MOLECULAR METHODS SURVEY OF HARMFUL ALGAE IN SHIPS' BALLAST WATER

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Molecular Methods Survey of Harmful Algae in Ships' Ballast Water. (May 2014)

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Harmful algal blooms (HAB) are increasing in frequency and diversity in the Gulf of Mexico and along the Texas coast. In 2016, the U.S. EPA and U.S. Coast Guard will require monitoring of ballast water discharged from all foreign ships calling at ports U.S. territorial waters. Archived samples from ships' ballast water and Texas bays and ports were screened with polymerase chain reaction (PCR) assays specific for several species causing harmful algal blooms. Previous analysis of these archived samples has detected 13 different genera and species of harmful algae known to be transported via ships' ballast water. Thus far, *Karenia brevis*, *Pfiesteria piscicida*, and *Microcystis* spp. have been detected thus far in upper Galveston Bay and the Port of Texas City and in the Upper Laguna Madre and the Port of Brownsville. *P. piscicida* has been detected in 7 out of 14 ballast water samples and *Alexandrium* spp. in 9 samples from ships calling at the Port of Houston, the 2nd busiest port in the U.S. Hypotheses to be tested by this study:

H_1: Higher concentrations of HABs will be present in ballast water that is less than 10 days old, i.e. taken up or exchanged less than 10 days prior to discharge at the Port of Houston.

Objective 1: To determine if age of ballast water influences the detectability of HABs with quantitative PCR.

H2: Ports and bays receiving ballast water discharged from ships that have not conducted a ballast water exchange in the open ocean, i.e. containing 'coastal water' will contain a greater number or a higher diversity of potential HAB species.

Objective 2: To use PCR assays specific for different species of HABs to determine if the potential diversity of HABs in Texas port and bay waters can be linked to sources of ships' ballast water.

NOMENCLATURE

HAB	Harmful Algal Bloom
BWE	Ballast Water Exchange
PSP	Paralytic Shellfish Poisoning
ASP	Amnesic Shellfish Poisoning
DSP	Diarrhetic Shellfish Poisoning
SST	Sea Surface Temperature
PCR	Polymerase Chain Reaction

CHAPTER I

INTRODUCTION

The production of harmful algal blooms by harmful algae (HABs) affects coastal economies, public health, and biodiversity of marine life (Lewitus et. al, 2012). Harmful algae have released toxins of which accumulate in several marine fishes, mammals and also invertebrates (Anderson, 2009). While these fauna can potentially become seafood for coastal communities, human populations are at risk of suffering from paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), and toxic aerosols brought to land from the coast (Lewitus et. al, 2012). The increasing presence of these HAB events can reduce toxin-free resources such as seafood, and prelude the marine fisheries to economical vulnerability. For coastal ecosystems, these HAB events can cause oxygen depletion, alterations in nutrient chemistry, and loss of biodiversity of marine life (Anderson, 2009).

In past decades, HABs have affected every coastal community in the world (Anderson, 2009). Natural disasters such as hurricanes along with changes in sea surface temperature (SST) contribute to the prolonged occurrence of HAB events. However, increased anthropogenic activities have triggered the appearance of blooms exponentially (Anderson, 2009). Coastal eutrophication due to effluent substances from land are adding to ecosystems continuously, as well as increased transportation of ships' ballast water from port to port (Anderson, 2009). The combination of larger ballast tanks, accelerated international commerce, and shorter transit times contribute to the increasing rate of successful HAB invasions in coastal bays and estuaries (Cohen and Carlton, 1998). Several HAB species are increasing in frequency and global

distribution and it is estimated that while 300 species can discolor aqueous environments, 90 are considered harmful due to the production of toxins (Bowers et al., 2000). Some of the algae leading these HAB invasions are *Karenia brevis, Microcystis spp., Alexandrium spp.,Dinophysis spp.* and *Pfiesteria piscicida*. All of these organisms are dinoflagellates except for the genus *Microcystis*.

The genus *Alexandrium* is a dinoflagellate known to cause PSP, and the toxin responsible for this poisoning is saxitoxin. PSP inhibits neuromuscular functions and has been reported to cause paralysis, headaches, fever, dizziness, and a range of neurological effects (Bowers et al., 2000). PSP is considered a global threat as the toxins released by this method are capable of navigating throughout any coastal ecosystem and affecting numerous trophic levels (Anderson et al., 1994). This genus has been a threat on the east coast of the U.S. estuaries, Canada, and also European waters along Spain and the UK (Anderson et al., 1994).

Dinophysis spp. is a dinoflagellate that produces the toxin okadaic acid and pectenotoxin groups, and ultimately causes DSP (Fux et al., 2011). Accumulation of these toxins can occur in marine life and DSP has the potential to spread throughout fishing industries and ultimately to coastal communities. With a recent bloom in 2008 on the Texas coast of *Dinophysis spp.* and the closure of shellfish harvesting in this region, this species is considered high-risk as pertaining to public health (Fux et al., 2011).

