BACTERIA IN BALLAST WATER: THE SHIPPING INDUSTRY'S CONTRIBUTIONS TO THE TRANSPORT AND DISTRIBUTION OF MICROBIAL SPECIES IN TEXAS

A Thesis

by

ELIZABETH B. NEYLAND

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2009

Major Subject: Biology

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Approved by:

Co-Chairs of Committee,	Robin Brinkmeyer
	Susan Golden
Committee Members,	Michael Benedik
	Antonietta Quigg
Head of Department,	Uel J. McMahan

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ABSTRACT

Bacteria in Ballast Water: The Shipping Industry's Contributions to the Transport and Distribution of Microbial Species in Texas. (August 2009)

Elizabeth B. Neyland, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Robin Brinkmeyer
Dr. Susan Golden

The transportation of organisms in the ballast water of cargo ships has been recognized as a source of invasive species despite current control measures. Pathogenic bacteria in the ballast tank have been studied but the total diversity of the ballast tank bacterial community has not been examined. This study is the first to characterize the total bacterial community within a ballast tank by constructing a clone library from a ballast water sample from a cargo ship in the Port of Houston, amplified ribosomal rDNA restriction analysis (ARDRA) and phylogenetic analysis. Bacterial communities in Texas ports and bays were also examined using denaturing gradient gel electrophoresis (DGGE), looking at both temporal and spatial variations for effects of deballasting activity.

This ballast tank bacterial community had a high level of diversity (95%) with the clone library only representing 40% of the total community of the tank. Most probable originating habitats of the ballast bacteria were: marine pelagic (40%), estuarine (37%), coastal (6%), freshwater (3%) and other (14%), even though this ballast

tank was exchanged with pelagic water. Predominate groups were alpha- and gammaproteobacteria, a few betaproteobacteria and bacteriodetes, and one each of verrucomicrobia, planctomycetes and actinobacteria, but no pathogens were detected. The data reveals a ballast tank that consists of half marine-pelagic, half port bacteria, revealing a low efficacy of exchange control methods and potentially invasive bacteria.

The bacterial communities of five ships that exchanged ballast water in the Pacific Ocean shared on average 50% similarity. Two ships that exchanged ballast water in temperate latitudes were more similar than three other ships that exchanged in tropical latitudes, showing a correlation between location of exchange and community similarity.

The bacterial communities of the Ports of Houston and Galveston exhibit stable, seasonal successions over one year. The port and bay systems of Texas exhibited spatial variations in bacterial communities related to salinity levels. Both experiments did not show evidence of community disruption by deballasting activities. This study shows that ballast water is a viable vector for invasive bacterial transport, although impact on Texas estuarine systems seems minimal.

DEDICATION

To my dad, mom, and brother, Mathew: Thank you for your support, encouragement and love throughout this process.

To Jeremiah: I couldn't have made it through this without you!

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A very special thank you to the shipping agent in the Port of Houston who collected ballast water samples for us, and without whom none of the ballast work in the project could have been done.

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NOMENCLATURE

BWE	Ballast Water Exchange
IMO	International Maritime Organization
PCR	Polymerase Chain Reaction
ARDRA	Amplified Ribosomal rDNA Restriction Analysis
DGGE	Denaturing Gradient Gel Electrophoresis

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CHAPTER I

INTRODUCTION

Introduction

Usage of Ballast Water

The use of ballast water in the shipping industry began in the 1870's (Gollasch et al., 2000; National Research Council, 1996). Ballast is "any solid or liquid, including sediment, placed in a ship to increase the draft, to change the trim, to regulate the stability, or to maintain stress loads within acceptable limits" (National Research Council, 1996). During a ship's voyage, ballast is used to compensate for cargo loss. Since the need is quite variable and can be weather dependent, ships use water as ballast material to avoid the dangers and difficulties of solid ballast. Ballast serves many safety purposes including: controlling the submergence of the propeller, rudder and hull, providing transverse stability and reducing stress levels on the hull. Under heavy weather conditions, the ship's officer determines ballast levels to control stability and maintain manageability of the vessel. Ballast tanks are filled when the cargo is off-loaded from a ship to compensate for the lack of weight. Once the ship has reached the next port and is loading new cargo on board, the ballast water is emptied (Fig. I. 1). The size and configuration of the ship affects the amount of ballast water that is transported

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This thesis follows the style of Limnology and Oceanography

during a voyage. Large cargo tankers can hold over 200,000m^3 or 50,000 metric tons of ballast (McGee et al., 2006; National Research Council, 1996).

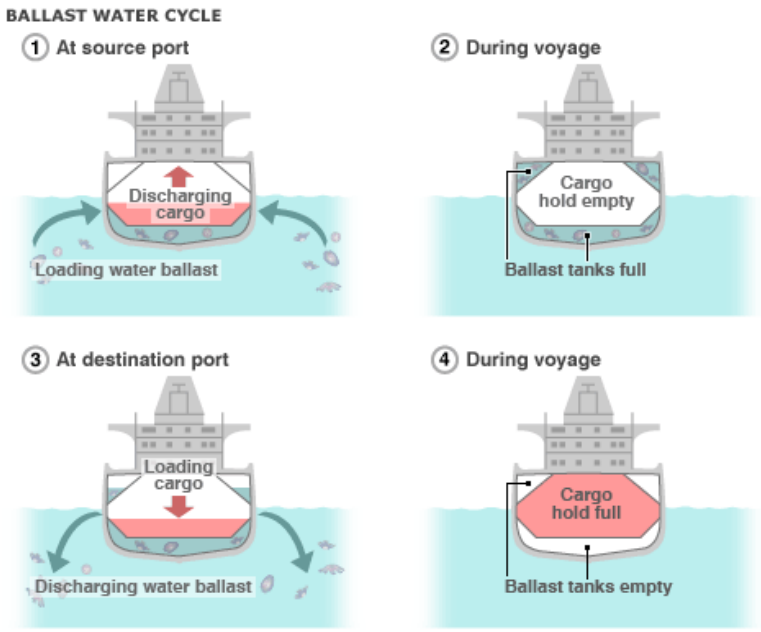

Fig. I. 1. Ballast water uptake and discharge in cargo ships. (www.globallast.imo.org)

An estimated 10 billion tons of ballast water is currently transported world-wide annually, and with it, an estimated 3,000 to 4,000 eukaryotic species (Gollasch et al., 2000). Collectively, all the ports in the United States of America received over 70 million metric tons of ballast from foreign ports in 2000 (Ruiz et al., 2000). McGee et al. (2006) calculated that over 60 million metric tons of ballast water was discharged over a four and a half year period in Alaskan waters. In 2007, the state of Texas received

31,225,589 metric tons of ballast discharge from all ship types (National Ballast Information Clearinghouse, 2008). Of this, cargo ships contributed 1,679,333 metric tons, with 845,374 metric tons that came from coastwise (domestic) sources and 833,959 metric tons that originated overseas. The largest port in Texas and one of the biggest worldwide, the Port of Houston received 253,736 metric tons of domestic and 467,653 metric tons of overseas ballast discharge. A smaller Texas port, the Port of Galveston received 11,522 metric tons from domestic sources and 14,523 metric tons of overseas ballast discharge (National Ballast Information Clearinghouse, 2008).

Bacteria in Ballast Water

In both lakes and oceans, a single milliliter of water may harbor approximately 1,000,000 bacteria (Dobbs and Rogerson, 2005). In view of the thousands of tons of ballast water transported in a single cargo vessel, billions of bacteria inevitably find their way into ballast tanks. Bacteria have several advantages to surviving long journeys including high reproductive rates (asexual reproduction and rapid growth), simpler nutrient requirements and broader tolerances to physical conditions than eukaryotes, and the ability to form resting states when conditions become unfavorable (Dobbs and Rogerson, 2005; Drake et al., 2007). All of these advantages point to the invasive potential of bacteria transported in ballast water. In fact, ballast water transport is currently the leading vector for alien aquatic species transfer and is responsible for most historical and recent aquatic bioinvasions (Burkholder et al., 2007; Murphy et al., 2004).

In twenty-eight ships sampled from the U.S. Military Sealift Command and the Maritime Administration, the average bacterial abundance per tank was 3.05×10^{14} cells per liter (Burkholder et al., 2007). Among these bacteria, several pathogens were found, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Listeria monocytogenes* and *Mycobacterium* spp. In the Chesapeake Bay, average bacterial concentration in ballast water was 8.3×10^{8} per liter (Ruiz et al., 2000). Moreover, 93% of ships examined arriving into Chesapeake Bay from foreign ports contained the toxigenic *Vibrio cholerae* 01 and 0139 serotypes. Dividing cells were visible under the microscope, indicating that some of the bacteria were viable upon arrival (Ruiz et al., 2000). *V. cholerae* 0139 was also found in the ballast water of ships in Mobile, Alabama originating from an area in South America that was experiencing a cholera epidemic (Drake et al., 2007).

Several methods of control have been tested to reduce or eradicate microbial ballast water populations. These techniques have included filtration, UV irradiation, biocides, deoxygenation and thermal treatments without complete success (Dobbs and Rogerson, 2005). Often, these treatments either fail to effectively reduce the microbial population or render the water unsuitable for discharge. Currently, the most common measure to control bacteria is the act of mid-ocean ballast water exchange (Murphy et al., 2004). In 1991, the International Maritime Organization established voluntary guidelines for ships arriving from beyond the 'exclusive economic zone' (EEZ), which extends about 200 miles offshore, to discharge ballast water in depths greater than 2000 m and re-fill with mid-ocean water (Murphy et al., 2004). The salinities of the open oceans are stable, the Pacific and Atlantic Ocean at 33 and 35 ppt, respectively (Murphy

et al., 2004), whereas the salinities of the receiving ports are typically much lower (<10 ppt) and highly variable. The justification of mid-ocean ballast water exchange is to discharge organisms into the inhospitable and nutrient poor oligotrophic open ocean water.

Marine Bacterial Communities and Invasive Species Concerns

Marine bacterial communities have been well characterized in recent years (Azam and Worden, 2004; Casamayor et al., 2000; Glockner et al., 2000; Morris et al., 2002; Nold and Zwart, 1998), especially with the growing application of molecular methods (using the 16s ribosomal subunit gene) that allows for the analysis of communities of uncultured microorganisms (Fig. I. 2). Differing patterns of bacterial distribution exist between fresh and marine, pelagic and benthic waters (Nold and Zwart, 1998; Methé et al., 1998). The marine pelagic zone is dominated by the α- and γ-proteobacteria, but lacks β-proteobacteria, which are predominant in freshwater, and to some extent, estuarine systems where fresh water wedges exist (Bouvier and del Giorgio, 2002). In estuaries, the Delaware for example, Bacteriodetes and α-proteobacterial fractions are dominant and trends have been observed that both α- and γ-proteobacteria increase as salinity increases, with a decrease in β-proteobacteria (Castle and Kirchman, 2004; Bouvier and del Giorgio, 2002). There are even distinctions between marine bacterial communities occurring on aggregates of particulate organic matter versus those that are free-living. For example, members of the Planctomycetes and the Bacteriodetes predominate on marine snow (Delong et al. 1993). While the

concept of biogeography for bacterial species is debated among microbial ecologists, there are specific phylogenetic clusters that are habitat-specific. Most notable are the SAR clusters characterized from the Sargasso Sea (Giovannoni et al., 1990) which include the SAR86 clade (γ-proteobacteria) and SAR11 clade (α-proteobacteria). *Pelagibacter ubique*, within SAR11, was later found to comprise roughly one third of all prokaryotic cells in marine surface waters in the subtropical zone (Morris et al., 2002). Another example, the *Roseobacter* clade affiliated (RCA) group which is found only in marine waters extending from temperate to polar regions but not in tropical and subtropical regions (Selje et al., 2004). Other groups that characterize distinct marine communities include the Chloroflexi (SAR202), Fibrobacter (SAR406), Planctomycetes,

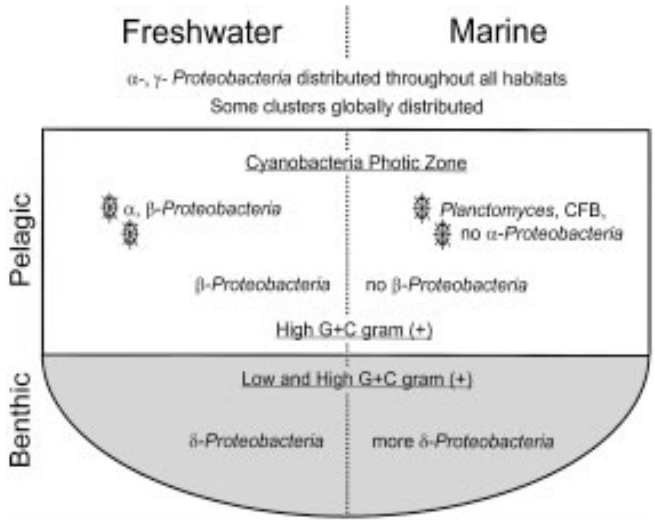

Fig. I. 2. Location in the water column of typical aquatic bacterial phyla. (Nold and Zwart, 1998).

Actinobacteria, Bacteroidetes and Cyanobacteria (Giovannoni and Stingl, 2005). While many physical (e.g. temperature, barometric pressure) and chemical factors (e.g. carbon and nutrient availability, pH) influence the composition of marine bacterial communities, the dominant determinant seems to be salinity, as demonstrated by Lozupone and Knight (2007). Because the salinity of water in a ballast tank would remain constant throughout transport, it is feasible to believe that bacteria could be easily relocated to distant geographic regions as long as the salinity is similar to that of their area of origin.

Invasive species are legally defined as a "non-native species whose presence in an ecosystem does or is likely to cause environmental or economic harm" (Union of Concerned Scientists, 2007). Harmful bacteria are defined as being capable of causing disease or death to humans or aquatic life (Burkholder et al., 2007). Eukaryotic invasive species are well-documented, but little is known about bacterial invaders (Union of Concerned Scientists, 2007). One documented example is the June 1998 widespread outbreak of gastroenteritis that occurred from the consumption of Galveston Bay oysters containing an exotic serotype (03:K6) of *Vibrio parahaemolyticus* (DePaola, 2003). Ballast water discharge was thought to be the source of the bacterium. Although common in Asia, this strain of *V. parahaemolyticus* had not been previously isolated in the U. S. (DePaola, 2003). Surprisingly, there are no published studies of the diversity of bacterial communities in Texas estuaries except for two that examine seasonal variation in serotypes of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in Galveston Bay (Lin et al., 2003; Myers et al., 2003).

Study Area

The Ports of Houston and Galveston are located in the northern and southern sections of Galveston Bay respectively (Fig. I. 3). The Port of Houston is the top port in the nation in foreign waterborne commerce, the 2nd largest national port in area and the 14th largest in the world in terms of tonnage (Port of Houston, 2009). In the summer and fall of 2005, Louisiana and Mississippi ports were damaged or lost due to Hurricanes Katrina and Rita respectively resulting in increased commerce to Texas ports. With increased ship traffic arriving to major Texas ports, concerns about bacterial invaders and their impacts on ecosystem health have elevated. An important component of the study described in this thesis is to establish which bacterial species are already present in Texas waters. Because the bacterial communities in the Texas estuaries and port systems have not been previously characterized, this study provides novel information for future research.

The eight bay systems and ten ports included in this study span from Sabine Lake in the northernmost region of the Texas gulf coast (between Texas and Louisiana), to the Port of Brownsville, in the southernmost region of the Texas gulf coast, between Texas and Mexico (Table I. 1). Physical and hydrological parameters for the bay systems can be seen in Table I. 2, showing average water temperature, salinity and monthly river flow as well as area measurements. Sabine Lake (including Sabine Pass) lies in the Sabine River basin, and receives freshwater inflow from both the Sabine and the Neches Rivers (Fig. I. 4). The three ports that are located in the Sabine system are the Ports of Beaumont, Arthur, and Orange. The Port of Orange is the easternmost port in Texas at

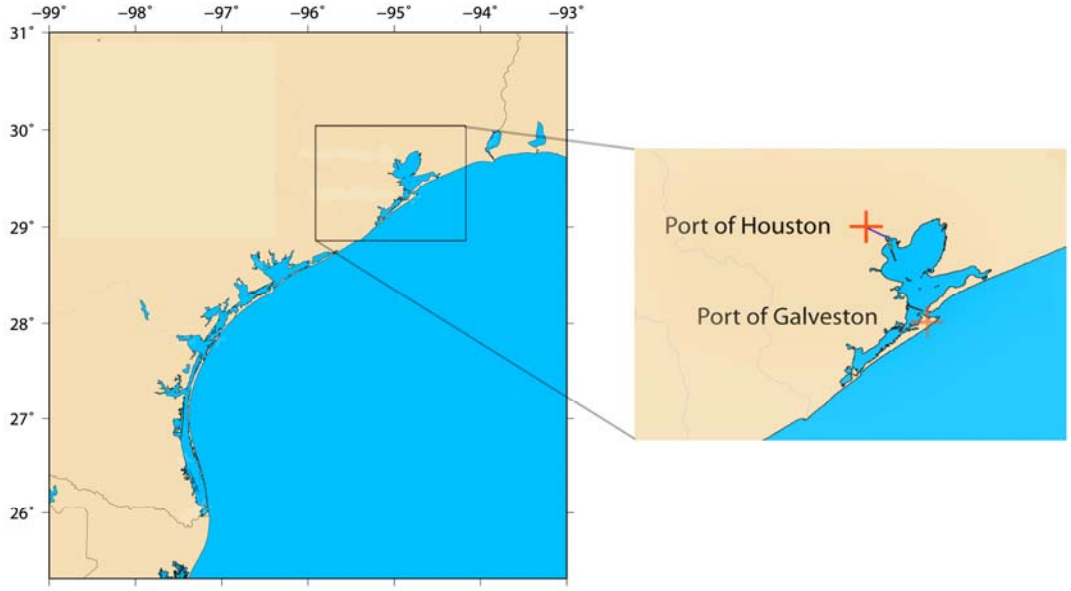

Fig. I. 3. Locations of the Ports of Houston and Galveston.

the border with Louisiana, located 12 miles above the Sabine River outlet into Sabine Lake. The Port of Beaumont is 42 miles inland along the Sabine-Neches ship channel. It is important, both commercially, and militarily, as it is the 2nd largest U. S. military port in the world (Port of Beaumont, 2009). Port Arthur lies on the west bank of Sabine Lake, near the mouth of the Neches River. Galveston Bay lies in three coastal basins, and one river basin: the Neches-Trinity Coastal basin to the east, the Trinity-San Jacinto Coastal basin and the San Jacinto River basin to the north, and the San Jacinto-Brazos Coastal basin to the west. Galveston Bay receives freshwater mainly from the San Jacinto and the Trinity Rivers. Two major ports are found in Galveston Bay: the Ports of Houston and Galveston. Both of these ports are discussed in a later section.

Table I. 1. Location (latitude and longitude) of bay and port sampling sites in this study.

	Latitude/Longitude					Latitude/Longitude
Bay	**Station 1**	**Station 2**	**Station 3**	**Station 4**	**Port**	**Station**
Sabine Lake	29° 57.226N 93° 49.986W	29° 53.459N 93° 51.260W	29° 50.075N 93° 50.075W	N/A	Beaumont	30° 04.903N 94° 05.549W
Sabine Pass	29° 42.992N 93° 51.316W	N/A	N/A	N/A	Orange	30° 05.462N 93° 43.917W
Galveston Bay	29° 21.308N 94° 45.31W	29° 18.633N 94° 52.28W	29° 32.358N 94° 34.81W	N/A	Port Arthur	29° 52.191N 93° 55.913W
Lavaca Bay	28° 38.360N 96° 36.725W	28° 41.053N 96° 37.661W	N/A	N/A	Houston	29° 45.079N 95° 05.653W
Copano Bay	28° 07.983N 97° 00.679W	28° 07.768N 97° 00.828W	28° 06.828N 97° 01.553W	28 04.108N 97 06.716W	Galveston	29° 18.813N 94° 47.189W
Aransas Bay	28° 03.896N 97° 01.943W	28° 00.767N 97° 03.094W	N/A	N/A	LaVaca	28° 38.587N 96° 32.960W
Nueces Bay	27° 52.961N 97° 20.618W	27° 50.250N 97° 22.857W	N/A	N/A	Corpus Christi	27° 48.697N 97° 23.702W
Corpus Christi Bay	27° 44.081N 97° 21.637W	27° 46.443N 97° 23.238W	27° 50.977N 97° 21.162W	N/A	Mansfield	26° 33.365N 97° 25.733W
Baffin Bay	27° 19.165N 97° 40.737W	27° 17.307N 97° 39.673W	N/A	N/A	Isabel	26° 03.615N 97° 12.890W
					Brownsville	25° 57.121N 97° 24.177W

Table I. 2. Physical and hydrologic parameters of Texas bays and estuaries (modified from Thronson and Quigg, 2008).

	Total EDA area (km^2)	Estuary area (km^2)	Average depth (m)	Estuary volume (m^3x10^9)	Average summer salinity (psu)	Tide height (m)	Tidal prism volume (m^3x10^9)	Average monthly river flow (m^3 day^{-1})	Average annual water temp. (°C)
Sabine Lake	12,444	265	1.9	0.660	7.7	0.24	0.125	44,055,103	31.7
Galveston Bay	11,505	1,456	2.0	2.707	18	0.16	0.449	24,279,600	29.5
Corpus Christi Bay	5,063	571	2.2	1.280	30	0.21	0.230	492,324	29.7
Aransas Bay	6,945	524	2.5	0.847	19	0.21	0.121	586,665	29.7
Baffin Bay	8,774	239	2.0	0.311	52	0.03	0.014	72,149	30.1

EDA: estuarine drainage area defined by NOAA's Coastal Assessment Framework; represents the sum of land and water area for the watershed.

Fig. I. 4. Texas river and coastal basins. U. S. Geological Survey, 2009.

LaVaca Bay is the most western and inland section of the larger Matagorda Bay, and lies within two costal basins and one river basin: the LaVaca River basin to the north, the Colorado-LaVaca coastal basin to the east, and the LaVaca-Guadalupe coastal basin to the west. The major source of fresh water into LaVaca Bay is the LaVaca River. Port LaVaca is located on the east bank of LaVaca Bay in an area known as Point Comfort, across the bay from the city proper (Port LaVaca, 2009).

The Aransas Bay system is comprised of Copano Bay, inland and to the west, and Aransas Bay, to the east, opening to the Gulf. This bay system lies in the San Antonio-Nueces coastal basin, but does not receive any major river inflows of

freshwater. The Corpus Christi Bay system is comprised of Nueces Bay, inland and to the west, and Corpus Christi Bay, which opens to the Gulf. This bay system lies within two coastal basins and one river basin: The San Antonio-Nueces coastal basin to the north, the Nueces River basin to the west, and the Nueces-Rio Grande coastal basin to the south. The major source of freshwater inflow comes from the Nueces River. The Port of Corpus Christi is located within the city proper, along the west bank of the Corpus Christi Bay, where it connects to the Nueces Bay. It is significant in its import of large amounts of crude and gas oil (Port of Corpus Christi, 2009). Corpus Christi Bay is separated from the Gulf of Mexico by a series of barrier islands, the nearest of which is Mustang Island. Port Aransas is located on the north shores of Mustang Island, and is mainly a tourist destination.

The last bay system examined in this study is Baffin Bay, which lies in the Nueces- Rio Grande coastal basin, and lacks any major river source. Barrier islands lining the south Texas coast create a system known as the Laguna Madre, which extends from just above Baffin Bay down to the southern edges of Texas, where Padre Island comes close to the mainland, resulting in the Brazos Santiago Pass. About midway between Baffin Bay and the end of the Laguna Madre is Port Mansfield. Far from being considered an industrial port, Port Mansfield is mainly a recreation spot for fishing (Port Mansfield, 2009). On the south edge of the Laguna Madre is Port Isabel, ending the series of Texas bay systems. The Port of Brownsville is located at the southernmost tip of Texas at the end of a 17 mile channel to the Gulf of Mexico. This port is an important shipping port as it connects trade between Mexico and the United States, and is where

land transportation in Mexico meets with the Gulf Intracoastal Waterway (Port of Brownsville, 2009).

Very little is known about the endemic bacterial populations along the Texan portion of the Gulf Coast of Mexico. Studies to date are limited to Galveston Bay and have focused only on pathogenic *V. vulnificus* (Lin et al., 2003; Lin and Schwarz, 2003; Vanoy et al., 1992), *V. cholerae* (Davis and Sizemore, 1982), *V. parahaemolyticus* (Myers et al., 2003), as well as salmonellae and fecal coliforms Goyal et al., 1977).

Previous studies of ballast water-transported bacteria have not examined the total bacterial diversity in the ballast water, nor did they examine the potential influence of these 'stowaways' on the microbial communities at the individual ports. The primary goals of this study are to 1) characterize bacterial community diversity in ballast tanks of commercial cargo ships docking at the Port of Houston and their influence upon Galveston Bay and 2) compare observed ballast water bacterial taxa with communities present in major Texas estuaries and ports using cultivation-independent molecular methods. This study will also provide the first look at the genetic fingerprints or signatures of bacterial communities in the Texas estuary systems. These data will be important to assess if and how the ballast water bacteria influence the endemic populations. This study is the first of its kind to characterize the total bacterial communities of ballast water and Texas estuaries, rather than just calculating abundance or targeting only pathogenic strains.

Research Objectives

For my Master's research, I characterized bacterial communities found in the ballast tanks of international cargo ships using cultivation-independent DNA fingerprinting and compared these fingerprints between ships and to endemic populations in Texas ports and estuarine systems. This was accomplished through two objectives:

Objective 1: Characterize Bacterial Communities in Ballast Water.

Previous studies on ballast water bacteria concentrated on human pathogenic species, but ignored the total community diversity. This study sought to determine total bacterial diversity in ships' ballast water. The bacterial members found in ballast water were categorized into most probable originating environments. From these environmental signatures, I attempted to define the potential invasive species arriving in ballast water based on their ability to survive in brackish Port of Houston water. Little is known about the community dynamics of a ballast water tank. Due to ballast water exchange measures, ballast water originating from port is theoretically completely exchanged with open-ocean water and consequently, the bacterial communities should be those found in upper water column of the pelagic zone of the ocean.

Hypothesis 1: Ballast water bacterial communities will resemble those of open-ocean, upper water column communities and exhibit low diversity.

To test this hypothesis, a 16s rRNA clones library was created from a sample of water from a commercial cargo ship ballast tank arriving in the Port of Houston. Clones containing 16s rRNA genes were further analyzed by amplified ribosomal rDNA restriction analysis (ARDRA) fingerprinting to determine total diversity and unique clones were sequenced. Sequences were then compared to an online database of bacterial genes (GenBank) and analyzed to determine phylogenetic relatedness. Because some groups of bacteria are only found in certain aquatic habitats e.g. coastal, estuarine, pelagic, temperate, tropical, subtropical, or polar, it may be possible to make inferences about the environment and dynamics of the water taken up for ballast. For example, β-proteobacteria are predominately found in freshwater habitats, and are sometimes seen in coastal waters (Nold and Zwart, 1998; Giovannoni and Stingle, 2005). However, they are not found in open-ocean waters. The presence of these groups in ballast tanks would suggest that ballast water exchange occurred in a port rather than beyond the EEZ 200 miles offshore. Using this bacterial analysis as a proxy, I attempted to deduce the origin of the ballast water.

There are also several parallels between ballast tank habitats. First, because of ballast water exchange measures, the bacterial community in a tank following exchange should be that of upper water column of the pelagic zone of the ocean. So each tank should have similar "starting" communities, following ballast water exchange. Secondly, although physical design of the tank varies between ships, all tanks are self-contained, do not mix with extra-tank environments during travel, and do not allow sunlight into the tank. Therefore, since these ballast water bacterial communities theoretically start with

similar composition, the parallel selection pressures should favor the same bacterial lineages, resulting in similar communities at the end of the voyage.

To test this argument, this study compared ballast water bacterial community compositions between cargo ships where ballast water exchange occurred in proximate geographical locations.

> *Hypothesis 2:* Bacterial communities in ships' ballast water will have parallel compositions to others where open-ocean ballast water exchange occurred in proximate geographical locations.

For community comparisons, polymerase chain reaction (PCR) amplification of the 16s rRNA gene followed by denaturing gradient gel electrophoresis (DGGE) was used to create a molecular fingerprint profile of the community structure for comparison of major banding trends. Bands were excised for DNA sequencing and phylogenetic analyses. Ballast water bacterial communities were readily discernable in DGGE gels. Since this process separates amplified genes based on nucleotide composition, each band should represent a unique member of the community. By comparing the community fingerprint of each ballast tank to others, it can be determined if there is a common pattern or if certain species are more likely to be found in ballast water.

*Objective 2: Assess If and How Ballast Water Discharge Affects Natural Bacterial P**opulations in Texas Ports and Estuaries.***

A main concern about bacterial species transport is the effect the foreign bacteria will have on the endemic populations. Bacteria play a very significant role in the marine food web and impact nutrient cycles such as carbon and nitrogen. Studying community dynamics and changes over the seasons can help shed light on these bacterial niches. Without knowledge of endemic bacterial species temporal and spatial patterns, we cannot predict the impact of invasive ballast water bacteria. For Texas ports and estuary systems, it was important to determine if bacterial populations varied month to month with port activity, or displayed a natural community succession throughout the year. To examine temporal and seasonal community successions, the Ports of Houston and Galveston were sampled on a monthly basis from June 2006 to June 2007. Hydrological parameters were measured, including temperature, salinity and dissolved oxygen levels.

Hypothesis 3: Bacterial communities in the Ports of Houston and Galveston will exhibit temporal variations related to changes in temperature and salinity.

Changes in port bacterial communities were examined by PCR-DGGE. Previous studies have shown that distinct seasonal succession in bacterial communities (Kent et al., 2004; Kan et al., 2006a), including the identification of three major seasonal patterns: winter (December and January), spring (February to May), and summer-fall (June to November) in the Baltimore Inner Harbor (Kan et al., 2006b). Since the Ports of

Houston and Galveston were sampled on a monthly basis for the duration of the project, the port communities can be visualized over a significant time span to determine how quickly changes occur and if these changes have any correlation to deballasting activity. While many other variables are present, general statements may be made about the effect of ballast water dumping on port bacterial communities. For example, if the port of Houston bacterial community changes more quickly than the port of Galveston community, it may be speculated that this change is influenced by the higher commercial activity level of the port of Houston to Galveston.

