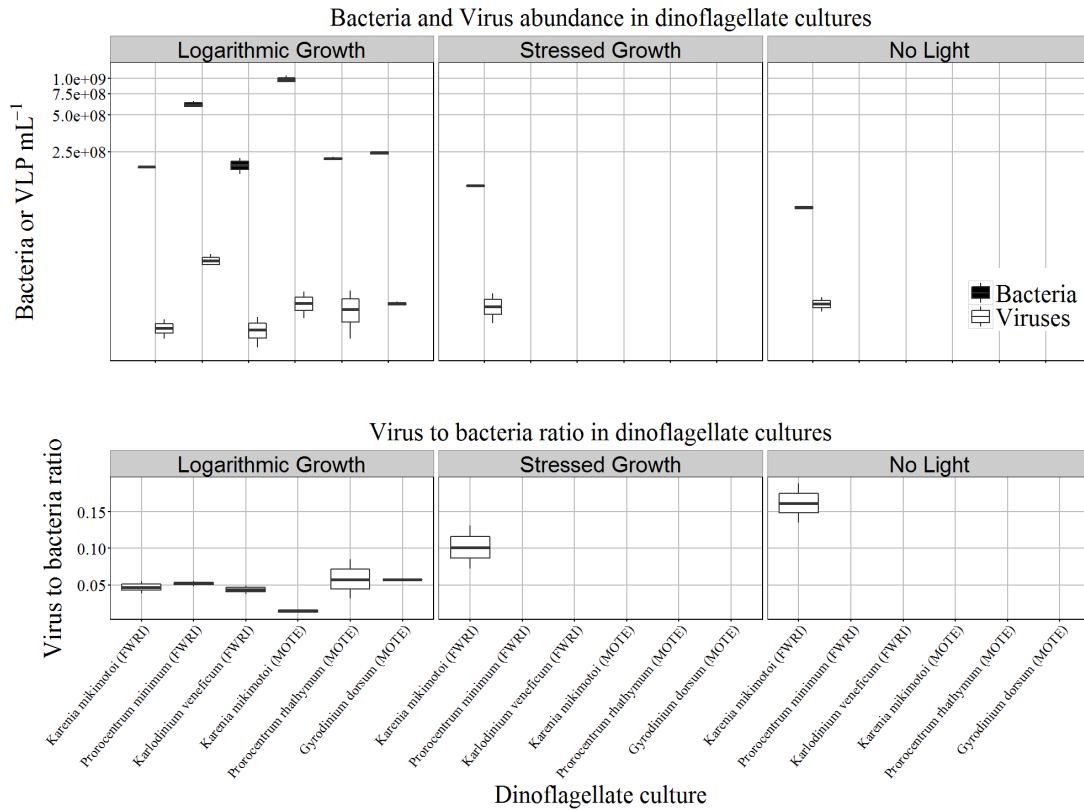
Figure 6.3: Abundance of bacteria (Black boxes) and virus-like particles (VLPs, white boxes) and the virus to bacteria ratio in different dinoflagellates cultured at Florida Fish and Wildlife Research Institute (FWRI) and MOTE Marine Laboratory (MOTE). The top and bottom of the boxes represent the first and third quartiles (25th and 75th percentiles) and whiskers extending to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Cultures were grown in batch.

Viral abundance

Viral abundance in FWRI cultures were on the order of 10^6-10^7 VLPs mL^{-1} and all VBRs were <0.35, indicating that viruses were not a significant component of the microbial community. The *K. brevis* strains Wilson, Sarasota, and Charlotte Harbor had no significant differences between growth treatments for either VLP count or VBR (Table 6.3, Fig. 6.2). Counts of VLP in the Jacksonville strain showed significant differences between growth treatments (GR > ST > NL), however VBR for all treatments was <0.05 indicating no change in viral influence within the culture. There were no significant differences in VLP or VBR between growth treatments in *K. mikimotoi* cultures, though both were trending up (Table 6.3). The second highest concentration of VLPs in all cultures was *P. minimum*, but a VBR of 0.05 indicated that despite a high abundance of VLPs viral-mediated lysis was not a significant source of mortality in the culture (Table 6.3). Similar patterns were observed in *K. veneficum* cultures: VLP concentration was >10^6 mL^{-1} and VBR was <1.

MOTE *K. brevis* New Pass VLP counts in GR and ST growth treatments were not significantly different from one another (Table 6.3). The ST* New Pass culture had significantly more VLPs (37.59x10^6 VLPs mL^{-1}) than the New Pass GR treatment but the VBR was not significantly different. The MOTE Wilson strain had a VLP abundance of 9.37x10^6 mL^{-1}, which was not significantly lower than GR treatments of FWRI Wilson. The Wilson "LT" strain VLP abundance was not significantly different between growth treatments and VBR was <1 for both GR and ST treatments. The VLP and VBR for Wilson "LT" were also not significantly different from either Wilson clone (FWRI or MOTE). Wilson "LT" had significantly

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lower VBR than FWRI Wilson cultures in ST treatments but no difference in VLP abundance (Table 6.3). The Sarasota culture demonstrated no significant differences in VLP or VBR for any growth treatments, and had a consistently low VBR. Counts of VLP and the VBR in the MOTE culture of *K. mikimotoi* in GR treatments were not significantly different from the FWRI culture (Table 6.3). The non-*Karenia* dinoflagellates *P. rhathymum* and *G. dorsum* had VLP concentrations of 1.26×10^7 mL^{-1} and 8.50×10^6 mL^{-1} respectively. The VBRs were 0.06 and 0.04 for *P. rhathymum* and *G. dorsum*, respectively (Table 6.3).

Bacterial production and growth

Carbon production and BGR calculated from ^{3}H-Leucine incorporation measure protein synthesis in the community and individual cell level, and indicate metabolic costs to the bacteria in their given environment. Carbon production in bacterial size fractions was significantly higher in GR treatments in MOTE cultures New Pass and Manasota Key; other cultures showed no significant differences between growth treatments (Table 6.4, Fig. 6.4). Comparing cultures in the same growth treatment showed that carbon production was highest in MOTE Manasota Key and FWRI Wilson culture in GR treatments and FWRI Wilson had the highest carbon production ST treatments. The Leu-BGR was consistently high in both growth treatments of FWRI Wilson, though it was significantly lower than Jacksonville in GR treatments (Table 6.4, Fig. 6.4). All three MOTE cultures had higher Leu-BGR in the GR treatments. Leucine production measurements show active bacterial

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metabolism in the FWRI Wilson culture and MOTE cultures in GR growth treatments.

Carbon production calculated from TdR incorporation showed significantly more active communities in Manasota Key cultures (MOTE), and consistent production in both FWRI cultures (Fig. 6.4). Measurements of TdR-BGR were similar to production except for MOTE cultures having similar BGR in ST treatments. Production measured from TdR incorporation show actively dividing microbial communities in all cultures, with the highest production in the FWRI Wilson culture and MOTE cultures showing shifts in production based on growth treatment.

The Leu:TdR, a measure of the energetic requirements of the microbial community within a sample, was ≤10 in all strains and growth treatments, indicating all culture environments had low energetic costs. Significant differences between growth treatments were found in three of the five cultures sampled: Wilson (FWRI), New Pass, and Manasota Key (Table 6.4, Fig. 6.5). In the FWRI Wilson strain, Leu:TdR was highest in ST growth and lowest in NL treatments. The ST treatments had Leu:TdR which was significantly higher in the MOTE Wilson "LT" strain and significantly lower in Manasota Key. Despite significant differences between strain growth treatments, Leu:TdR was consistently low, demonstrating that all culture environments were energetically favorable for microbial production and cellular division.

Table 6.4: Bacterial production measured from five different strains of *K. brevis* in multiple growth treatments. Leu:TdR represents the ratio of protein (Leu production) to nucleic acid synthesis (TdR production) within a given population, and therefore how hostile a particular environment may be for growth (del Giorgio et al., 2011).

Strain	Lab	Treat	Leu μg C L^{-1} h^{-1}	Leu-BGR pmol Leu cell^{-1} h^{-1} x10^{-8}	TdR μg C L^{-1} h^{-1}	TdR-BGR pmol TdRcell^{-1} h^{-1} x10^{-8}	Leu:TdR
Wilson	FWRI	GR	12.51±2.64	4.01±0.87	20.41±8.75	1.01±0.43	4.62±2.55
		ST	22.60±4.93	12.92±3.74	13.72±1.35	1.19±0.05	10.85±3.29
		NL	9.37±6.30	2.97±2.00	33.25±9.10	1.63±0.46	1.86±1.42
Wilson "LT"	MOTE	GR	4.06±0.19	0.73±0.01	9.67±0.12	0.27±0.02	2.72±0.13
		ST	4.30±0.40	0.20±0.02	4.42±0.63	0.03±0.01	6.38±1.17
New Pass	MOTE	GR	5.09±0.72	0.13±0.02	4.80±0.11	0.02±0.001	6.86±0.90
		ST	3.39±0.20	0.03±0.001	2.67±0.13	0.004±0.0003	8.21±0.43
Manasota Key	MOTE	GR	16.64±0.45	2.28±0.43	25.28±0.66	0.54±0.09	4.26±0.10
		ST	4.56±0.59	0.06±0.01	11.44±0.68	0.02±0.002	2.58±0.28
Jacksonville	FWRI	GR	10.89±2.52	0.71±0.18	18.27±3.01	0.18±0.02	3.99±1.38
		ST	7.15±3.94	1.42±0.79	24.78±0.46	0.52±0.26	3.66±3.98
		NL	14.31±1.77	1.74±0.24	22.99±1.89	0.41±0.02	4.07±0.86
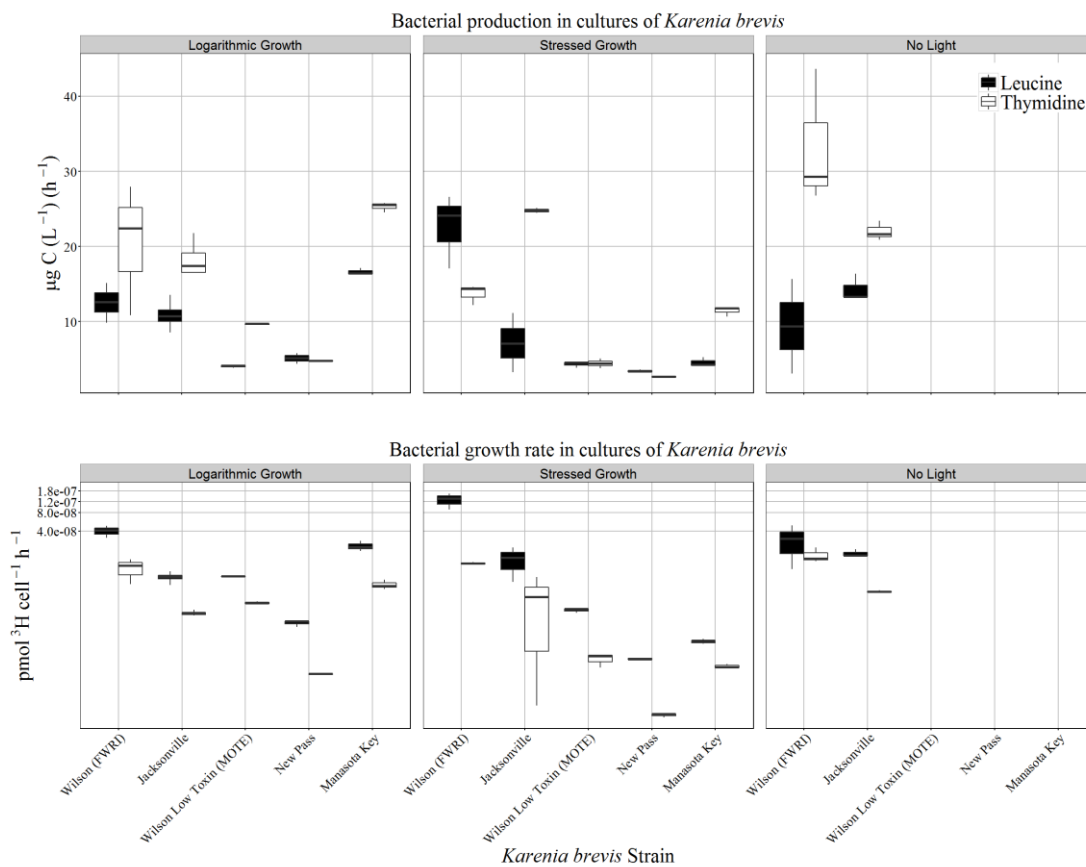
Figure 6.4: Bacterial production (μg C L^{-1} h^{-1}) and bacterial growth rate (BGR, cell specific isotope uptake x10^{-8}) in *Karenia brevis* cultures measured by uptake of ^{3}H-Leucine (Black boxes) and Thymidine (White boxes). Measurements were taken in logarithmic growth (GR, left column), stressed growth (ST, center column), and no light treatments (NL, right column). All measurements represent the bacterial size fraction (0.2-3 μm). The top and bottom of the data boxes represent the first and third quartiles and whiskers extend to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Cultures were grown in batch.
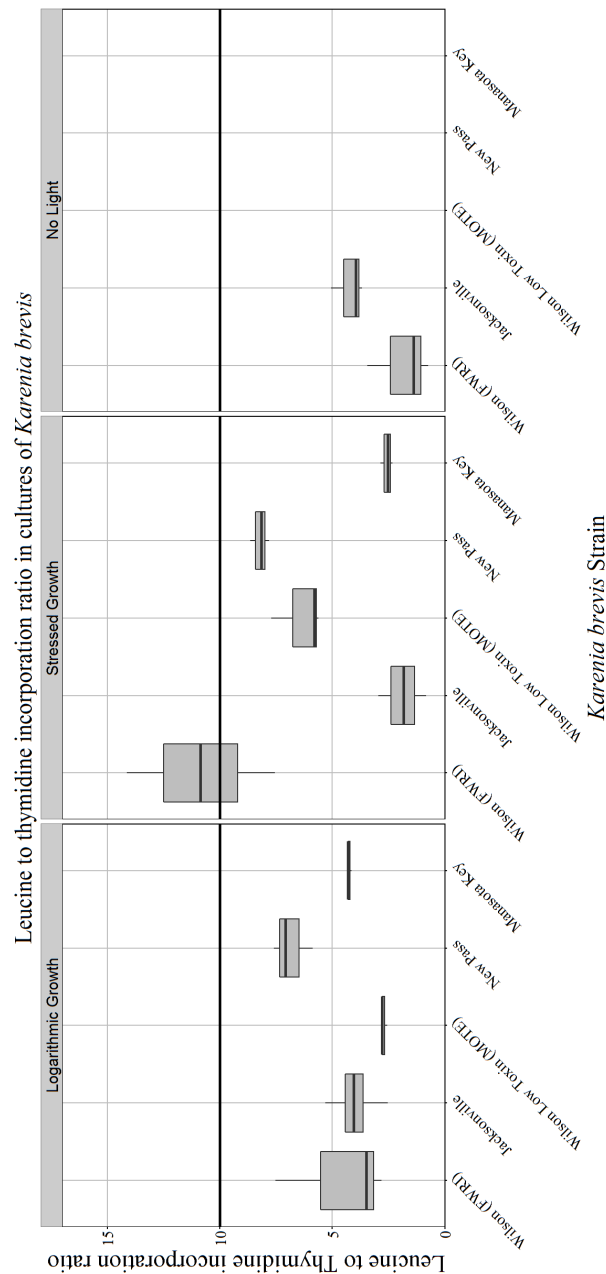
Figure 6.5: The Leucine to Thymidine incorporation ratio of bacteria (0.2-3 µm) in cultures of *Karenia brevis*. The black line represents the threshold for balanced (above) or unbalanced (below) growth (del Giorgio et al., 2011). The top and bottom of the data boxes represent the first and third quartiles and whiskers extend to the highest and lowest values in within 1.5 * IQR, where IQR is the inter-quartile range. Cultures were grown in batch.