K. brevis produces brevetoxins and has caused mass fish kills, along with accumulation in tissues of other marine life. This species produces blooms annually on the west coast of Florida and

continuously impacts ecosystems and coastal communities (Tomlinson et al., 2004). These blooms have occurred since the 1980's in the Gulf of Mexico, extending from Texas to Florida (Tomlinson et al., 2004). The magnitude of these blooms have prompted the use of a forecast system within the western region of Florida, and currently test for *K. brevis* cell concentrations daily (Tomlinson et al., 2004).

P. piscicida is another dinoflagellate that has been largely detected in the Atlantic Ocean, causing major fish kills in 1997 in the Pocomoke, Manokin, and Chicamacomico rivers in Maryland (Bowers et al., 2000). Exposed individuals have experienced burning skin, memory loss, headache, etc., and due to the extremities of this species a monitoring program is active in the Atlantic coast (Bowers et al., 2000). Maryland, Virginia and North Carolina are a few recognized states of this program, and bimonthly samples are collected for enumeration of *Pfiesteria spp.* (Bowers et al., 2000). In the years before 1998, *Pfiesteria spp.* presence was reported in the Gulf of Mexico, and a study in 2001 detected this species in Texas for the first time (Tengs, 2001). Therefore, the known range of occurrence currently extends beyond the east coast. This species has caused high controversy in the scientific community due to its complex life cycle, nutritional modes, and interactions among several ecosystems (Rublee et al., 2005). Despite the difficulty in culturing this dinoflagellate, and often detection, efforts have been made to raise awareness involving the seafood industry and public health (Rublee et al., 2005). It has been suggested that one-tenth of environmental samples in the U.S. contain the genus *Pfiesteria,* however the life cycle stage of this organism dictates the repercussions it can release into an ecosystem at a given time (Tengs, 2001).

Microcystis spp. is a blue-green algae producing cyanotoxins, and an example is microcystin which causes liver damage. Other effects on humans include pneumonia, nausea, headaches, diarrhea, vomiting and fever. Beginning in the mid-1990's, blooms of Microcystis have occurred in Lake Erie and is considered a cosmopolitan species and produces microcystin, a cyanobacterial toxin (Rinta-Kanto et al., 2005). The cyanobacterial blooms produced by this genus causes problems in water quality and can directly affect aquatic life and humans (Rinta-Kanto et al., 2005). Factors influencing these blooms include high quantities of nutrients, increased populations of coastal communities, and fluctuations in the concentration of carbon, nitrate, chlorophyll *a*, and dissolved phosphorus (Rinta-Kanto et al., 2005). To date, satellite images, toxin levels, chlorophyll sampling and quantitative PCR have been efficient methods in detecting this genus in Lake Erie specifically (Rinta-Kanto et al., 2005).

In 2005, the Coastal Health Lab (PI Brinkmeyer) and the Phytoplankton Ecology Lab (PI Quigg) initiated a long-term collaborative study to examine introduction of HABs into Galveston Bay via ships' ballast water. Thus far, only qualitative analyses have been used to determine the diversity of HAB species in ballast water samples collected from ships calling at the Port of Houston. Analysis of 18S rRNA from 13 ballast water samples from vessels that took on ballast water at locations across the North Atlantic Ocean from the Port of Malboa, West African coast to the Port of Houston, Texas (Table 1) have detected at least 13 different HAB species within the dinoflagellate genera *Alexandrium, Exuviella, Gyrodinium, Heterocapsa, Karlodinium, and Pfiesteria* and *Scrippsiella Actinocyclus, Ditylum, Nitzschia, Stephanopyxis* and *Thalassiosirales* (Steichen et al. *in review*).

This study will use existing quantitative PCR assays to enumerate HABs in archived ballast water and Texas bays and ports samples available from the Brinkmeyer lab. Assays will target HAB species detected previously in the same ballast water and Galveston Bay samples (Steichen et al., 2012; Steichen et al. *in review*) as well as species known to occur at many of the global ports of departure.

CHAPTER II

METHODS

Frozen DNA extracted from ballast water in ships calling at the Port of Houston in 2007 and 2008 and from water collected at several Texas ports and bays , in 2007 (Figure 1), were screened with polymerase chain reaction (PCR) assays for HAB genera and species. Traditional PCR assays for *Pfiesteria piscicida*, *Dinophysis* spp., *Karenia brevis*, *Microcystis* spp., and *Alexandrium* spp. were conducted to pre-screen samples prior to analysis with quantitative PCR (Table 1). *In Silico* specificity of primer pairs was confirmed using the Blastn tool of GenBank.