Again, little to nothing is known of the endemic bacterial populations along the Texas gulf coast. The last component of this study is to examine spatial variations in bacterial communities of Texas ports and bays during the spring and summer of 2007.

> *Hypothesis 4*: Bacterial communities will exhibit variations related to geographic proximity, and be more similar in closely located port and bay systems.

Port and estuary samples were examine by PCR-DGGE as objectives two and three. Denaturing gradient gel electrophoresis was used to compare molecular fingerprint profiles or 'signatures' of bacterial communities, showing trends and shifts in communities between geographic locations and seasonality in bacterial composition and abundance by absence/presence of a band and brightness of the band, respectively. Due to the monetary and time expense of this massive sampling effort, only two seasons were sampled, the spring and summer of 2007. Both the temporal and spatial comparisons

shed light on the impact of deballasting activities in ports on native bacterial communities.

Total Materials and Methods

This study utilized both field-based ecological and laboratory-based molecular techniques to examine and compare the bacterial communities of the Texas estuaries and ballast water of international cargo ships. Typically less than 1% of bacteria can be cultivated from environmental samples (Amann et al. 1995). Since the 1990's, cultivation-independent molecular methods are the norm for microbial ecological studies that examine total bacterial diversity (Diez et al., 2001; Edenborn and Sexstone, 2007; Muyzer et al., 1993; Muyzer et al., 1998). For this study, the established methods of polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), 16S rRNA clone library construction and amplified rDNA restriction analysis (ARDRA) were used.

Sample Collection from Texas Bay Systems, Ports and Ballast Water

Eight estuaries and ten ports were selected for sampling (Fig. I. 5). Permission for collection was obtained from all port authorities. Sabine Pass and Sabine Lake are counted as the same estuary system. The first set of samples was collected in spring 2007. During this trip, the Port of Corpus Christi was sampled, but not the Port of Aransas due to a lack of port access. The second sampling season in summer of 2007 was cut short due to logistical and weather related problems and only a portion of the

state ports and estuaries were sampled from Port LaVaca to Port of Brownsville. Water samples were collected off of accessible boat ramps and fishing piers into acid-washed brown bottles or 4L gas tanks. Bacteria in water samples were immediately collected onto 0.22 Sterivex μm filters (Millipore) and frozen at -20°C for transport back to the lab at Texas A&M University in Galveston (TAMUG). Once back at the lab, the samples were frozen at -80°C until later processing. In addition to water samples, other hydrological parameters were measured, including temperature, salinity, conductivity, and dissolved oxygen levels. Two to four stations per bay system were taken, depending upon accessibility points. In the results section, all stations per system are averaged together for hydrological parameters, and all samples from all stations were combined for DGGE analysis resulting in one DGGE lane/ bay system.

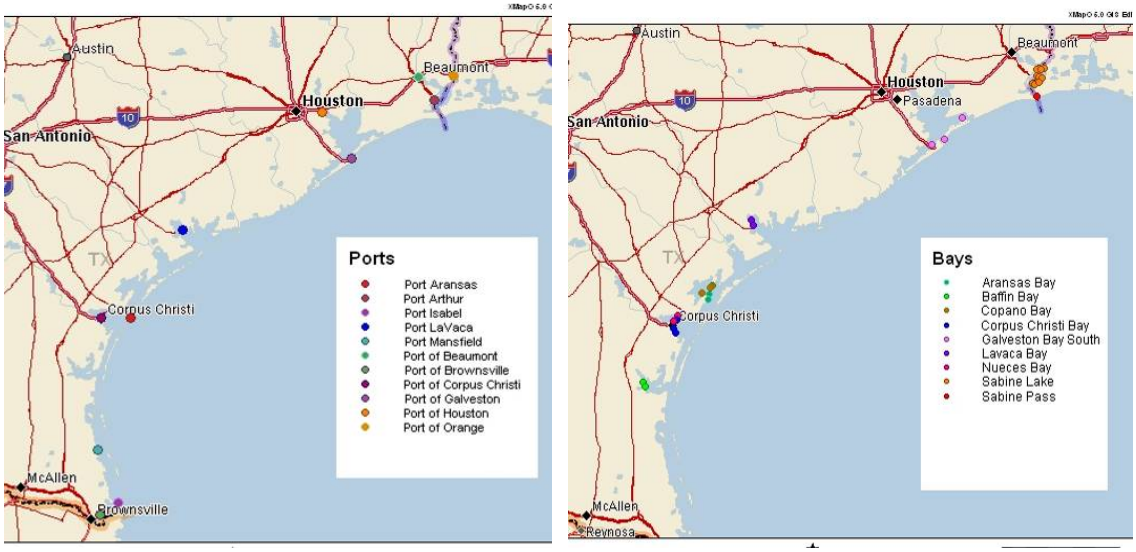

Fig. I. 5. Texas ports (left) and bays (right) sampled during this study.

The Ports of Houston and Galveston (Fig. I. 6) were sampled at the beginning of every month from June 2007 to June 2008 to examine seasonal shifts in bacterial populations and to determine if there are any correlations in diversity change with the date a sampled cargo ship was in port. Water samples were collected in acid-washed brown bottles or 4L gas tanks. Bacteria in water samples were collected onto 0.22 μm Sterivex filters (Millipore) and immediately frozen at -20°C at TAMUG. In addition to water samples, other hydrological parameters were measured, including temperature, salinity, conductivity, and dissolved oxygen levels.

Ballast water was collected from incoming cargo ships in the Port of Houston with the assistance of a shipping agent. The locations at which the exchanges occurred are indicated in Fig I. 7. Only ships analyzed in this study are included on the maps. Information collected from ships includes date and location (latitude and longitude) of the most recent water exchange of sampled ballast tank. Due to privacy agreements between the principle investigators of this study and the shipping agent who facilitated the sample collection, the names of the ships and shipping companies cannot be disclosed. Immediately after a ballast sample was collected, it was transported to TAMUG, filtered and stored at -80°C.

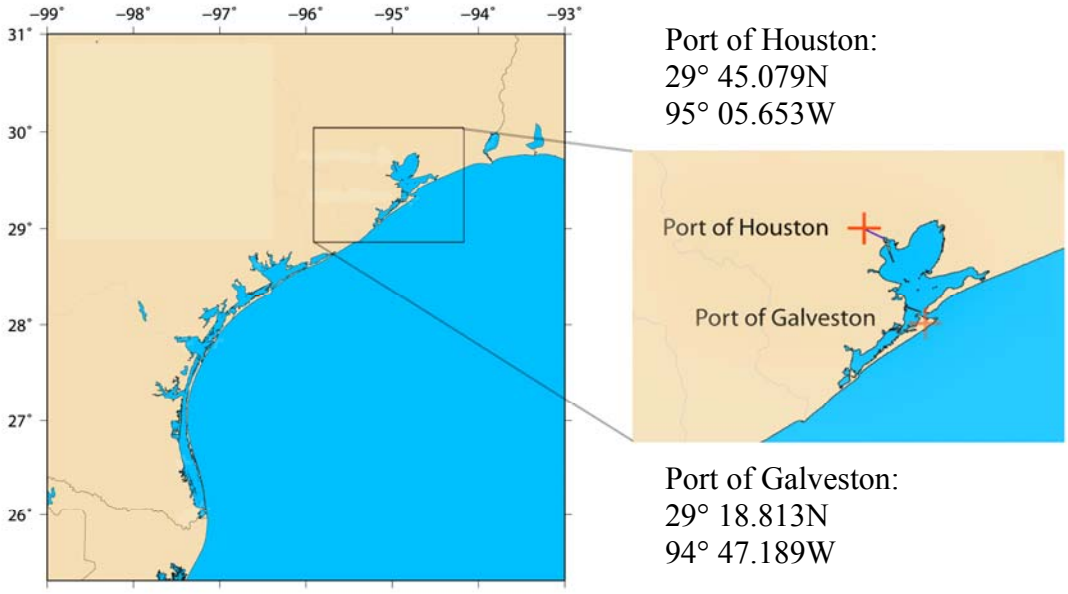

Fig. I. 6. Locations of the Ports of Houston and Galveston.

Fig. I. 7. Location of ballast water exchange in ships sampled in this study.

Nucleic Acid Extraction

Bacterial nucleic acids were extracted from the frozen filters using a hexadecyltrimethylammonium bromide (CTAB)/chloroform-isoamyl alcohol method (Doyle and Doyle 1990) adapted for Sterivex cartridge filters. Filter cartridges were opened using an ethanol-sterilized hand saw and filters where placed inside a 50 ml Falcon tube. The filter was completely covered with 10 ml of 3% CTAB using a sterile pipette and vortexed briefly. The falcon tube containing the filter and CTAB were put in a 65°C water bath for 2 hours, with vortexing every 15 minutes. CTAB is a strong detergent that lyses cells and rapidly denatures DNAases and RNAases. Sterile forceps were used to remove the filter; 10 ml of chloroform isoamyl alcohol was added to create a white emulsion solution. Tubes were vortexed for 5-7 seconds and then centrifuged at 4°C at 12,000rpm for 15 minutes. The aqueous phase was extracted using a sterile pipette and placed in a new, sterile 50ml Falcon tube. DNA was precipitated by adding two-thirds of the total aqueous volume of 100% isopropanol (-20°C) to the tubes and vortexing for 5-7 seconds. Tubes were stored at 4°C overnight, and then centrifuged at 4°C at 12,000 rpm for 20 minutes to pellet DNA. The isopropanol was decanted and DNA was washed with 200 ul of 80% ethanol (from freezer). Tubes were suspended upside down to dry overnight. The DNA pellet was suspended in 200 ul of LT buffer, incubated overnight at room temperature, then placed in 2.0 ml microcentrifuge tubes and stored at -20°C for later analysis. In some cases, where a multitude of samples rendered the CTAB method unwieldy, the commercial PowerPlant™ DNA Isolation Kit

(MoBio Inc., Solana, CA) was used, but yielded less DNA. All ballast water samples were extracted using the above-described CTAB/chloroform-isoamyl alcohol method.

DNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Purity was measured by examining the ratio of 260nm (at which DNA absorbs UV light) with the 280nm (at which proteins absorb UV light). A 260 nm: 280 nm ratio between 1.8 and 2.0 is considered "pure" DNA with little to no protein contamination. Similarly, organic compounds tend to absorb UV light around the 230nm wavelength, and a 260:230 ratio between 1.8 and 2.0 indicates little to no organic contamination. Protein contamination is a known PCR inhibitor, so any sample with a 260:280 ratio below 1.8 was cleaned using a commercial kit (Promega Wizard PCR Cleanup System, Madison, WI).

Polymerase Chain Reaction (PCR)

Genes that encode 16s rRNA were amplified using PCR with standard bacterial primers 341F and 907RM (Integrated DNA Technologies, Coralville, IA) containing a 30 bp long GC-rich clamp for denaturing gradient gel electrophoresis and primers 8F and 1492R for clone library construction and amplified ribosomal DNA restriction analysis (Table I. 3). The 16s rRNA gene is commonly used in bacterial phylogenetics and microbial ecology because it is ubiquitous in all prokaryotic organisms with conserved regions for universal analysis, but also has variable regions for more fine-scaled analysis at the family and genus levels (Case et al., 2007).

For primers 341F and 907RM, a touchdown PCR was performed. Touchdown PCR utilizes a decreasing scale of annealing temperatures, each with two repetitions, in order to target the maximum number of organisms in an environmental sample and reduce the formation of artificial byproducts during the amplification. The touchdown PCR cycles went as follows:

Initial denaturation	94°C	5 minutes
Annealing step	65 – 50°C	1 minute
Elongation step	72°C	3 minutes
Repeat entire cycle twice for each annealing temperature		
Final elongation	72°C	20 minutes

The final elongation was extended to 20 minutes to prevent artifactual double bands during PCR in later DGGE analyses (Janse et al., 2004). A limitation of DGGE is that only amplicons of about 500 base pairs in length can be separated and a 30-50 base pair GC-rich clamp is needed to act as a high temperature melting domain to increase sequence variance detection from 50% to 100% (Muyzer et al., 1998).

Table I. 3. Primers used in this study.

Primer	Specificity	Method	Sequence 5' -3'	Reference
GM5-clamp (341F)	Bacteria	PCR-DGGE	cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc gcc tac ggg agg cag	Casamayor et al., 2000
907RM	Most known organisms	PCR-DGGE	ccg tca att cmt ttg agt tt	Casamayor et al., 2000
8F	Bacteria	16S rRNA clone library & ARDRA	aga gtt tga tcc tgg ctc ag	Lane 1991
1492R	Archaea and most Bacteria	16S rRNA clone library & ARDRA	ac ggy tac ctt gtt acg act t	Lane 1991

Clone Library Construction

Extracted DNA intended for ARDRA was amplified by the universal primers 8F and 1492R in a traditional PCR. These primers amplify an approximately 1400 base pair region of the 16s rRNA gene allowing for greater phylogenetic resolution downstream. 16S rRNA clone libraries of ballast water samples were created from PCR products ligated into a vector used to transform competent *E. coli* cells (pGEM-T Easy Vector System, Promega). Transformed *E. coli* was grown up overnight on selective agar (ampicillin and x-gal) and 150 positive DNA insert colonies (white positive vs. blue negative) were screened by gel electrophoresis for appropriate DNA insert size. Plasmids meeting the selection criteria were extracted from harvested colonies and stored at -20°C until later analysis.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Each clone underwent amplified ribosomal DNA restriction analysis to develop a genetic fingerprint, the set of which was used to examine diversity of the sample. The amplicons were digested using the restriction enzymes HaeIII and RsaI (Promega) and run on an 8% polyacrylamide gel. HaeIII is a high-frequency cutting restriction enzyme that has a high average number of restriction sites and comes from the organism, *Haemophilius aegyptius*. RsaI was used as a secondary restriction endonuclease to increase fingerprint resolution. RsaI come from *Rhodobacter sphaeroides*. The recognition sequences for both of these enzymes are:

HaeIII 5'GG^CC3'

RsaI 5'GT^AC3'

The digest resulted in banding patterns based on differential enzyme cutting due to sequence variation. The gels were examined using GelCompar (Applied Maths, Sint-Martens-Latern, Belgium) looking for repeating patterns. The software normalizes the gels and performs a pair-wise comparison using a neighbor joining algorithm to establish similarity values for each clone sequence. Clones with similarity values ≥ 97% were considered the same species and one clone from each pattern was sequenced and compared to the GenBank database to establish identity.

Sequence data was analyzed with the ARB software package (ARB, 2007). Only clones with sequences longer than 1000 base pairs were used for tree reconstruction. Clone sequences were initially aligned using the ClustalW algorithm and individually

checked against an *E. coli* reference, then later verified and adjusted using phylum-specific references.

Phylogenetic trees were reconstructed using maximum-likelihood analyses. Filters for phylogenetic subdivisions and/or groups which consider only 50% conserved regions were applied to exclude highly variable positions. Partial sequences were later added to existing trees by a special algorithm included in the ARB software that does not change the tree topology based on almost complete sequences. In addition to clone sequences, closest matched clone sequences and closest cultured sequences from GenBank records were added to increase phylogenetic resolution. Also included in the trees were cultured bacteria representatives that have been found in ballast tanks previously (Ruiz et al, 2000, Drake et al., 2005, Burkholder et al., 2007) and GenBank members that were closest matches to unpublished ballast tank clone sequences (Burkholder et al., 2007).

Bootstrap values were calculated for each tree with 1,000 replicates using the DNAPARS maximum parsimony tool in the PHYLIP package included in the ARB program.

Species Diversity

To examine species diversity within the clone library, both Simpson (1-D) and Shannon (H') Indices of Diversity were calculated along with evenness (J'). To examine total theoretical diversity of the entire ballast tank, rarefaction curves were interpolated using the Analytic Rarefaction freeware program (Analytic Rarefaction, 2009) followed

by a coverage calculation (C). Coverage values give an estimate of the percentage of the total population that has been successfully sampled, and is calculated by the formula:

$$C = 1-(n_i/N)$$

where N is the total number of ARDRA patterns detected, and n_i is the number of patterns that only appeared once.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (Muyzer et al., 1998) was used for comparison of bacterial communities in ballast water, ports and bays. DGGE separates PCR amplified products onto a vertical acrylamide gel based on nucleotide composition. The gel contains a linear increasing gradient of the denaturants urea and formamide. Variations in a fragment will cause the DNA to melt at unique concentrations of the denaturants, forming distinct bands, and creating a fingerprint structure for any given sample. In this study, gels were made of 8% polyacrylamide (37.5:1, acrylamide:bis-acrylamide), and ranged from 28 – 64% concentration gradient of urea and formamide, with a layer 0% denaturant concentration at the top of gel to form the wells. Fifteen clones were selected from the clone library constructed during this study and used as a standard ladder for comparisons between separate gels. These fifteen clones represented the major bacterial phyla (Table I. 4). Two microliters of each clone were mixed together in a 2.0 ml microcentrifuge tube. One microliter of DGGE ladder was amplified in an identical manner as the water samples. A BioRad D-Code universal

mutation system was used to cast and run denaturing gels. Gels were cast using an acrylic gradient former on top of a stir plate and a peristaltic pump.

Table I. 4. Clones used to create DGGE ladder.

Clone Number	Bacterial Phylum
48	actinobacteria
129	alphaproteobacteria
199	alphaproteobacteria
203	alphaproteobacteria
4	betaproteobacteria
28	betaproteobacteria
59	betaproteobacteria
47	bacteriodetes
15	bacteriodetes
66	gammaproteobacteria
118	gammaproteobacteria
212	gammaproteobacteria
123	planctomycetes
37	planctomycetes
128	verrucomicrobia

N,N,N',N'-Tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) are needed to polymerize acrylamide. TEMED accelerates the formation of free radicals by the APS that will react with the acrylamide monomers and catalyze polymerization. Bis-acrylamide will cross-link the growing polymers resulting in a porous gel (Mentor, http://www.bio-rad.com/LifeScience/pdf/Bulletin_1156.pdf). 10% APS (36 ul) and TEMED (7 ul) were added to both denaturing acrylamide solutions. The more concentrated denaturing acrylamide (64%) was placed in the left hand chamber and the less concentrated denaturing acrylamide (28%) was placed in the right hand

chamber with a mini stirring bean. Both chamber gates were opened and the solution was pumped into casting setup (glass plates with spacers clamped together). Once the denaturing gradient gel was solidified, 25ul of 10% APS and 5ul of TEMED were added to the 0% denaturant acrylamide, and half of the solution was carefully pipetted into the casting setup. The comb was added, and the remainder of the acrylamide solution was added. Gels sat for at least two hours before use.

Gels were submerged in a tank of 1X TAE buffer and run at 60°C for 18 hours. Gels were removed from the system in complete darkness and placed into a SYBR-Gold staining bath for 30 minutes. Afterwards, gels were quickly documented under UV light using a gel documentation system (BioRad, Hercules, CA), and then immediately transferred to a blue light transilluminator (Clare Chemical Research, Dolores, Co). Blue light (400 - 500 nm) does not nick DNA and can be used for longer analysis times.

An ethanol-sterilized razor blade was used to excise single bands from the gel and stored in 1.5ml Eppendorf tubes at -20°C. Gel slices were then put into 50ul of PCR-grade water overnight to elute DNA. Eluted DNA was reamplified using the same primers, and DGGE gels were run again, with only one band per lane, to ensure that only a single band had been excised and successfully reamplified. The gel was documented as before and transferred to the blue light transilluminator where each band was excised using an ethanol-sterilized razor blade. Gel slices were again eluted overnight in PCR-grade water, and reamplified the next day with the same primers not having the GC-clamp on the forward primer (341f). PCR product size was confirmed on agarose gels. Samples were purified (Cycle-Pure, E.Z.N.A Omega Bio-Tek, Inc., Norcross, GA) to

remove any remnants from PCR (excess nucleotides, buffer, etc.) and 0.5 ul of forward primer was added to each sample. Samples were sequenced at Yale University DNA Analysis Facility. Sequences were compared to the online GenBank database to determine identity. Due to time limitations, the only DGGE sequences included in this study are from only the spring 2007 Texas ports and bays sampling season.

Statistical Analysis

Images of denaturing gels were imported in TIFF format into the BioNumerics software program (Applied Maths). Bands were scored as present or absent based on a threshold of 5% of the total density (the most intense band in the gel). Gels were normalized using the positions of the standard bands. Clustering analysis was performed using Unweighted Pair Group method with Arithmetic Mean (UPGMA) with the Dice coefficient, which creates a pairwise distance matrix for all bands based on binary data (presence/absence of band) and constructs a dendrogram based on similarity values. The overall protocol is summarized in Fig. I. 8.

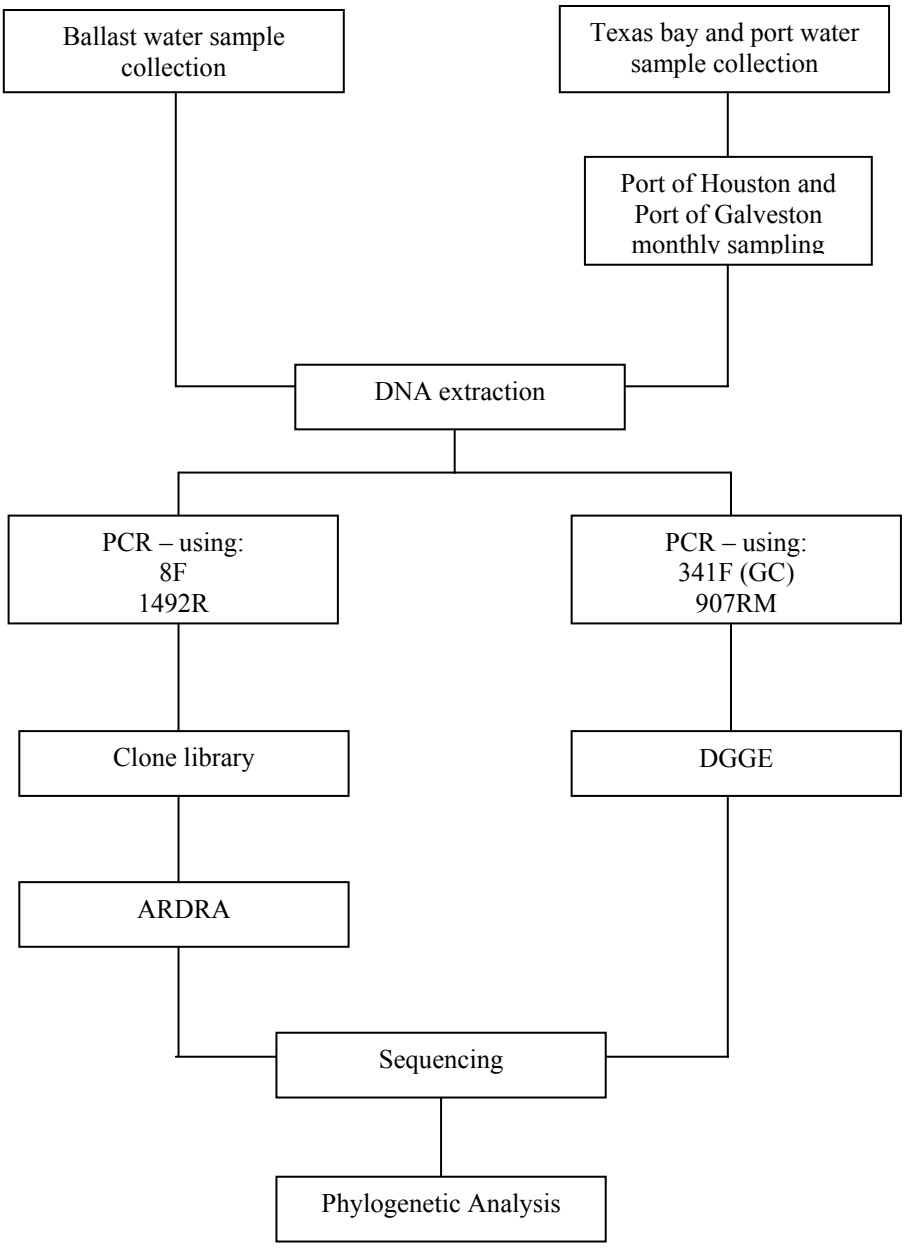

Fig. I. 8. Summary of methods used in this study.

CHAPTER II

DIVERSITY OF BACTERIA IN THE BALLAST TANK OF A COMMERCIAL CARGO SHIP IN THE PORT OF HOUSTON, TEXAS

Introduction

Usage of Ballast Water

The use of ballast water in the shipping industry began in the 1870's (Gollasch et al., 2000; National Research Council, 1996). Ballast water serves many safety purposes, including: controlling the submergence of the propeller, rudder and hull, providing transverse stability, and reducing stress levels on the hull. The size and configuration of the ship affects the amount of ballast water that is transported during a voyage. Large cargo tankers can hold over 200,000m^3 or 50,000 metric tons of ballast (McGee et al., 2006; National Research Council, 1996). An estimated 10 billion tons of ballast water is currently transported world-wide annually, and with it, an estimated 3,000 to 4,000 eukaryotic species (Gollasch et al., 2000). Collectively, all the ports in the United States of America received over 70 million metric tons of ballast from foreign ports in 2001 (Ruiz et al., 2000). McGee et al. (2006) calculated that over 60 million metric tons of ballast water was discharged over a four and a half year period in Alaskan waters. In 2007, the state of Texas received 31,225,589 metric tons of ballast discharge from all ship types (National Ballast Information Clearinghouse, 2008). Of this, cargo ships contributed 1,679,333 metric tons, with 845,374 metric tons that came from coastwise (domestic) sources, and 833,959 metric tons that originated overseas. The Port of

Houston, the largest port in Texas and one of the largest worldwide, received 253,736 metric tons of domestic and 467,653 metric tons of overseas ballast discharge.

In both lakes and oceans, a single milliliter of water may harbor approximately 10^6 bacteria (Dobbs and Rogerson, 2005). In view of the thousands of tons of ballast water transported in a single cargo vessel, billions of bacteria inevitably find their way into ballast tanks. Bacteria have several advantages to surviving long journeys including high replication rates, simple nutrient requirements, broad tolerances and strategies for surviving unfavorable conditions including the ability to form resting states (Dobbs and Rogerson, 2005; Drake et al., 2007). All of these factors point to the invasive potential of bacteria transported in ballast water. In fact, ballast water transport is currently the leading vector for alien aquatic species transfer and is responsible for most historical and recent aquatic bioinvasions (Burkholder et al., 2007; Murphy et al., 2004).

Several methods of control have been tested to reduce or eradicate microbial ballast water populations. These techniques have included filtration, UV irradiation, biocides, deoxygenation, and thermal treatments without complete success (Dobbs and Rogerson, 2005). Often, these treatments either fail to effectively reduce the population, or render the water unsuitable for discharge. Currently, the most common measure to control bacteria is the act of mid-ocean ballast water exchange (Murphy et al., 2004). In 1991, the International Maritime Organization established voluntary guidelines for ships arriving from beyond the 'exclusive economic zone' (EEZ), which extends about 200 miles offshore, to discharge ballast water in depths greater than 2000 m and re-fill with mid-ocean water (Murphy et al., 2004). The salinities of the open oceans are stable, the

Pacific and Atlantic Ocean at 33 and 35 ppt, respectively (Murphy et al., 2004), whereas the salinities of the receiving ports are typically much lower (<10 ppt) and highly variable. The justification of mid-ocean ballast water exchange is to discharge organisms into the inhospitable and nutrient poor oligotrophic open ocean water.

A survey of the scientific literature revealed only a few studies on the bacterial species present in ships' ballast tanks: these were mostly limited to pathogenic serotypes (Drake et al., 2005; Drake et al., 2007; Burkholder et al., 2007; Ruiz et al., 2000). In twenty-eight ships sampled from the U.S. Military Sealift Command and the Maritime Administration, the average bacterial abundance per tank was 3.05×10^8 cells per liter (Burkholder et al., 2007). Among these bacteria, several pathogens were found, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Listeria monocytogenes*, and *Mycobacterium* spp. In the Chesapeake Bay, average bacterial concentration in ballast water has been reported as 8.3×10^8 per liter (Ruiz et al., 2000). Moreover, 93% of ships examined arriving into Chesapeake Bay from foreign ports contained the toxigenic *Vibrio cholerae* 01 and 0139 serotypes. Dividing cells were visible under the microscope, indicating that some of the bacteria were viable upon arrival (Ruiz et al., 2000). Like Chesapeake Bay, ballast water has been implicated in the introduction of pathogenic bacteria to Galveston Bay. In June 1998, a widespread outbreak of gastroenteritis occurred in the U.S. from the consumption of Galveston Bay oysters containing an exotic serotype (03:K6) of *Vibrio parahaemolyticus* (DePaola, 2003).

The Port of Houston is the top port in the nation in foreign waterborne commerce, the 2nd largest national port in area and the 14th largest in the world in terms

of tonnage (Port of Houston, 2009). With increased ship traffic arriving to major Texas ports, concerns about bacterial invaders and their impacts on ecosystem health as well as the estimated $10 million per year oyster and fishing industries have elevated (Galveston Bay Foundation, 2009).

Previous studies of ballast water-transported bacteria did not examine the total diversity of bacteria, nor did they examine the potential influence of these 'stowaways' on the microbial communities at the individual ports. This study seeks to examine the total bacterial community in the water of a ship's ballast tank to determine how it correlates to known marine, pelagic microbial communities. Since ballast water exchange occurs outside of the EEZ, bacteria in ballast tanks should be similar to communities typical of pelagic waters. It is important to determine if mid-ocean ballast water exchange is adequate enough to prevent the transport of coastal or estuarine bacteria to other port systems in which they may survive and flourish. This chapter focuses on the first hypothesis:

> *Hypothesis 1:* Ballast water bacterial communities will resemble those of open-ocean, upper water column communities and exhibit low diversity.