Bacterial community composition

A total of 2,050,547 OTUs were identified from the twelve cultures sampled, with an average of 43,629 OTUs per sample. Weighted UniFrac distances revealed that bacterial communities associated with the Wilson and Wilson "LT" strain of *K. brevis* do not cluster well with one another (Fig. 6.6) but are more similar to each other than to other strains of *K. brevis* (Fig. 6.7) despite being cultured in different labs. Communities in non-Wilson strains of *K. brevis* cultured at FWRI clustered in all growth treatments, with the Charlotte Harbor strain forming its own cluster in all growth treatments (Fig. 6.7). Bacterial communities in the Manasota Key and New Pass strains cultured at MOTE clustered together and were different than communities associated with other *Karenia* species and strains, but showed consistent communities across growth treatments. The two *K. mikimotoi* bacterial communities clustered together across both labs and growth treatments (Figs. 6.6 and 6.7). Bacterial communities from non-*Karenia* cultures contained unique assemblages (Fig. 6.7); *P. rhathymum* clustered with senescing *K. brevis* Wilson despite being cultured at a different laboratory (MOTE vs. FWRI, Fig. 6.6).

Communities cultured at FWRI and MOTE and individual strains of *K. brevis* were found to be significantly different from one another (ANOSIM, R=0.23 and 0.53, respectively). Two PCoAs of communities were conducted: one including bacterial production data which limited analysis to the Wilson (FWRI), Jacksonville (FWRI), Wilson Low Toxin (MOTE), New Pass (MOTE), and Manasota Key (MOTE), and another with all cultures.

Bacterial community clustering in dinoflagellate cultures
based on weighted UniFrac distance calculations

Growth phase

■ Logarithmic
▲ Stressed Growth
● No Light
◆ Maintained

Strain or Species

● Wilson (FWRI)
● Jacksonville (FWRI)
● Sarasota (FWRI)
● Charlotte Harbor (FWRI)
● Karenia mikimotoi (FWRI)
● Karlodinium veneficum (FWRI)
● Wilson Low Toxin (MOTE)
● New Pass (MOTE)
● Manasota Key (MOTE)
● Karenia mikimotoi (MOTE)
● Prorocentrum rhathymum (MOTE)
● Gyrodinium dorsum (MOTE)
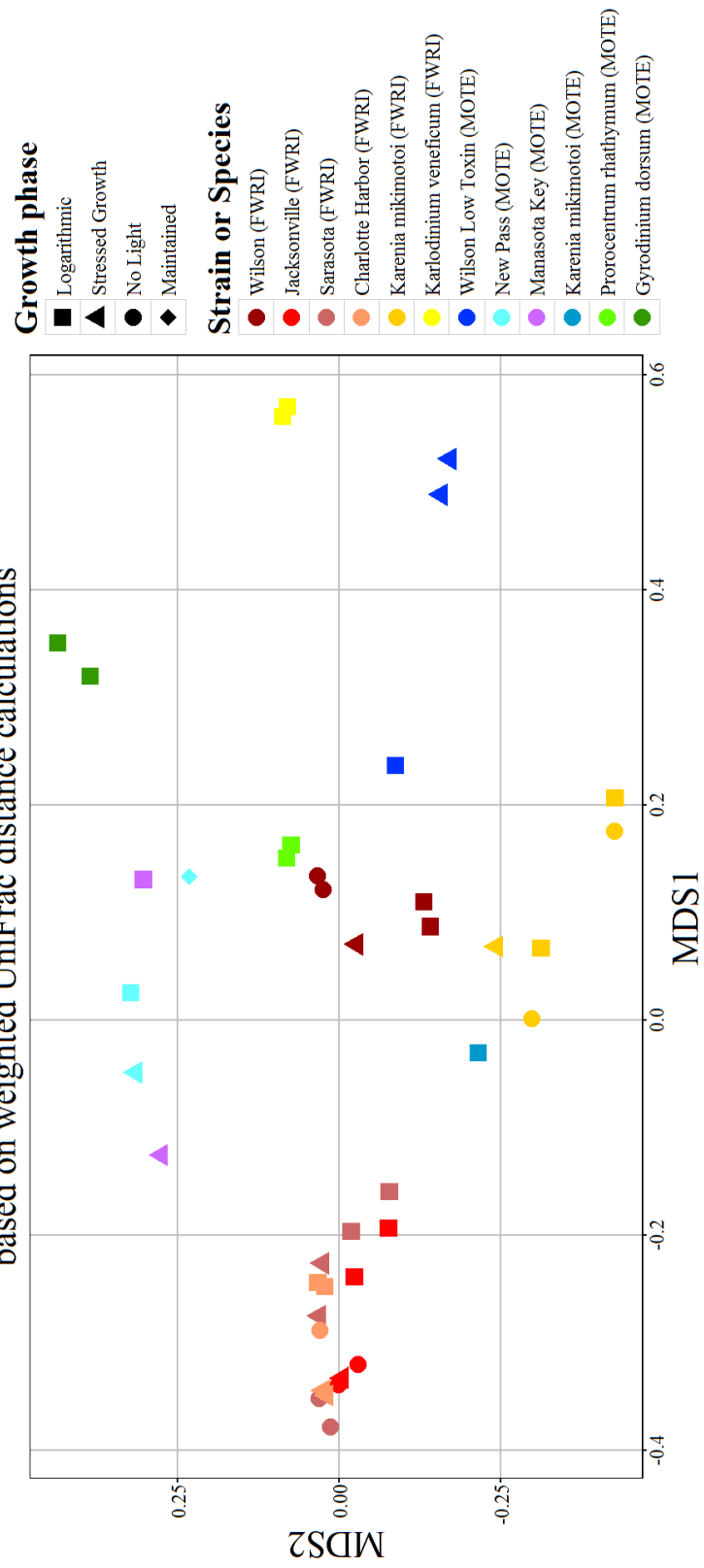
Figure 6.6: Clustering of bacterial OTUs from dinoflagellate cultures grown at FRWI (red and yellow points) and MOTE (blue, purple, and green points). All cultures were available in GR treatments (squares) but many cultures were also available in ST (triangles) and NL (circles) treatments. One culture of *Karenia brevis* New Pass had been left unattended for several months (ST*, diamond). Bacterial OTUs were classified according to the Greengenes May 2013 taxonomic database (McDonald et al., 2012) and UniFrac distance calculations were done using QIIME (Caporaso et al., 2010). Stress of the plot was 0.126.

Bray Distance

0.0   0.2   0.4   0.6   0.8   1.0

MOTE_Manasota_Key_Stat_1
MOTE_Manasota_Key_Stat_2
MOTE_Manasota_Key_Log_1
MOTE_Manasota_Key_Log_2
MOTE_New_Pass_Scen_1
MOTE_New_Pass_Stat_2
MOTE_New_Pass_Stat_1
MOTE_New_Pass_Log_1
MOTE_New_Pass_Log_2
MOTE_P_rhathymum_Log_1
MOTE_P_rhathymum_Log_2
MOTE_G_dorsum_Log_1
MOTE_G_dorsum_Log_2
FWRI_K_veneficum_1
FWRI_K_veneficum_Log_2
FWRI_K_mikimotoi_Stat_2
FWRI_K_mikimotoi_Stat_1
FWRI_K_mikimotoi_Scen_2
FWRI_K_mikimotoi_Log_1
FWRI_K_mikimotoi_Scen_1
FWRI_K_mikimotoi_Log_2
MOTE_K_mikimotoi_Log_2
FWRI_Sarasota_Log_2
FWRI_Sarasota_Log_1
FWRI_Jacksonville_Log_2
FWRI_Jacksonville_Log_1
FWRI_Sarasota_Scen_2
FWRI_Sarasota_Scen_1
FWRI_Jacksonville_Stat_1
FWRI_Jacksonville_Scen_2
FWRI_Jacksonville_Scen_1
FWRI_Sarasota_Stat_2
FWRI_Sarasota_Stat_1
FWRI_Charlotte_Log_2
FWRI_Charlotte_Log_1
FWRI_Charlotte_Scen_1
FWRI_Charlotte_Stat_1
FWRI_Charlotte_Stat_2
MOTE_Wilson_LT_Log_2
MOTE_Wilson_LT_Stat_1
MOTE_Wilson_LT_Stat_2
FWRI_Wilson_Log_1
FWRI_Wilson_Log_2
FWRI_Wilson_Stat_1
FWRI_Wilson_Scen_1
FWRI_Wilson_Scen_2

Sites
hclust (*, "average")

Average linked dendogram no rare OTUs braydist

Figure 6.7: Average linked dendrogram of bacterial communities in cultures of dinoflagellates. Calculations are based on weighted UniFrac distances provided by QIIME and analyzed using a hierarchical clustering algorithm within the {stats} package in R (both version 3.0.1). All labels use the same format: laboratory_strain/species_growth treatment_replicate. Log= GR treatments, Stat= ST treatments, Scen: NL treatments.

The PCoA including bacterial production explained 50.08% and 21.65% of the variation among samples along the first and second principal axes (PC1 and PC2), respectively (Fig. 6.8). Abundance, production, and irradiance data (CCA, data not shown) explained 60.02% of the variation within the PCoA. The first and second Eigenvalues (CCA1, CCA2) were the only significant axes (ANOVA, $p<0.05$). The primary constraining variables within CCA1 were dinoflagellate abundance (-0.84), bacterial abundance (-0.59), and Leucine production (0.66). For CCA2 dinoflagllate abundance (0.51) and Thymidine production (-0.50) were the main variables (Fig. 6.8). Of these variables, only dinoflagellate abundance was significant (ANOVA, $p=0.01$).