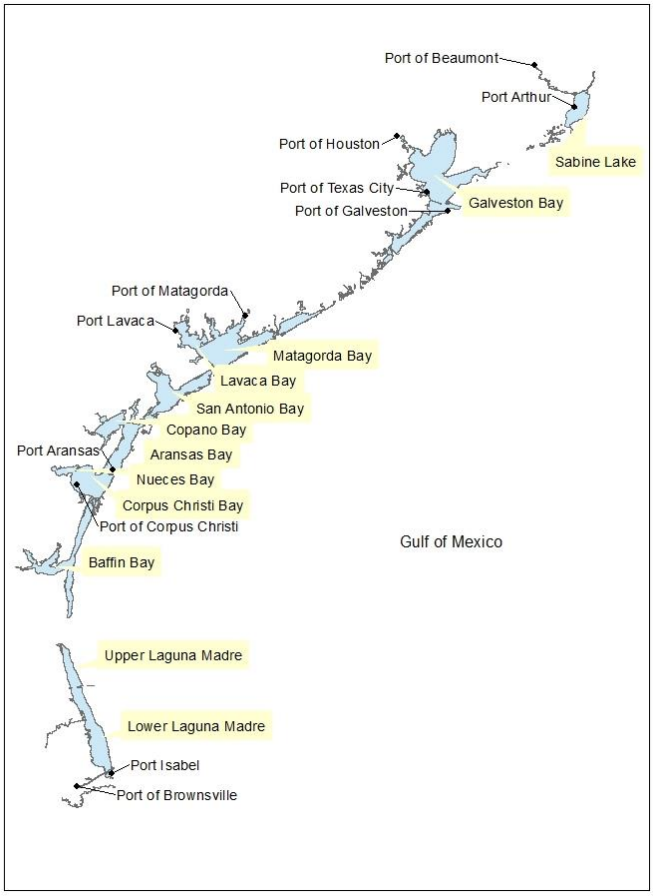

Figure 1: Map of Texas bays and ports

Table 1. Quantitative PCR primers for HAB Assays used in this study

Target Species	Primer	5' to 3' Sequence	Gene	Reference
Alexandrium spp.	AlexF AlexR	YGATGAAGAATGCAGCAAMATG CAAGCAHACCTTCAAGMATATCC	5.8S rRNA	Galluzzi et al. 2004
Karenia brevis	KbrevF KbrevR	TGAAACGTTATTGGGTCTGT AGGTACACACTTTCGTAAACTA	28S rRNA	Gray et al. 2003
Pfiesteria piscicida	PfiestF PfiestR	CAGTTAGATTGTCTTTGGTGGTCAA TACCATATCACTTTCTGACCTATCA	18S rRNA	Bowers et al. 2000
Dinophysis spp.	DIN_F DIN_R	ATGAGGGAAAGGTGAAAA CTTACGCACAAGCATAAC	28S rRNA	Kavanagh et al. 2010
Mycrocystis spp.	MIC16SF MIC16SR	AAAGCGTGCTACTGGGCTGTA CCCTTTCGCTCCCCTAGCT	16S rRNA	Baxa et al. 2010

Prior to PCR, DNA concentration and quality was determined with a spectrophotometer (ND 1000; Nanodrop Corp). The PCR reaction volumes were 20 µl and contained 0.5 µM each primer pair, X dNTPs, 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.05 × BSA, and 2-4 µl DNA. The thermocycler conditions were as follows: 95℃ denaturation for 10 minutes, then 40 to 50 cycles of 95℃ denaturation for 50 sec, annealing temperatures of x to 60℃ (according to assay) for 20 sec, and elongation at 72℃ for 10 sec using a Mastercycler Pro S (Eppendorf). PCR amplification products were checked on 3% agarose gels run in 1 × Tris-Acetic Acid-EDTA buffer at 100 V for 30 minutes to 1 hour.

PCR amplification products are currently undergoing DNA sequencing to confirm specificity of the assay targets. Samples testing positive for HABs have also been amplified with genus and species specific PCR primers for the 18S rRNA gene and are undergoing DNA sequencing for a second confirmation of the assays.

For quantitative PCR standard curves, assay amplification products were cleaned with a QiaQuick PCR purification kit (Qiagen) and ligated into pGEM plasmids according to

manufacturer instructions (pGEM-T Easy Vector System, Promega). Plasmids with inserts were used to create a ten-fold dilution series for the standard curve. HAB cells were enumerated on a Smartcycler (Cepheid) using the intercalating dye, SYBR Green (IQ Sybr Green Supermix; BioRad). Sybr green is intercalated into PCR amplicons with each cycle and Smartcycler measures the progressive increase in fluorescence from the dye.

CHAPTER III

RESULTS

Initial Screening with traditional PCR

Thirty-three samples from Texas bays and ports were screened for HAB species using genus or species specific PCR assays. The dinoflagellates *P. piscicida* and *K. brevis* were detected in water collected at the Port of Texas City, in Galveston Bay. *P. piscicida* was also detected in upper Galveston Bay. Water collected at the Port of Brownsville as well as the Upper Laguna Madre also contained *P. piscicida* as well as *Microcystis* spp. (Table 2).

P. piscicida was detected in 7 out of 14 ballast water samples. *Alexandrium* spp. was detected in 9 of the ballast water samples and dinophysis in only one sample. (Table 3). *K. brevis* and *Microcystis* spp. were not detected in any of the ballast water samples.