Materials and Methods

Sample Collection

Ballast water was collected from a cargo ship in the Port of Houston with the cooperation of the ship captain. According to the ship's records, ballast water exchange occurred 29 days before docking at the Port of Houston at a location 750 miles offshore of the west coast of Africa in the Atlantic Ocean at 06° 57.3S, 008° 15.3W (Fig. II.1). Due to privacy agreements between the principle investigators of this study and the shipping agent who facilitated the sample collection, the name of the ship, shipping company, and date the ship docked in the Port of Houston cannot be disclosed. The sample nomenclature is based upon the ocean and latitude, longitude where ballast water exchange occurred (AO 06° 57.3S, 008° 15.3W). The sample was transported to TAMUG in Galveston, TX. Upon arrival, the sample was gently shaken to thoroughly mix the bacteria, and any other particulates that may have settled, then filtered onto a 0.22µm cartridge filter (3 replicates; Sterivex) using a peristaltic pump, and then frozen at -80°C.

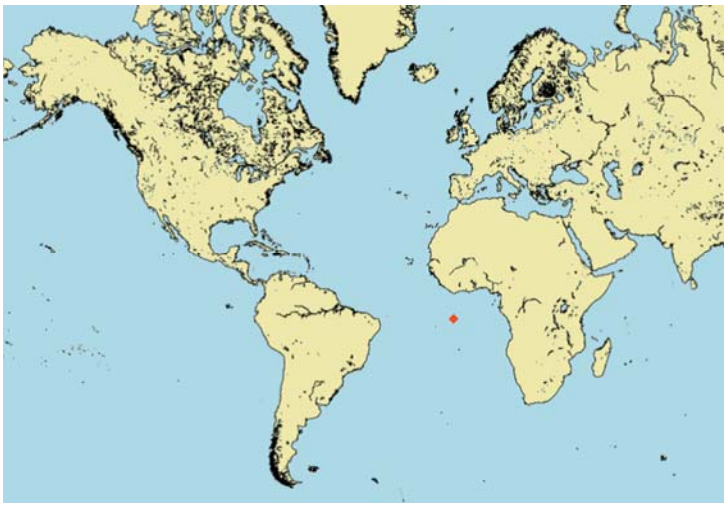

Fig. II. 1. Location (06° 57.3S, 008° 15.3W) of ballast water exchange by the ship examined in this study.

Nucleic Acid Extraction

Bacterial nucleic acids were extracted from the frozen filters using a CTAB/chloroform-isoamyl alcohol method (Doyle and Doyle 1990) adapted for cartridge filters. The three filter cartridges were opened using an ethanol-sterilized hand saw and filters where placed inside separate 50ml Falcon tubes. DNA purity and quantity was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The initial 260:280 ratio was below 1.8 so each sample was cleaned one time, which raised the ratio to above 1.8.

Polymerase Chain Reaction (PCR)

16s rRNA genes were amplified using PCR with standard bacterial primers 8F and 1492R for clone library construction and amplified ribosomal DNA restriction analysis (Table II. 1). Pure water and an *E. coli* sample were used for negative and positive controls, respectively. The 16s rRNA gene is commonly used in microbial ecology because it has conserved regions for universal analysis, but also has variable regions for more fine-scaled analysis at the family and genus levels (Case et al., 2007).

Table II. 1. Primers used for clone library and ARDRA.

Primer	**Specificity**	**Method**	**Sequence 5' -3'**	**Reference**
8F	Bacteria	16S rRNA clone library & ARDRA	aga gtt tga tcc tgg ctc ag	Lane 1991
1492R	Archaea and most Bacteria	16S rRNA clone library & ARDRA	ac ggy tac ctt gtt acg act t	Lane 1991

Clone Library Construction

Extracted DNA intended for ARDRA was amplified by the universal primers 8F and 1492R in a traditional PCR. These primers amplify an approximately 1400 base pair region of the 16s rRNA gene allowing for greater phylogenetic resolution downstream. A 16S rRNA clone library of the ballast water sample was created from PCR products ligated into a vector used to transform competent *E. coli* cells (pGEM-T Easy Vector System, Promega). Transformed *E. coli* was grown up overnight on selective agar (ampicillin and x-gal) and 150 positive DNA insert colonies (white positive vs. blue

negative) were screened by gel electrophoresis for appropriate DNA insert size. Plasmids meeting the selection criteria were extracted from harvested colonies and stored at -20°C until later analysis.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Each clone underwent amplified ribosomal DNA restriction analysis to develop a genetic fingerprint which was then used to examine diversity of the sample. The amplicons were digested using the restriction enzymes HaeIII and RsaI (Promega) and run on an 8% polyacrylamide gel. HaeIII is a high-frequency cutting restriction enzyme that has a high average number of restriction sites and comes from the organism, *Haemophilius aegyptius*. RsaI was used as a secondary restriction endonuclease to increase fingerprint resolution. RsaI come from *Rhodobacter sphaeroides*. The recognition sequences for both of these enzymes are:

HaeIII 5'GG^CC3'

RsaI 5'GT^AC3'

The digest resulted in banding patterns based on differential enzyme cutting due to sequence variation. The gels were examined using GelCompar (Applied Maths) looking for repeating patterns. The software normalizes the gels and performs a pair-wise comparison using a neighbor joining algorithm to establish similarity values for each clone sequence. Clones with similarity values ≥ 97% were considered the same species and one clone from each pattern was sequenced at Yale University and compared to the GenBank database to establish identity.

Phylogenetic Analysis

Sequence data was analyzed with the ARB software package (ARB, 2007). Clone sequences were initially aligned using the ClustalW algorithm and individually checked against an *E. coli* reference, then later verified and adjusted using phylum-specific references.

Phylogenetic trees were reconstructed using maximum-likelihood analyses. Filters for phylogenetic subdivisions and/or groups which consider only 50% conserved regions were applied to exclude highly variable positions. Partial sequences were later added to existing trees by a special algorithm included in the ARB software that does not change the tree topology based on almost complete sequences. In addition to clone sequences, closest matched clone sequences and closest cultured sequences from GenBank records were added to increase phylogenetic resolution. For the beta-proteobacteria tree, at least one cultured representative from each family was included because the occurrence of clones was low. Also included in the trees were cultured bacteria representatives that have been found in ballast tanks previously (Ruiz et al, 2000, Drake et al., 2005, Burkholder et al., 2007) and GenBank members that were closest matches to unpublished ballast tank clone sequences (Burkholder et al., 2007). Bootstrap values were calculated for each tree with 1,000 replicates using the DNAPARS maximum parsimony tool in the PHYLIP package included in the ARB program.

To examine species diversity within the clone library, both Simpson (1-D) and Shannon (H') Indices of Diversity were calculated along with evenness (J'). To examine

total theoretical diversity of the entire ballast tank, rarefaction curves were interpolated using the Analytic Rarefaction freeware program (Analytic Rarefaction, 2009) followed by a coverage calculation (C). Coverage values give an estimate of the percentage of the total population that has been successfully sampled, and is calculated by the formula:

$$C = 1-(n_i/N)$$

Where N is the total number of ARDRA patterns detected, and n_i is the number of patterns that only appeared once.

Analysis of almost complete 16S rRNA clone sequences using ARB and the GenBank databases determined the lowest phylogenetic lineage of each clone. Additionally, the most likely habitat associated with the closest 16S rRNA relative (cultured isolate or environmental clone) was used to classify each clone in our study as either marine pelagic, coastal, estuarine, or freshwater based upon typically associated bacterial groups (Nold and Zwart, 1998; Acinas et al., 1999; Cottrell and Kirchman, 2000; Eilers et al., 2000; Castle and Kirchman, 2004; Bouvier and del Giorgio, 2002; Giovannoni and Stingl, 2005). In this study, coastal is defined as associated with marine coastal zones but not estuaries, and endosymbionts were categorized as “other”, since these organisms are not free-living in the upper water column.

Accession Numbers

All sequences were submitted to GenBank under accession numbers: FJ666139-FJ666209.

Results

A total of 83 clones were generated from ballast water sample AO06° 57.3S, 008° 15.3W. ARDRA revealed 60 unique banding patterns or 16S rRNA Operational Taxonomic Units (OTU) that were then sequenced for phylogenetic analysis. The Simpson's Index of Diversity was calculated to be 0.97, indicating that any two clones from the library have a 97% chance of being two different OTU. The Shannon Diversity Index determined high diversity of OTUs with a H_{max} of 4.09 and H' of 3.88. Evenness (J') was calculated to be 0.95, indicating an even species distribution among the total population. Rarefaction analysis revealed in a steep curve (Fig. II. 2), indicating that the clone library did not detect all the diversity in the ballast tank. Similarly, coverage was calculated to be 40%; that is to say, only 40% of the total diversity of this ballast tank was represented in the clone library created in this study and 60% remains unknown.

The predominant groups of bacteria found were from the alpha- and gamma-proteobacteria. Other groups detected were the betaproteobacteria, planctomycetes, actinobacteria, bacteroidetes and verrucomicrobia (Table II. 2). Thirteen clones had a 95-99% similarity to a cultured isolate in GenBank (Table II. 3) that were used to infer carbon or energy source for ballast water bacteria. Three of the cultured isolates are degraders of hydrocarbons, alkanes or other organic compounds, along with one clone that was most closely related to a Dibenzofuran-degrading clone (Table II.2).

The ‘originating’ habitat of the ballast water bacterial community closest relatives, determined by comparison to the ARB and GenBank databases, was predominantly marine pelagic (40%), followed by estuarine (37%), other (14%), coastal (6%), and finally freshwater (3%) (Table II. 2; Fig. II. 3).

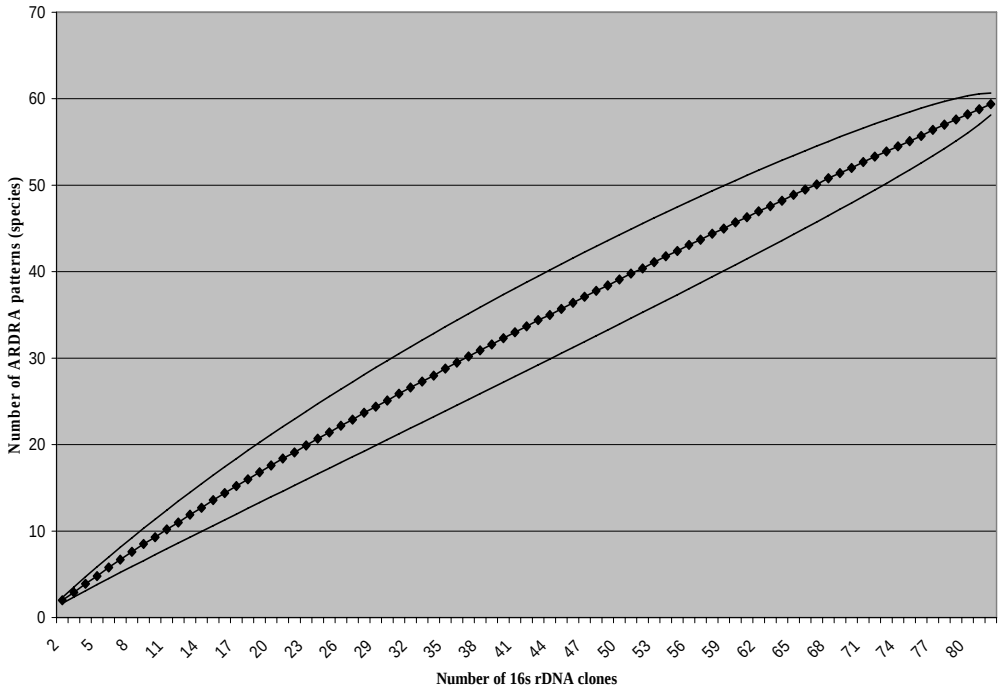

Fig. II. 2. Rarefaction curve for clone library AO06’57.3S008’15.3W.

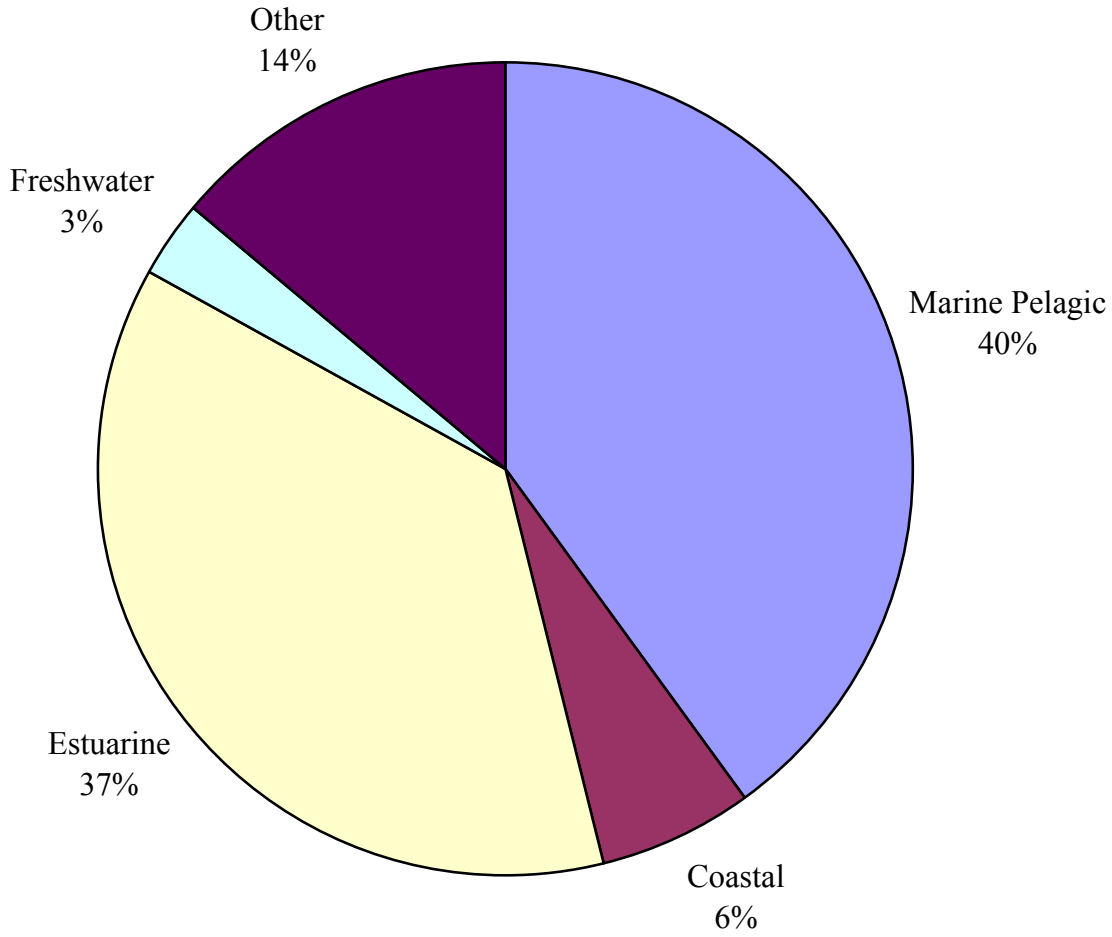

Fig. II. 3. Habitat characterization of ballast tank bacterial community members.

Table II. 2. 16S rRNA affiliation and habitat classification of clone library AO06’57.3S008’15.3W.

Phylum	Habitat	Clone	Lowest Defined Lineage	Closest Relative/origin	Accession Number	% Similarity
α-Proteobacteria	Marine Pelagic	67		Clone 205 from 200 m depth in N. Aegean Sea	EU373875	95
	Marine Pelagic	139		clone P9X2b7E12 from seafloor lava at the Loi'hi Seamount, HI	EU491201	93
	Marine Pelagic	201		clone P9X2b7E12 from seafloor lava at the Loi'hi Seamount, HI	EU491201	93
	Marine Pelagic	54	*Rhodospirillaceae*	clone ZA2526c from a trans Atlantic cruise	AF382105	91
	Marine Pelagic	60		clone b115 from Konigsfjorden, Svalbard	EU919810	95
	Marine Pelagic	61		clone P9X2b7E12 from seafloor lava at the Loi'hi Seamount, HI	EU491201	91
	Marine Pelagic	88		clone P9X2b7E12 from seafloor lava at the Loi'hi Seamount, HI	EU491201	93
	Marine Pelagic	22		clone S23_377 from Cocos Island, equatorial Pacific	EF572278	91
	Coastal	129	*Rhodobacteraceae*	isolate JH10_C12 from intertidal flat Gangwha, S. Korea	AY568770	97
	Estuarine	203	*Rhodobacteraceae*	*Pseudoruegeria aquimaris* strain SW-255	DQ675021	96
	Estuarine	68	*Rhodobacteraceae*	Rhodobacterales clone: pItb-vmat-58.	AB294959	96
	Estuarine	58	*Rhodobacteraceae*	*Thalassobius aestuarii* from the Yellow Sea (Pechili Bay), Korea	DQ535898	95
	Estuarine	105		clone 3C002614 from Chesapeake Bay, MD	EU801348	97
	Estuarine	23		alpha proteobacterium DG1252 dinoflagellate associated	DQ486492	95
	Estuarine	234		clone 3C002932 from Chesapeake Bay, MD	EU801596	96
	Estuarine	25		clone 3C002413 from Chesapeake Bay, MD	EU801176	97
	Freshwater	211	*Rickettsiales*	clone K2-S-24 16S from Lake Kauhako, HI	AY344373	94
	Other	199	*Rhodobacteraceae*	clone STX_15f from coral having black band disease	EF123331	97
	Other	206		clone ELB16-121 from Lake Bonney Antarctica	DQ015800	92
	Other	233		clone ctg_NISA166 from deep sea octacoral	DQ396148	96
	Other	31	*Rhodobacteraceae*	clone ctg_CGOF319 from deep sea octacoral	DQ395608	96
γ-Proteobacteria	Marine Pelagic	224	*Alteromonadaceae*	*Alteromonas* sp. CF11-5 from deep sea sediment, China	FJ170012	99
	Marine Pelagic	150	*Thiotrichales*	Dibenzofuran-degrading bacterium DBF-MAK isolated from seawater	AB086228	99
	Marine Pelagic	212	*Alcanivoraceae*	*Alcanivorax dieselolei* strain PR56-2 from Southwest Indian Ocean deep sea water	EU440990	99
	Marine Pelagic	228		clone 6C232252 from seawater 250 miles from Panama City	EU804347	95
	Marine Pelagic	239		clone 6C232252 from seawater 250 miles from Panama City	EU804347	96

Table II. 2. Continued.

Phylum	Habitat	Clone	Lowest Defined Lineage	Closest Relative/origin	Accession Number	% Similarity
	Marine Pelagic	52	*Alteromonadaceae*	*Marinobacter* sp. P78 from deep sea sediment	EU864262	99
	Marine Pelagic	75	*Alcanivoraceae*	*Alcanivorax indicus* strain B114 isolated from Indian Ocean	EF583624	91
	Marine Pelagic	124		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	97
	Marine Pelagic	144		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	97
	Marine Pelagic	202		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	97
	Marine Pelagic	208		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	97
	Marine Pelagic	27		clone S23_1272 from Cocos Island, equatorial Pacific	EF573173	97
	Marine Pelagic	30		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	95
	Marine Pelagic	49		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	96
	Marine Pelagic	6		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	97
	Marine Pelagic	90		clone S23_215 from Cocos Island, equatorial Pacific	EF572116	98
	Coastal	98		clone DR550SWSAEE23 from subsurface sediment in Kalahari Shield, South Africa	DQ354722	92
	Coastal	218		clone Fitz1_7 from Great Barrier Reef calcareous sediments	DQ256678	98
	Estuarine	9	*Pseudomonadaceae*	Gamma proteobacterium CL-CB467 associated with cyanobacteria, Korea	EF988654	95
	Estuarine	131		clone CB22C01 from Chesapeake Bay, MD	EF471694	99
	Estuarine	20		clone B13 from Singapore seawater, China	EU010137	99
	Estuarine	29	*Pseudomonadaceae*	Gamma proteobacterium CL-CB467 associated with cyanobacteria, Korea	EF988654	99
	Estuarine	45	*Pseudomonadaceae*	Gamma proteobacterium CL-CB467 associated with cyanobacteria, Korea	EF988654	99
	Estuarine	204		clone D8S-33 from the Yellow Sea (Pechili Bay) sediment, Korea	EU652559	93
	Estuarine	118	*Alteromonadaceae*	*Marinobacter flavimaris* strain SW-145 from the Yellow Sea (Pechili Bay) seawater, Korea	AY517632	99
	Estuarine	43	*Thiotrichales*	*Cycloclasticus spirillensus* from Boston Harbor, MA	AY026915	93
	Estuarine	66	*Idiomarinaceae*	*Idiomarina baltica* strain SS-01 from Palk Bay sediments, India	EU624441	99
	Other	57	*Halomonadaceae*	clone ctg_NISA102 from deep sea octacoral	DQ396041	93
	Other	17	*Halomonadaceae*	clone ctg_NISA091 from a deep sea octacoral	DQ396289	95
	Other	110	*Oceanospirillaceae*	clone 1.17 from echinoid lesions	AM930462	92
β-Proteobacteria	Estuarine	4	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	97

Table II. 2. Continued.

Phylum	Habitat	Clone	Lowest Defined Lineage	Closest Relative/Origin	Accession Number	% Similarity
	Estuarine	28	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	98
	Estuarine	53	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	99
	Estuarine	59	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	99
	Estuarine	82	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	99
	Estuarine	113	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	99
	Estuarine	205	*Burkholderaceae*	clone 1C227376 from Newport Harbor, RI	EU799733	99
Actinobacteria	Freshwater	48		clone K2-30-12 from volcanic crater Lake Kauhako, HI	AY344421	98
Bacteriodetes	Marine Pelagic	15	*Sphingobacteriales*	Uncultured Cytophaga sp. clone: BD1-15 from deep sea sediments	AB015524	95
	Marine Pelagic	96	*Sphingobacteriales*	clone 149H54 from sediments in N. Bering Sea	EU925914	91
	Other	240	*Flavobacteriaceae*	clone B2706_G2 from Caribbean reef sponge	EF092235	98
	Other	134	*Cryomorphaceae*	clone PEACE2006/31_P3 from Bay of Biscay, Spain/Portugal	EU394563	91
Planctomycetes	Coastal	37		clone 7F15 from coastal seawater	AF029079	93
Verrucomicrobia	Estuarine	128		clone CB from Bohai Bay, China	FJ155059	95

Table II. 3. Clones with 95-99% 16S rRNA sequence similarity to cultured isolates in GenBank. Carbon and energy sources of isolates were used to interpret substrate utilization of clones.

Class	Habitat	Clone Number	Accession Number	Nearest Cultured Isolate in GenBank/Carbon Utilization	% Similarity	Query Coverage
Gamma	Coastal	224	FJ170012	Alteromonas sp. CF11-5 16S ribosomal RNA gene, partial sequence/chemoorganotroph	99	100
Gamma	Estuarine	45	EU143369	Cellvibrio sp. J115 16S ribosomal RNA gene, partial sequence/ chemoorganotroph	98	95
Gamma	Estuarine	29	EU143369	Cellvibrio sp. J115 16S ribosomal RNA gene, partial sequence/ chemoorganotroph	97	96
Gamma	Marine	212	EU440990	Alcanivorax dieselolei strain PR56-2 16S ribosomal RNA gene/branched alkene degrader (oil carbon source)	99	100
Gamma	Marine	150	AY026915	Cycloclasticus spirillensus 16S ribosomal RNA gene/PAH degrader (oil carbon source)	97	99
Gamma	Marine	17	AM403724	Halomonas sp. EP33 16S rRNA gene/ chemoorganotroph	95	99
Gamma	Marine	66	EU624441	Idiomarina baltica strain SS-01 16S ribosomal RNA gene/ chemoorganotroph	99	100
Gamma	Marine	118	AY517632	Marinobacter flavimaris strain SW-145 16S ribosomal RNA gene, partial sequence/ chemoorganotroph	99	100
Gamma	Marine	52	EU864262	Marinobacter sp. p78 16S ribosomal RNA gene, partial sequence/ chemoorganotroph	99	99
Alpha	Coastal	129	DQ535898	Thalassobius aestuarii strain TF-212 16S ribosomal RNA gene, partial sequence/ chemoorganotroph	97	97
Alpha	Marine	203	DQ675021	Pseudoruegeria aquimaris strain SW-255 16S ribosomal RNA, partial/ chemoorganotroph	96	99
Alpha	Marine	68	DQ675021	Pseudoruegeria aquimaris strain SW-255 16S ribosomal RNA, partial sequence/ chemoorganotroph	96	98
Alpha	Marine	58	DQ675021	Pseudoruegeria aquimaris strain SW-255 16S ribosomal RNA, partial sequence/ chemoorganotroph	95	99

The alphaproteobacteria–32% of bacteria in ballast water were α-proteobacteria. Habitat analysis, based upon the closest phylogenetic relative and its origin, determined that most (38%) were typical of marine pelagic waters, 33% from estuarine, 5% from coastal, 5% from freshwater, and 19% other (Table II.2; Fig. II. 4a). Four clones clustered as marine pelagic due to their relatedness (91-93%) to other 16S rRNA genes detected at the Loi'hi Seamount in the Hawaii Archipelago. The other clones classified as marine pelagic were dispersed throughout the phylogenetic tree and determined to be marine pelagic by their relatedness to 16S rRNA genes or isolates from open ocean or deep sea sediment samples. One clone could be clearly classified as coastal due to its close relatedness to bacteria from an intertidal flat of Ganghwa, S. Korea. Seven clones were classified as estuarine clustering with *Thalassobius aestuarii* isolated from the Yellow Sea (formerly Pechili Bay), Korea or 16S rRNA genes detected in Chesapeake Bay. One clone was classified as freshwater, and was most closely related to bacteria from Lake Kauhako, HI. Four could not be habitat classified and were designated as 'other'. One of these was most closely related to 16S rRNA genes from Lake Bonney, Antarctica. Interestingly, the other three were most closely related to bacteria associated with corals, with one from a corals having black band disease.

The gammaproteobacteria – 45% of bacteria in ballast water were identified as gammaproteobacteria. Of these, 53% were classified as marine pelagic, 30% as estuarine, 7% as coastal, and 10% other (Table II.2; Fig. II.4b).

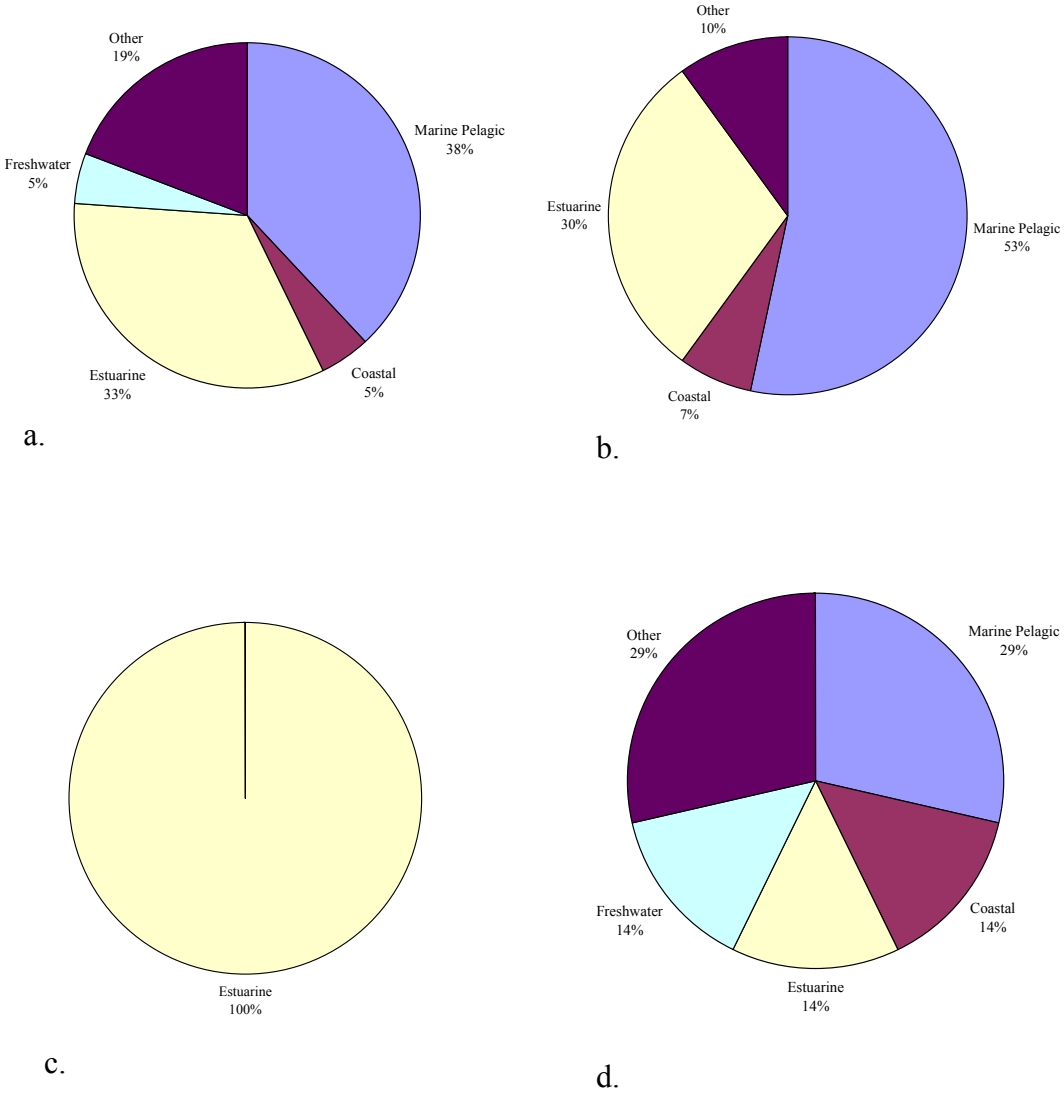

Fig. II. 4. Habitat Characterization of each major bacterial group: (a) α-proteobacteria, (b) γ-proteobacteria, (c) β-proteobacteria, and the (d) non-proteobacteria phyla.