The PCoA of all samples only had one significant axis (PC1) which explained 61.52% of the variance. Axis PC2 explained 19.27% of variation but was not significant based on broken stick analysis and Monte-Carlo simulations of Eigenvalues (it was significant based on Kaiser's criterion, Fig. 6.9). Only 18.37% of this variation is explained by abundance and irradiance data (CCA, data not shown) and the first two axes were found to be significant (CCA1 and CCA2, ANOVA, $p=0.005$). Dinoflagellate abundance and virus abundance were the primary components of CCA1 (-0.8993 and -0.5969, respectively) and irradiance was the primary component of CCA2 (0.9436). Dinoflagellate abundance and irradiance were both significant (ANOVA, $p=0.01$).
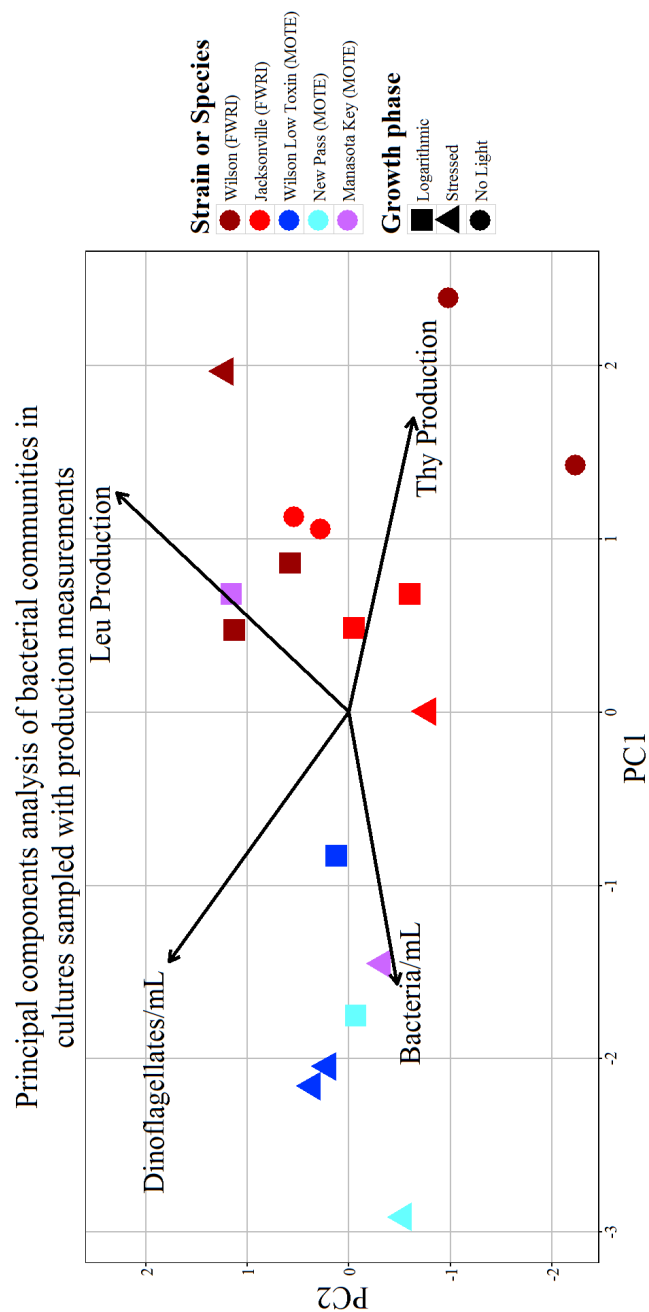
Figure 6.8: Clustering of bacterial communities in cultures of *Karenia brevis* using Principal Components Analysis (PCoA). Point colors represent the strain of *K. brevis* and laboratory in which it was cultured, the point shape represents the conditions in which the culture was grown. Samples separated significantly (p=0.05) along the first and second principal component axes (PC1 and PC2) which explained 80.80% of the variation (PC1 61.52%, PC2 19.27%).
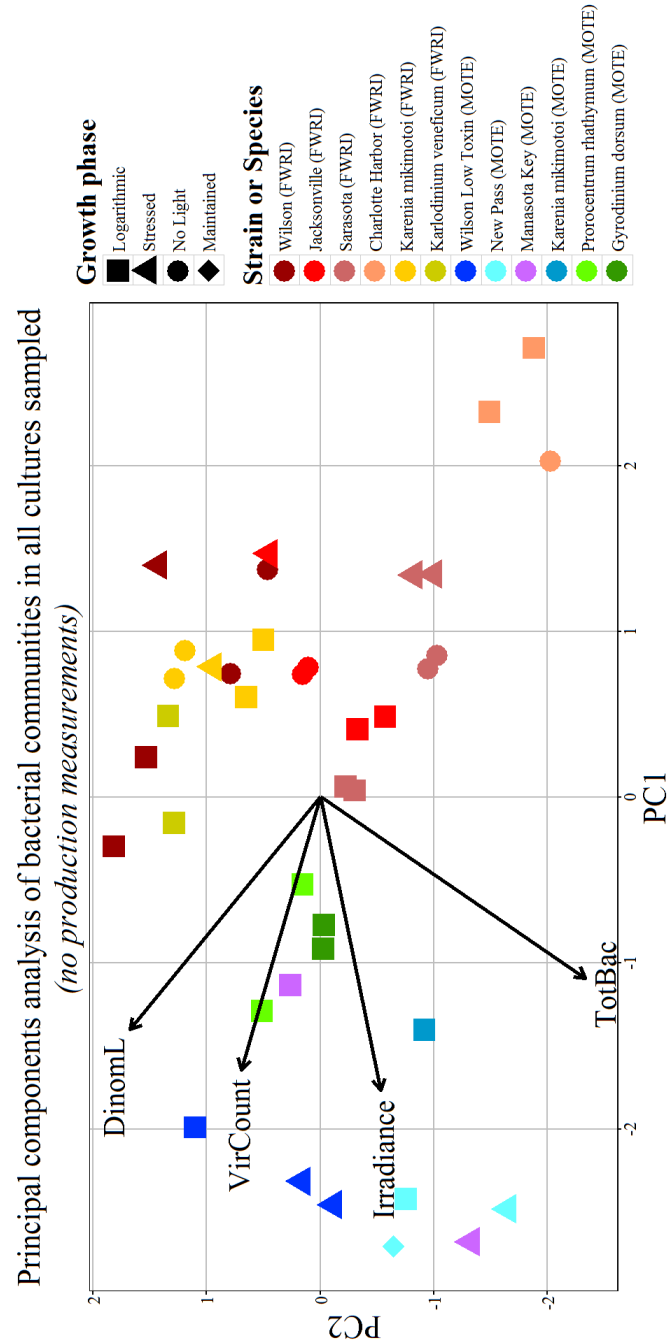
Figure 6.9: Clustering of bacterial communities using Principal Components Analysis (PCoA). Point colors represent the strain of *K. brevis* and laboratory in which it was cultured, the shape of the point represents the growth treatment of the culture at time of sampling. Samples separated significantly (p=0.05) along the first principal component axes (PC1) which explained 54% of the variation. Axis PC2 was not significant but explained 25.51% of total variation.

The Wilson strain (FWRI) had *Cryomorphaceae*, *Planctomycetes*, and *Rhodobacteraceae* OTUs as a large component of the community in all growth treatments (13-42%, 16-26%, and 11-15% for GR, ST, and NL treatments respectively). The NL treatment communities of the Wilson Strain were also represented by a higher proportion of *Altermonodales* (~14% or OTUs compared to <8% in GR and ST, Fig. 6.10). None of these groups had a significant indicator value (p = 0.05) in cultures of the Wilson strain. The *Cryomorphaceae* and *Planctomycetes* were both negatively correlated with bacterial abundance (Spearman = -0.54 and -0.66, respectively) and positively correlated with the virus to bacteria ratio (VBR, 0.69 and 0.64) and cell-specific leucine and thymidine incorporation (Leu-BGR 0.65 and 0.72, TdR-BGR 0.59 and 0.75, respectively, p < 0.05). The *Planctomycetes* also had significant correlations with PSU (0.56), temperature (-0.56), and irradiance (-0.56). The *Rhodobacteraceae* were not significantly correlated with any measured parameter.

In contrast, the other *K. brevis* strains cultured at FWRI, Jacksonville, Sarasota, and Charlotte Harbor, had communities dominated by *Flavobacteriaceae* in all growth treatments (>50% of identified OTUs). This group as well as the *Flammeovirigaceae* and *Rhodobacteraceae* contained significant indicator OTUs in all growth treatments. The *Flavobacteriaceae* had strong correlations to environmental conditions (Spearman's -0.73 for temperature and irradiance, and 0.73 for PSU) and was also the only group with a correlation to the abundance of *K. brevis* (Spearman's = -0.54). The *Flammeovirgaceae* had the strongest correlation to VBR (-0.54) but was also positively correlated to bacterial abundance, irradiance, and

temperature and negatively correlated to PSU and Leu- and TdR-BGR (all

correlations < 0.50). No measurements were correlated with *Rhodobacteraceae*.

In the Wilson "LT" strain (MOTE) *Rhodobacteraceae* were the dominant

bacterial group in both GR and ST growth treatments (34% and 27%, respectively).

The groups *Flavobacteriaceae* and unclassified *Alphaproteobacteria* (Fig. 6.10) were

17% and 14% of the total identified OTUs, respectively, in GR treatments, but

comprised only 6% in the ST treatment in which *Rhodospirillales* (17%) and

Verrucomicrobia (11%) became more dominant. The Wilson "LT" strain also had the

highest occurrence of SAR11 of any cultures sampled; 4% of OTUs in ST treatments

were identified as SAR11 compared to <1% in the six other stains containing a

SAR11 OTU (Fig. 6.10). Interestingly, none of these groups contained significant

indicator OTUs within Wilson Low Toxin cultures. The *Rhodospirillales* were

correlated with TdR incorporation and production (Spearman's -0.62) as well as

Leu:TdR (0.49).

The MOTE culture of *K. brevis* New Pass had groups *Flavobacteriales,*

Flammeovirgaceae, Saprospirales, and the bacterium *Thalassospira xiamenensis*

representing 11-26%, 17-19%, 13-23%, and 13-20%, respectively, of the OTUs in

GR and ST growth treatments (Fig. 6.10). The ST* culture was more difficult to

classify as 61% of OTUs would only classify to the *Alphaproteobacteria sp.* level; the

next largest group were the *Saprospirales* (16%). The Manasota Key strain also had a

large proportion of OTUs classified as *Flavobacteriales* (18-34%) and *Thalassospira*

xiamenensis (17-33%). The ST treatment of the Manasota Key strain also had

Balneolaceae sp. comprising 15% of

Bacterial community composition in cultures of *Karenia brevis*

Karenia brevis strain

Percentage of sequences in OTUs classified by major taxonomic groups

Legend:
- Other Bacteria
- Verrucomicrobia
- Other Proteobacteria
- Other Gammaproteobacteria
- Alteromonadales
- Other Alphaproteobacteria
- Rickettsiales
- SAR11
- Rhodospirillales
- Rhodobacteraceae
- Hyphomonadaceae
- Thalassospira xiamenensis
- Planctomycetes
- Other Cyanobacteria
- Flavobacteriales
- Flavobacteriaceae
- Cryomorphaceae
- Flammeovirgaceae
- Saprospirales
- Balneolaceae

Figure 6.10: Community composition of bacteria found within cultures of *Karenia brevis* depicted as the percentage of OTUs classified by major taxonomic groups. Different strains of *K. brevis* were isolated and cultured by Florida Fish and Wildlife Research Institute (FWRI) and MOTE Marine Laboratory, all growth treatments were sampled at the same time. A total of 1,400,082 sequences were classified into 679 OTUs using Mothur (Schloss et al., 2009) and the Greengenes taxonomic database (McDonald et al., 2012). The "Maintained" culture (ST*) strain New Pass had been in culture at MOTE for ~4 years at the time of sampling; the same culture in GR and ST treatments were descendents of the ST* culture.

the OTUs, which was the only *K. brevis* strain in which this group was >10% (Fig. 6.10). The *Flavobacteriales* group had an indicator value of 0.95 and *Thalassospira xiamenensis* an indicator value of 0.90 for both New Pass and Manasota Key cultures (in GR, ST, and ST* treatments). Several *Flammeovirigaceae* OTUs were significant indicator taxa but only in the Manasota Key cultures, which had a smaller proportion of *Flammeovirigaceae* than the New Pass strain (Fig. 6.10). Temperature, irradiance, and PSU had the strongest correlation with *Thalassospira xiamenensis* (Spearman's 0.64, 0.64, and -0.64, respectively) but was also correlated to bacterial abundance (0.55), BGR (-0.43 for both Leu- and TdR-BGR), and VBR (-0.40).