Table 2. HABs detected in Texas Bays and Ports

Bay or Port	*Pfiesteria piscicda*	*Dinophysis* spp.	*Karenia brevis*	*Microcystis* spp.	*Alexandrium* spp.
Sabine Lake					
Galveston Bay North	X				
Galveston Bay South					
Matagorda Bay					
Lavaca Bay					
Copano Bay					
Nueces Bay					
Aransas Bay					
Corpus Christi Bay					
Baffin Bay					
Upper Laguna Madre	X			X	
Lower Laguna Madre					
Port Arthur					
Port of Beaumont					
Port of Galveston					
Port of Texas City	X		X		
Port of Houston					
Port of Lavaca					
Port of Corpus Christi					
Port of Matagorda					
Port Isabel					
Port of Brownsville	X			X	

Table 3. HABs detected in Ships Ballast Water

Ship Name Abbreviation	Ballast Water Source	*Ballast Water Age (d)*	*Pfiesteria piscicda*	*Dinophysis* spp.	*Karenia brevis*	*Microcystis* spp.	*Alexandrium* spp.
1. Gab	Coast of Angola						X
2. Tex	S. Coast of Cuba	2	X				X
3. Ang	Mid-south Atlantic	5					
4. Cong	W. Coast of Brazil	8	X	X			X
5. Rot	Mid-south Atlantic	5	X				X
6. Med	W. Coast of Morocco	10	X				X
7. Cob	Caribbean Sea	2					X
8. Euro	Mid-south Atlantic	5					
9. Hou	S. Coast of Cuba	2	X				
10. Bold	Pensacola, Fl	1					X
11. Assm	Black Sea	15	X				X
12. Rod	Mid-south Atlantic	5	X				X
13. Pac Cel	North of Fiji	15					
14. Cyp	Mid-south Atlantic	5					

Examples of gels indicating the positive presence of DNA in the PCR products:

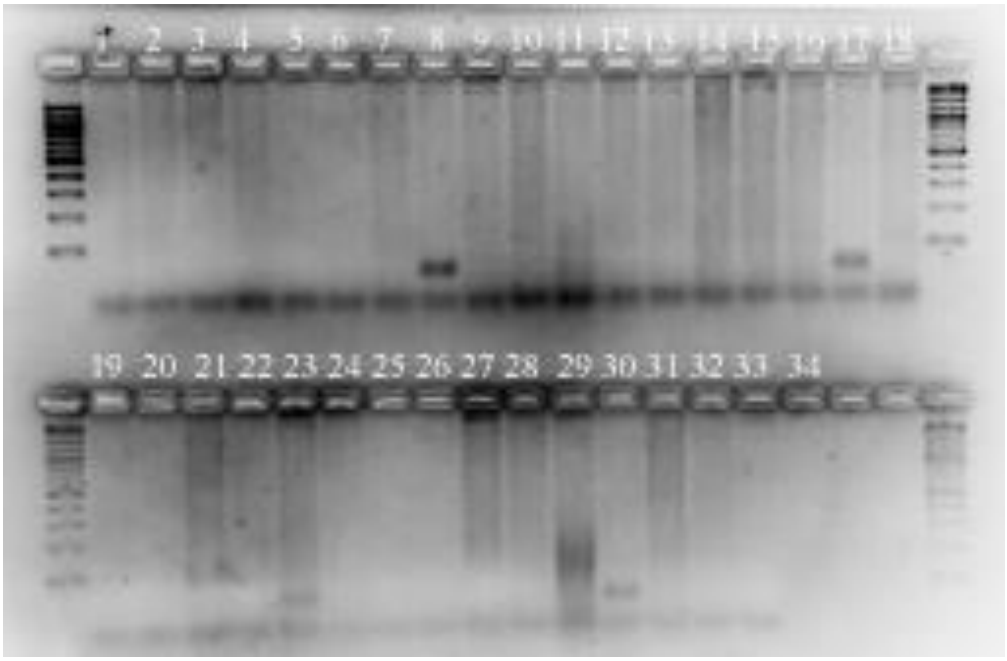

Figure 2: Ports & bays sample amplicons for *Pfiesteria piscicda* PCR assay.

Bands less than 200 bp appear in this gel in wells 8, 17, 23, and 30.