Nine clones were closely related to cultured isolates in the GenBank database (Table II. 3) with five that are known marine genera, *Marinobacter, Idiomarina, Cycloclasticus, Alcanivorax, Halomonas. Marinobacter*, *Alcanivorax*, and *Cycloclasticus* are all degraders of organic hydrocarbons. *Alcanivorax* and *Cycloclasticus* are known specifically to degrade polyaromatic hydrocarbons (PAH) (McKew et al., 2007a; Kasai et al., 2002). Eleven of the clones did not fall into any identifiable taxon group, however, the nearest matches on GenBank were all either from water surrounding Coco's Island, off the west coast of Costa Rica, or 250 miles off the west shore of Panama. These two sites are relatively close together, over 200 miles off equatorial Central America in the Pacific Ocean, and so were classified as marine pelagic. These clones also clustered together tightly on the gamma proteobacterial tree with only 1-2% differences between sequences. The other 6 clones classified as marine pelagic were most closely related to 16S rRNA genes from deep sea sediments or known open ocean bacteria. Only two clones were classified as coastal due to their relatedness to bacteria associated with the Great Barrier Reef calcareous sediments and subsurface sediment from the Kalahari Shield, South Africa. The seven clones classified as estuarine were most closely related to bacteria from estuaries (i.e. Chesapeake Bay, Newport Harbor, Singapore, the Yellow Sea, and from cultures of cyanobacteria, most likely isolated from an estuary or freshwater). Two clones were classified as 'other'. One was most closely related to bacteria associated with deep sea coral and the other was most closely related to bacteria associated with wall lesions on echinoids.

The betaproteobacteria – all seven clones that were determined to be betaproteobacteria fell into the same cluster in the family *Burkholderiaceae*, of the order *Burkholderiales*, classifying the entire group as estuarine (Fig. II. 4c). This cluster was most closely related to bacteria detected in Newport Harbor, RI associated with the Narragansett Bay estuary (Shaw et al., 2008).

Non-proteobacteria – The rest of the clones making up 12% of the library were non-proteobacterial and could be classified as marine pelagic (29%), 14% as coastal, 14% as estuarine, and 29% other (Table II.2; Figs. II.4d). The only Actinobacterial clone was most closely related to bacteria from Lake Kauhako, HI, a freshwater volcanic crater lake. One planctomycete clone was identified and most closely related to an endosymbiont of a sponge and so was classified as coastal. One verrucomicrobia clone was identified and most closely related to a clone from Chinese bay soil and was classified as estuarine. Four bacteroidete clones were identified. Two clones were classified as marine and determined to be in the order *Sphingobacteriales*, and related to deep-sea or Bering Sea sediments (accession numbers AB015587 and EU925914, respectively). Another clone fell into the family *Cryomorphaceae* and was most closely related to a clone from the Bay of Biscay between Spain and France (accession number EU394563) and classified as estuarine- The last clone fell into the family *Flavobacteriaceae* and was most closely related to an endosymbiont of the reef sponge *Axinella corrugata* (accession number EF092235), classifying it as a coastal clone. Figures II.5-II.8 show the results of the phylogenetic analyses.

Fig. II. 5. Phylogenetic tree of alphaproteobacteria diversity in ballast water sample '06° 57.3S, 008° 15.3W' and closest 16S rRNA relatives used to classify habitat. The scale bar indicates 10% estimated sequence divergence.

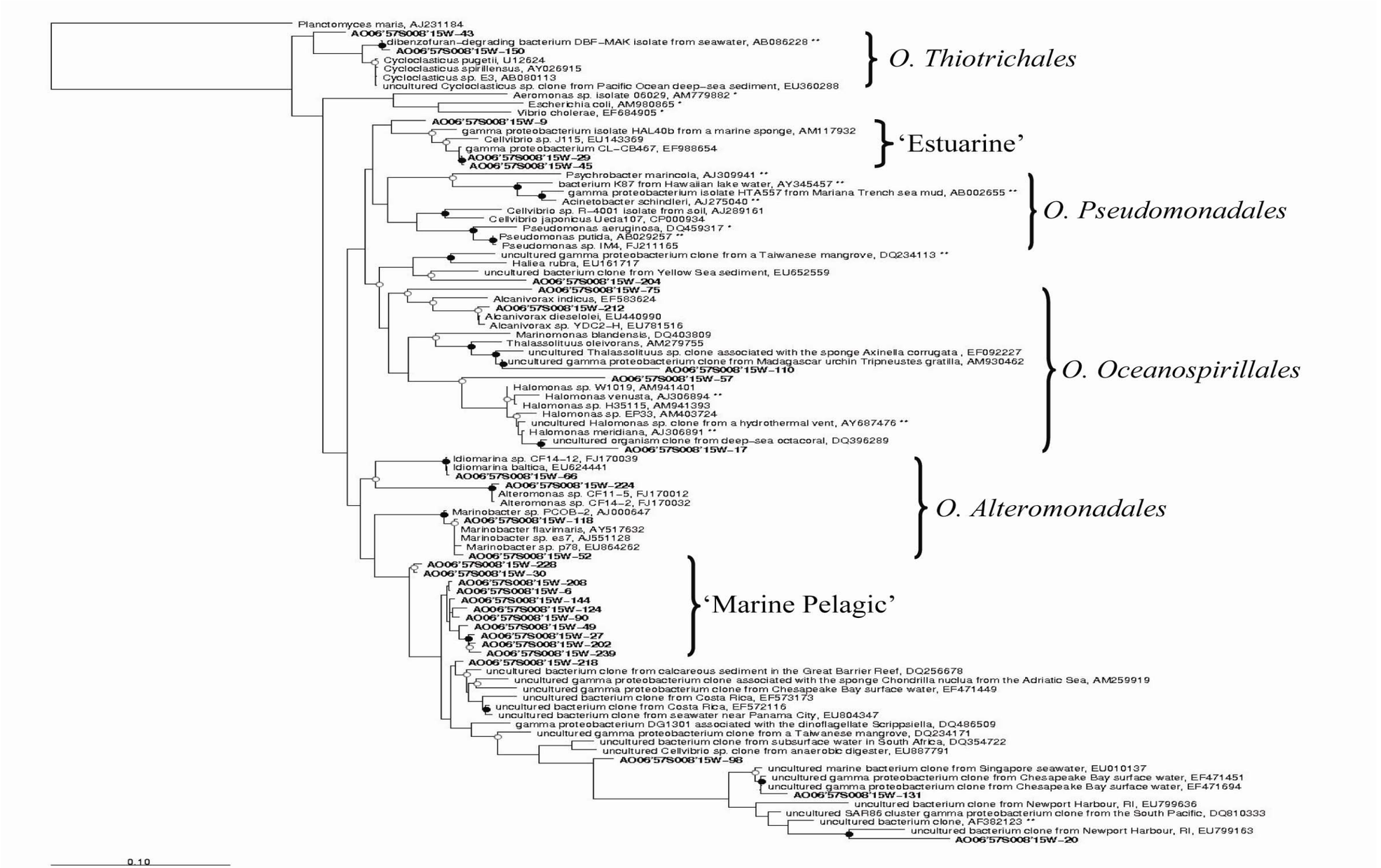

Fig. II. 6. Phylogenetic tree of gammaproteobacteria diversity in ballast water sample '06° 57.3S, 008° 15.3W' and closest 16S rRNA relatives used to classify habitat. The scale bar indicates 10% estimated sequence divergence.

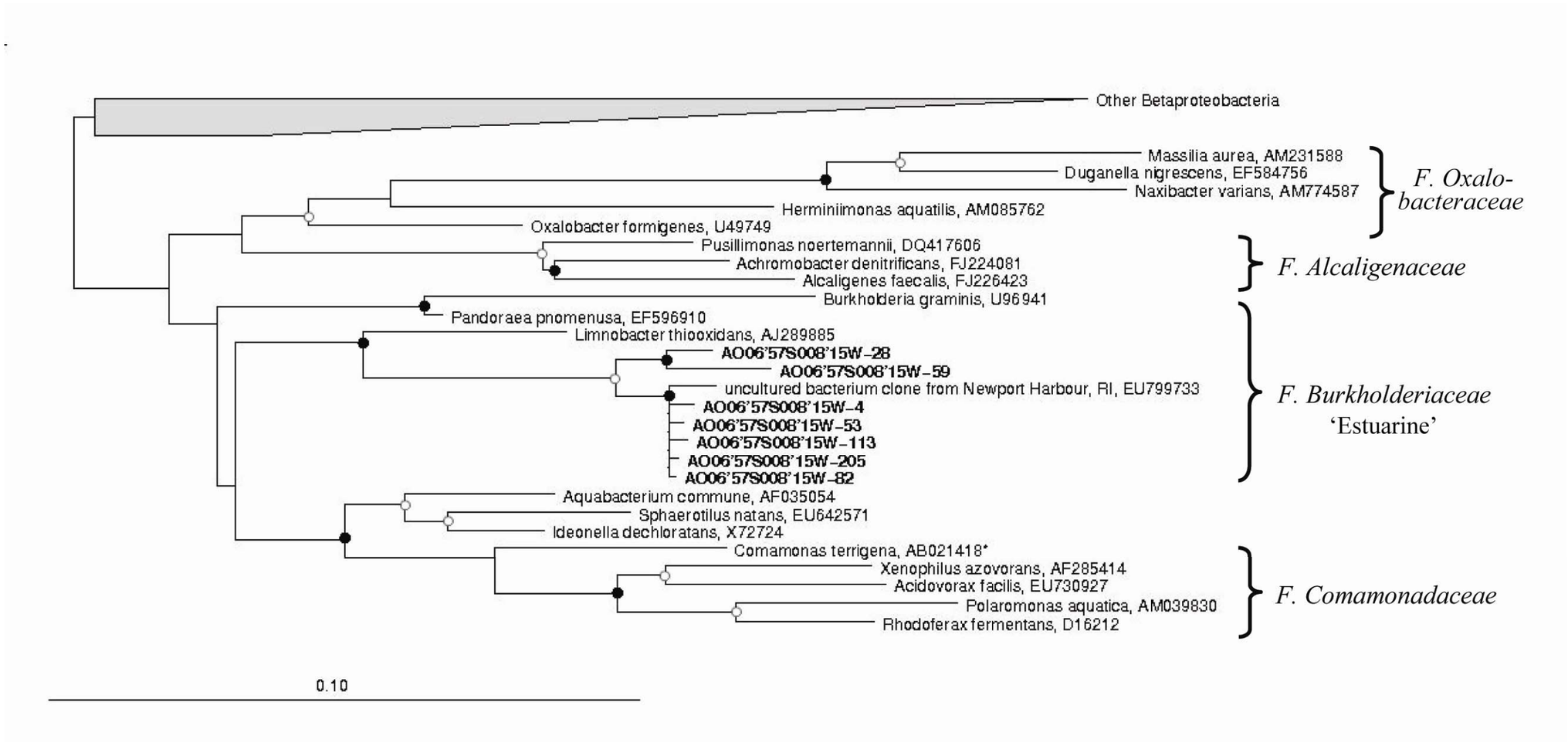

Fig. II. 7. Phylogenetic tree of betaproteobacteria diversity in ballast water sample '06° 57.3S, 008° 15.3W' and closest 16S rRNA relatives used to classify habitat. The scale bar indicates 10% estimated sequence divergence.

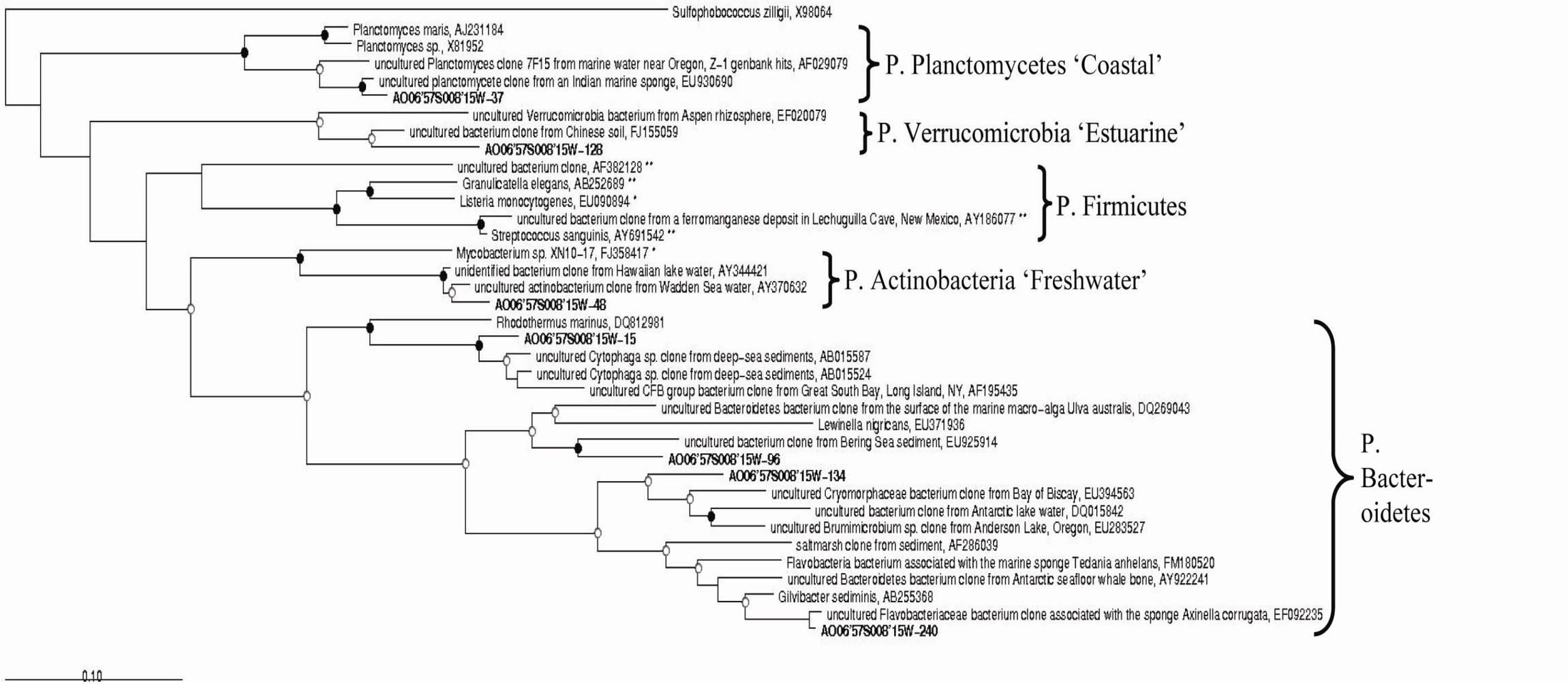

Fig. II. 8. Phylogenetic tree of all other bacterial phyla diversity in ballast water sample '06° 57.3S, 008° 15.3W' and closest 16S rRNA relatives used to classify habitat. The scale bar indicates 10% estimated sequence divergence.

Discussion

The presence of bacteria in the ballast water tank in this study, along with previous studies that have found bacteria in ballast water, indicates a risk of invasion when deballasted into port water if the bacteria survive. Because of concerns for community dynamics of aquatic ecosystems, it was imperative to look beyond pathogenic bacteria harmful to eukaryotic organisms and into the total diversity of microbial immigrants. As stated previously, because a tanker can hold up to over 50,000 metric tons of ballast water, high levels of bacteria in the hundreds of ships in commercial trade presents a very serious invasive threat. This chapter tested the first hypothesis:

> *Hypothesis 1:* Ballast water bacterial communities will resemble those of open-ocean, upper water column communities and exhibit low diversity.

Diversity of Ballast Water Bacteria

No direct comparison of the diversity of the ballast water in this study to other ballast tanks is possible since this is the first study to examine overall bacterial diversity in a ship's ballast tank. Our *a priori* assumptions were that ballast tank bacterial diversity would be relatively low and unevenly distributed due to lack of primary production and thus fresh DOM so that during the months-long journey and competition for these vanishing nutrients would have favored the survival of only a few species. Even though the diversity within the clone library was high, the low coverage value and

steep rarefaction curve indicate that the total bacterial community of this ballast tank was not fully detected. Since no direct comparison to other ballast tank studies was possible, we examined diversity in representative clone libraries from coastal waters and the open ocean. The rarefaction curve from this study was compared to rarefaction curves created from clone libraries of bacteria from surface waters of the Mediterranean Sea (attached and free-living) (Acinas et al., 1999), the Californian Coast (Cottrell and Kirchman, 2000), the Oregon Coast (Rappe et al., 2000), and the North Sea (Eilers et al., 2000) (Fig. II. 9). Both of the Mediterranean clone libraries were very small (5-8 OTUs), but the curves are quite different between the attached (curve B: Fig. II. 9) and free-living bacteria (curve C: Fig. II. 9). The attached bacteria curve leveled off at around 18 clones, showing that the total diversity was represented. However, the free-living curve is still very steep, indicating that the total bacterial diversity of that habitat was not successfully sampled in the 16 clones. In the attached clone library, all clones but one belonged to the gammaproteobacteria, and the free living clone library was composed of alphaproteobacteria (53%), gammaproteobacteria (35%), and 6% (one clone only) each of cyanobacteria and bacteriodetes. Interestingly, the diversity of the North Sea (curve E: Fig. II. 9) was predominated by the gammaproteobacteria (92.5%), including SAR86. The alphaproteobacteria made up only 7.5%, and the epsilonproteobacteria constituted the remaining 2%. However, with 53 clones, the diversity has been successfully sampled as the curve has leveled out. Even the Californian coastal waters curve (curve D: Fig. II. 9), predominated by alphaproteobacteria (27%) and gammaproteobacteria (49%), containing clones belonging to the betaproteobacteria, planctomycetes, bacteriodetes,

verrucomicrobia, firmicutes, actinobacteria and chloroplasts, shows successful diversity sampling. The curve begins to decrease in slope around clone #30, and even further decreases around clone #60, while the rarefaction curve of the clone library in this study (curve A) is still steep at 82 clones. The Oregon coastal waters curve (Curve F), with the highest number of clones (105), showed the same pattern of slope change as the Californian coastal water, and consisted of only the alphaproteobacteria (21%), gammaproteobacteria (19%), betaproteobacteria (9%) and chloroplasts (51%). Curve A is in fact, the steepest of all five curves, showing that the diversity level in the ballast tank of this study is higher than the surface waters of all three marine environments. The low diversity of curves E and B can most likely be explained by the particular habitat characteristics. The North Sea is much colder than the Mediterranean Sea, the Californian and Oregon coasts, or the South Atlantic, which would support fewer organisms than warm waters.

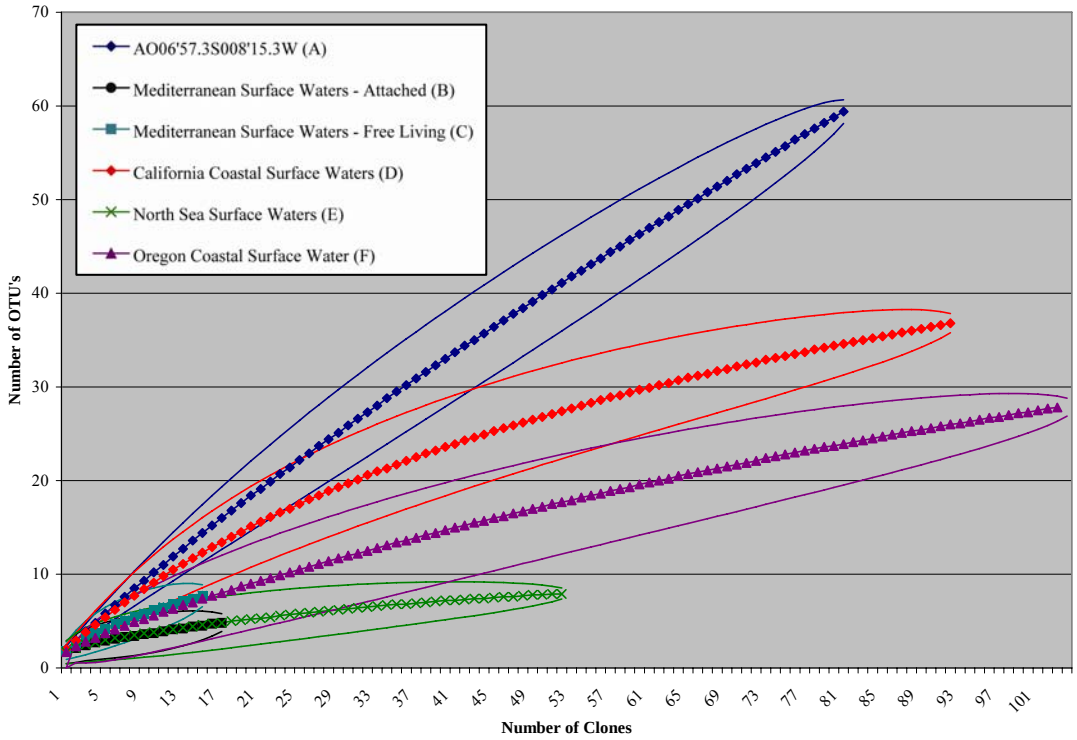

Fig. II. 9. Rarefaction curves of clone libraries from (A) ballast water (this study), (B, C) Mediterranean surface water (Acinas et al., 1999), (D) California coastal surface water (Cottrell and Kirchman, 2000), (E) North Sea surface water (Eilers et al., 2000), and (F) Oregon coastal surface water (Rappe et al., 2000). Smooth lines of the same color on either side of each curve represent the upper and lower 99% confidence intervals.

To further this comparison, diversity indices were calculated for each of these other clone libraries (Table II. 4). The Shannon and Simpson indices of diversity confirmed that the ballast tank clone library (present study) had the highest level of diversity, and well has the most even distribution of species.

Table II. 4. Clone library comparisons of ballast tank diversity with coastal and pelagic surface water diversity. H' = Shannon Index of diversity; H_{max} = maximum diversity level for data set; %H = percentage of H_{max} detected by H'; J' = Evenness; 1-D = Simpson's Index of diversity; C = Coverage value for clone library.

Clone Library	H'	H_{max}	%H	J'	1-D	C
AO06'57.3S008'15.3W	3.88	4.09	95%	0.95	0.97	40%
Mediterranean Surface Waters – Attached	0.56	1.61	35%	0.35	0.67	40%
Mediterranean Surface Waters – Free Living	0.64	2.08	31%	0.31	0.85	69%
California Coastal Surface Waters	3.28	3.61	91%	0.91	0.96	51%
North Sea Surface Waters	1.09	3.99	27%	0.27	0.66	63%
Oregon Coastal Surface Water	2.23	3.33	67%	0.67	0.72	84%

These extreme differences in diversity may indicate that the bacterial community of this ballast tank is a consortium of marine environments i.e. the original port community, the pelagic community received upon BWE, and possibly a community unique to ballast tanks. If the diverse ballast water bacteria from this study were released into and survived in any of these environments, the natural populations could be overtaken by competition for resources.

Origin of Ballast Water Bacteria

This study relied on the phylogenetic analysis and BLAST results to determine the most likely endemic habitat for each clone from the ballast tank water. It is impossible to determine the exact origin of the bacteria found in this ballast tank, however the analysis of nearest 16S rRNA relative either as environmental clones or cultured isolates as a proxies allows for reasonable interpretation. If we know the habitat of that most related bacterium, the aquatic environment of the ballast tank clone is likely comparable.

The similar percent compositions between marine pelagic bacteria (40%) and the combined coastal, estuarine and freshwater environments (46%) is a strong indication that the original port water was not completely exchanged in the open ocean by the ship sampled and ballast water exchange is not a completely efficient form of invasive species control.

Many factors may contribute to incomplete exchange, including inclement weather, limited time, and the individual structural design of the ballast tanks allowing inoculates to remain within the tank, either attached to algae or picoeukaryotes, or within biofilms. Biofilms are organic matrices that can form on any surface within the tank submerged in water, providing a protective refuge from stress factors. While containing fewer organisms than the ballast water, biofilms have been shown to harbor bacteria, virus-like particles and microalgal cysts (Drake et al., 2005). These biofilms may produce large amounts of exopolymer secretions, which protect the organisms from mechanical or chemical removal and predation (Drake et al., 2007). These protective

films may act as “safe houses” for bacteria during transport, with bacteria becoming resuspended in ballast water after multiple flushings and released into new environments.

If the ship sampled had filled its ballast tanks in a port(s), then the turbid water in that system may have led to the deposition of many sediment particles and their associated bacteria in the ballast tank. During transport, the sediment would have settled to the bottom of the tank along with a lot of the estuarine bacteria, and while ballast exchange may have mixed up the sediment, it was not sufficient to thoroughly flush it out. Bacteria indigenous to the port habitat would most likely have little trouble surviving in the similar conditions found in the Port of Houston. Competition for available nutrients would inevitably favor some species over others. Thirteen of the ballast tank clones were closely related to known chemoorganotrophs able to utilize a variety of hydrocarbon and other chemicals for their carbon and energy sources. With the high commercial activity level of the port and abundance of petro-chemical refineries within ports, oil-degrading bacteria could flourish. Moreover, these bacteria may utilize chemicals and hydrocarbons associated with ballast water to survive and even persist in this environment. *Alcanivorax*, found in this study, degrades the branched alkanes and are very prominent in oil-impacted environments (McKew et al., 2007b). Another important fraction of crude oil is the aromatic hydrocarbons, which are more toxic and present a longer-term environmental problem. *Cycloclasticus*, also found in this study, degrades polynuclear aromatic hydrocarbons (PAHs) (McKew et al., 2007a) and is already present in the Gulf of Mexico (Head et al., 2006). Its presence in the Gulf of

Mexico may indicates a hospitable environment for the genus. Introduced *Cycloclasticus* could survive and flourish along with the endemic members. Both *Alcanivorax* and *Cycloclasticus* rely almost exclusively on oil as a carbon source (Yakimov et al., 2007). It is well known that hydrocarbon degradation is limited by the availability of inorganic nutrients, mainly nitrogen and phosphorus (Leahy and Colwell, 1990). The addition of nutrients increases degradation rates. In environments were *Alcanivorax* levels are undetectable, addition of oil increases population levels to 30%. When nutrients are then added to the system, population levels increase to 70 – 90% (Head et al., 2006). McKew et al (2007b) found that there is possibly a synergistic relationship between *Alcanivorax* and *Cycloclasticus*, as PAH degradation rates increased following addition of *Alcanivorax* and nutrients, but *Alcanivorax* cannot degrade PAH. It was speculated that this is due to an extracellular glucose lipid biosurfactant, produced by *Alcanivorax borkumensis*, that increases the bioavailability of PAHs for degradation by *Cycloclasticus*. In addition to nutrients, mixing actions, such as waves and wind, cause oil and water emulsions to form, which are very important for hydrocarbon uptake by bacteria. During the process of mid-ocean ballast exchange, this very mixing would be very prevalent, allowing for oil-degrading bacteria already in the ballast tank to take advantage of the new carbon source. Also, it was found that oil-degrading bacteria themselves from both marine and sediment cultures were capable of strong emulsifying abilities (Leahy and Colwell, 1990). Although studies of non-pathogenic bacteria in ballast water are scarce, one study (Burkholder et al., 2007),

detected the same dibenzofuran-degrading bacterium (AB086228) as our study indicating that this ability may be beneficial to survival in the ballast tank environment.

Bacteria detected by this study included members of the alpha-, gamma- and betaproteobacteria, actinobacteria, planctomycetes, verrucomicrobia, and bacteroidetes. The alpha- and gamma-proteobacteria are known ubiquitous groups in both freshwater and marine ecosystems (Nogales et al., 2007, Nold and Zwart, 1998) and are known to dominate the marine pelagic zone. Interestingly, we did not detect bacteria any of the SAR bacterial clades (i.e. SAR11 or SAR86; Giovannoni et al. 1990; Morris et al. 2002), found throughout the world in marine pelagic waters. A possible explanation is the exchange of ballast water near the coast or that the ballast tank environment is inhospitable to these highly oligotrophic groups (Rappe et al. 2000). Most of the groups detected represent bacteria associated with particles and appear to be highly adaptable to a range of physico-chemical conditions (i.e. salinity, pH, temperature, light).

In the alphaproteobacteria, one member of the family Rhodospirillaceae was found (clone 54, Table II. 2). Rhodospirillaceaens are typically found in anaerobic aquatic environments and are photoorganotrophic. They grow anaerobically in the presence of light, but in the absence of light, they can grow aerobically (Pfenning, 1978). These bacteria could be introduced into the ballast tank during intake of turbid waters, with the bacteria originally among the anaerobic muds at the bottom of estuaries. Although generally not pathogenic, the ability to survive in the tank could make this family a common component of ballast water bacteria resulting in multiple introductions and potential bioinvasions (see Chapter IV).

One member of the order Rickettsiales was found (clone 211, Table II. 2). This is of concern because this order consists of parasitic or mutualistic bacteria, and contains the pathogen Rickettsia, the organism responsible for the Rocky Mountain spotted fever. Many of the alphaproteobacteria belonged to the family Rhodobacteraceae, an ecologically diverse group of bacteria that are mainly aquatic, require NaCl for growth, and encompasses photoheterotrophs and chemoorganotrophs. *Pseudoruegeria aquimaris* is a gram-negative rod that is found in saline waters, where it gets it name, isolated from the coastal waters in the East Sea, Korea (Yoon et al., 2007). *Thalassobius aestuarii* was isolated from tidal flat sediment in Ganghwa Island, Korea, and tends to prefer lower salinities than *Pseudoruegeria aquimaris*, is a strict aerobe, but would still survive ballast tank conditions (Yi and Chun, 2006).

Members of the gammaproteobacteria included *Alteromonas*, an open-ocean or coastal bacterium. The *Cellvibrio* found in this study (clones 29 and 45) did not fall in with the rest of the Pseudomonadaceae family, so this classification is dubious. Typically, *Cellvibrio* are aerobic soil bacterium, but the validity of this genus has been debated over time, and not all bacteria labeled *Cellvibrio* belong to this group (Blackall et al., 1985). The two clones falling into the genus have been omitted from this discussion because their ecology and lifestyle are not certain. *Halomonas* contains over 20 separate species and thrives in hypersaline (>30ppt) waters, usually cultured between 30-150ppt. However, many of the species can grow in salinities between 5-30ppt, although most have optimums with a high-end range of over 50ppt. *Idiomarina baltica* and *Marinobacter flavimaris* are two halophilic, marine bacteria that would be present in

the upper water column of the pelagic habitat (Brettar et al., 2003; Yoon et al., 2004). The last two gammaproteobacteria found, *Alcanivorax* and *Cycloclasticus* possess the capability to degrade components of crude oil, and will be discussed in detail later.