The dinoflagellate *K. mikimotoi* had similar groups as the *K. brevis* strains also cultured at FWRI (Fig. 6.11). The *Flavobacteriaceae, Planctomycetes,* and *Rhodobacteraceae* were dominant in all three growth phases (23-36%, 17-31%, and 13-21%, respectively). When cultured at MOTE *K. mikimotoi* communities were primarily *Flavobacteriaceae* (37%) and unclassified *Gammaproteobacteria* (24%). The *Rhodobacteraceae* had significant indicator OTUs in both FWRI and MOTE cultures of *K. mikimotoi*. Culturing conditions were correlated to the percentage of *Planctomycetes* OTUs (Spearman's -0.57 for irradiance and temperature and 0.57 for PSU). There were no correlations between *Rhodobacteraceae* and any measured parameter.
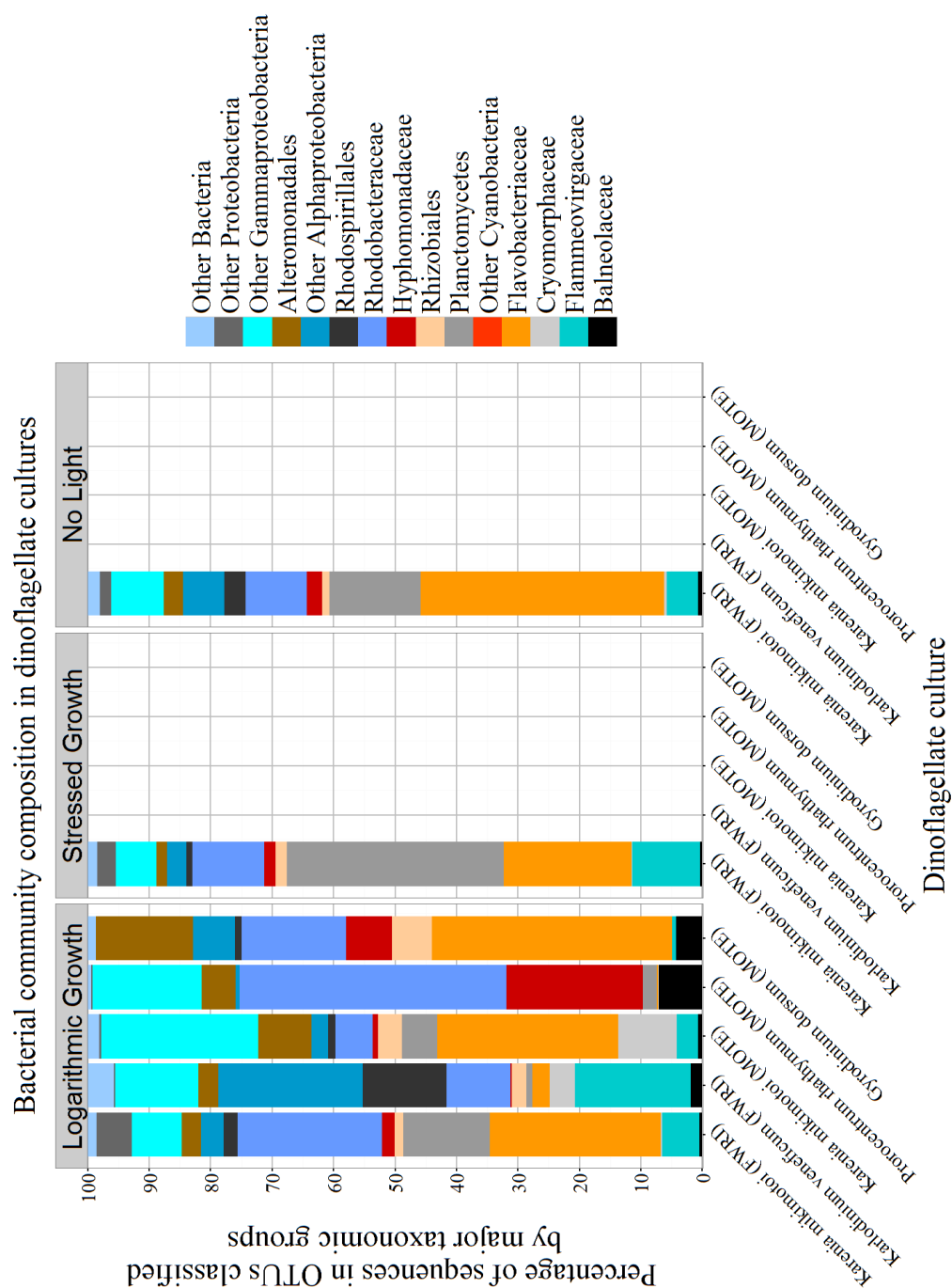
Figure 6.11: Bacterial community composition, expressed as a percentage of major taxonomic groups, in dinoflagellate cultures grown at Florida Fish and Wildlife Research Institute (FWRI) and MOTE Marine Laboratory. A total of 649,871 sequences were classified into 679 OTUs using Mothur (Schloss et al., 2009) and the Greengenes taxonomic database (McDonald et al., 2012).

The non-*Karenia* dinoflagellates all had differing bacterial communities. Unclassified *alphaproteobacteria* (23%) were the most abundant group in *Karlodinium veneficum* cultures (FWRI, Fig. 6.10). In *P. minium* and *G. dorsum* cultures (MOTE) *Rhodobacteracea* comprised 45% of all OTUs in the former, and *Flavobacteriaceae* 41% in the latter. None of the significant indicator OTUs were represented in any non-*Karenia* dinoflagellate culture from either laboratory. However, the percentage of *Flavobacteriaceae* OTUs was negatively correlated with the abundance of the dinoflagellates in culture (Spearman's -0.72).

Discussion

A survey of commonly used experimental cultures of *Karenia brevis*, *Karenia mikimotoi*, and four other dinoflagellates maintained at FWRI and MOTE found co-occurring bacterial communities within cultures were different between cultures and different from naturally occurring communities on the WFS. Cultures of *K. brevis* were dominated by members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex and *Alphaproteobacteria* and were missing several bacterial groups commonly found on the WFS and within blooms of *K. brevis*. The primary bacteria within the CFB group could further identify strains grown at FWRI vs. MOTE indicating the laboratory culturing conditions have an effect on community composition. The Wilson clone had a wholly unique microbial community and may no longer be a good "type strain" for *K. brevis* as the associated bacteria no longer represent bacterial communities found within blooms. It is also possible that the

composition of the bacterial community within cultures has an impact on toxin production, as the Wilson "low toxin" strain sampled in this study also had an associated bacterial community which differed from both Wilson and more recent isolates. On the whole, non-axenic cultures should have their bacterial communities more closely examined to better understand potential *Karenia*-bacteria interactions and help prevent biological activity of associated bacteria from being attributed to the dinoflagellate.

Culture preparation and conditions effectively controlled viruses

All cultures were begun with an inocula of existing cultured material into 0.2μm pre-filtered, autoclaved seawater. Most filtration is not capable of removing all viruses (Boehme et al., 1993), and additional treatment such as UV irradiation or autoclaving can alter the nutrient composition of water in addition to deactivating viruses (Noble and Fuhrman, 1997, M. Garrett, pers. comm.). Viral abundances were significantly lower than the abundance of bacteria in all cultures and growth phases, evidenced by both the low total abundances as well as the extremely low VBR (<0.3 in all cultures). This value is lower than that found in oligotrophic offshore waters of the Gulf of Mexico whose nutrient levels are far below that of the cultures in this study (Boehme et al., 1993). This relatively low abundance of viruses limits the possibility of viral lysis as a source of mortality within cultures (Jacquet et al., 2005). The VBR remained steady or decreased across all growth treatments, except *K. mikimotoi* grown at FWRI, which suggests minimal viral influence on the growth and abundance of dinoflagellates or bacteria in culture. Given the high abundance and

production of bacteria within cultures, it is likely that a majority of viruses were free-phages and not contained within host genomes (prophage) which is often the case when host growth is poor or stressed (Williamson et al., 2002).

Viral-induced mortality of both bacteria and phytoplankton has been shown in both environmental and cultured conditions but was not a major factor in the cultures in this study. Given that the cultures sampled within this study are maintained for experimental use, minimizing viral influence on cultures by limiting viral abundance is a good way to prevent the rapid termination of cultures both within the collection and during experiments.

It should also be noted that cultures are also maintained without zooplankton or protist grazers (M. Garrett, pers. comm.) which are also potential sources of bacterial mortality (Fuhrman, 1999). Grazing rates of heterotrophic nanoflagellates can be four times higher than viral-induced mortality (Fonda Umania et al., 2010) High rates of mortality which could not be attributed to viral lysis were not observed suggesting that exclusion methods were effective at excluding both zooplankton and heterotrophic nanoplankton (Stoecker et al., 2008). Two other possible sources of bacterial mortality were bacteriophages (Fuhrman, 1999) and *K. brevis* mixotrophy (Glibert et al., 2009), yet neither appear to have been an important component of bacterial mortality within this experiment.

Laboratory "signatures" exist in microbial communities of cultured phytoplankton

Bacterial communities within cultures formed clusters by the laboratory in which they were cultured as well as by species or strain of dinoflagellate. Both NMDS and PCoA demonstrate the laboratory maintaining the cultures can have an effect on the microbial community (Fig. 6.11) and possibly how that community functions (Fig. 6.10). It was interesting that culture lineage was obvious despite laboratory influence on bacterial communities. If the same species was cultured at different laboratories (Ex. *K. brevis* Wilson and *K. mikimotoi*) they would cluster across laboratories whereas different strains of *K. brevis* cultured independently at each laboratory (Ex. Jacksonville and Sarasota) would cluster better with strains from the same laboratory. Non-*Karenia* dinoflagellates would also cluster more closely with cultures from the same laboratory but not between laboratories (Fig. 6.7).

Given that both the Wilson "Low Toxin" and *K. mikimotoi* cultured at MOTE were descended from cultures obtained from FWRI, the clustering across laboratories demonstrates that the bacterial community persists for multiple generations despite a change in culturing conditions (Schäfer et al., 2002). It is therefore possible for a laboratory to start their own culture collection and maintain a microbial community similar to the culture and laboratory of origin. The difference in clustering between the Wilson and Wilson "Low toxin" strains provides an ideal starting point for further study differentiating differences between the microbial communities that could lead to differences in toxin production (Bates et al., 1995, Hold et al., 2001).

The *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex was prominent in all *Karenia spp.* and *Alphaproteobacteria* were dominant in non-*Karenia* cultures. The Wilson "Low Toxin" strain appears to be the exception; bacterial communities were dominated by *Alphaproteobacteria* rather than *Bacteroidetes*. The percentage of OTUs classified as *Bacteroidetes* in Wilson "Low Toxin" were half that of any other culture in both GR and stationary treatments. The *Alphaproteobacteria* represented 15-29% more of the OTUs for the same comparison.

The prominence of *Alphaproteobacteria* and members of the CFB group has been observed in a bloom of *K. brevis* on the WFS (Jones et al., 2010), in cultures of toxic dinoflagellates (Perez et al., 2008, Moustafa et al., 2010), and in numerous diatom cultures (Pinhassi et al., 2004). Cultures of differing genus or species (non-*Karenia*) had unique microbial communities which could not be compared across laboratories due to different species availability at different laboratories and only one strain of each species available (Schäfer et al., 2002). Community "signatures" were noticeable between cultures of the same genus (*Karenia*). Differences were determined by the dominant member of the *Alphaproteobacteria* and CFB groups; the proportion of these groups often correlated with microbial production and/or culturing conditions.

The Wilson clone has a unique microbial community

As the oldest and most frequently used isolate (Wilson and Collier, 1955, Lidie et al., 2005) the Wilson clone (FWRI) represents over 50 years of compounding "bottle effects." The differences observed in bacterial community clustering (Fig. 6.6)

and composition (Fig. 6.10) between growth treatments of *K. brevis* Wilson and other strains of *K. brevis* are significant. In Wilson cultures *Cryomorphaceae* are the dominant *Bacteroidetes* in all treatments and there is also a high proportion of *Planctomycetes.* The *Cryomorphaceae* have been identified within cultures and blooms of phytoflagellates before, but not in association with toxic species (Pinhassi et al., 2004, Jasti et al., 2005). Cultures of the okadaic acid-producing dinoflagellates *Prorocentrum lima*, *P. hoffmanianum*, and *P. rhathymum* have all been found to contain multiple OTUs of *Planctomycetes* (Perez et al., 2008).

In long-term cultures of diatoms (~2 months in batch culture) bacterial communities have been observed to remain similar in composition with only minor changes in relative abundance of each group observed after 10 days (Schäfer et al., 2002). Since replicate batch cultures were separately cultured to obtain GR, ST, or NL treatments, all were sampled after the same amount of time in culture and likely excluded any temporal changes in the bacterial community. Given the amount of time the Wilson clone has been maintained in culture the bacterial community described here represents a stable assemblage of associated bacteria.

While the abundance of *K. brevis* Wilson (FWRI) decreased between GR and ST growth treatments, bacterial abundance and production fluctuated (Figs. 6.1, 6.2, and 6.4). The *Cryomorphaceae* and *Planctomycetes* were a large proportion of bacteria in GR and ST treatments and positively correlated with Leu- and TdR-BGR, indicating they were most likely the active microbes associated with healthy cultures of *K. brevis* Wilson. Bacterial growth was balanced and thymidine production high, indicating these bacteria were efficiently using available nutrients for cell division

(Fig. 6.5, del Giorgio et al., 2011). In ST treatments the environment was becoming hostile to both bacteria and *K. brevis*; dinoflagellate and bacteria abundance decreased and bacterial metabolism was very active (high leucine production) but not matched by cell division (Leu:TdR ~10). Previous studies have found that metabolic activity was dominated by a limited number of opportunistic bacteria (Massana et al., 2001, Zubkov et al., 2001). This indicates the change in growth efficiency, increase in metabolic costs, and decrease in cell abundance between GR and ST cultures was most likely due to loss of these groups (especially the *Cryomorphaceae*). As *K. brevis* abundance further declined in NL treatments *Hyphomonadaceae* and *Altermonadales* become more dominant. As these groups are typically free-living and associated with the uptake of amino acids, they would be well suited for increased growth and production utilizing the decaying *K. brevis* (Fandino et al., 2001). This is a prime example of opportunistic species taking advantage of the changing structure of the microbial community due to a loss of two numerically and metabolically dominant bacteria.