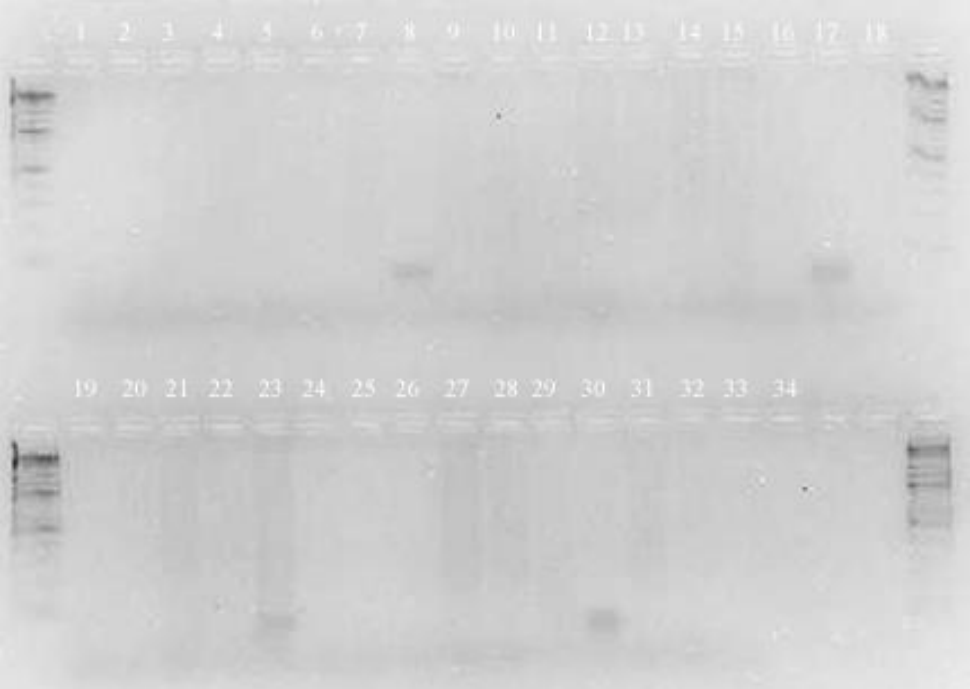

Figure 3: Ports & bays sample amplicons for *Microcystis* spp. PCR assay.

Positive bands of less than 200 bp appear in Wells 8, 17, 23, and 30.

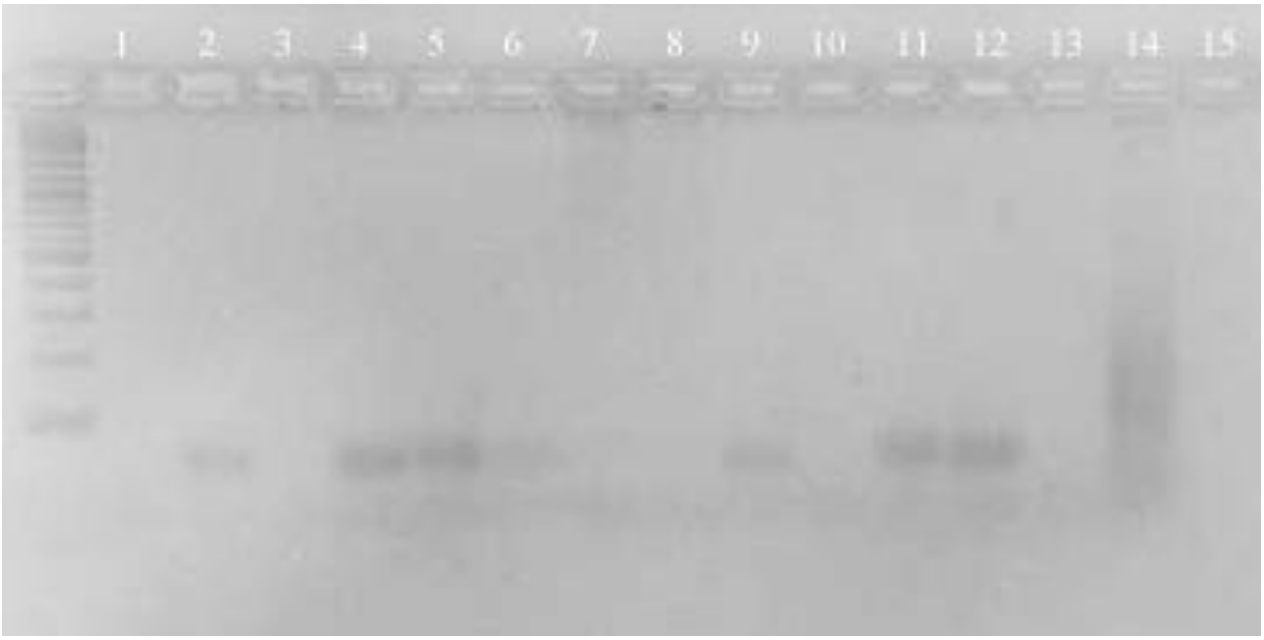

Figure 4: Ballast water sample amplicons for *Pfiesteria piscicda* PCR assay.

Bands of less than 200 bp appear in the wells 2, 4, 5, 6, 9, 11, and 12 for a total of 7.