Although typically soil or freshwater inhabitants (Bouvier and del Giorgio, 2002), there have been betaproteobacteria found in ballast water. A specimen of *Comamonas terrugina*, a member of the family *Comamonadaceae*, was cultured by Drake and colleagues (2005), and is a bacterium commonly found in seawater aquaria and marine microfouling films (Drake et al, 2005, Zambon et al., 1984). The betaproteobacteria found in this study are more closely related to the genus *Limnobacter*, in the family *Burkholderiaceae*. *Limnobacter* species have been found in freshwater lake sediments (Spring et al., 2001) and natural mineral water (Loy et al., 2005). However, a clade of beta-proteobacteria known as the OM43, as well as clones belonging to the *Burkholderiaceae* family have been found off the coast of Oregon (Rappé et al., 2000, Morris et al., 2006), so it is possible that the clones found in this study are of estuarine or coastal origin.

The presence of actinobacteria is not surprising as they have been found in previous studies, consist of a very large phylum, and are found in many diverse environments, including marine waters (Glockner et al., 2000, Ventura et al., 2007). The ecology and lifestyles of these bacteria are diverse, inclining the bacteria to survival in most aquatic habitats, including open-ocean as well as coastal and estuarine, as well as within a ballast tank.

Three groups that were found are the first in ballast water literature. These are the planctomycetes, the verrucomicrobia and the bacteroidetes. The planctomycetes are found in both aquatic and terrestrial habitats, both free living and endosymbiotic (Wagner and Horn, 2006). Importantly, they are players in the nitrogen cycles of the ocean, contributing to 50% of oceanic nitrogen loss due to the abilities of anaerobic oxidation of ammonium and converting nitrate and nitrate into N_2 (Wagner et al., 2006, Strous et al., 2006). They also are active in the sludge of a wastewater treatment plant (Chouari et al., 2003), most probably due to their nitrogen-altering abilities. The presence of these bacteria in the ballast tank is neither surprising nor indicative either way of oceanic or estuarine origin, but may play a role in nutrient availability in the ballast tank, providing nitrogen sources for the tank inhabitants, prokaryotic and eukaryotic.

The verrucomicrobial clone is an oddity. While it is true that verrucomicrobia are found in marine sediments, water and algae (Yoon et al., 2008, Wagner and Horn, 2006), the nearest GenBank relatives were clones derived from soil habitats. Verrucomicrobia are immensely important in soil environments, and tend to live in association with eukaryotes (Wagner and Horn, 2006). The presence of this group, particularly being closest to soil relatives, lends further evidence that the community in this ballast tank contains estuarine as well as marine pelagic members.

The bacteroidetes are one of the most abundant groups of aquatic bacteria, and have been found in coastal seawater, oceanic water, sea ice, and freshwater (O'Sullivan et al., 2004, DeLong et al., 1993). In one study examining Plymouth coastal seawater in

the United Kingdom, bacteroidetes made up 66% of bacteria found (O'Sullivan et al., 2004). Only four of the clones fell into the bacteroidetes. This group is typically associated with phytoplankton and the presumed lack of fresh, labile, dissolved organic material may explain the limited representation in the ballast tank.

While we detected no human pathogenic bacteria in the ballast tank, we did detect several clones that were closely related to black band coral and echinoid disease associated bacteria. Previous studies of bacteria isolated from ballast water have also implicated ballast water with known marine invertebrate diseases. Aguirre-Macedo et al (2008) found *Serratia marcescens* and *Sphingomonas spp*., two bacteria known to be associated with white pox and white plague type II coral disease. White pox disease causes white lesions that cover the entire coral, damaging tissue. White plague disease, type II, causes coral tissue to slough off, leaving a white band of coral skeleton at a rapid rate, quickly killing small coral communities. Black band coral disease is similar to the white plague disease, and is characterized by a single black band that migrates down the coral, leaving the coral skeleton behind, but is caused by the association of cyanobacteria and sulfur-oxidizing and reducing bacteria (Richardson, 1998; Cooney et al., 2002). Transport and introduction of these coral pathogens into Texas Gulf waters could cause considerable damage to the Flower Garden Banks, a collection of coral reefs about 150 miles offshore of Galveston. Another non-human disease brought on by bacteria is the bald-sea urchin disease. This disease is characterized by the formation of lesions on the outer surface of the sea urchin, starting at the base of select spines, and eventually causing the entire epidermis to become necrotic and deteriorate (Jangoux, 1987). Several

bacteria are connected with this disease, including *Vibrio anguillarum*, *Aeromonas salmonicida*, *Vibrio parahaemolyticus*, *Exiguobacterium* sp., as well as many other alpha- and gammaproteobacteria and bacteriodetes (Becker et al., 2007; Becker et al., 2008; Jangoux, 1987). While *Vibrio parahaemolyticus* is already present in Texas waters, further introduction of it, or any other of these bacteria could be detrimental to the echinoderm populations.

All the pathogens found in previous studies (Burkholder et al., 2007; DePaola et al., 2003; Drake et al., 2005; Drake et al., 2007; Ruiz et al., 2000) were not found in this clone library. The closest clone to being a pathogen was in the order *Rickettsiales,* which includes the genus *Rickettsia*, the bacterium known for causing spotted fever. Also not detected in this study were the cyanobacteria. Found in at least one study (Burkholder et al., 2007), these organisms dominate the top layers of marine water, where light is readily available for photosynthesis. Absence of this group may be due to die-off in a dark ballast tank with no available light over a month-long journey.

It is possible that the bacteria detected in this clone library consists of bacteria that posses the right phenotypic traits for survival in the ballast tank, and may compose a typical "ballast tank community", that can be found in most ballast tanks. Bacteria that can survive the dark, tumultuous travel by "hiding out" in biofilms or in sediment deposits would persist within the tank environment and proliferate. Additional studies of this type will be required for comparison to determine if this hypothesis is correct.

Further studies are necessary for assessing the true potential threat of ballast water tank bacterial communities. Multiple ships from different origins might contain

entirely different bacterial communities. Some ships, traveling among ports of the same country (i.e. same geographic waters), are not required to undergo ballast exchange beyond the EEZ and can deballast at the port. Other parameters of the ballast tanks should be examined, such as pH, salinity and nutrient levels to understand conditions conducive to bacterial survival. Because 16S rRNA studies can only determine that a bacterium was in the tank, but not necessarily alive, viability studies on ballast tanks are needed to examine if any of the bacteria that survive these long journeys can indeed survive in arriving port water. The presence of bacteria that posses the capability to use oil as a carbon source does not provide conclusive evidence that oil degradation is occurring. Reverse transcription of mRNA that codes for the enzymes involved would be a possible method to determine the metabolic activity of these bacterial groups.

This study provides evidence that bacteria in the ballast tank of commercial cargo ships are still a viable invasive threat. This threat is recognized by the Environmental Protection Agency (EPA), which is considering the requirement of commercial permits for ballast water discharge (Lovell and Drake, 2009). These permits would continue the practice of BWE beyond the EEZ, but also that ships that remain in coastal waters would be required to exchange ballast water at least 50 knots offshore. In addition to the standard ballast water exchange practices, bacterial and other invasive species eradication methods are still in development.

CHAPTER III

SPATIAL AND TEMPORAL COMMUNITY COMPARISONS IN BALLAST WATER, THE PORTS OF HOUSTON AND GALVESTON, AND TEXAS BAY AND PORT SYSTEMS

Introduction

Texas Ports and Estuaries

Very little is known about the endemic bacterial populations along the Texan portion of the Gulf Coast of Mexico. Studies to date are limited to Galveston Bay and have focused only on pathogenic *V. vulnificus* (Lin et al., 2003; Lin and Schwarz, 2003; Vanoy et al., 1992), *V. cholerae* (Davis and Sizemore, 1982), and *V. parahaemolyticus* (Myers et al., 2003), as well as salmonellae and fecal coliforms Goyal et al., 1977). One goal of this study was to provide an unbiased 'first look' at bacterial communities in Texas bays and estuaries and to determine if there are differences in community composition from bay to bay.

The eight bay systems and ten ports included in this study span Sabine Lake in the northernmost region of the Texas gulf coast (between Texas and Louisiana), to the Port of Brownsville, in the southernmost region of the Texas gulf coast, between Texas and Mexico.

Sabine Lake (including Sabine Pass) lies in the Sabine River basin, and receives freshwater inflow from both the Sabine and the Neches Rivers (Fig. III. 1). The three ports that are located in the Sabine system are the Ports of Beaumont, Arthur, and Orange. The Port of Orange is the easternmost port in Texas at the border with

Louisiana, located 12 miles above the Sabine River outlet into Sabine Lake. The Port of Beaumont is 42 miles inland along the Sabine-Neches ship channel. It is important, both commercially, and militarily, as it is the 2nd largest U. S. military port in the world (Port of Beaumont, 2009). Port Arthur lies on the west bank of Sabine Lake, near the mouth of the Neches River.

Galveston Bay lies in three coastal basins and one river basin: the Neches-Trinity Coastal basin to the east, the Trinity-San Jacinto Coastal basin and the San Jacinto River basin to the north, and the San Jacinto-Brazos Coastal basin to the west.

The bay receives freshwater mainly from the San Jacinto and the Trinity Rivers. Two major ports are found in Galveston Bay: the Ports of Houston and Galveston. The Port of Houston is a 25 mile-long complex located at the end of the Houston Ship Channel, which extends all the way through Galveston bay into the Gulf of Mexico. Galveston Island is a barrier island found at the southern end of Galveston Bay. The body of water between Galveston Island and the Texas mainland is West Bay. The Port of Galveston lies along the north shore of the barrier island, lining shipping channel created between Galveston Island and the smaller Pelican Island. Both of these ports are discussed in a later section.

Fig. III. 1. Texas river and coastal basins. U. S. Geological Survey, 2009.

LaVaca Bay is the most western and inland section of the larger Matagorda Bay, and lies within two costal basins and one river basin: the LaVaca River basin to the north, the Colorado-LaVaca coastal basin to the east, and the LaVaca-Guadalupe coastal basin to the west. The major source of fresh water into LaVaca Bay is the LaVaca River. Port LaVaca is located on the east bank of LaVaca Bay in an area known as Point Comfort, across the bay from the city proper (Port Lavaca, 2009).

The Aransas Bay system is comprised of Copano Bay, inland and to the west, and Aransas Bay, to the east, opening to the Gulf. This bay system lies in the San Antonio-Nueces coastal basin, but does not receive any major river inflows of

freshwater. The Corpus Christi Bay system is comprised of Nueces Bay, inland and to the west, and Corpus Christi Bay, which opens to the Gulf. This bay system lies within two coastal basins and one river basin: The San Antonio-Nueces coastal basin to the north, the Nueces River basin to the west, and the Nueces-Rio Grande coastal basin to the south. The major source of freshwater inflow comes from the Nueces River. The Port of Corpus Christi is located within the city proper, along the west bank of the Corpus Christi Bay, where it connects to the Nueces Bay. It is significant in its import of large amounts of crude and gas oil (Port of Corpus Christi, 2009). Corpus Christi Bay is separated from the Gulf of Mexico by a series of barrier islands, the nearest of which is Mustang Island. Port Aransas is located on the north shores of Mustang Island, and is mainly a tourist destination.

The last bay system examined in this study is Baffin Bay, which lies in the Nueces- Rio Grande coastal basin, and lacks any major river source. Barrier islands lining the south Texas coast create a system known as the Laguna Madre, which extends from just above Baffin Bay down to the southern edges of Texas, where Padre Island comes close to the mainland, resulting in the Brazos Santiago Pass. About midway between Baffin Bay and the end of the Laguna Madre is Port Mansfield. Far from being considered an industrial port, Port Mansfield is mainly a recreation spot for fishing (Port Mansfield, 2009). On the south edge of the Laguna Madre is Port Isabel, ending the series of Texas bay systems. The Port of Brownsville is located at the southernmost tip of Texas at the end of a 17 mile channel to the Gulf of Mexico. This port is an important shipping port as it connects trade between Mexico and the United States, and is where

land transportation in Mexico meets with the Gulf Intracoastal Waterway (Port of Brownsville, 2009).

Ports of Houston and Galveston

The Port of Houston is located at the end of the Houston Ship Channel in the San Jacinto River Basin and the Port of Galveston is located on the southern edge of Galveston bay. The Port of Galveston spans the north shore of the Galveston Island, along the shipping channel created between the barrier island and the smaller Pelican Island. (Fig. III. 2). The Port of Houston is the top port in the nation in foreign waterborne commerce, the 2nd largest national port in area and the 14th largest in the world in terms of tonnage (Port of Houston, 2009). In the year 2007, the Port of Houston received 253,736 metric tons of domestic and 467,653 metric tons of overseas ballast discharge, and the Port of Galveston received 11,522 metric tons from domestic sources and 14,523 metric tons of overseas ballast discharge (National Ballast Information Clearinghouse, 2008).

In the summer and fall of 2005, Louisiana and Mississippi ports were damaged or lost due to Hurricanes Katrina and Rita respectively resulting in increased commerce to Texas ports, specifically into the Port of Houston. With increased ship traffic arriving to major Texas ports, concerns about bacterial invaders arriving with these ships and their impacts on ecosystem health have elevated. However, little is known of the endemic bacterial populations of the port of Houston. Several studies have examined

pathogenic *Vibrio* bacteria in Galveston bay but the predominant bacterial groups and seasonal patterns are still a mystery.

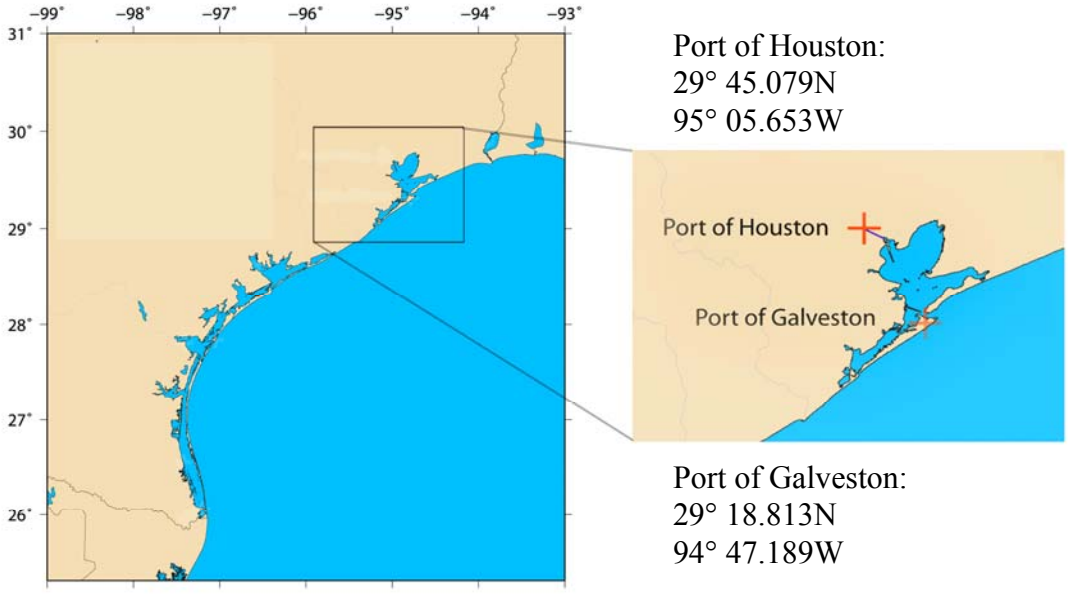

Fig. III. 2. Locations of the Ports of Houston and Galveston.

Ballast Water

The use of ballast water in the shipping industry began in the 1870's (Gollasch et al., 2000; National Research Council, 1996). Ballast is "any solid or liquid, including sediment, placed in a ship to increase the draft, to change the trim, to regulate the stability, or to maintain stress loads within acceptable limits" (National Research Council, 1996). During a ship's voyage, ballast is used to compensate for cargo loss; since the need is quite variable and can be weather dependent, ships use water as ballast material to avoid the dangers and difficulties of solid ballast. The derivation of the word "ballast" in Middle Dutch is "useless load" revealing the aversion of ship owners to using ballast. However, ballast water serves many safety purposes, including: controlling the submergence of the propeller, rudder and hull, providing transverse stability, and reducing stress levels on the hull. Under heavy weather conditions, the ship's officer determines ballast levels to control stability and maintain manageability of the vessel. The ballast tanks are filled when the cargo is off-loaded from a ship to compensate for the lack of weight. Once the ship has reached the next port and is loading new cargo on board, the ballast water is emptied. The size and configuration of the ship affects the amount of ballast water that is transported during a voyage, and can range from as little as a few cubic centimeters to hundreds of thousands of cubic meters. Large cargo tankers can hold over 200,000m^3 or 50,000 metric tons of ballast (McGee et al., 2006; National Research Council, 1996). An estimated 10 billion tons of ballast water is currently transported world-wide annually, and with it, an estimated 3,000 to 4,000 eukaryotic species (Gollasch et al., 2000).

In both lakes and oceans, a single milliliter of water may harbor approximately 1,000,000 bacteria (Dobbs and Rogerson, 2005). In view of the thousands of tons of ballast water transported in a single cargo vessel, billions of bacteria inevitably find their way into ballast tanks. Bacteria have several advantages to surviving long journeys including high reproductive rates (asexual reproduction and rapid growth), simpler nutrient requirements and broader tolerances to physical conditions than eukaryotes, and the ability to form resting states when conditions become unfavorable (Dobbs and Rogerson, 2005; Drake et al., 2007). All of these advantages point to the invasive potential of bacteria transported in ballast water. In fact, ballast water transport is currently the leading vector for alien aquatic species transfer and is responsible for most historical and recent aquatic bioinvasions (Burkholder et al., 2007; Murphy et al., 2004).

In the previous chapter, I demonstrated that bacterial diversity in the ballast tank is high. Part of this chapter will examine ballast tank bacterial communities between five ships that engaged in ballast water exchange in the Pacific Ocean. There are several parallels between ballast tanks. First, because of ballast water exchange measures, the community in a tank following exchange should be that of upper water column of the ocean. Therefore, each tank should have similar "starting" communities, following ballast water exchange. Secondly, while physical design of the tank varies between ships, all tanks are self-contained, do not mix with extra-tank environments during travel, and do not allow sunlight into the tank. If starting with similar communities, these parallel selection pressures should favor the same bacterial lineages, resulting in resembling communities at the end of the voyage.

Bacterial Communities and Invasive Species Concerns

While many physical (e.g. temperature, barometric pressure) and chemical factors (e.g. carbon and nutrient availability, pH, dissolved oxygen) influence the composition of marine bacterial communities, the dominant determinant seems to be salinity, as demonstrated by Lozupone and Knight (2007). Because the salinity of water in a ballast tank would not be affected by transport, as temperature would, it is feasible to believe that bacteria could be easily relocated to distant geographic regions as long as the salinity is relatively similar to that of their point of origin.

Invasive species are legally defined as a "non-native species whose presence in an ecosystem does or is likely to cause environmental or economic harm" (Union of Concerned Scientists, 2007). Harmful bacteria are defined as being capable of causing disease or death to humans or aquatic life (Burkholder et al., 2007). In Texas, eukaryotic invasive species are well-documented, but little is known about bacterial invaders (Union of Concerned Scientists, 2007). Moreover, there are no published studies of the diversity of bacterial communities in Texas estuaries except for examining pathogenic bacteria in Galveston Bay, as mentioned previously. The shipping industry in Texas has been in place since the 1800's, so it is impossible to determine the original bacterial community, but cataloging them now provides a starting framework for invasive bacterial studies in the future. This chapter is divided into three areas of focus: a spatial comparison of ballast water communities, a temporal comparison of communities from the Ports of Houston and Galveston over a one year period, and a spatial comparison of bacterial communities in many of Texas bay systems and ports. The first goal was to

determine if ballast tanks carry similar populations of bacteria that can be used as indicator organisms for invasive activity. The alternate aspect of invasive organisms is the endemic population. It is important to characterize the endemic communities and their population dynamics to monitor invasive effects of transported bacteria. It is possible that transported species do not affect the endemic populations in a significant or invasive manner. This dataset is the starting point for future studies, providing the initial framework for invasive studies focused around ballast water bacterial transport. Each of these areas will be examined by testing one of three hypotheses:

> *Hypothesis 2:* Bacterial communities in ships' ballast water will have parallel compositions to others where open-ocean ballast water exchange occurred in proximate geographical locations.

> *Hypothesis 3*: Bacterial communities in the Ports of Houston and Galveston will exhibit temporal variations related to changes in temperature and salinity.

> *Hypothesis 4*: Bacterial communities will be similar in closely located port and bay systems, but will show greater variations in further systems.

Materials and Methods

Sample Collection

Ballast water was collected from five ships that engaged in ballast water exchange in the Pacific Ocean, three in the North Pacific and two in the South Pacific (Fig. III. 3). Information collected from ships includes date and location (latitude and longitude) of the last ballast tank exchange. Due to privacy agreements between the principle investigators of this study and the shipping agent who facilitated the sample collection, the names of the ships and shipping companies cannot be disclosed. Ballast samples were labeled according to the ocean and latitude and longitude of ballast water exchange and abbreviated for format.

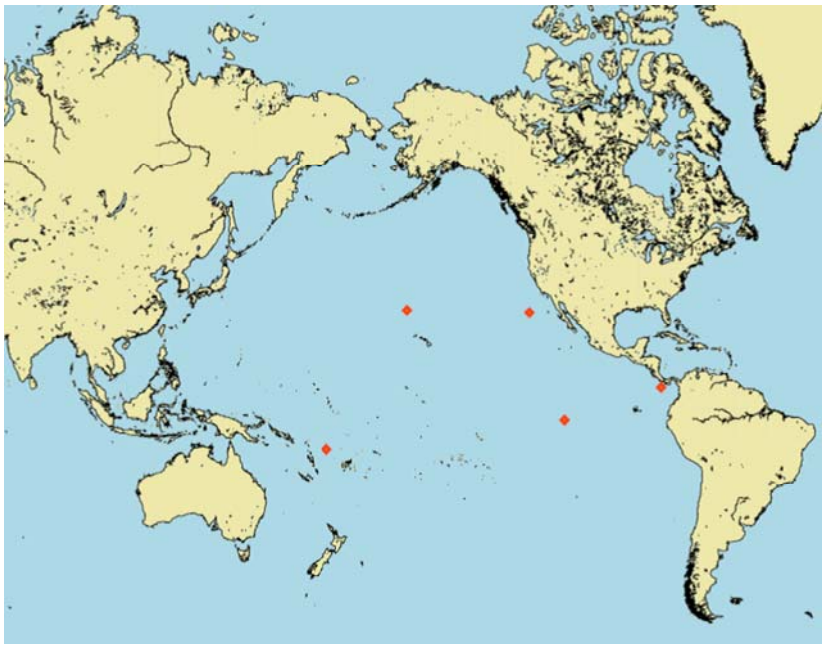

Fig. III. 3. Site of ballast water exchange for all five ships examined in this experiment.

For example, one ballast water sample was exchanged at 29° 30.0N and 163° 40W, in the Pacific Ocean. The full sample name is PO29'30N163'40W, abbreviated to PO29N163W. Immediately after a ballast sample was collected by the shipping agent, it was transported to TAMUG, filtered and stored at -80°C.

The Ports of Houston and Galveston were sampled at the beginning of every month from June 2007 to June 2008 to examine seasonal shifts in bacterial populations and to determine if there are any correlations in diversity change with the date a sampled cargo ship was in port. Water samples were transported to the lab at Texas A&M University in Galveston (TAMUG). Once back at the lab, the samples were collected onto 0.22 Sterivex μm filters (Millipore) and immediately frozen at -80°C until later processing. In addition to water samples temperature and salinity, were also measured.

Eight estuaries and ten ports were selected for sampling (Fig. III. 4). Permission for collection was obtained from all port authorities. Sabine Pass and Sabine Lake are counted as the same estuary system. The first set of samples were collected in spring 2007 between March 3 and March 16th, 2007, with the exception of Galveston Bay, which was sampled November 21, 2006, and the Ports of Houston and Corpus Christi, which were sampled on April 26th and 19th, respectively. During this trip, the Port of Corpus Christi was sampled, but the Port of Aransas was omitted due to delay of port access permission. The second sampling season in summer of 2007 was cut short due to logistical and weather related problems and only a portion of the state estuaries were sampled from LaVaca Bay to Port of Brownsville between July 9 and 12th, 2007, with the exception of the Ports of Mansfield, Isabel and Brownsville, which were sampled on

August 8th, 2007. The northern ports of Beaumont through Galveston were sampled on June 29th, 2007. Bacteria in water samples were collected onto 0.22 µm Sterivex filters (Millipore) and immediately frozen at -20°C for transport back to the lab at Texas A&M University in Galveston (TAMUG). Once back at the lab, the samples were frozen at -80°C until later processing. In addition to water samples, temperature and salinity were measured using a Hydrolab water sonde (Hach Environmental, Loveland, CO). During the second sampling season in the summer of 2007, problems with the Hydrolab prevented the acquisition of all data except salinity for bays Copano, Aransas, Nueces and Corpus Christi, and Port Aransas. This will be discussed below.

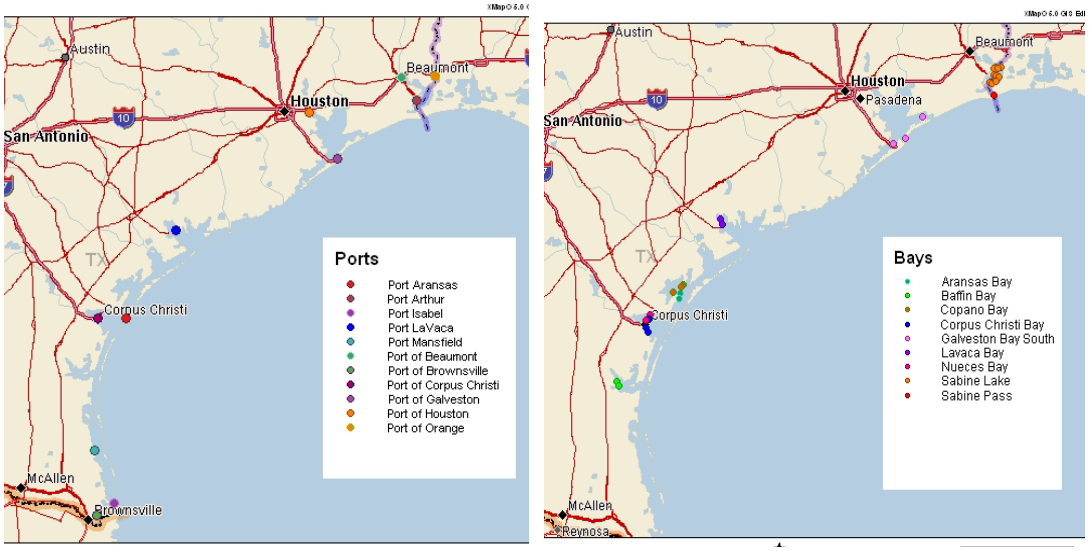

Fig. III. 4. Texas ports (left) and bays (right) sampled during this study.

DNA Extraction

Bacterial nucleic acids were extracted from the frozen filters using a CTAB/chloroform-isoamyl alcohol method (Doyle and Doyle, 1990) that was adapted for the Sterivex cartridge filters. Briefly, after opening the cartridges, filters were immediately submerged in 3% CTAB. Following a two hour incubation at 65°C with periodic vortexing, chloroform-isoamyl alcohol was added, the sample centrifuged at 10,000 g at 4° C for 15 min, and the aqueous layer extracted. DNA was precipitated overnight at 4°C with isopropanol then centrifuged (10,000 g at 4° C for 20 min), washed with ethanol, and dried overnight. The dried pellet was re-suspended in LT buffer, and DNA was quantified by spectrophotometry (NanoDrop 1000).

Polymerase Chain Reaction (PCR)

16s rRNA genes were amplified using PCR with standard bacterial primers 341F and 907RM containing a 30 bp long GC-rich clamp for denaturing gradient gel electrophoresis (Table III. 1). The 16s rRNA gene is commonly used in bacterial phylogenetics and microbial ecology because it is ubiquitous in all prokaryotic organisms with conserved regions for universal analysis, but also has variable regions for more fine-scaled analysis at the family and genus levels (Case et al., 2007).

A limitation of DGGE is that only amplicons of about 500 base pairs in length can be separated and a 30-50 base pair GC-rich clamp is needed to act as a high temperature melting domain to increase sequence variance detection from 50% to 100% (Muyzer et al., 1998). For the application of the amplicons for denaturing gradient gel

electrophoresis, a touchdown PCR was performed. Touchdown PCR utilizes a decreasing scale of annealing temperatures, each with two repetitions, in order to target the maximum number of organisms in an environmental sample and reduce the formation of artificial byproducts during the amplification. The touchdown PCR cycles went as follows:

Initial denaturation	94°C	5 minutes
Annealing step	65 – 50°C	1 minute
Elongation step	72°C	3 minutes
Repeat entire cycle twice for each annealing temperature		
Final elongation	72°C	20 minutes

The final elongation was extended to 20 minutes to prevent artifactual double bands during PCR in later DGGE analyses (Janse et al., 2004).

Table III. 1. Primers used for denaturing gradient gel electrophoresis.