Cultures lack prominent bacteria found along the WFS

There were several bacterial groups found inside and outside blooms of *K. brevis* in freshly collected samples on the WFS that were absent within cultures. The *Betaproteobacteria*, *Deltaproteobacteria,* and SAR406 were completely absent from all cultures but observed across the WFS (Chapter 4). The absence of these groups is not surprising; numerous studies have noted their absence in dinoflagellate cultures (Perez et al., 2008) as well as other phytoplankton cultures originating from coastal

environments (Jones et al., 2010). In contrast, SAR406 is typically found within deep-ocean environments (Eloe et al., 2011), and the appearance of SAR406 in surface samples is associated with upwelling currents along the WFS (Walsh et al., 2006). Culturing conditions are designed to mimic surface conditions (high light and surface temperatures) and would therefore select against a group ideally suited for deep water such as SAR406. The role of these groups in bloom initiation and maintenance is unknown, but their absence from all cultures suggests there is not a critical relationship between SAR406 and bloom initiation or maintenance.

All cultures sampled in this study had low abundances, or a total lack, of cyanobacteria and *Pelagibacteraceae* (SAR11). Both of these groups are well known components of oligotrophic ecosystems, such as the WFS, but both comprise less than half a percent of identified OTUs. It is possible that some SAR11 was classified as *Alphaproteobacteria sp.*, however the percentage of *Alphaproteobacteria spp.* OTUs were typically lower than that observed in samples from bloom on the WFS in 2001 (Jones et al., 2010) and 2009 (Chapter 4).

All data collected in this study point toward cultures missing key components of typical WFS bacterial communities (Jones et al., 2010, Chapter 4). Given that shifts in bacterial community composition have been observed in as few as 16 hours, it is not surprising that these cultures no longer contain "natural" communities (Ferguson et al., 1984). A survey of bacterial diversity within diatom cultures found similar differences between communities in cultures and native waters, with *Alphaproteobacteria* and *Bacteroidetes*/*Flavobacteria* consistently dominant in all cultures (Amin et al., 2012). As such, studies utilizing cultures of *K. brevis* as proxies

for studies of bloom ecology, mixotrophy, and allelopathy (Prince et al., 2008, Glibert et al., 2009, Sinclair et al., 2009, Prince et al., 2010) have not accounted for the "true" culture history i.e. differences in bacterial composition from that found in the waters at the time of isolation (Hiesler et al., 2008).

Microbial metabolism in phytoplankton cultures should not be overlooked

Microbial production within cultures was higher in the free-living bacteria (0.2-3µm) than in the attached bacteria (>3µm), accounting for ~92% and ~90% of measured leucine and thymidine production, respectively (range 82.43-99.30% and 81.85-98.61, data not shown). This is opposite of that found in studies of natural phytoplankton populations where the attached bacteria were responsible for up to 92% of bacterial productivity (Rooney-Varga et al., 2005).

Compared to naturally occurring blooms of *K. brevis*, bacterial community production in cultures (all growth phases) was much higher than in both initiation (logarithmic) and stationary bloom phases (Chapter 3). Use of media to promote growth of phytoplankton will also promote bacterial growth; given the higher surface to volume ratio and abundance of bacteria compared to *K. brevis* bacteria have a competitive advantage for available nutrients (Michelou et al., 2011). The low Leu:TdR in all cultures (except the Wilson clone grown in the ST treatment) indicate the microbial communities are in an energetically favorable environment and poised to respond to any changes or treatment (del Giorgio et al., 2011). Any such response should be faster than that of the dinoflagellate. In cultures of the toxic dinoflagellate *Alexandrium tamarense* changes in gene expression signature between nutrient-

limiting and nutrient-replete conditions were greater in the bacterial community than the dinoflagellate (Moustafa et al., 2010).

The BGR in cultures compared to that measured in blooms suggest the higher production in cultures was due to higher bacterial abundance in cultures (10^7-10^8 bacteria mL^{-1}) than in blooms on the WFS (10^6 bacteria mL^{-1}, Chapter 3). Within the same bacterial assemblage *Alphaproteobacteria* can dominate uptake of amino acids and the CFB-group can dominate consumption of chitin and protein (Cottrell and Kirchman, 2000).This study failed to take into account differential uptake of nutrient pools by the major phylogenetic groups and therefore cannot determine if total community production within a particular growth phase was due to a select group(s) of bacteria or if the nutrient media used were the cause of community differences between cultures grown at FWRI and MOTE (Massana et al., 2001).

Synergistic interactions between bacteria and phytoplankton require an exchange of compounds in a mutually beneficial manner. Given that this study did not track any changes in the dissolved or particulate organic matter pools, changes in community composition as a result of available nutrients could not be assessed. However, CFB bacteria have a preference for organic polymers (Suzuki et al., 2001) and the high proportion of these bacteria in *K. brevis* cultures suggests that complex compounds produced by *K. brevis* are broken down by CFB bacteria in exchange for vitamins or trace elements (Fandino et al., 2001, Amin et al., 2012). On the other hand, *Alphaproteobacteria* demonstrate a competitive advantage for uptake of amino acids (Cottrell and Kirchman, 2000). This potential competitive interaction for free amino acids may affect toxin production by *K. brevis* which utilizes free amino acids

in brevetoxin synthesis pathways (Shimizu and Wrensford, 1993). It is possible that the higher proportions of *Alphaproteobacteria* in the Wilson "Low Toxin" strain are responsible for the reduced toxin concentrations compared to the standard Wilson clone as well as other *K. brevis* strains. Testing this hypothesis may help variation in toxin concentrations in experimental cultures of *K. brevis*.

Bacterial community composition and metabolism within cultures need to be better characterized

Interactions between bacteria and phytoplankton can fall under three main categories: synergistic, competitive, and parasitic (Amin et al., 2012). These interactions can occur both in the environment and within isolated cultures. This complicates conclusions based on experimental results that fail to take such interactions into account. Removing bacteria to create axenic cultures can eliminate interactions and lead to overestimation (competitive interactions) or underestimation (synergistic interactions) of any measured rates; utilizing non-axenic cultures would have the opposite result.

Given that no axenic cultures of *K. brevis* are currently maintained by any laboratory and the dominance of relatively few bacterial groups within those cultures there is most likely a synergistic relationship within cultures between *K. brevis* and co-occurring bacteria. Members of the *Alphaproteobacteria* and *Bacteroidetes* (CFB group) were prominent in all cultures, typically >70% of the community but never less than 50%. These groups have been observed to be the primary phyla associated with both toxic and non-toxic dinoflagellates (Hold et al., 2001) as well as diatoms

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(Pinhassi et al., 2004). Difference in bacterial communities between cultures can be attributed to differences in the primary groups comprising each of these phyla.

Synergistic interactions are typically driven by exchange of vitamins, trace elements, and dissolved carbon and nitrogen compounds (Amin et al., 2012). The preference of the CFB group for organic polymers and their high proportion within most *K. brevis* culture communities suggests compounds produced by *K. brevis* are utilized by the CFB group in exchange for either vitamins or trace elements. As many CFB bacteria are themselves, or related to, surface-associated bacteria, a synergistic relationship is highly likely (Hold et al., 2001). Even with most bacteria being classified as free-living, many could occupy the phycosphere of *K. brevis* cells which would facilitate the exchange of compounds.

There also appears to be a competitive interaction between the *Alphaproteobacteria* and *K. brevis* which affects the toxicity of the culture and possibly blooms on the WFS. Brevetoxin production by *K. brevis* requires free amino acids yet *Alphaproteobacteria* demonstrate a strong affinity for free amino acids as well. If a competitive relationship does exist, determining the threshold(s) for competitive dominance is critical and could impact bloom mitigation strategies (Heil and Steidinger, 2009).

Given the numerous potential interactions between bacteria and phytoplankton, better ecological determinations of bacteria in non-axenic cultures of toxic phytoplankton needs to be a renewed priority in harmful algal bloom research. This study has demonstrated that numerous strains of the toxic dinoflagellate *K. brevis* possess different microbial communities which have different metabolic

capabilities in different growth conditions. These dominant bacteria appear to have both synergistic and competitive relationships with *K. brevis* that may be important in bloom formation, maintenance, and toxicity along the WFS. Further exploration of bacterial communities within cultures will help determine which compounds serve as the currency as well as the rates and thresholds of any relationships. These results indicate communities in nutrient-replete conditions yet any such relationships will be altered in a short time-span by changes in the environment (Wholers-Zöllner et al., 2011). The effect of temperature and nutrient stoichiometry will both need to be better characterized within the context of *Karenia*-bacteria interactions in order to put such interactions into context that could yield useful insight for bloom monitoring, mitigation, and management (Lenes and Heil, 2010).

Use of laboratory cultures as a proxy for naturally occurring populations remains a viable tool for phytoplankton (including harmful algae) research; however bacterial communities cannot be overlooked. Bacteria occur throughout the oceans and relationships between phytoplankton and bacteria are well established. Phytoplankton monocultures should be viewed as microcosms for bloom ecosystems in which bacteria are key players. Such an approach should clarify biological rates and pathways as well as help differentiate relationships which impact phytoplankton life histories in an ecologically and management-relevant context.

Chapter 7: Synthesis

Evaluation of research objectives

Characterize the function of bacteria within natural blooms of *Karenia brevis* on the West Florida Shelf through *in situ* measurements

Bacterial production measurements are an accepted method to measure heterotrophic production both *in situ* and in experimental treatments (Fuhrman and Azam, 1982, Bell, 1993, Kirchman, 1993). By using two forms of tritium (^{3}H-leucine and ^{3}H-thymidine) additional parameters can also be quantified which can be used to infer functionality within the bacterial community (Franco-Vidal and Morán, 2010, del Giorgio et al., 2011). By utilizing both ^{3}H-Leucine and ^{3}H-Thymidine the following measurements were obtained: heterotrophic bacterial production, protein synthesis, nucleic acid synthesis, cell-specific growth rates (requiring bacterial cell counts), and community physiological state (growth balance). The length of incubations for production measurements, 1 hour, were designed to minimize "bottle effects" (Ferguson et al., 1984) as well as prevent uptake of the isotope by larger size fractions, as evidenced by minimal production in larger size fractions (Simon and Azam, 1989). This provided insight into bacterial community growth and mortality within different bloom stages *K. brevis* and allowed for inferences regarding *K. brevis* mixotrophy to supplement nutrient requirements.

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Whether the specific aspects of bacterial production, and therefore carbon metabolism, can translate into the function of individual, or groups of, bacteria within the ecosystem is still not clear, especially since understanding of connections between different aspects of bacterial metabolism are still being determined (del Giorgio et al., 2011). Until such connections are better understood through methods such as transcriptomics and single-cell genomics, *in situ* bacterial production measurements will only provide information on whole-community dynamics. This thesis has highlighted several communities which may contain specific species and/or groups of bacteria that may play significant roles in blooms, cultures, and growth dynamics of *K. brevis*. Unfortunately, production measurements were not capable of fractioning production to support any hypotheses or conclusions regarding individual components of bacterial communities.

Whole community production measurements were successful for multiple size fractions (with the use of GF/F filters in 2008 being the exception) and appear to have been limited to the bacterial community. Replicate treatments from both field and laboratory studies shows repeatable measurements with acceptable variance for both isotopes. Measurements were within ranges observed for similar filters sizes in like-environments (del Giorgio et al., 2011) but higher than previous observations on the West Florida Shelf (WFS) which used larger pore sizes (Heil et al., 2004). Separating measurements by year, and therefore bloom stage, was also useful for scientific comparisons; pooling data showed similar results to other studies but separated data had significant differences between bloom and non-bloom stations as well as bloom stage (no bloom, initiation, and maintenance).

Determine if viruses are playing a role in *Karenia brevis* bloom dynamics, and if so, if viruses are acting directly on *K. brevis* or on associated bacteria

Viral concentrates are an effective way to obtain viruses for a better characterization of viral communities as well as use in infection studies (Winter et al., 2004, Wommack et al., 2010). Three critical errors in experimental design may have impacted the effectiveness of this method and the initial experimental setup; 1) A pre-filtration step was used before viral concentration but did not adequately address the impact of pore-size on the viral community. Up to two-thirds of the viral particles may have been removed by the 0.2 μm pore size used in pre-filtration, reducing the efficiency of the concentration step which followed (Boehme et al., 1993); 2) Due to the small volume of viral concentrate produced and the number of treatments in the experiment, no concentrate remained for direct counts of the concentrate. The viral concentration may have entirely failed, however without a count of VLPs in the concentrate the study was not able to determine if the concentrate significantly increased VLPs at the start of the experiment; 3) The length of incubation was likely too short to allow for a complete lytic cycle to have occurred, assuming viruses in the concentrate successfully infected bacteria in the treatments (Bratbak et al., 1993).

Despite the above errors in experimental design, other data produced by the viral concentrate experiment were able to address the primary hypothesis; that viruses were acting upon bacteria co-occurring with *K. brevis* and not the dinoflagellate directly. Bacterial production, bacteria and dinoflagellate abundance, and community

composition data supported this hypothesis while also reinforcing the need to further explore bacteria-*Karenia* interactions.