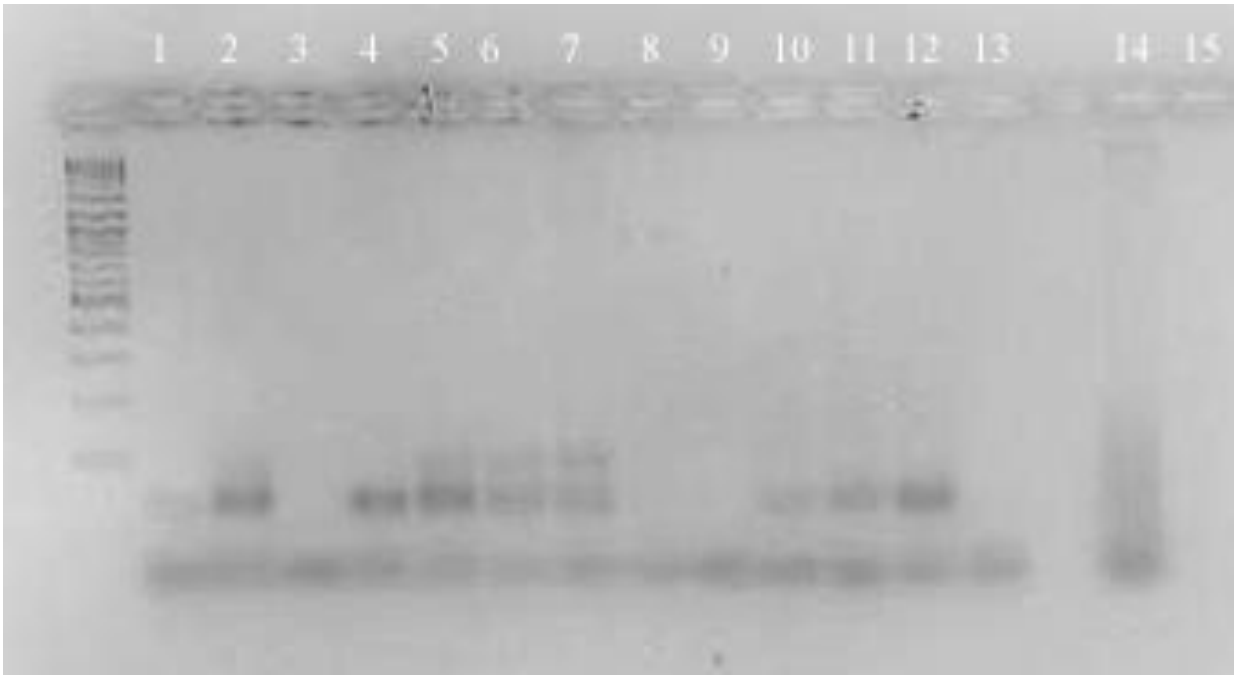

Figure 5: Ballast water sample amplicons for *Alexandrium* spp. PCR assay.

Bands of less than 200 bp appear in wells 2, 4, 5, 6, 7, 10, 11, 12, and possibly 14 for a total of 9.

Quantitative PCR

Quantitative PCR (qPCR) assays were conducted with ballast water samples testing positive for HABs with traditional PCR screening. Concentrations of *Pfiesteria piscicida* ranged from 0.26 to 0.31 cells/ml (Table 4).

Table 4. Cell counts for *Pfiesteria piscicida* in ships' ballast water.

Ship Name Abbreviation	Cell count/ml
Tex	0.29
Cong	0.31
Rot	0.29
Med	0.29
Hou	0.30
FJ2	0.26
FJ6	0.27

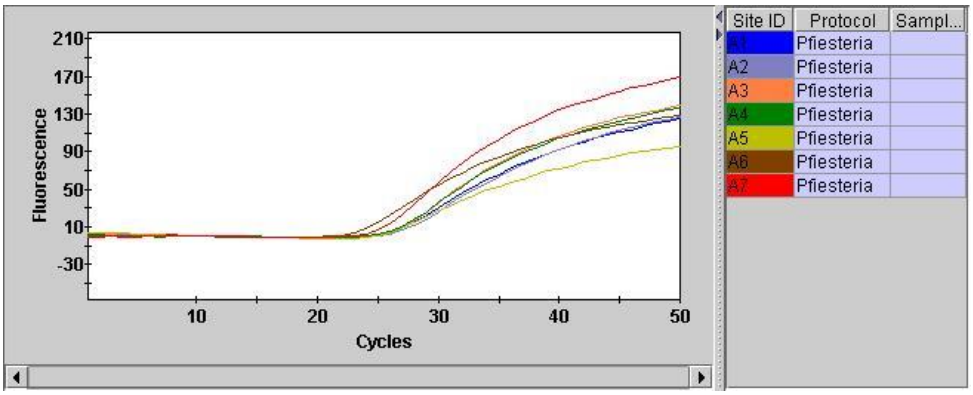

Figure 6. Amplification curves for ballast water samples for the *Pfiesteria piscicida* qPCR assay.

Concentrations of *Alexandrium* ranged from 0.28 to 0.35 cells/ml. Only one sample tested positive for presence of *Dinophysis* spp. with traditional PCR screening. Enumeration with qPCR determined that it contained 0.5 cells/ml (Table 6).

Table 5. Cell counts for *Alexandrium* spp. in ships' ballast water.