Primer	**Specificity**	**Method**	**Sequence 5' -3'**	**Reference**
GM5-clamp (341F)	Bacteria	PCR-DGGE	cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc gcc tac ggg agg cag	Casamayor et al., 2000
907RM	Most known organisms	PCR-DGGE	ccg tca att cmt ttg agt tt	Casamayor et al., 2000

Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (Muyzer et al., 1998) can be used for comparison of bacterial communities in ballast water, ports and bays. DGGE separates

PCR amplified products onto a vertical acrylamide gel based on nucleotide composition. The gel contains a linear increasing gradient of the denaturants urea and formamide. Sequence variations in a fragment will cause the DNA to melt at unique concentrations of the denaturants, forming distinct bands, and creating a fingerprint structure for any given sample. In this study, gels were made of 8% polyacrylamide, and ranged from 28 – 64% concentration gradient of urea and formamide, with a layer with 0% concentration of denaturants at the top of gel. Fifteen clones were selected from clone libraries constructed during this study and used as a standard (see Chapter I). Gels were run at 60°C for 18 hours at 100V. Gels were removed from the system in complete darkness and place into a SYBR-Gold staining bath for 30 minutes. Afterwards pictures were taken of the gels, and then gels were moved to a blue light table. A sterile razor blade was used to excise single bands from the gel and these were stored in 1.5ml Eppendorf tubes at -20°C. Gel slices were then put into 50ul of PCR-grade water overnight to elute DNA. Eluted DNA was reamplified using the same primers, and DGGE gels were run, with only one band per lane. The gel was photographed as before and moved to a blue light table where each band was excised using a sterile razor blade and stored at -20°C overnight. Gel slices were again eluted overnight in PCR-grade water, and reamplified the next day with the same primers, only without the GC-clamp on the forward primer. PCR products were run on a gel to check for DNA, then purified and sequenced. Due to time constraints of this project, only the sequences for the spring 2007 Texas bay and port systems will be reported.

Fingerprint Analysis

Images of denaturing gels were imported in TIFF format into the BioNumerics software program (Applied Maths). Bands were scored as present or absent based on a threshold of 5% of the total density (the most intense band in the gel). Gels were normalized using the positions of the standard bands. Clustering analysis was performed using Unweighted Pair Group method with Arithmetic Mean (UPGMA) with the Dice coefficient, which creates a pairwise distance matrix for all bands based on binary data (presence/absence of band) and constructs a dendrogram based on similarity values. The Dice coefficient was chosen over an alternative Pearson correlation value that is based upon densitometric curves for each gel. The Dice coefficient only counts the bands as absent or present, while the Pearson correlation value scores each band based upon brightness (abundance). Because each lane was a combination of several PCRs of the same water sample, amplication bias should have been minimal, but still a possibility. Choosing the Dice coefficient over the Pearson correlation value sought to further decrease possible bias, because the band brightness may not specifically been a factor of the abundance of the species in the environment, but rather how well the primers bound to it or even staining differences due to different batches of SYBR-Gold.

Results

Ballast Water

Ballast water samples from five ships that engaged in ballast water exchange in the Pacific Ocean were run in parallel on denaturing gradient gels. Latitudes and longitudes of purported exchange locations are presented in Table III. 2. Three ships exchanged in the North Pacific, and the other two in the South Pacific. The dates of sample collection were between May 2007 to January 2008. The two ships from the South Pacific exchanged ballast water near Fuji (PO13S172E) and about 1,500 knots west of the Galapagos (PO4S114W), both ships exchanged ballast water above 14°S latitude. One North Pacific ship collected ballast water off the Southwest coast of Panama (PO6N83W), at about 6°N latitude. The other two North Pacific ships exchanged ballast water above 28°N latitude, one north of Hawaii (PO29N163W), the other of the west coast of the Baja Peninsula (PO28N125W).

Visual inspection of denaturing gels revealed that all five ships contained different communities, with very few common bands (Fig. III. 5. A). Cluster analysis revealed a strong similarity in bacterial communities (62.5%) between the two South Pacific exchange locations (ships PO13S172E and PO4S114W) (Fig. III. 5. B). Ballast water from ship PO6N83W grouped with ships PO13S172E and PO4S114W at 41.4% similarity to form ballast water 'Group A'. The two North Pacific ships (PO29N163W and PO28N125W) grouped together with a 50% bacterial community composition similarity to form ballast water 'Group B' (Fig. III. 5. B). These groupings occurred according to location of ballast water exchange as well as date of collection. The three

ships in Group A were collected in the fall and winter months of October and November of 2007 and January 2008, while the two ships in Group B were collected in the summer months of May and June, 2007. These temporal variations maybe responsible for the differences in communities as well as geographic distance.

Table III. 2. Ballast water samples collected in the Pacific Ocean.

Ballast Sample	Date Received	Latitude	Longitude	Geographic Region	Geographic Description
PO29N163W	5/5/2007	29° 30.0N	163° 40W	North Pacific	North of Hawaii
PO28N125W	6/8/2007	28° 55.0N	125° 48.00W	North Pacific	West of Baja Peninsula
PO6N83W	1/23/2008	06° 20.0N	083° 00.0W	North Pacific	South West coast of Panama
PO13S172E	10/1/2007	13° 09.1S	172° 20.0E	South Pacific	North of Fuji
PO4S114W	11/15/2007	04° 19.1S	114° 52.2W	South Pacific	1,500 knots west of Galapagos Is.

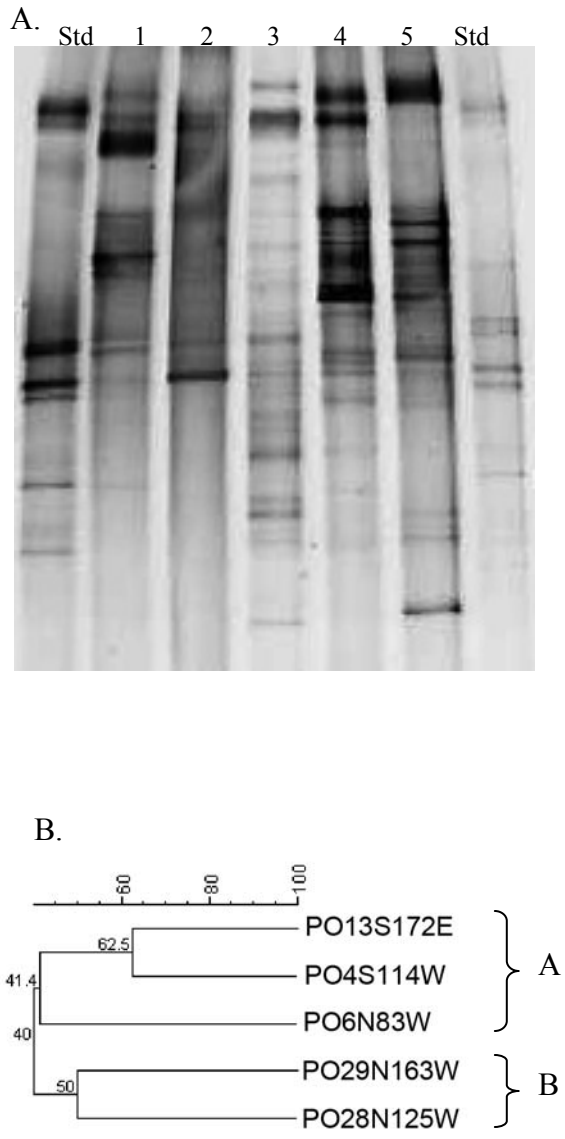

Fig. III. 5. A. DGGE fingerprint of Pacific Ocean ballast water bacterial communities. Std=standard, 1= PO29N163W, 2=PO28N125W, 3=PO6N83W, 4= PO13S172E, 5=PO4S114W. B. Cluster analysis of Pacific Ocean ballast water bacterial community DGGE profiles. Group A: ships whose latitude spanned from 7°N to 14°S, across the equator. Group B: ships whose latitude was greater than 28°N.

Ports of Houston and Galveston

Temperature in the Port of Houston varied across seasons, with the highest temperatures (27-29°C) in the summer months and the lowest temperatures (12-16°C) in the winter months (Table III. 3. A, Fig. III. 6). These values were inversely proportional to the levels of dissolved oxygen. The lowest dissolved oxygen levels (4-5mg/L) occurred in the summer, and the highest levels (11-13mg/L) occurred in the winter months. Salinity was lowest in the summer (3-6ppt) and higher, but variable, in the winter (8-15ppt) (Fig. III. 7).

Bacterial communities displayed seasonal shifts (Fig III. 8. A), although one band (i.e. one species) was present all year round. Cluster analysis revealed four seasonal groupings (Fig. III. 8. B). In the spring and early summer months (April through June), communities stayed relatively stable, sharing an 89% community composition similarity. The spring months, April and May shared a 92% community similarity, while the early summer month of June for both 2007 and 2008 shared a 94% similarity. July and August made up the late summer cluster, sharing a 94% similarity in bacterial communities. The fall months consisted of September, October and November. In September, when salinity increased dramatically (3ppt in August to 10ppt in September), the DGGE profiles showed a noticeable decline in bacterial diversity. October and November communities shared a 92% similarity, and the September bacterial community was 86% similar to the October/November cluster. The winter months (January and February) increased in community diversity as salinity began to decrease, with the highest diversity seen in April, after which, the communities appeared

Table III. 3. Hydrological data for the: A. Port of Houston, and B. Port of Galveston, from June 2007 to June 2008.

A. Port of Houston

Lat/Long:
29° 45.079N
95° 05.653W

Season	Month	Oxy (mg/L)	Temp (ºC)	Salinity (ppt)
Early Summer	June	4.68	22.39	4.31
Late Summer	July	4.60	28.76	2.92
	August	3.75	28.83	3.41
Fall	September	N/A	N/A	10.00
	October	6.16	29.18	8.99
	November	9.10	21.78	15.43
Winter	December	11.17	15.93	10.78
	January	11.34	15.47	14.78
	February	12.72	12.07	8.49
	March	10.70	16.14	7.62
Spring and Early Summer	April	10.38	21.34	9.33
	May	5.13	23.43	5.12
	June	5.27	29.10	5.89

B. Port of Galveston

Lat/Long:
29° 18.813N
94° 47.189W

Season	Month	Oxy (mg/L)	Temp (ºC)	Salinity (ppt)
Summer and Fall	June	7.00	27.02	24.16
	July	6.35	29.60	25.35
	August	5.55	29.72	13.68
	September	N/A	N/A	20.00
	October	8.06	28.52	22.14
Winter	November	11.31	21.64	25.97
	December	12.38	17.03	25.05
	January	14.69	13.90	24.10
	February	12.49	14.58	25.17
	March	12.17	15.28	15.79
Spring	April	10.50	22.14	17.87
	May	6.16	23.14	19.26
Summer and Fall	June	5.41	28.93	23.69

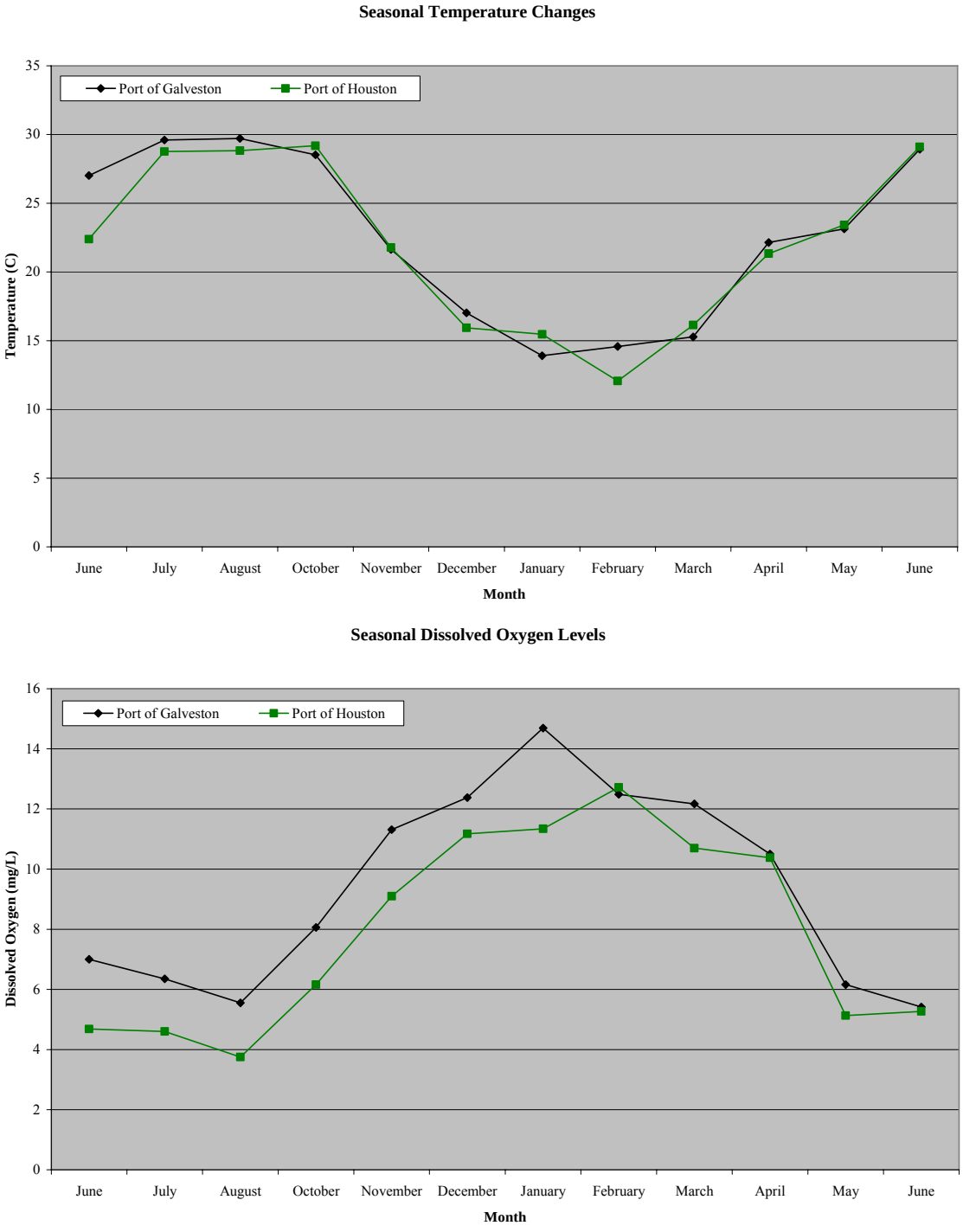

Fig. III. 6. Temperature and dissolved oxygen levels in the Ports of Houston and Galveston between June 2007 and June 2008.

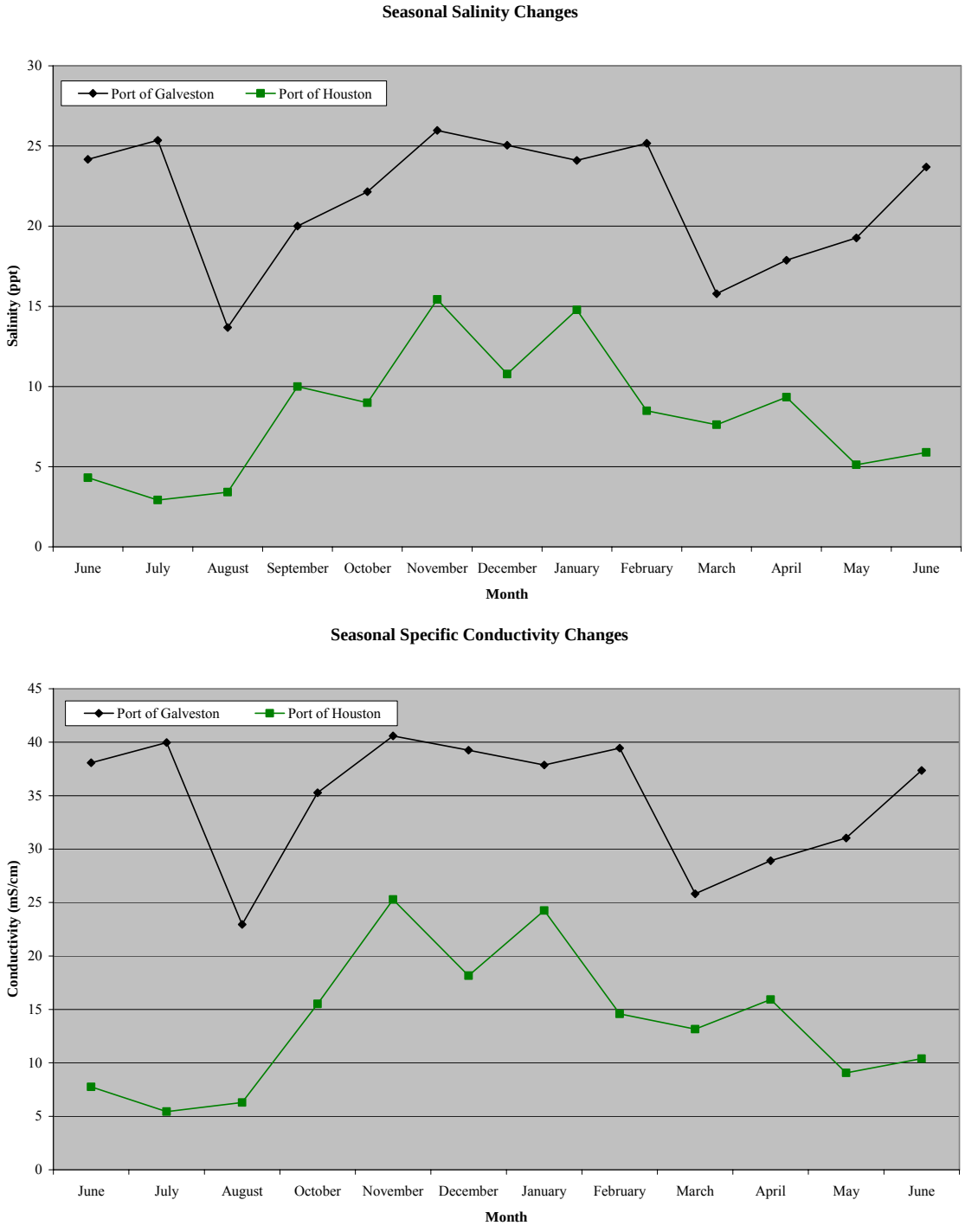

Fig. III. 7. Salinity and specific conductivity levels in the Ports of Houston and Galveston between June 2007 and June 2008.

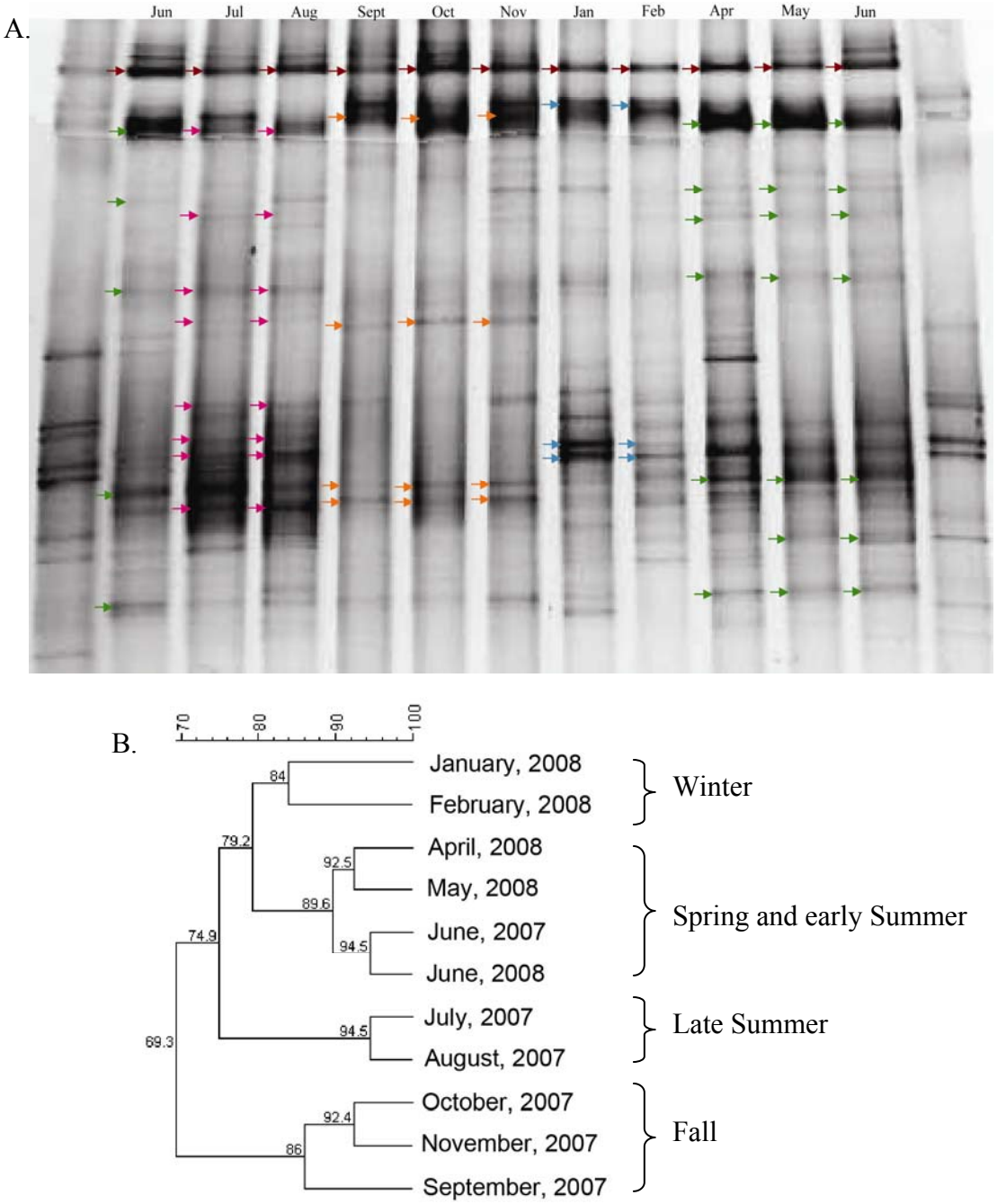

Fig. III. 8. Port of Houston bacterial community shifts between June 2007 and June 2008. A. DGGE fingerprint analysis. Red arrows=bands present all year, blue arrows=winter months, green arrows=spring and early summer months, pink arrows=late summer months, and orange arrows=fall months. B. Cluster analysis of the fingerprints of each month.

to decline in diversity, but stabilize in May throughout the rest of the summer, when the salinity remained low. The months of December and March did not successfully amplify for the Port of Houston samples, and were excluded from the cluster analysis, but based upon time, temperature and, are assumed to fall within the winter cluster.

The Port of Galveston was similar to the Port of Houston in seasonal temperature variations (Table III. 3. B, Fig. III. 6), but had higher salinity. The highest temperatures were in the summer (27-30°C) and the lowest temperatures occurred in the winter (14-17°C). Salinity levels were appreciably higher in the Port of Galveston than the Port of Houston. Dissolved oxygen Unlike the Port of Houston, salinity and specific conductivity levels in the Port of Galveston were highest (between 24-26ppt) in both the summer and winter (Fig. III. 7). In the spring months (March-May), salinity dropped to between 16-19ppt, and again dropped in the late summer-early fall to between 14-22ppt, with the most dramatic drop from 25ppt in July to 14ppt in August.

The bacterial communities also displayed seasonal shifts (Fig III. 9. A), but had two bands (i.e. species) that were present year round. Communities were most stable in the summer months, but variable in the winter. Highest levels of diversity occurred in the winter months of December-February when dissolved oxygen levels and salinity were the highest, but then decreased dramatically in March, when the salinity again plunged from 25-16ppt.

Cluster analysis split the Port of Galveston community fingerprints into only three seasonal groups (Fig. III. 9. B). The winter group was comprised of the months of December through March, with an 85% similarity. November and December shared a

93% similarity, with January being the next closest, sharing an 89% similarity. February and March were more similar to each other (93%) than the rest of the winter months. April and May comprised the spring group, with 86% similarity, and June through October comprised the summer and fall cluster. In March, when salinity dropped about 10ppt, the DGGE fingerprint showed a dramatic decrease in diversity. Salinity steadily increased until June of 2008, where it appears the bacterial communities stabilized. June and July of 2007 had similar salinities and communities. Diversity was lowest in the month of August, when salinity fell from 25-14ppt and dissolved oxygen was low (6mg/L), and the DGGE fingerprint shows an almost complete disruption of the community, resulting in a new, different population. In September, when salinity increased again to 20 ppt, the community observed in August disappeared, and a new community similar to July appeared. However, in October through December, when salinity was at it highest, new bands began to appear showing high levels of diversity (> 6 number of species) until March.

Both ports showed a correlation between salinity and bacterial diversity. However, this correlation was completely opposite between the ports. The Port of Houston experienced a decrease in diversity, from about 20 to 12 species, as salinity increased in the winter, whereas the Port of Galveston showed an increase in diversity, from 15 to 30 species, during the winter months with higher salinity.

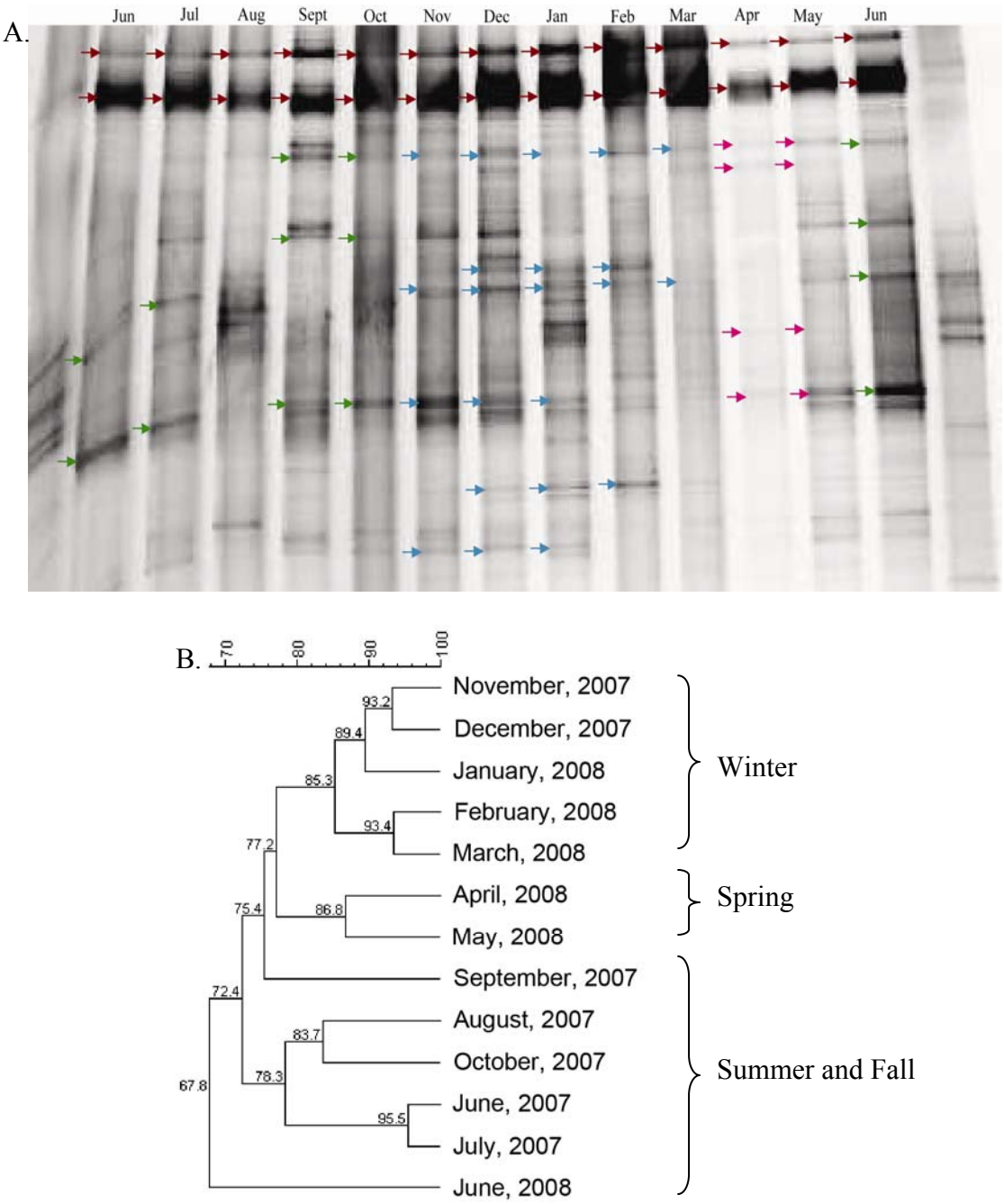

Fig. III. 9. Port of Galveston bacterial community shifts between June 2007 and June 2008. A. DGGE fingerprint analysis. Red arrows=bands present all year, blue arrows=winter months, pink arrows=spring months, and green arrows=summer and fall months. B. Cluster analysis of the fingerprints of each month.

Texas Ports and Estuaries

Bacterial communities were variable between all systems in both spring and summer sampling seasons. Statistical analyses showed that bay and port communities were mainly differentiated between the northern systems (between Sabine Lake and Galveston or LaVaca Bays) and southern systems (Copano Bay to the Port of Brownsville), with a general trend of an increase in salinity from north to south. Number of sample sites varied between systems, and an average was taken for all hydrological data for each system.

Spring data set –The effecting abiotic factor was salinity. While temperature, pH, and dissolved oxygen levels remained relatively stable, salinity and specific conductivity fluctuated between ports (Table III. 4 and Fig. III. 10. A). All ports north of Galveston had salinities below 8ppt, while the other, southern ports had salinities around 13ppt. The bays showed a similar hydrological trend, with temperature, pH, and dissolved oxygen levels remaining fairly stable (Fig. III. 10. B). Salinity and specific conductivity were lowest in the Northern-most bays system, Sabine Lake, and highest in the Southern-most Baffin Bay system.

Upon visual inspection of DGGE bands, bacterial communities did look more similar to those observed in adjacent systems, sharing similar bands in both the bays and the ports (Fig. III. 11. A and Fig. III. 12. A). Cluster analysis categorized the three Sabine area ports and the Port of Houston together into Group A, with the ports of Galveston through Brownsville into Group B (Fig. III. 11. B). Cluster analysis categorized the bays into three groups, aligning with salinities (Fig. III. 12. B). Group A

consisted of Sabine Lake, Sabine Pass, and Galveston Bay, all with a salinity below 16ppt. Sabine Lake and Sabine Pass had salinities around 7ppt and shared a 93% similarity, whereas Galveston Bay had a salinity of 15.65 and was only 74% similar to the Sabine sites. Group B contained only LaVaca Bay, and had a salinity of 18ppt. Group C contained the other five bays, which had salinities of 19ppt or greater.