Classify the bacteria communities within cultures of *Karenia brevis* which are maintained for scientific research

Next generation sequencing technologies were used to explore community composition of bacteria co-occurring with *K. brevis* in culture as well as within naturally occurring blooms on the WFS. Both 454 Tag pyrosequencing and Illumina MiSeq platforms were effective methods: millions of sequences were classified into hundreds of unique operational taxonomic units (OTUs). Bioinformatics pipelines used also allowed for the diversity of communities to be compared statistically, OTUs analyzed for indicator species, and sequences analyzed for algicidal and algicidal resistance-conferring bacteria. This built upon previous studies of the bacteria community associated with blooms of *K. brevis* (Jones et al., 2010) as well as investigations into specific bacteria capable of lysing, or preventing lysis of, *K. brevis* in culture (Roth et al., 2008a).

Given previous studies of bacteria associated with bloom forming algae (toxic and non-toxic, Hold et al., 2001, Amin et al., 2012) and the ecological role of some groups of bacteria (Cottrell and Kirchman, 2000, Fandino et al., 2001), classification of bacterial communities co-occurring with *K. brevis* on the WFS and in cultures led to testable hypotheses regarding interactions between bacteria and *K. brevis*. The level of classification and whole-community sequencing approach did not allow for

any such interactions to be clearly identified or explored, but identified several groups which should be considered for further study.

Conclusions

Methods used in these studies were appropriate at addressing all research objectives

It is essential to give methods proper consideration, especially considering the specific nature of some of the objectives and hypotheses being investigated. Some methods used were both appropriate and effective while others were not able to fully address the objectives. Valuable data was obtained with all methods used in both field (shipboard) and laboratory experiments, with measurements similar to those observed previous on the WFS or in similar ecosystems.

Primary production measurements demonstrated both high primary production and high chlorophyll-specific production within a bloom of *K. brevis* in size fractions that include *K. brevis* (>3.0 µm) and bacterioplankton (0.2-3.0 µm). Bacterial production measurements captured heterotrophic production in multiple size classes as well as information regarding the metabolic state of said size classes.

Few studies use both tritiated leucine and thymidine despite it being well understood that each represents a different component of bacterial metabolism (Franco-Vidal and Morán, 2010). Previous measurements of bacterial production within *K. brevis* blooms only used ^{3}H-Leucine (Heil et al., 2004). Due to the

recognition that using both isotopes provided more information regarding metabolic state, both isotopes were used starting with the ECOHAB: *Karenia* process cruises in order to address multiple research objectives. In 2009, the ECOHAB: *Karenia* project began to standardize size-fractionation for all measurements. This led to the discovery that most bacteria measured in *in situ* and laboratory experiments were free-living (Eloe et al., 2011) and not attached as has been observed for some dinoflagellate species (Gasol et al., 2005). These measurements were critical in addressing the first objective of this thesis, providing supporting data for the second and third objectives.

Evidence that viruses are capable of terminating algal blooms through direct infection of bloom-forming phytoplankton (Bratbak et al., 1993) was the basis for such studies being conducted on *K. brevis* (Paul et al., 2002). Unfortunately, *K. brevis* cultures are maintained with co-occurring bacterial communities and therefore by failing to remove bacteria from cultures prior to inoculation with a lytic agent (viral concentrate), previous studies could not demonstrate viral infection of *K. brevis* without microscope images of phages within or nearby *K. brevis* cells. Such evidence was not obtained, leading to the hypothesis that viruses were acting upon the bacterial community and not *K. brevis* directly (Paul et al., 2002). A second attempt at this experiment did not terminate the *K. brevis* culture (I. Hewson, pers. comm.). Neither of these studies measured any bacteria-specific parameters, and they therefore were not able to determine if bacteria were indeed the target of concentrated viruses nor how that could impact *K. brevis* bloom dynamics.

In order to investigate the bacteria-lysis hypothesis of *K. brevis* bloom termination, an experiment was designed to measure both bacterial metabolism and

community composition in cultures of *K. brevis* using a viral concentrate from the WFS. The method used to concentrate viruses in the second study was replicated (after training in the Hewson laboratory, Cornell University) but did not provide enough viral concentrate for all experimental treatments and a viral count of the concentrate. This was a problem as no significant difference in viral abundance was observed between treatments. After further research, it was determined that there were two steps in the concentration procedure during which something may have gone wrong: pre-filtration of seawater to be concentrated may have removed a significant portion of the viral community (Boehme et al., 1993) and the concentration itself may have failed (Wommack et al., 2010). Without a direct count of the concentrate (and possibly the pre-filtrate) it could not be determined if either of those steps disrupted the concentration procedure. Other measurements taken during this experiment provided additional support that viruses were acting upon bacteria, but the questions surround the concentrate may overshadow such evidence when presented to the scientific community at large.

A second set of treatments were employed in the virus study to attempt to demonstrate viral lysis of bacteria: these treatments were cultures of *K. brevis* pre-filtered through a GF/F filter (0.07 μm) to remove *K. brevis* while allowing the co-occurring bacteria to pass through to the filtrate. This method was used after prior attempts to filter and re-suspend *K. brevis* in bacteria-free water (0.2 μm filtered seawater) were shown to damage and ultimately kill *K. brevis* cells. Shear stress has been shown to affect growth and toxicity of dinoflagellates in previous studies (Juhl et al., 2001, Stoecker et al., 2006a). The GF/F filtration was meant to only remove *K.*

brevis but also removed ~88% of the bacteria as well. This also changed the composition of the bacterial community that passed through the filter, presenting a significantly different community for inoculation with a viral concentrate. These treatments were therefore not useful in determining if viral lysis of bacteria was altering bacterial community structure and leading to termination of *K. brevis* blooms. Valuable information was still obtained, however, and the second objective of this thesis was still extensively explored, however further studies are necessary to adequately test the hypothesis regarding viral lysis within *K. brevis* blooms.

High-throughput sequencing was used to classify bacterial communities along the WFS and within cultures of *K. brevis* and other dinoflagellates. These technologies produce large datasets in electronic files which can then be compared to other databases (when available) and stored for comparison to future studies. The two platforms used, 454 and Illumina, produced different amounts of data as well as different community compositions. Some of these differences can be attributed to the sequencing platforms (Quince et al., 2009, Werner et al., 2012) but the choice to use two different primers (one for each platform) for amplification of extracted DNA most likely had the strongest influence on the results (Liu et al., 2007). These differences did not adversely affect downstream analyses of data however, as community clustering and indicator analyses produced similar results with both platforms.

Methods were chosen based upon reproducibility of results, proper training, prior studies, and cost. In most cases the methods used produced consistent results which addressed the objectives of the ECOHAB: *Karenia* project and this thesis. In

the cases where results were not as expected, a literature review found probable explanations that can be used to improve future studies. Despite experiments that could have been improved, the methods used produced results that allowed for conclusions to be drawn regarding all objectives.

Bloom stage needs to be identified when studying *Karenia brevis*

Three blooms were observed during the 4 year study period of the ECOHAB: *Karenia* project, one each in 2007, 2008, and 2009. The 2008 bloom was defined as being in initiation phase. Biomass was relatively low and cell concentrations were increasing (highest abundance observed on the last day of sampling, Table 3.1) as the bloom moved from offshore to nearshore environments (physical environment aggregating the bloom, Walsh et al., 2003). Furthermore, there was no prior detection of this bloom by any regional monitoring programs. The 2009 bloom was defined as a maintenance/stationary bloom due to its offshore location (serving to disperse the bloom, Weisberg et al., in press), low to moderate concentrations of *K. brevis* present, and high numbers of lipid bodies within cells (which have been shown to indicate older cells in stationary phase, Steidinger, 1979). While it was likely that data were going to be compared across years, this fortuitious finding provided an additional biologically relevant context for the comparison.

The bloom in initiation phase had higher primary production, bacterial production and bacterial growth rates and lower virus to bacteria (VBR) and Leucine to Thymidine (Leu:TdR) ratios than the blooms in maintenance phase. Had all data

been grouped together the variance would have increased and overwhelmed any patterns, including differences between bloom stages, and between bloom and non-bloom waters from the same year. Furthermore, placing results into the context of a specific bloom stage led to conclusions that may have otherwise been overlooked.

Results from *in situ* measurements periods when there was no bloom (2010 research cruise) and blooms in initiation (2008 research cruise) and maintenance (2009 research cruise) phase indicated that the bacterial community on the WFS is nutrient limited, poised for rapid growth when blooms are initiating, and metabolically stressed and subject to viral attack in maintenance phase. A nutrient limited community is to be expected during non-bloom periods as the WFS is an oligotrophic ecosystem (Heil et al., 2007); the highest bacterial abundance and production rates were observed near and within estuaries. Within the initiating bloom bacterial production and growth were higher than stations outside the bloom; this fit with previous observations of blooms on the WFS (Heil et al., 2004). The Leu:TdR indicated that the bacterial community had balanced growth, and thus was efficiently using nutrients for both metabolism and cell division. If bacteria were still nutrient limited, the Leu:TdR would be expected to be high, and production measured by TdR would not be significantly different from non-bloom (nutrient limited) samples (del Giorgio et al., 2011). In the maintenance phase bloom, not only were production measurements significantly lower than outside the bloom, the Leu:TdR indicated bacteria in a hostile environment (del Giorgio et al., 2011), and the VBR was increasing. This suggested that not only was the bacterial community energetically stressed, but also becoming more susceptible to viral infection (Jacquet et al., 2005).

These differences not only highlight the dynamic nature of *K. brevis* blooms, but also highlight the changes in ecological roles of associated organisms such as bacteria and viruses. Despite years of research on *K. brevis* blooms on the WFS, few studies determine the stage of the bloom(s) sampled. This may be because many blooms are sampled only after they are detected, thus they are well into initiation and possibly into maintenance phase (Brand et al., 2012). It may also be due to resource availability limiting sampling so that even long-term blooms are only sampled a few times, such as when first identified or when reaching a concentration or chlorophyll maximum (Vargo et al., 2004). The differences between blooms in initiation and maintenance phases in this study emphasize the need to classify *K. brevis* bloom stage. Not only because significantly different values in many parameters are observed, but because by not classifying blooms stage scientists are not allowing for the study of the entire evolution of a bloom, from before formation through the termination and a return to normal community composition. It may be that all the studies combined have completely characterized a bloom, but without knowing the bloom stage of each study, scientists cannot piece together the data into an ordered, coherent picture of *K. brevis* blooms on the WFS. Moving forward, those doing research should note the growth phase of both naturally occurring and cultured *K. brevis* so that a combined effort can understand bloom dynamics for the entirety of a single bloom event.

Bacterial community composition is important to consider in *Karenia brevis* bloom dynamics

Cultures of *K. brevis* have been maintained since 1953 (Wilson and Collier, 1955) and the one common characteristic of all *K. brevis* isolates is the presence of co-occurring bacteria (non-axenic cultures, Schaeffer et al., 2007). This is interesting considering that algal-bacteria interactions are well document (Amin et al., 2012), yet few studies which use cultures of *K. brevis* address this and reviews of *Karenia* research fail to acknowledge this issue (Brand et al., 2012). There are also numerous studies which document ways that bacteria co-occurring in phytoplankton cultures can alter results of both physiological and molecular studies (Bates et al., 1995, López-Lozano et al., 2002).

By investigating both naturally occurring blooms and cultures of *K. brevis*, significant differences in bacterial community composition were found. These differences were not limited to only between cultures and natural blooms, but also between different strains of *K. brevis* cultured at the same, and different, laboratories. One example is the *K. brevis* Wilson isolate, the "type strain" which was first isolated in 1953. The bacteria community found in cultures of *K. brevis* Wilson was different from that of all other cultures and blooms observed. The Wilson bacterial community actually grouped better with the communities in cultures of the dinoflagellates *Karenia mikimotoi* and *Prorocentrum rhathymum* than with those in other strains of *K. brevis*. Given this large difference in community composition, the Wilson clone is probably NOT a good "type strain" to use in experiments; any conclusions or data obtained using a culture of the Wilson clone should be reviewed with this in mind and

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compared to similar measurements from a more recent isolate or natural blooms for ecological validity.

This study was only able to investigate bacterial communities when no bloom was present (2010) and during a bloom in maintenance phase. Therefore, no conclusions can be made regarding bacterial community composition in different bloom stages. As bacteria are capable of utilizing different nutrient sources, it is likely that different bacteria are dominant, or at the very least metabolically active during different bloom stages (Cottrell and Kirchman, 2000).

It is very likely that multiple interactions, both synergistic and competitive, exist between bacteria and *K. brevis* (Hold et al., 2001). Two bacterial strains which exhibit algicidal activity towards *K. brevis* (Roth et al., 2008b) and one that confers resistance to such algicidal activity (Roth et al., 2008a) were found on the WFS in bloom and non-bloom waters. While the methods used only allowed for the detection of these bacteria, laboratory studies have shown that these bacteria are capable of quickly terminating or prolonging a bloom, depending on the strain. If these bacteria individually reach threshold abundances, they could alter the growth and toxicity of blooms (Shimizu and Wrensford, 1993, Fandino et al., 2001, Suzuki et al., 2001). The first step was identifying and understanding the relationship between the algicidal bacteria and *K. brevis* as well as between algicidal and resistance-conferring bacteria. This study was a second step by determining that such bacteria occurred across the WFS and within natural blooms of *K. brevis*.