Ship Name Abbreviation	Cell count/ml
Rot	0.33
Med	0.35
Berg	0.28

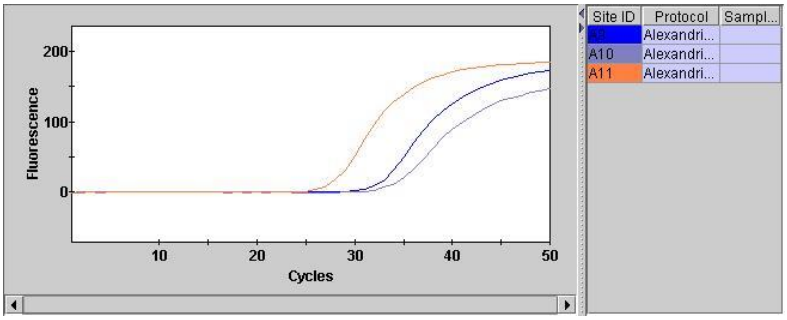

Figure 7. Amplification curves for ballast water samples for the *Alexandriu*m spp. qPCR assay.

Table 6. Cell counts for *Dinophysis* spp. in ships' ballast water.

Ship Name Abbreviation	Cell count/ml
Cong	0.5

CHAPTER IV

DISCUSSION

This study is still ongoing, however the detection thus far of 4 HAB genera/species with PCR assays is promising as a tool for rapid screening of bay and ships' ballast water samples.

HABs in Bays and Ports

Only two HAB species *Pfiesteria piscicida* and *K. brevis* were detected with traditional PCR at the Port of Texas City and in Galveston Bay. This is surprising since there are several busy ports on the bay, including the Port of Houston. The Port of Houston is the busiest port in the U.S. in terms of foreign tonnage (~5 $\times 10^5$ t yr^{-1}), the second-busiest in terms of overall tonnage (~7 $\times 10^5$ t yr^{-1}), and the tenth busiest in the world. Each year >8,000 vessels call at the Port of Houston (www.portofhouston.com).

P. piscicida and *Microcystis* spp. were detected in the Upper Laguna Madre as well as at the nearby Port of Brownsville. These HAB species are thought to be more likely to occur in bays or estuaries with low water residence times. The Laguna Madre has a water residence time of approximately 1 year. The Laguna Madre has already experienced a "brown tide," caused *Aureoumbra lagunensis*, that is a persistent and recurring HAB in the Laguna Madre. Detection of *Microcystis* spp. in bay and port waters may be an indicator of eutrophication processes. *Microcystis* is usually found in freshwater lakes but can also occur in estuaries, as seen in (Table 4) along the east coast of the US.

Interestingly, *Dinophysis* spp. was not detected in Aransas Bay or in Port Aransas in 2007 samples. In 2008, an extensive *Dinophysis* spp. bloom occurred in that area and was thought to have been caused by discharge of ship ballast water collected from overseas (Table 4). However, in March 2014, the oyster waters were closed to harvesting due to a *Dinophysis* spp. bloom. To my knowledge, *Dinophysis* has not previously been detected in Galveston Bay.

Historically, *K. brevis* has occurred along the entire Texas coast, though it is highly present in the north and southwest coastal waters of Florida (Table 4). The implications of *K.brevis* spreading throughout the Gulf of Mexico is alarming as this species affects fishing industries, bioaccumulates in marine life, and produces harmful toxins and aerosols. *Alexandrium monilata* blooms have occurred infrequently along the Texas coast since the early 1930's. In this study, no *Alexandrium* spp. were detected with PCR.

HABs in ships' ballast water

The predominant HAB species detected in ballast water were *P. piscicida* and *Alexandrium* spp. *P. piscicida* was detected in 7 out of 14 samples at concentrations of 0.26 to 0.31 cells/ml. *Alexandrium* spp. was detected in 9 out of 14 samples at similar concentrations of 0.28 to 0.35 cells/ml. And *Dinophysis* spp. was detected in one sample (0.5 cells/ml). These concentrations may seem to be low, however when one considers that ships carry millions of liters of ballast water, the cell counts can potentially be in the millions.

With the occurrence of *Alexandrium* along the east coast of the U.S., in due time it is likely this algae could be carried further around the world due to ship transport. As mentioned before, this

genus produces the harmful saxitoxins and is responsible for several neurological effects on humans and coastal communities.

All the ballast water samples analyzed by this study were supposedly taken on in the open ocean where there should be very few algae or other organisms. However, the high number of samples testing positive for HABs indicates that the practice of ballast water exchange to rid ballast tanks of coastal organisms is not efficient. The history of a ship's movements undoubtedly influences the organisms taken up in ballast water. For example, *Pfiesteria piscicida* was detected in vessel 'FJ6,'a 7 ton container cargo ship. According to the ship's records, ballast water was taken on in the mid South Atlantic. Prior to docking at the Port of Houston, this ship called at ports in Mexico, Belgium, the Netherlands, United Kingdom, and France (Table X). *Pfiesteria* spp. has been previously detected in ships arriving at the Chesapeake from Belgium, The Netherlands, and the United Kingdom (Doblin et al. 2002).

I was not able to prove or disprove my hypotheses with this study. Analysis of more samples is needed to determine if the age of ballast water influences the PCR detection of HABs. However, based upon my results thus far, it appears that ballast water that is less than 10 days old may contain a higher number or diversity of HABS. One complication is that dinoflagellates form cysts that allow them to survive adverse conditions and the age of ballast water may not matter in this case.