Summer data set – During the summer sampling season, Port Aransas was sampled instead of the Port of Corpus Christi, but no hydrological data except salinity could be obtained. Temperature remained stable, being slightly higher in the South Texas ports, and dissolved oxygen levels remained stable until the Port of Brownsville, at which it increased 9mg/L above the nearby Port Isabel. However, the Port of Brownsville failed to amplify, and is omitted from the community cluster analysis. Salinity and specific conductivity displayed erratic changes between ports and bays (Table III. 5 and Fig. III. 13).

Table III. 4. Hydrological data for: A. Texas bays, and B. Texas ports, from the spring 2007 sampling season.

A. Texas Ports

Location	Temp (ºC)	Cond (mS/cm)	Salinity (ppt)	pH	Oxygen (mg/L)
Port of Beaumont	15.91	15.09	7.54	7.05	7.51
Port of Orange	17.10	0.16	0.07	7.36	7.45
Port Arthur	16.23	11.78	6.71	6.66	7.42
Port of Houston	22.39	7.75	4.31	7.96	4.68
Port of Galveston	19.31	28.65	17.52	7.73	8.58
Port LaVaca	19.26	23.08	13.88	7.34	6.86
Port of Corpus Christi	21.29	49.62	32.64	7.82	6.42
Port Mansfield	23.10	50.03	32.90	7.96	6.10
Port Isabel	21.41	50.12	32.76	7.81	6.33
Port of Brownsville	20.67	55.37	36.78	7.60	4.35

B. Texas Bays

Location	Temp (ºC)	Cond (mS/cm)	Salinity (ppt)	pH	Oxygen (mg/L)
Sabine Lake	16.78	2.23	1.10	6.98	8.13
Sabine Pass	16.86	10.37	5.90	7.14	7.97
Galveston Bay	14.87	25.73	15.65	8.10	9.11
Lavaca Bay	20.56	29.37	18.14	7.52	7.02
Copano Bay	20.55	30.34	18.83	7.86	8.21
Aransas Bay	20.00	35.15	22.65	7.65	6.67
Nueces Bay	21.06	52.09	34.35	7.71	7.27
Corpus Christi Bay	21.04	50.73	33.26	7.83	7.08
Baffin Bay	21.36	62.87	42.40	7.47	5.37

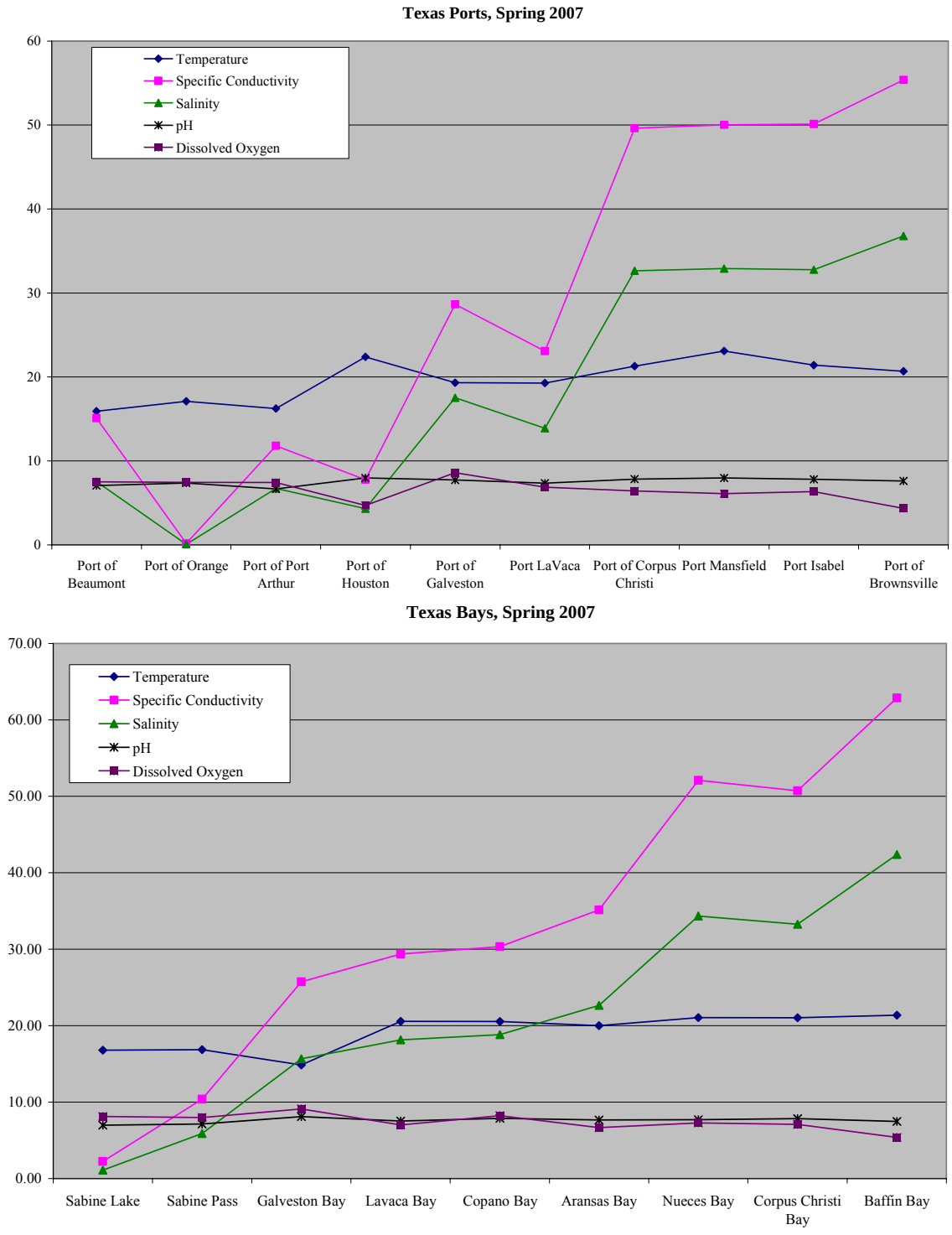

Fig. III. 10. Variations in hydrological data between Texas ports and bays for the spring of 2007. Ports and bays are ordered from north to south.

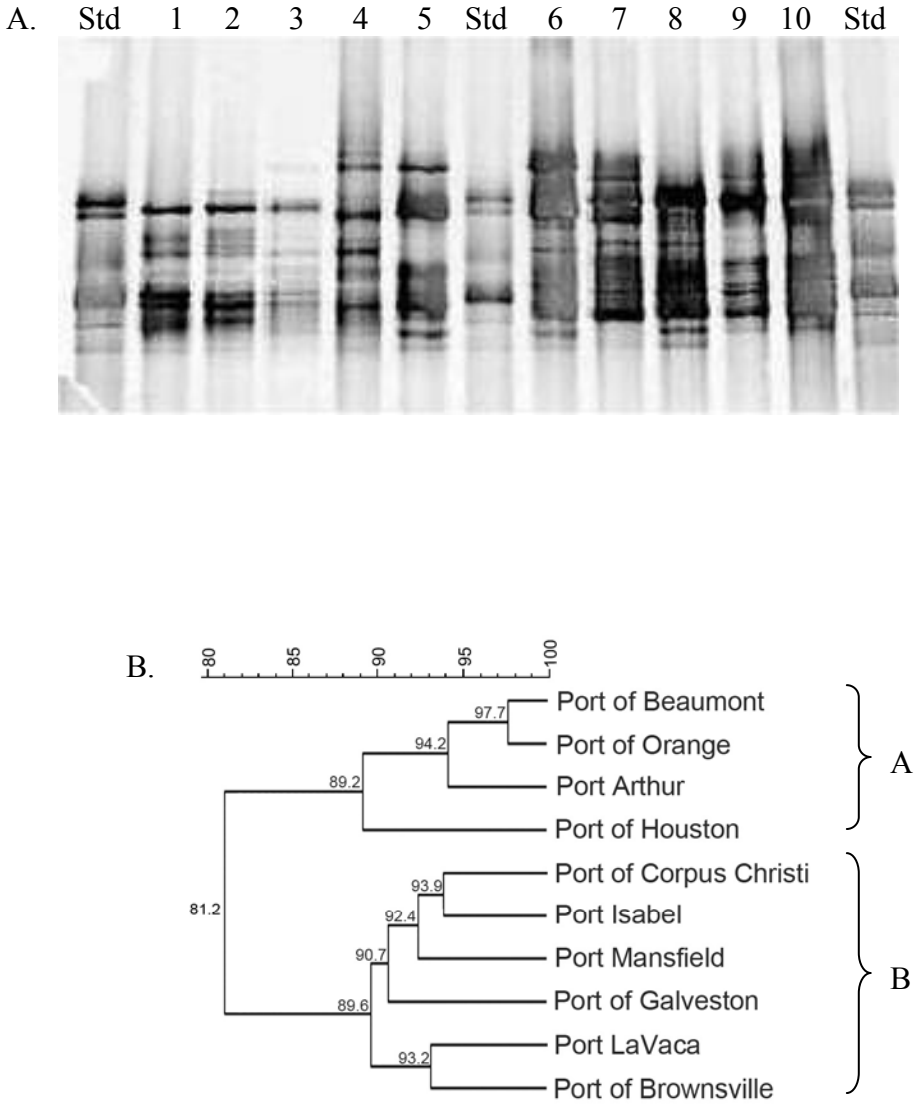

Fig. III. 11. Texas ports bacterial community shifts in the spring of 2007. A. DGGE fingerprint analysis. Std=standard, 1=Port of Beaumont, 2=Port of Orange, 3=Port Arthur, 4=Port of Houston, 5=Port of Galveston, 6=Port LaVaca, 7=Port of Corpus Christi, 8=Port Mansfield, 9=Port Isabel, and 10=Port of Brownsville. B. Cluster analysis of the fingerprints of each port.

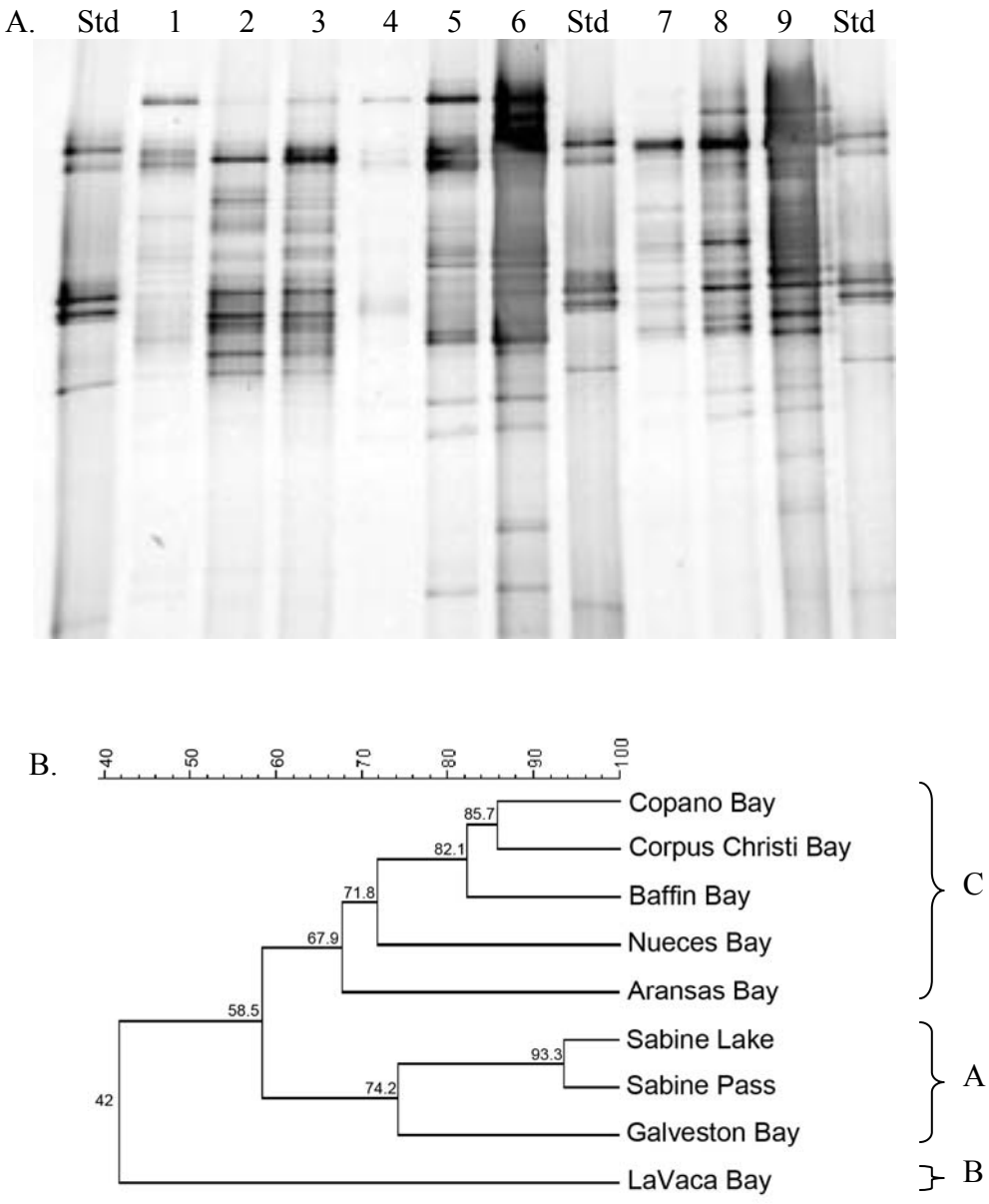

Fig. III. 12. Texas bays bacterial community shifts in the spring of 2007. A. DGGE fingerprint analysis. Std=standard, 1=Galveston Bay, 2=Sabine Lake, 3=Sabine Pass, 4=LaVaca Bay, 5=Copano Bay, 6=Aransas Bay, 7=Corpus Christi Bay, 8=Nueces Bay, and 9=Baffin Bay. B. Cluster analysis of the fingerprints of each bay.

Table III. 5. Hydrological data for: A. Texas bays, and B. Texas ports, from the summer 2007 sampling season.

A. Texas Ports

Location	Temp (ºC)	Cond (mS/cm)	Salinity (ppt)	pH	Oxygen (mg/L)
Port of Beaumont	29.56	0.29	0.14	5.83	73.90
Port of Orange	30.70	0.31	0.15	7.27	97.20
Port of Port Arthur	29.71	19.26	11.45	5.96	82.30
Port of Houston	28.76	5.45	2.92	4.60	59.10
Port of Galveston	29.60	39.97	25.35	6.35	95.70
Port LaVaca	29.31	1.73	0.86	4.92	62.80
Port Aransas	N/A	N/A	18.00	N/A	N/A
Port Mansfield	32.94	40.95	25.97	4.83	75.10
Port Isabel	30.94	54.62	35.95	7.38	121.00
Port of Brownsville	32.60	47.08	30.37	16.36	266.20

B. Texas Bays

Location	Temp (ºC)	Cond (mS/cm)	Salinity (ppt)	pH	Oxygen (mg/L)
Lavaca Bay	29.31	0.35	0.17	5.45	70.80
Copano Bay	N/A	N/A	4.25	N/A	N/A
Aransas Bay	N/A	N/A	6.50	N/A	N/A
Nueces Bay	N/A	N/A	7.50	N/A	N/A
Corpus Christi Bay	N/A	N/A	16.33	N/A	N/A
Baffin Bay	29.89	48.62	31.71	5.89	89.25

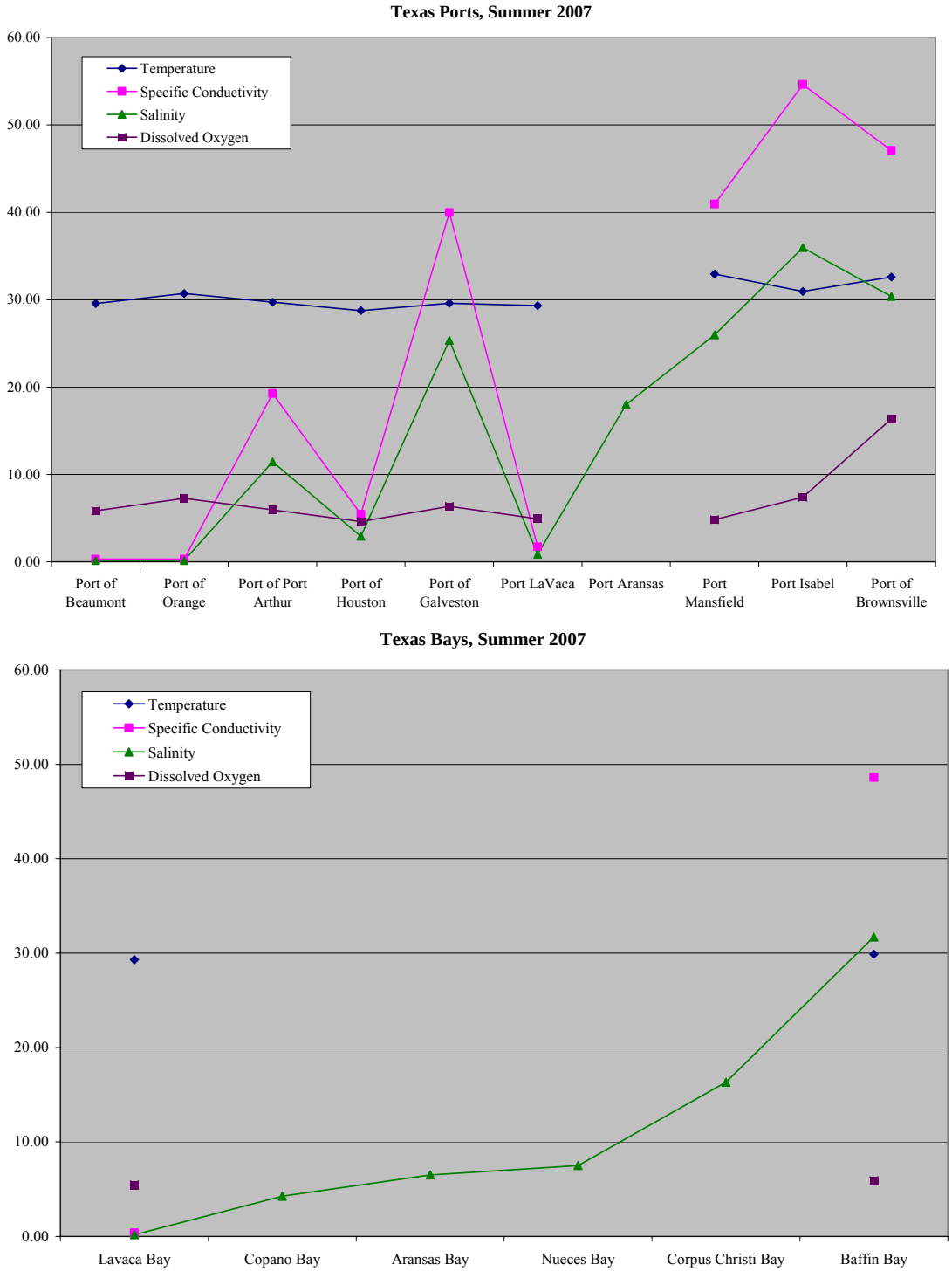

Fig. III. 13. Variations in hydrological data between Texas ports and bays for the summer of 2007.

Again, upon visual inspection of DGGE bands, bacterial communities did look more similar to those immediately next to them, sharing similar bands in both the bays and the ports (Fig. III. 14. A and Fig. III. 15. A). Cluster analysis revealed two main groups among the ports based on salinity (Fig. III. 14. B). The first group was split into two because the disparity in salinity was high. Group A, the Ports of Beaumont, Orange, and surprisingly, LaVaca, all had salinities below 1ppt. Port LaVaca (and the entire LaVaca bay system) had abnormally low salinity due to a massive freshwater inflow described by the Port Authorities. Group B consisted of the Port of Houston and Port Arthur with salinities ranging from 2-12ppt. Group C was comprised of the Ports of Galveston, Aransas, Mansfield and Isabel with salinities over 17ppt. The Port of Brownsville had a salinity of 30ppt, and presumably would have been a part of this last group.

The bays sampled in the summer included only the southern half of the state's systems, from LaVaca to Baffin Bay, and only salinity could be measured for the middle bays (Copano, Aransas, Nueces and Corpus Christi). Salinity exhibited a gradual increase as the bays went further south. Temperature and dissolved oxygen levels were able to be measured in the northern-most bay of this data set, LaVaca Bay, and the southern-most bay, Baffin Bay.

Although variations could have occurred through the bays, both end bays were fairly equal, which would be consistent with the other data sets. Cluster analysis created two separate groups for this data set (Fig. III. 15. B). Group A contained only LaVaca Bay, while Group B contained all the other southern bays. This was expected as the spring data set clustered the same bays together, apart from the northern bay systems. LaVaca Bay was the most distant from the other bays with a salinity of 0.2ppt. This is again due to the large freshwater influx into the system. Salinities of Copano, Aransas and Nueces Bay ranged from 4-7.5ppt. The salinity of Corpus Christi Bay was much higher at 16ppt, and Baffin Bay even higher with 32ppt. But this was not apparent in the clustering analysis. The bays were more similar to those of nearest geographical location, with the exception of Aransas Bay being slightly more similar to Baffin Bay than Copano Bay.

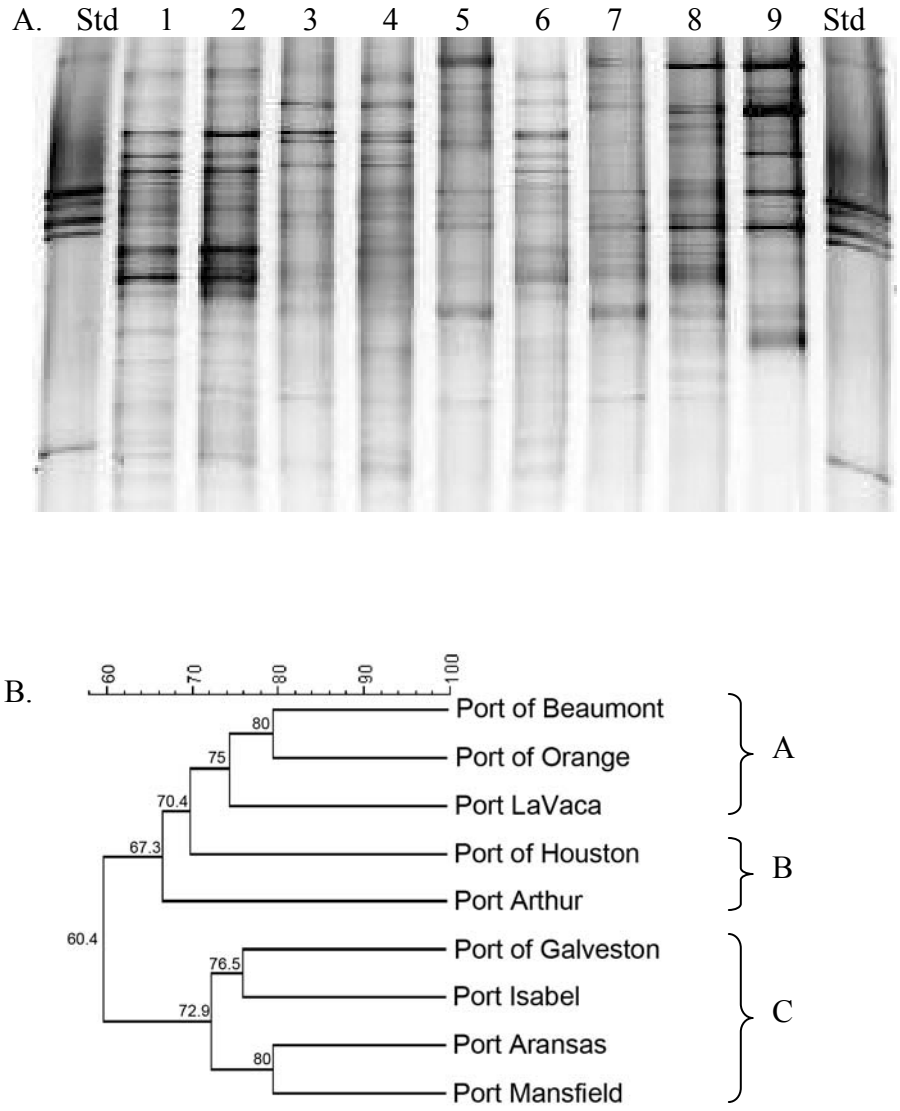

Fig. III. 14. Texas ports bacterial community shifts in the summer of 2007. A. DGGE fingerprint analysis. The Port of Brownsville could not be amplified and is omitted from the analyses. Std=standard, 1=Port of Beaumont, 2=Port of Orange, 3=Port Arthur, 4=Port of Houston, 5=Port of Galveston, 6=Port LaVaca, 7=Port Aransas, 8=Port Mansfield, and 9=Port Isabel. B. Cluster analysis of the fingerprints of each port.

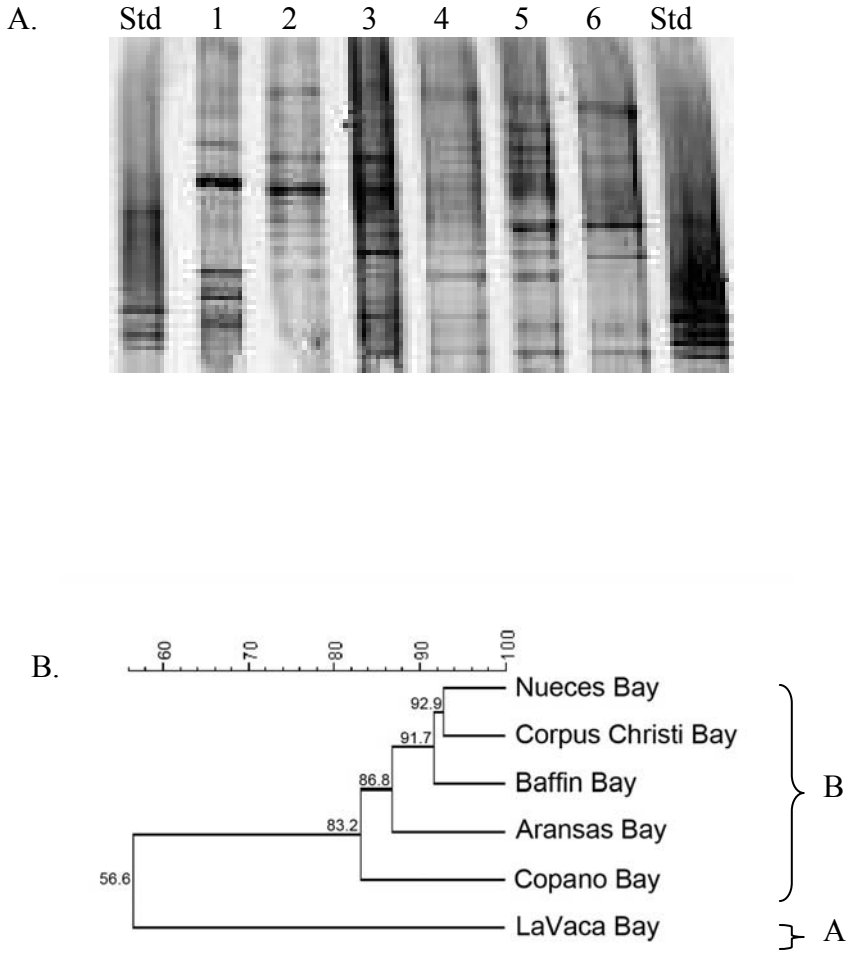

Fig. III. 15. Texas bays bacterial community shifts in the summer of 2007. A. DGGE fingerprint analysis. The northern systems of Sabine Lake and Galveston bay were omitted from the analyses. Std=standard, 1=LaVaca Bay, 2=Copano Bay, 3=Aransas Bay, 4=Corpus Christi Bay, 5=Nueces Bay, and 6=Baffin Bay. B. Cluster analysis of the fingerprints of each bay.

Spring sequences – 67 total sequences were obtained for the spring data set (Table III. 6). Of these, the most prominent groups of bacteria were the α-proteobacteria (31% of total sequences), followed by the actinobacteria making up 28% of the total sequences. (Fig. III. 16). The other 41% of sequences consisted of the cyanobacteria (13%), the bacteriodetes (12%), the β- and γ-proteobacteria (10% and 3%, respectively), and two eukaryotic plastids (3%), and varied between systems (Fig. III. 17). While all three ports in the Sabine system along with Sabine Lake contained different community structures, all phyla found in them were present in the Sabine Pass sample, as expected, where all the waters from these sites are combined before exiting to the Gulf of Mexico. The only gammaproteobacteria were found in Aransas and Nueces Bay, which is surprising considering that this group is typically dominant in most environments.

There were no a bacterial phyla that were present throughout all systems.

Table III. 6. DGGE sequences from Texas bays and ports in the Spring of 2007.

System	Phylum	Band	Nearest Match on GenBank	Accession Number	Sequence Length	% Similarity
Sabine Lake	*a-Proteobacteria*	12B	Uncultured alpha proteobacterium clone PIB-8	AM888002	499	98
Sabine Lake	*Bacteriodetes*	12G	Uncultured Bacteroidetes bacterium clone IRD18G07	AY947969	474	93
Sabine Pass	*Actinobacteria*	13C	Uncultured actinobacterium clone STH11-11	DQ316375	199	96
Sabine Pass	*a-Proteobacteria*	13A	Uncultured bacterium clone L2W-61	AJ966125	324	95
Sabine Pass	*a-Proteobacteria*	13G	Uncultured alpha proteobacterium clone CB11E01	EF471707	492	99
Sabine Pass	*Bacteriodetes*	13H	Uncultured Bacteroidetes bacterium clone PI_RT146	AY580658	262	95
Sabine Pass	*b-Proteobacteria*	13I	Polynucleobacter necessarius subsp. necessarius STIR1	CP001010	165	98
Port Arthur	*Actinobacteria*	3D	Uncultured actinobacterium clone SIMO-2123	AY711489	524	98
Port Arthur	*Bacteriodetes*	3B	Uncultured Bacteroidetes bacterium clone PI_RT146	AY580658	518	97
Port of Beaumont	*a-Proteobacteria*	1A	Uncultured bacterium clone N06Jan-7	EU442980	521	99
Port of Beaumont	*Bacteriodetes*	1C	Uncultured Flexibacteraceae bacterium clone LiUU-3-217	AY509280	410	96
Port of Beaumont	*b-Proteobacteria*	1D	Polynucleobacter necessarius subsp. asymbioticus	AB470466	380	97
Port of Beaumont	*b-Proteobacteria*	1E	Uncultured Burkholderiales bacterium clone DSV2Q1u76	EU631386	96	100
Port of Orange	*Actinobacteria*	2F	Uncultured actinobacterium clone TH3-71	AM690962	488	100
Port of Orange	*Bacteriodetes*	2C	Uncultured Bacteroidetes bacterium clone SOC1 2B	DQ628942	379	92
Port of Orange	*b-Proteobacteria*	2D	Uncultured beta proteobacterium SBR1001 clone DDR2W1u39	EU634832	182	96

Table III. 6. continued.