As this was a preliminary investigation into the composition of bacterial communities associated with *K. brevis*, any bacteria-algae interactions are

hypothetical. There is a scientific basis for such interactions and many have been identified and quantified in laboratories for bacteria-diatom interactions (Figure 7.1, Amin et al., 2012) as well as bacteria-*Karenia* interactions (Figure 7.2, Roth et al., 2008a, Van Dolah et al., 2009). This stresses the need to further investigate the bacterial community, and possible bacteria-*Karenia* interactions, in both naturally occurring blooms and isolated cultures of *K. brevis*. If bacteria are overlooked, conclusions regarding *K. brevis* are not accounting for any affects bacteria may be having on the biogeochemistry of the ecosystem as well as the dinoflagellate itself. Further studies should include a microbial component to ensure a complete picture of *K. brevis* blooms and bloom dynamics.

Bacteria could be used to help predict the transition between bloom stages

The survey of bacterial communities on the WFS and in cultures of *K. brevis* identified several bacterial groups which may be useful in bloom detection, monitoring, and forecasting. The dominant group in all samples (environmental and cultures) were the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, which likely have a synergistic interaction with *K. brevis* in which the dinoflagellate provides complex compounds to the bacteria (Suzuki et al., 2001) in exchange for vitamins or
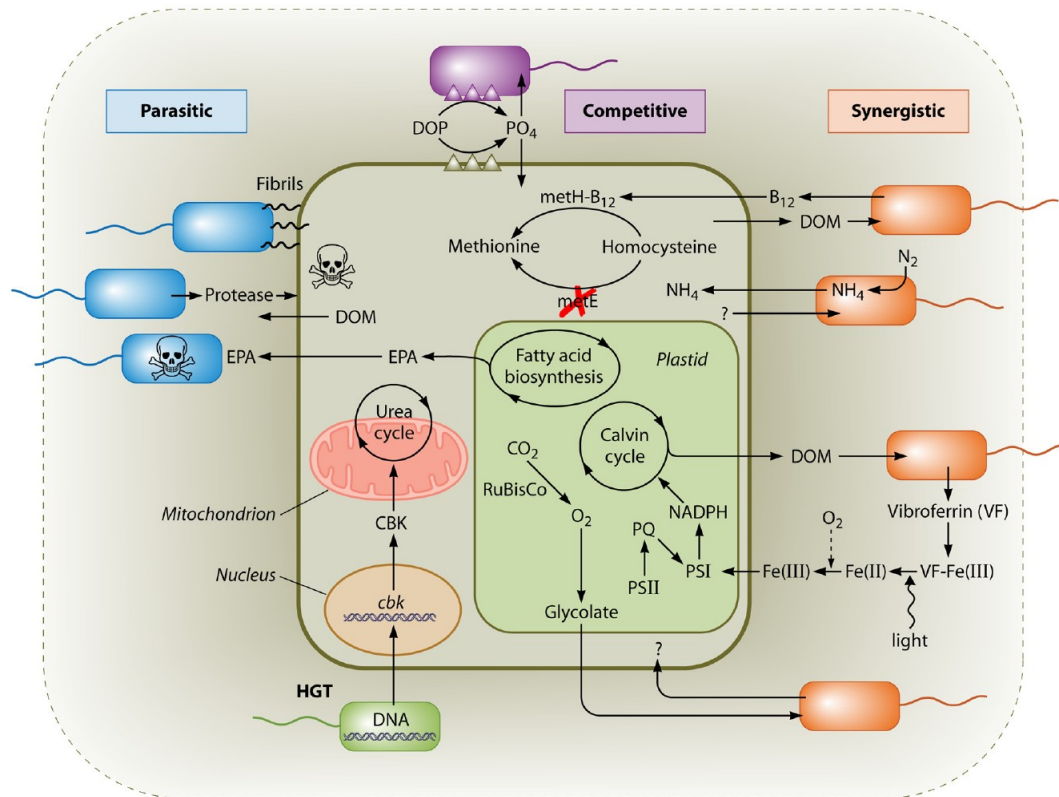
Figure 7.1: Known interactions between diatoms and bacteria. Interactions are classified as parasitic (blue), competitive (purple), or synergistic (orange). Horizontal gene transfer is also possible between diatoms and algae, and shown here (green). Small rectangles with flagella are bacteria, the large dark green rectangle represents a diatom cell, the smaller green rectangle is a diatom plastid, and the background gradient surrounded by a dashed line is the phycosphere (an area of influence surrounding the cell which stimulates bacterial growth). Key reactions which dictate bacteria-diatom interactions are depicted within and between involved cells. From Azam et al. (2012).
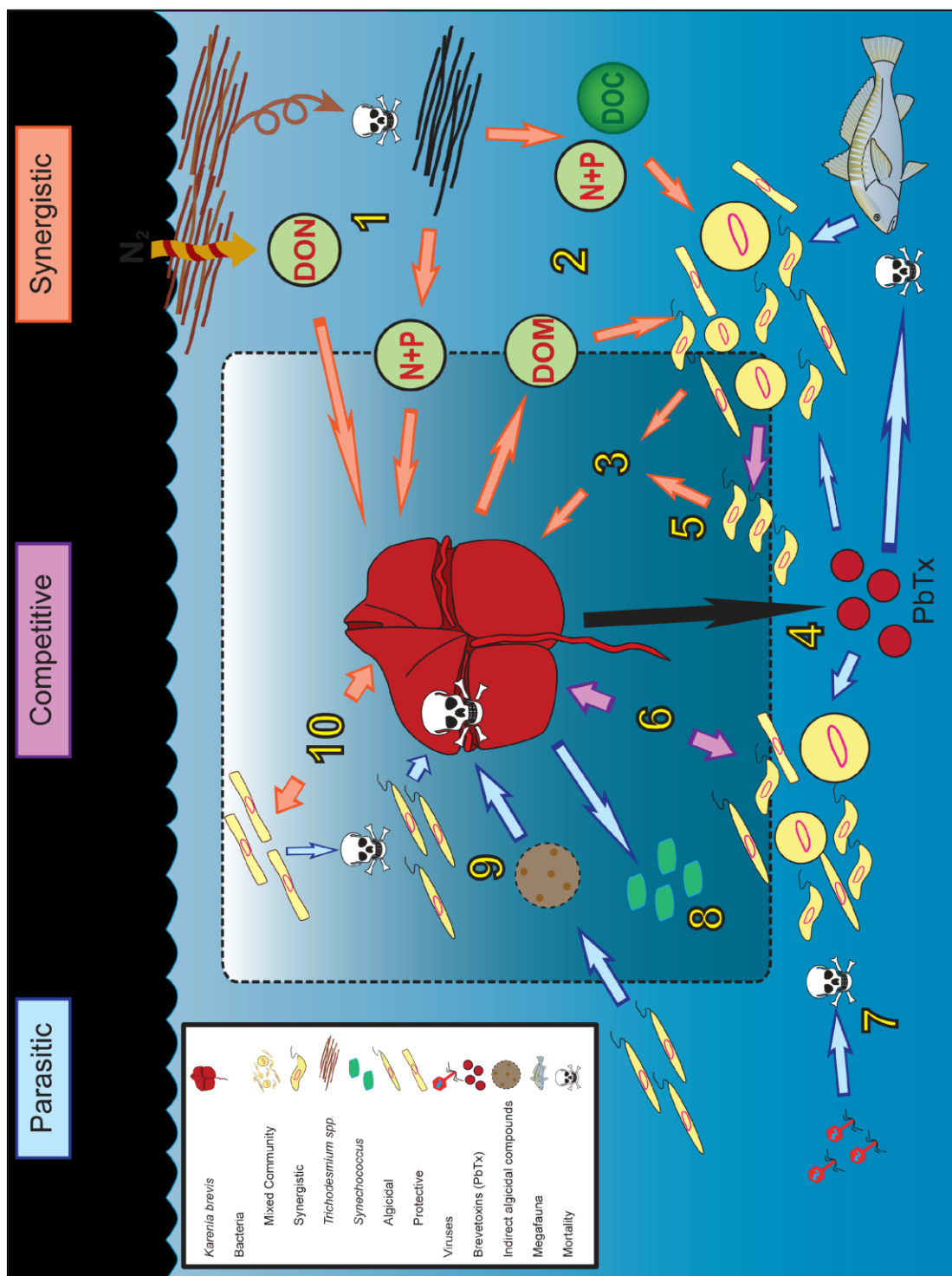
Figure 7.2: An overview of potential interactions involving bacteria and *Karenia brevis* in nearshore environments. Interactions can take place inside or outside the phycosphere of *K. brevis* cells (dashed area near the cells) and fall into one of three primary categories: parasitic (blue arrows), competitive (purple arrows), or synergistic (orange arrows). 1- nitrogen fixation by *Trichodesmium* spp. can provide nitrogen (DON) which can be taken up directly by *K. brevis* (Mulholland et al., 2006, Sinclair et al., 2009) or provided by remineralization of decaying biomass (Lenes and Heil, 2010). 2- Organic matter from *K. brevis* and decaying *Trichodesmium* spp. increases productivity within the bacterial community. 3- A highly productive bacterial community provides nutrients or other growth factors which promote *K. brevis* growth (Chapter 3). 4- Brevetoxins released by *K. brevis* can lead to fish and marine vertebrate kills which promote bacterial growth, directly alter the function of the bacterial community (Chapter 4) or 5- select for certain bacteria within the community (Sipler, 2009). 6- Bacteria and *K. brevis* compete for common resources. 7- Viral infection of the bacterial community leads to bacterial lysis which can eliminate synergistic bacteria and/or competition between bacteria and *K. brevis* (Chapter 5). 8- *K. brevis* can graze upon the cyanobacteria *Synechococcus* to supplement nutrient requirements (Glibert et al., 2009). 9- Algicidal bacteria can directly and indirectly lyse *K. brevis*, leading to termination of blooms (Jones et al., 2010). 10- Bacteria provide protection to *K. brevis* from algicidal bacteria (Roth et al., 2008) potentially due to synergistic interactions with *K. brevis*. Modeled after Amin et al. (2012).

trace elements (Fandino et al., 2001, Amin et al., 2012). The second most dominant group was the *Alphaproteobacteria*, which likely compete with *K. brevis* for amino acids (Cottrell and Kirchman, 2000). Making up a small portion of environmental samples were SAR406, typically a deep-water bacteria, the algicidal strains S03 and 41-BDG2, and bacterial strain D38BY which is antagonistic to strain S03. All of these bacteria appear to have a relationship to *K. brevis* blooms that may be limited to specific bloom stages or transitions between stages.

It was not surprising to find the CFB group or *Alphaproteobacteria* in any study, as these bacteria are often found in surface waters and phytoplankton cultures (Perez et al., 2008, Jones et al., 2010, Moustafa et al., 2010). The presence of SAR406 in surface waters was surprising because this Phylum has previously been identified in deep (below the deep chlorophyll maximum) waters to the near the sea floor (Gordon and Giovannoni, 1996, Gallagher et al., 2004). The presence of SAR406 in the surface has been found to be linked to spring phytoplankton blooms resulting from stratification following mixing events in the Sargasso Sea. The algicidal and antagonistic bacterial strains were originally isolated from *Karenia* blooms on the WFS in the Gulf of Mexico, but it was not determined if these strains would be found when a bloom was not present (Roth et al., 2008a). These observations can be utilized to formulate a hypothesis for bacterial community dynamics throughout the full cycle of a *K. brevis* bloom.

A primary hypothesis for formation of *K. brevis* blooms on the WFS involves seasonal upwelling of near-bottom water of the middle to outer WFS (Walsh et al., 2006) where localized and relatively weak upwelling aggregates *K. brevis* within

frontal zones (Walsh et al., 2003). The presence of SAR406 in surface waters

indicates such upwelling is taking place, and that the water originates from deep

offshore sources. Thus, if SAR406 is present in surface water and frontal zones are

also present along the WFS, conditions would be favorable for a *K. brevis* bloom to

initiate.

Bloom initiation would be supported by highly productive CFB bacteria

which are growing efficiently. In recent isolates of *K. brevis*, the CFB group was

~63% of the bacterial communities all cultures in logarithmic growth (GR, Chapter 6)

and a large proportion in an initiating bloom. Studies have found that metabolic

activity was dominated by a limited number of opportunistic bacteria (Massana et al.,

2001, Zubkov et al., 2001), and given that whole community bacterial production was

high, it is likely the CFB bacterial accounted for a large proportion of this production.

As the bloom enters into maintenance phase, similar to those observed in this study,

there will be relatively equal proportions of CFB and *Alphaproteobacteria*. At

MOTE, the isolates of *K. brevis* which were allowed to continue after being used to

establish a new generation in logarithmic growth saw a decrease in CFB bacteria and

an increase in *Alphaproteobacteria* as the age of the culture increased. The Wilson

culture (grown at FWRI) was observed to have a decrease in *Cryomorphaceae* (a

member of the CFB group), bacterial growth efficiency, and cell abundance between

logarithmic and ST treatments. These decreases were mirrored by increases in the

Alphaproteobacter Hyphomonadaceae, which are capable of utilizing amino acids

released by decaying *K. brevis* (Fandino et al., 2001). When *K. brevis* was removed

from a culture the proportion of *Alphaprotobacteria* increased and CFB bacteria

decreased significantly (Chapter 5). This trend would continue as the bloom shifts from senescing to termination, with *Alphaproteobacteria* becoming the dominant bacteria within the ecosystem.