Table 7: Occurrences of *P. piscicida*, *Dinophysis spp.*, *K. brevis*, *Microcystis spp.*, and *Alexandrium spp.* in US and foreign waters

Location	*P. piscicida*	*Dinophysis* spp.	*K. brevis*	*Microcystis* spp.	*Alexandrium* spp.	Reference
Chesapeake Bay, MD	**X**					Bowers et al., 2000
Albermarle-Pamlico Sound, NC	**X**					Bowers et al., 2000
Flanners Beach, NC	**X**					Tengs, 2001
Carolina Pines, NC	**X**					Tengs, 2001
Oslofjord, Norway	**X**					Tengs, 2002
Gulf of St Lawrence, Canada		**X**			**X**	Cembella, 1989, Anderson, 1997
Catalonia, Spain		**X**			**X**	Vila et al., 2001
Port Aransas, Texas		**X**				Campbell et al., 2009
Eel Pond, Woods Hole, MA		**X**				Fux et al., 2011
Bay of Fundy, MA		**X**			**X**	Fux et al., 2011, Anderson, 1997
Reloncavi Estuary, Chile		**X**				Fux et al., 2011
SW Florida			**X**			Hu et al., 2005
Tampa Bay, Florida			**X**			Stumpf et al., 2003
Charlotte Harbor, Florida			**X**			Stumpf et al., 2003
Lake Erie				**X**		Rinta-Kanto et al., 2005
Southern Finland				**X**		Rantala et al., 2006
Lake Ontario				**X**		Hotto et al., 2007
San Francisco Bay estuary				**X**		Lehman et al., 2004
Huron River, Michigan				**X**		Lehman, 2007
Lake Oubeira, Algeria				**X**		Nasri et al., 2004
Cape Cod, MA					**X**	Anderson et al., 1994
Long Island, NY					**X**	Anderson et al., 1994
Gulf of Maine, NH					**X**	Anderson et al., 1994
Harbour Grace, New Foundland					**X**	Anderson et al., 1994
Killary Harbour, Ireland		**X**				Kavanagh et al., 2010
Bantry Bay, Ireland		**X**				Kavanagh et al., 2010
Helgoland, North Sea		**X**				Luckas et al., 2005

Table 8. Ports of Call for Container ship 'FJ6.'

Port of Call	Country	Arrival	Departure	Hours in Port
Houston	USA	5/10/07	5/24/07	337
Galveston Approach Anchorage	USA	2/18/07	2/25/07	180
Houston	USA	2/11/07	2/18/07	166
Galveston Approach Anchorage	USA	2/11/07	2/11/07	<2
Veracruz	Mexico	2/7/07	2/9/07	39
Veracruz Anchorage	Mexico	2/7/07	2/7/07	4
Galveston Approach Anchorage	USA	2/5/07	2/5/07	<2
Houston	USA	2/2/07	2/5/07	81
Galveston Approach Anchorage	USA	12/3/06	12/7/06	102
Houston	USA	11/23/06	12/3/06	223
Galveston Approach Anchorage	USA	11/23/06	11/23/06	<2
Galveston Approach Anchorage	USA	9/14/06	9/18/06	95
Houston	USA	9/1/06	9/14/06	319
Galveston Approach Anchorage	USA	9/1/06	9/1/06	<2
Houston	USA	6/16/06	6/29/06	299
Galveston Approach Anchorage	USA	6/16/06	6/16/06	<2
Houston	USA	3/30/06	4/10/06	280
Galveston Approach Anchorage	USA	3/29/06	3/29/06	9
Houston	USA	1/10/06	1/20/06	248
Galveston Approach Anchorage	USA	1/10/06	1/10/06	2
Antwerp	Belgium	11/12/05	11/15/05	83
Aberdeen (United Kingdom)	UK	11/8/05	11/10/05	49
Newhaven	UK	9/29/05	9/29/05	2
Terneuzen	Netherlands	9/29/05	9/29/05	<2
Antwerp	Belgium	9/25/05	9/29/05	83
Terneuzen	Netherlands	9/25/05	9/25/05	<2
Rotterdam	Netherlands	9/20/05	9/22/05	49
Rotterdam	Netherlands	9/19/05	9/20/05	31
Vlaardingen	Netherlands	9/19/05	9/19/05	2
Antwerp	Belgium	8/7/05	8/11/05	90
Antwerp	Belgium	8/7/05	8/7/05	<2
Aberdeen (United Kingdom)	UK	8/4/05	8/6/05	35
Antwerp	Belgium	6/7/05	6/9/05	52
Aberdeen (United Kingdom)	UK	6/4/05	6/5/05	35
Le Havre No 1 Anchorage	France	5/27/05	5/27/05	<2
Galveston Approach Anchorage	USA	4/2/05	5/27/05	1321
Galveston Approach Anchorage	USA	1/3/05	1/6/05	55

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