Where the algicidal and antagonistic bacteria fit in could be at the transition between bloom stages. Strains S03 and 41-DBG2 are algicidal towards *K. brevis* but require threshold concentrations before beginning to exhibit algicidal activity (Roth et al., 2008b). As both are CFB bacteria, they would be expected to be a component in initiating bloom communities, and capable of reaching the required thresholds. Once algicidal activity commences, the bloom could shift to maintenance phase. If strain D28BY is present it could confer resistance against the algicidal activity to *K. brevis* allowing for blooms to prolong maintenance phase (Roth et al., 2008a). This "arms race" between algicidal and resistance-conferring bacteria can extend indefinitely, and may be why some blooms have persisted for up to a year (Vargo et al., 2004, Steidinger, 2009). If the algicidal bacteria gain the upper hand, or nutrient pools to support the bloom run out, then the bloom would begin to senesce and promote shifts in the microbial community that ultimately might eliminates the bloom and potentially some of the bloom toxins (Shimizu and Wrensford, 1993).

By developing probes for certain members of the above groups and the specific strains already identified, it may be possible to determine which stage the bloom is in based on the bacteria present. This may also lead to the ability to predict transition between bloom stages by first observing changes in the bacterial community.

Viruses are capable of disrupting bacteria-*Karenia brevis* interactions

One potential bloom control mechanism is viral lysis, as blooms represent an ideal situation for viral infection to take place (Fuhrman, 1999). A study using cultures of *K. brevis* found a filterable lytic agent from the Gulf of Mexico which lysed the cultures but could not determine if viruses were causing dinoflagellate or bacterial mortality (Paul et al., 2002). A follow up study which focused on the bacterial community found that bacteria exposed to a viral concentrate had a higher rate of nucleic acid synthesis than control treatments (filtered seawater addition) but bacterial community composition was not significantly different. This suggests that when under viral attack, the decrease in metabolic function observed in bacterial communities is detrimental to *K. brevis* growth. Since the communities were dominated by *Flavobacteriales*, a member of the CFB group, the shift away from metabolism towards cell division would disrupt any synergistic interactions between the bacteria and *K. brevis* (Amin et al., 2012).

In naturally occurring blooms, more evidence for viral control of bacteria exists. Within an initiating bloom, the abundance of viruses actually decreased and bacterial production suggested balanced growth. Bacteria are known to produce nucleases which can degrade viral particles; within initiating blooms the high concentrations of bacteria with high rates of production could release enough nucleases to provide refuge from viral infection (Ammerman and Azam, 1991, Noble and Fuhrman, 1997). The bloom in maintenance phase displayed the opposite trend: viruses were increasing in abundance, bacterial production was decreasing, and bacteria were focusing on metabolic functions, possibly due to viral takeover of cell

machinery. Lysis of portions of the bacterial community could lead to bacteria succession, which would in turn promote transition between bloom phases including bloom termination.

Viruses are the most abundant entity in the oceans (Suttle, 2005) with a majority suited for bacterial infection (Steward et al., 2000). While there are viruses capable of infecting phytoplankton (Frada et al., 2008) it is more likely that viruses will have a closer link to the most abundant hosts, bacteria (Suttle, 1994, Hewson et al., 2006). Bacterial production data support this logic and given the high possibility of bacteria-*Karenia* interactions it is more likely that viral infection disrupts such interactions, to the detriment of *K. brevis,* through bacterial lysis than direct lytic infection of *K. brevis*.

Management recommendations

There have been numerous studies into the nutrient requirements of *K. brevis* and physical conditions which help to either promote or terminate bloom formation (Walsh et al., 2006, Vargo et al., 2008). In contrast, relatively few studies have investigated the bacteria communities which co-occur with both naturally occurring blooms and experimental cultures of *K. brevis* (Sipler, 2009, Jones et al., 2010). The studies undertaken as part of this Ph.D. thesis were focused on improving the state of knowledge regarding microbes surrounding *K. brevis* blooms so that future research, whether in the field, laboratory, or numerical model studies, do not overlook this dynamic and important component of the ecosystem.

It was fortitious that during the ECOHAB: *Karenia* project blooms in both initiation and maintenance phase were sampled. The differences observed in bacterial abundance and production highlighted the importance of placing data into the context of the biologically relevant parameter of bloom phase (Chapters 2 and 3). It would be ideal if mangers could require bloom studies to determine the phase of any bloom sampled so that: 1- data and conclusions from bloom studies are compared to research on blooms in the same bloom phase, and 2- multiple sampling efforts of blooms in different bloom stages can be combined to create an overall picture of the evolution of a bloom from initiation through termination.

Sampling efforts of naturally occurring blooms should also begin to investigate the microbiome associated with blooms. Dinoflagellates are not alone in the environment so sampling should therefore not focus soley on *K. brevis*. Bacterial samples are easy to obtain and technology has improved so that the sequencing cost per sample is decreasing and sequencing output (accuracy and number of sequences) is increasing. Furthermore, computing power and data management now allow for samples sequenced on different platforms, with different primers, from different studies to be compared an infinite number of times. This would allow for a long-term dataset to be established cataloging the bacterial community of multiple blooms. This study (Chapter 4) identified the bacterial group SAR406 as a potential indicator species of physical features that support existing bloom formation hypotheses. Further studies of the *K. brevis* bloom microbiome may identify additional bacterial species which may help indicate bloom stage or toxicity.

The possibility that viruses can contribute to *K. brevis* bloom termination suggests a potential avenue for bloom management (Chapter 5). This mechanism appears to act through bacteria associated with *K. brevis* and needs to be further explored. If a phage-host pair that leads to bloom termination is identified, experiments can be designed to test the possibility of large scale use of this to reduce the impact of *K. brevis* blooms on the WFS. Managers should consider supporting viral research that is exploring the use of viruses to terminate blooms of *K. brevis*.

Using cultures of *K. brevis* in scientific research is a great way to control variables to better understand the physiology of the dinoflagellate. However, the fact that all cultures of *K. brevis* are maintained with co-occurring bacteria has been overlooked. In essence this means that cultures are not a monoculture but rather a functioning ecosystem. A study of the bacteria contained within these cultures (Chapter 6) found that bacterial communities in cultures were different from those in naturally occurring blooms and different between strains of *K. brevis* isolated at different laboratories. Insitutions with culture collections should undertake an effort to catalog the microbiome of their cultures so that researchers will not only consider the effect bacteria may be having on their results, but compare the microbiome of their cultures to that found in naturally occurring blooms. This should provide a better characterization of the physiology of *K. brevis* and its microbiome. Researchers who use cultures should consider the effect of culture microbiome on their results as they represent a microcosm of natural blooms rather than one single organism.

A better characterization of microbial interactions within *K. brevis* blooms will provide more variables for use in numerical models. Mangers should enourage

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the collaboration between scientists that are measuring these variables and modelers so that models are created from a large pool of variables and rates. This should improve the ability of models to mimic blooms which could lead to a better ability to forecast bloom termination and toxicity.

Further research is needed into dynamics of microbes surrounding *K. brevis* blooms, but this can only occur with the continued support and collaboration between managers, stakeholders, and researchers. Managers and stakeholders should inform researchers what information they need to make timely decisions regarding human health and seafood safety. Researchers should in return provide scientific information in a timely and transparent manner with all results having been quality controlled and put into an ecosystem-relevent context. All three groups should work together to educate the public at large with focuses placed on multiple age groups, demographics, and communication media. Combined, continued strides in *K. brevis* research will be made which will help to reduce the harmful effects of this bloom on the ecosystem, economy, and health of West Florida.

Future Research

Culture collections should also catalog associated microbial communities

This was the first known attempt to classify bacterial communities co-occurring with *K. brevis* in cultures maintained at different laboratories. The results show that there are not only different bacterial communities in different isolated of

the same dinoflagellate, but that laboratories also influence the makeup of the communities in their collections over time. Since *K. brevis* is known to be maintained with co-occurring bacteria at more than the two laboratories, these communities need to be cataloged so that confounding interactions can be addressed in future studies. Failure to acknowledge or study these communities in previous studies may have already led to erroneous conclusions regarding the ecology and life history of *K. brevis* in culture as well as those making inferences about naturally occurring blooms. Given the differences observed between isolates from just two laboratories emphasizes the need to monitor bacterial communities in concert with other culture and experimental variables so that small-scale changes in the bacterial community are not overlooked (Gasol et al., 2005). Ideally, culturing facilities would catalog and monitor the bacterial community within their cultures so that in addition to information regarding the phytoplankton isolation and culturing there would be a bacterial community profile from which to compare and differences observed during experimentation.

Determine which members of the *Cytophaga-Flavobacterium-Bacteroides* interact with *Karenia brevis* and in what manner

The prominence of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex in the bacterial communities from all *K. brevis* studies suggests significant interactions exist between members of the CFB complex and *K. brevis*. Some of these interactions have been investigated and shown to include parasitic (algicidal) and synergistic (algicidal resistance) relationships (Roth et al., 2008a). Given the wide

variety of potential interactions and the dominance of this group when *K. brevis* is healthy, this bacterial group is a great place to start looking for other bacteria-*Karenia* interactions. Within a bloom *K. brevis'* sphere of influence would extend throughout the bloom area, providing a large area for exchange of materials between bacteria and the dinoflagellate (Amin et al., 2012). It has been shown that CFB bacteria have a preference for organic polymers (Suzuki et al., 2001) and it is likely that *Karenia* would need vitamins or trace elements in otherwise short supply on the oligotrophic West Florida Shelf (Fandino et al., 2001, Amin et al., 2012).

The metabolic diversity of bacteria make it difficult to think that interactions between CFB bacteria and *K. brevis* are limited to parasitic and parasite-resistance. Other relationships likely exist and the repeated dominance of the CFB community indicates these bacteria are a great place to start looking. Methods exists which are able to identify, classify, and quantify bacteria-algae interactions but in many cases such methods are "fishing expeditions" unless they have previously identified places to being searching so that investigations are productive. It is proposed that the CFB bacteria associated with *K. brevis* blooms and cultures be such a starting place for future investigations and classification of interactions between this harmful dinoflagellate and bacteria.

Investigate how viruses disrupt interactions between bacteria and *Karenia brevis*

Data indicate that viruses are most likely affecting bacterial communities within *K. brevis* blooms and not directly attacking the dinoflagellate. What remains to

be discerned is how viral lysis of bacteria leads to termination of *K. brevis*. Are beneficial bacteria being lost causing a critical nutrient or vitamin limitation to the dinoflagellate? Is lysis of bacteria releasing algicidal compounds which then affect *K. brevis* cellular integrity or growth? Are viruses altering the bacterial community structure so that bacteria which compete with *K. brevis* for resources become dominant? Support for the hypothesis that viruses were acting upon bacteria and not *K. brevis* has raised more questions than it answered. The answers however, will provide better details about the nature of viral infection on the WFS as well as interactions between *K. brevis* and associated bacteria. Methods need to be carefully considered for future studies as several insufficient methods have already failed to produce meaningful results. Viruses may be able to provide a means of bloom control if the mechanism by which viral infection leads to bloom termination is identified and fully understood.

Microbial interactions may be a key in understanding the ecology of *Karenia brevis* blooms

The dinoflagellate *Karenia brevis* is capable of significant ecological and economic impacts in Florida waters where blooms typically occur. Blooms and cultures of *K. brevis* were sampled to determine the composition, production, and possible ecological function of bacteria and virus communities associated with *K. brevis*. Bacterial communities on the WFS were similar inside and outside *K. brevis*

blooms, but primary and secondary (bacterial) production and bacteria and virus

abundances were different depending on bloom stage. Bloom stages need to be

identified so that discrete sampling events of different bloom stages can be combined

to characterize an entire bloom event from initiation through termination and the

return to a non-bloom community. Within an initiating bloom bacterial production

and mortality was high and viral abundance was low, suggesting that viral genomes

were either within host cells or bacterial mortality was due to mixotrophic grazing by

K. brevis. In a maintenance phase bloom the bacterial community was metabolically

stressed, subject to increased viral infection, and not subjected to mixotrophic

grazing. Bacterial communities associated with healthy *K. brevis* were dominated by

the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex; as *K. brevis* shifted to

stationary or senescing growth communities had higher proportions of

Alphaproteobacteria.

The SAR406 group, typically found in deep waters, was present in the surface

waters of the WFS which supports existing *K. brevis* bloom formation hypotheses

involving upwelling of deep waters from the mid to outer shelf. The CFB complex of

bacteria also need to be further investigated as they appear to be capable of numerous

interactions with *K. brevis*. Such interactions may be a vector of bloom control

through viral infection. Inoculating cultures of *K. brevis*, including associated

bacteria, with viral concentrates from the WFS showed differences in bacterial

production and growth which indicate viruses are acting upon the bacterial

community and not the dinoflagellates. Interactions between bacteria and *K. brevis*

need to be further elucidated and explored for a better understanding of the role of

each in dynamics of this harmful algal species. There may be a natural community succession amongst bacteria during blooms. Utilizing certain indicator species to indicate bloom stage and transition between stages may aid in bloom forecasting and detection efforts.

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