System	Phylum	Band	Nearest Match on GenBank	Accession Number	Sequence Length	% Similarity
Port of Galveston	*Actinobacteria*	5E	Uncultured actinobacterium clone CB01B07	EF471625	527	99
Port of Galveston	*a-Proteobacteria*	5A	Uncultured alpha proteobacterium clone CB22E02	EF471520	520	99
Port of Galveston	*a-Proteobacteria*	5B	Candidatus Pelagibacter ubique clone fosmid 01-003783	EU410957	500	99
Port of Galveston	*Cyanobacteria*	5C	Uncultured Synechococcus sp. clone CB22C09	EF471585	255	94
Port of Galveston	*Cyanobacteria*	5D	Synechococcus sp. MBIC10089	AB058226	516	98
Port of Houston	*b-Proteobacteria*	4E	Beta proteobacterium TEGF003	AB426582	544	99
LaVaca Bay	*a-Proteobacteria*	14A	Uncultured alpha proteobacterium clone CB22E02	EF471520	521	99
LaVaca Bay	*Cyanobacteria*	14C	Uncultured Synechococcus sp. clone CB11G10	EF471565	547	98
Port LaVaca	*Actinobacteria*	6B	Uncultured actinobacterium, clone TH3-71	AM690962	536	99
Port LaVaca	*Actinobacteria*	6F	Uncultured actinobacterium clone CB01B07	EF471625	251	96
Port LaVaca	*Actinobacteria*	6G	Uncultured actinobacterium clone CB01B09	EF471700	527	99
Port LaVaca	*a-Proteobacteria*	6A	Uncultured alpha proteobacterium clone CB22E02	EF471520	512	99
Port LaVaca	*Bacteriodetes*	6C	Uncultured Bacteroidetes bacterium clone CB11G06	EF471629	535	98
Port LaVaca	*Cyanobacteria*	6D	Uncultured Synechococcus sp. clone MPWIC_C06	EF414206	527	99
Port LaVaca	*Cyanobacteria*	6E	Uncultured Synechococcus sp. clone CB11D06	EF471563	525	99

Table III. 6. continued.

System	Phylum	Band	Nearest Match on GenBank	Accession Number	Sequence Length	% Similarity
Aransas Bay	*Actinobacteria*	16E	Uncultured actinobacterium clone CB01B07	EF471625	203	99
Aransas Bay	*Actinobacteria*	16G	Uncultured actinobacterium clone CB01B09	EF471700	522	99
Aransas Bay	*Actinobacteria*	16H	Uncultured actinobacterium clone CB51H12	EF471671	172	98
Aransas Bay	*a-Proteobacteria*	16B	Uncultured alpha proteobacterium clone CB11D07	EF471570	508	100
Aransas Bay	*Cyanobacteria*	16D	Synechococcus sp. KUAC 3041	EF152371	528	99
Aransas Bay	*g-Proteobacteria*	16F	Halomonas sp. MOLA 69	AM990844	60	100
Copano Bay	*Actinobacteria*	15F	Uncultured actinobacterium clone CB01E07	EF471485	508	99
Copano Bay	*Actinobacteria*	15G	Uncultured actinobacterium clone CB41H04	EF471594	534	99
Copano Bay	*Actinobacteria*	15H	Uncultured actinobacterium clone CB01B09	EF471700	536	99
Copano Bay	*a-Proteobacteria*	15A	Uncultured alpha proteobacterium clone CB22E02	EF471520	496	99
Copano Bay	*a-Proteobacteria*	15B	Uncultured alpha proteobacterium clone CB11D07	EF471570	496	99
Copano Bay	*a-Proteobacteria*	15C	Uncultured alpha proteobacterium clone CB22F02	EF471709	54	98
Copano Bay	*Cyanobacteria*	15E	Synechococcus sp. KUAC 3041	EF152371	515	99

Table III. 6. continued.

System	Phylum	Band	Nearest Match on GenBank	Accession Number	Sequence Length	% Similarity
Corpus Christi Bay	*Actinobacteria*	18G	Uncultured actinobacterium clone CB41H04	EF471594	528	99
Corpus Christi Bay	*a-Proteobacteria*	18A	Uncultured alpha proteobacterium clone CB11D07	EF471570	518	99
Corpus Christi Bay	*a-Proteobacteria*	18B	Uncultured alpha proteobacterium clone CONW25	AY828395	512	100
Corpus Christi Bay	*a-Proteobacteria*	18C	Rhodobacteraceae bacterium MOLA 108	AM990882	513	99
Corpus Christi Bay	*a-Proteobacteria*	18D	Uncultured alpha proteobacterium clone ATLC XRY-37	EU647622	82	100
Nueces Bay	*a-Proteobacteria*	17A	Uncultured alpha proteobacterium clone CB11D07	EF471570	444	98
Nueces Bay	*a-Proteobacteria*	17C	Rhodobacteraceae bacterium MOLA 108	AM990882	510	98
Nueces Bay	*a-Proteobacteria*	17D	Uncultured alpha proteobacterium clone CB11H08	EF471522	527	99
Nueces Bay	*Bacteriodetes*	17B	Uncultured Bacteroidetes bacterium clone CB11D01	EF471578	536	99
Nueces Bay	*g-Proteobacteria*	17E	Uncultured gamma proteobacterium clone T31_10	DQ436639	514	98
Port of Corpus Christi	*a-Proteobacteria*	7B	Uncultured alpha proteobacterium clone CB11A02	EF471724	88	100
Port of Corpus Christi	*Bacteriodetes*	7D	Uncultured Bacteroidetes bacterium clone CB11D01	EF471578	251	98
Port of Corpus Christi	*Cyanobacteria*	7G	Synechococcus sp. KUAC 3041	EF152371	530	99
Port of Corpus Christi	*Eukaryote - Chloroplast*	7C	Uncultured eukaryote clone S2-52; chloroplast	EF491368	533	99
Port of Corpus Christi	*Eukaryote - Chloroplast*	7E	Environmental clone OCS182, chloroplast gene for chloroplast RNA	AF001660	487	99

Table III. 6. continued.

System	Phylum	Band	Nearest Match on GenBank	Accession Number	Sequence Length	% Similarity
Baffin Bay	*Actinobacteria*	19H	Uncultured actinobacterium clone CB01B07	EF471625	213	98
Baffin Bay	*Actinobacteria*	19J	Agromyces sp. T33Y	AM983510	151	98
Baffin Bay	*Bacteriodetes*	19E	Balneola sp. MOLA 132	AM990906	28	96
Port Mansfield	*Actinobacteria*	8D	Uncultured Cellulomonas sp. clone VSJ5Q1u80	EU631294	53	100
Port Mansfield	*Actinobacteria*	8E	Uncultured actinobacterium clone CB01B09	EF471700	525	99
Port Mansfield	*Cyanobacteria*	8C	Synechococcus sp. KUAC 3041	EF152371	540	98
Port of Brownsville	*a-Proteobacteria*	10B	Uncultured alpha proteobacterium clone CB11D07	EF471570	282	97
Port of Brownsville	*b-Proteobacteria*	10G	Hydrogenophaga sp. JPB-3.10	EU652485	543	98

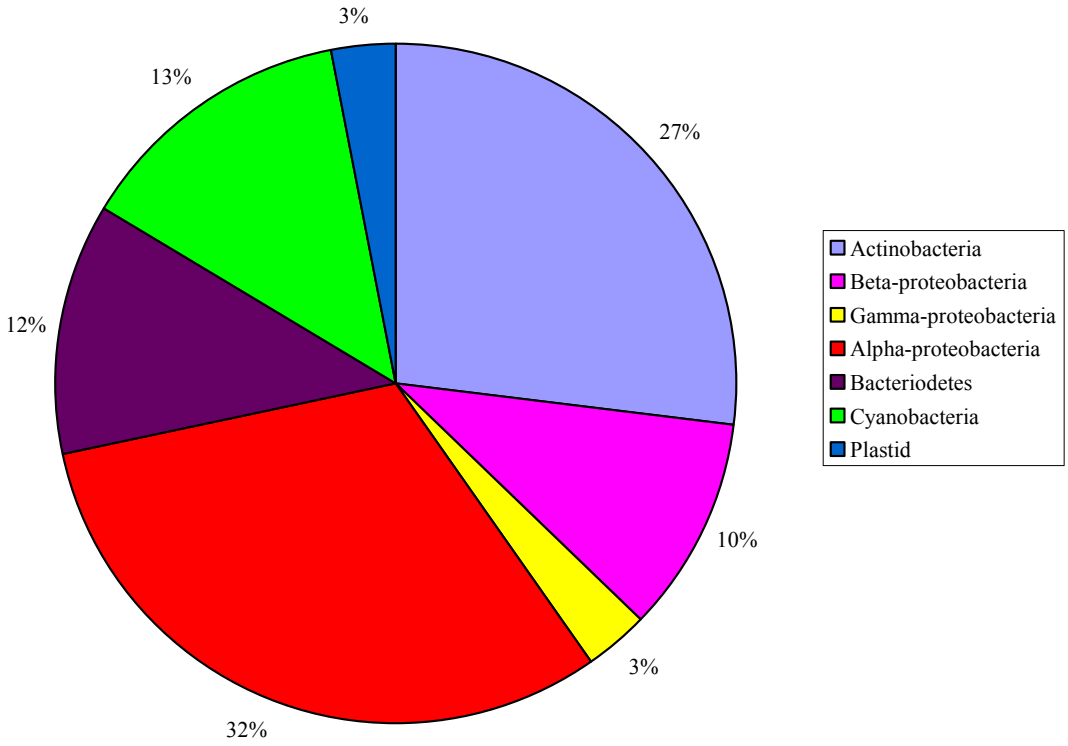

Fig. III. 16. Percentages of bacteria phyla found in Texas bays and ports in the spring of 2007.

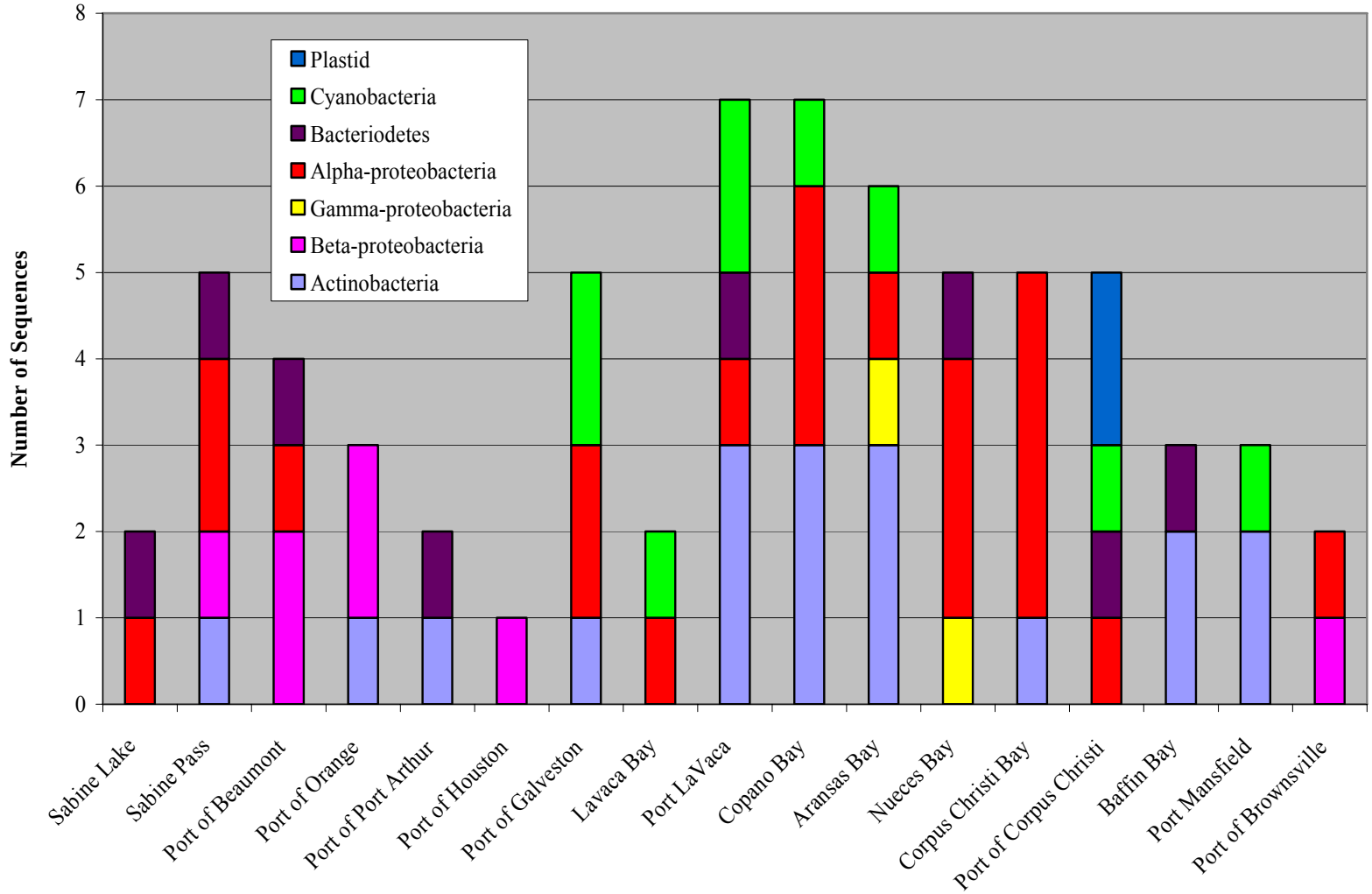

Fig. III. 17. Bacterial distribution in Texas bays and ports in the spring of 2007.

The cyanobacteria were not found until the Port of Galveston, then were absent in Nueces and Corpus Christi Bay then reappeared in the Port of Corpus Christi and Port Mansfield. This seems to indicate that I did not thoroughly sample the entirety of the population. Moreover, samples were taken from surface waters on the shore of each system, excluding bacteria in the middle of the system or below the surface waters.

Alternatively, the primers used for PCR contained sufficient mismatches to prevent annealing of cyanobacterial groups. Microscopic examination of samples for cyanobacteria has not yet been conducted but could help to explain this disparity. Because these represent a small subsample of the total population, inferences about community diversity cannot be made. Many of the bands excised failed to give readable sequences, and were omitted from the following Fig.s.

Ten organisms in GenBank matched up with more than one band in the spring DGGE analysis (Table III. 7). However, matching bands were not always in the same location. For example, band 2F, in bottom of the Port of Orange fingerprint matched the same actinobacterium as band 6B, in the upper area of the LaVaca Bay fingerprint. Five different bands all matched the same alphaproteobacterium, but were found in two different locations on the gel. All five bands were in the top of the gel, but three out of five were in the same lower position (15B, 17A, 10B), and the other two were in the same higher position (16B, 18A).

Many were in similar positions on the gel, and found in systems relatively close together, mostly separated into the same north/south distinctions. Bands found in the Sabine Lake systems did not match any bands south of the LaVaca Bay systems, but the southern systems shared up to 5 bands (Table III. 7).

Eleven sequences matched cultured isolates on GenBank, and five matched uncultured clones that were identified to the genus level (Table III. 8). The most abundant genus found was *Synechococcus*, a cyanobacterium ubiquitous in aquatic environments. Bacterial phyla seemed to stop migration in distinct areas of the gel so that identifications of bacterial groups without DNA sequencing were made. The α- proteobacteria denatured earliest in the upper regions of the gel, whereas the Actinobacteria denatured the latest in the bottom regions of the gel, with one exception of band 6B in Port LaVaca (Fig. III. 18). The bacteriodetes, cyanobacteria and β-proteobacteria all stopped in the middle region of the gel.

Table III. 7. Organisms found in multiple bands in the spring DGGE analysis.

Accession Number	Nearest Match on GenBank	Phylum	Band	Sequence Length	% Similarity	System
AM690962	Uncultured actinobacterium, clone TH3-71	*Actinobacteria*	2F	488	100	Port of Orange
			6B	536	99	Port LaVaca
AM990882	Rhodobacteraceae bacterium MOLA 108	*a-Proteobacteria*	18C	513	99	Corpus Christi Bay
			17C	510	98	Nueces Bay
AY580658	Uncultured Bacteroidetes bacterium clone PI_RT146	*Bacteriodetes*	13H	262	95	Sabine Pass
			3B	518	97	Port Arthur
EF152371	Synechococcus sp. KUAC 3041	*Cyanobacteria*	15E	515	99	Copano Bay
			16D	528	99	Aransas Bay
			7G	530	99	Port of Corpus Christi
			8C	540	98	Port Mansfield
EF471520	Uncultured alpha proteobacterium clone CB22E02	*a-Proteobacteria*	5A	520	99	Port of Galveston
			6A	512	99	Port LaVaca
			14A	521	99	LaVaca Bay
			15A	496	99	Copano Bay
EF471570	Uncultured alpha proteobacterium clone CB11D07	*a-Proteobacteria*	15B	496	99	Copano Bay
			16B	508	100	Aransas Bay
			18A	518	99	Corpus Christi Bay
			17A	444	98	Nueces Bay
EF471578	Uncultured Bacteroidetes bacterium clone CB11D01+B63	*Bacteriodetes*	7D	251	98	Port of Corpus Christi
			17B	536	99	Nueces Bay
EF471594	Uncultured actinobacterium clone CB41H04	*Actinobacteria*	15G	534	99	Copano Bay
			18G	528	99	Corpus Christi Bay
EF471625	Uncultured actinobacterium clone CB01B07	*Actinobacteria*	5E	527	99	Port of Galveston
			6F	251	96	Port LaVaca
			16E	203	99	Aransas Bay
EF471700	Uncultured actinobacterium clone CB01B09	*Actinobacteria*	6G	527	99	Port LaVaca
			15H	536	99	Copano Bay
			16G	522	99	Aransas Bay
			8E	525	99	Port Mansfield

Table III. 8. DGGE band sequences that matched 11 cultured isolates and 5 uncultured clones identified to the genus level.

Nearest Match on GenBank	Accession Number	% Similarity	Phylum	System	Band
Agromyces sp. T33Y	AM983510	98	*Actinobacteria*	Baffin Bay	19J
Balneola sp. MOLA 132	AM990906	96	*Bacteroidetes*	Baffin Bay	19E
Candidatus Pelagibacter ubique clone 01-003783	EU410957	99	*α-Proteobacteria*	Port of Galveston	5B
Halomonas sp. MOLA 69	AM990844	100	*γ-Proteobacteria*	Aransas Bay	16F
Hydrogenophaga sp. JPB-3.10	EU652485	98	*β-Proteobacteria*	Port of Brownsville	10G
Polynucleobacter necessarius subsp. asymbioticus	AB470466	97	*β-Proteobacteria*	Port of Beaumont	1D
Polynucleobacter necessarius subsp. necessarius STIR1	CP001010	98	*β-Proteobacteria*	Sabine Pass	13I
Synechococcus sp. KUAC 3041	EF152371	99	*Cyanobacteria*	Aransas Bay	16D
Synechococcus sp. KUAC 3041	EF152371	99	*Cyanobacteria*	Copano Bay	15E
Synechococcus sp. KUAC 3041	EF152371	99	*Cyanobacteria*	Port of Corpus Christi	7G
Synechococcus sp. KUAC 3041	EF152371	98	*Cyanobacteria*	Port Mansfield	8C
Synechococcus sp. MBIC10089	AB058226	98	*Cyanobacteria*	Port of Galveston	5D
Uncultured Cellulomonas sp. clone VSJ5Q1u80	EU631294	100	*Actinobacteria*	Port Mansfield	8D
Uncultured Synechococcus sp. clone CB11D06	EF471563	99	*Cyanobacteria*	Port LaVaca	6E
Uncultured Synechococcus sp. clone CB11G10	EF471565	98	*Cyanobacteria*	LaVaca Bay	14C
Uncultured Synechococcus sp. clone CB22C09	EF471585	94	*Cyanobacteria*	Port of Galveston	5C
Uncultured Synechococcus sp. clone MPWIC_C06	EF414206	99	*Cyanobacteria*	Port LaVaca	6D

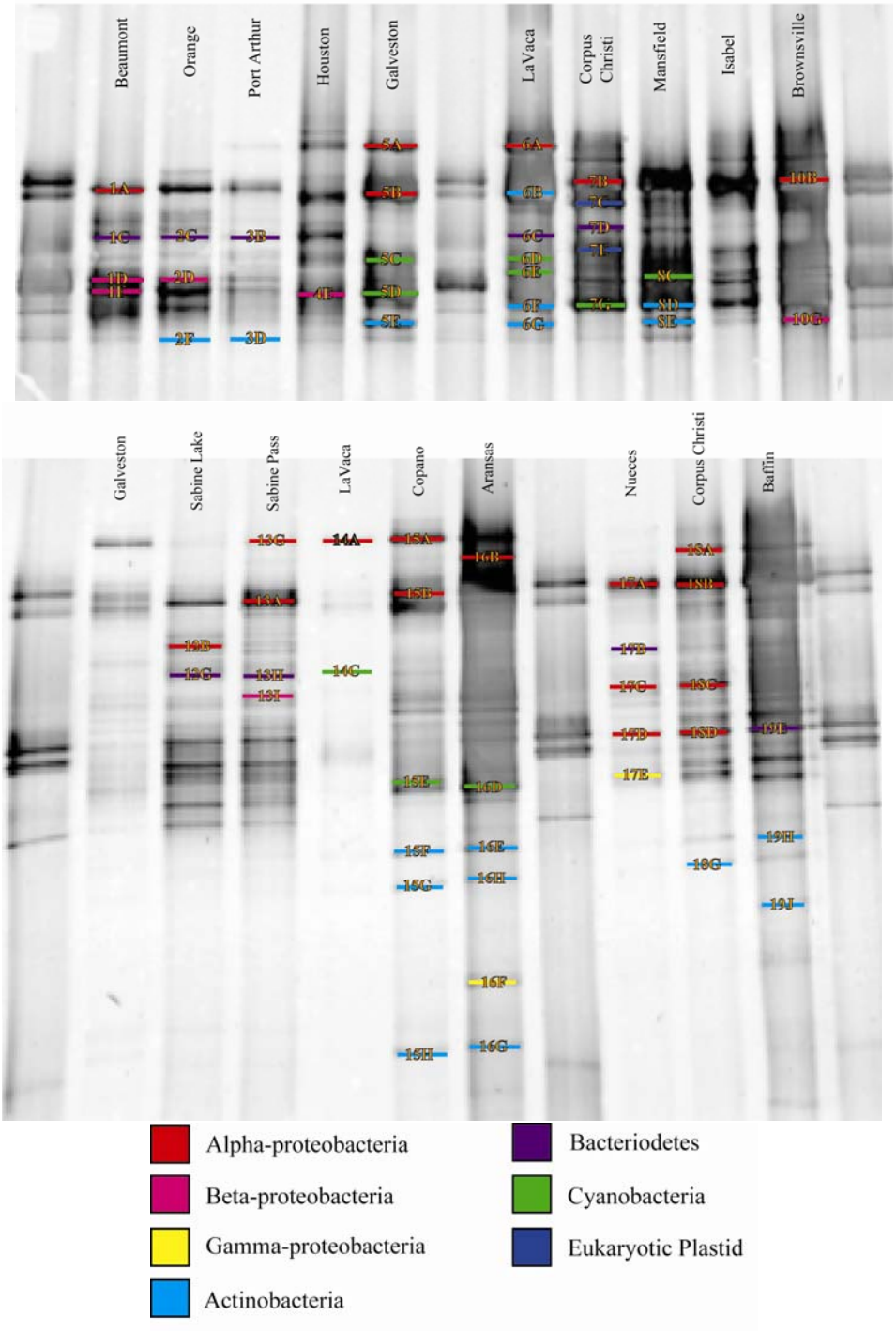

Fig. III. 18. Positions of sequenced bands from DGGE analysis of Texas ports and bays from the spring of 2007. Color of band corresponds to bacterial phylum of the band.

Discussion

Invasive bacteria represent a poorly studied field, due to the argument that the current bacterial populations almost certainly do not represent 'pre-anthropogenic influences' composition and are constantly changing, and that bacteria have the capacity to live in any environment that is survivable and lack a natural biogeography. The first limitation is unsurpassable because trading, specifically, maritime commerce, has been occurring in the Gulf for centuries with no documentation of bacteria present in the waters. This study cannot reconstruct the past, but has taken the first steps to address the impact of the introduction of bacteria into Texas estuarine systems as they are today. It is impossible to declare a species invasive if the endemic populations and their dynamics are unknown. This study looked at three areas of interest in the issue of ballast water transportation of bacteria into Texas estuaries: the ballast water itself, bacterial populations in the Ports of Houston and Galveston, and the bacterial inhabitants in the some of the Texas estuarine systems.

Ballast Water

Total bacterial communities in ballast water have never been studied. These communities were thought to be similar because of ballast water exchange measures, taking water from the upper water column of the pelagic zone of the ocean, not mixing with extra-tank environments during travel, and without sunlight entering the tank. These parallel conditions should have favored the same bacterial lineages, resulting in

resembling communities at the end of the voyage. This section of the study tested the second hypothesis:

> *Hypothesis 2:* Bacterial communities in ships' ballast water will have parallel compositions to others where open-ocean ballast water exchange occurred in proximate geographical locations.

From this data set, ships do appear to have more similar bacterial community compositions the closer they are geographically at the time of exchange. However, these communities were always less than 65% similar, demonstrating differences in bacterial members between ships. This data set was divided according to geographic location, but the collection dates of the ships also leads to the possibility that the differences could be caused by seasonal variations in the pelagic communities of the open ocean. Seasonal variation should be carefully considered, because as it was shown in the next two sections, salinity, temperature, and dissolved oxygen levels can drastically change the makeup of a microbial community. However, although no more similar than 65%, neither of the ships were less than 40% similar. Thus, the tanks are not completely different, indicating that these parallel selection pressures may indeed be impacting the ballast communities in a way that can be monitored, with the bacteria found in them useful as indicator species or proxies of ballast activity in an area. The five ships' ballast water exchange sites examined were distributed across the Pacific Ocean, so the presence of at least 40% similarity between ships is reasonably high. This data lacks the

support of sequences, allowing identification of the bands. Future work should include a much larger sample set of ballast water from ships from all around the globe, preferentially collected on or near the same date to exclude the seasonality variable in the populations.

Ports of Houston and Galveston

The bacterial communities in the Ports of Houston and Galveston are unknown. However, like other ecosystems, it was expected that they experienced seasonal shifts in composition as temperature, salinity and dissolved oxygen levels change over time. Since the sequence data has not yet been obtained, it is impossible to characterize the individual members of these communities, but DGGE analysis allowed observation of the expected seasonal shifts. This section of the study tested the third hypothesis:

> *Hypothesis 3*: Bacterial communities in the Ports of Houston and Galveston will exhibit temporal variations related to changes in temperature and salinity.

Both ports exhibited variations in temperature, dissolved oxygen, salinity and specific conductivity over the course of one year, in a seasonal manner. Both ports showed a correlation between salinity and bacterial diversity. However, this correlation was completely opposite between the ports. The Port of Houston experienced a decrease in diversity as salinity increased in the winter, whereas the Port of Galveston showed an increase in diversity during the winter months with higher salinity. The average salinity

in the Port of Galveston is much higher due to its proximal location to the Gulf of Mexico, whereas the Port of Houston has higher levels of freshwater from the San Jacinto River into the Houston Ship Channel. The communities in the Port of Galveston would be more acclimated to higher salinities, and probably flourished as salinity levels increased. With the exception of the month of August, 2007, when the salinity of the Port of Galveston dropped to an anomalous 13.6 ppt, the lowest salinity values in the port were higher than the highest salinity values of the Port of Houston. This would support the idea that the endemic bacterial populations of the Port of Galveston are markedly different in composition from the Port of Houston, despite location in the same bay system. The bacteria in the Port of Houston are most likely acclimated to lower salinities, almost bordering on freshwater levels, and would experience difficulty living in higher salinities, explaining why diversity decreased so sharply when salinity increased.

A goal of this section of the study was to determine if there were any visible effects of ballast water transport on bacterial populations in the communities living within a port. The Port of Houston receives hundreds of vessels every month, each potentially releasing a different cohort of bacteria into the port waters. This is highly speculative, but, if indeed these bacteria were invasive and able to out-compete the endemic population, each month's DGGE fingerprint would have been markedly different. These differences would not show a seasonal pattern, but rather the community would change on a more frequent basis according to the different ships deballasting in port. However, that is not the case seen in this one year of observation. Within each

season, the communities are relatively stable. Importantly, comparison of the June 2007 with the June 2008 communities revealed some of the same organisms reappearing in the same waters.

Further studies should examine seasonal patterns over the course of many years to determine if the same observations concerning salinity and diversity can be made. Additionally, sequencing of the bands in this study will add community characterization for these ports, providing the framework for a catalog of the endemic species of Galveston Bay. These port data will be compared to those of bacteria found in the ballast water of ships arriving into the Port of Houston (discussed in Chapter II), to determine how they contrast, and if the bacteria in the ballast water possess an invasive threat to the endemics, although preliminarily, it does not seem as though they do.

Texas Ports and Estuaries

To continue the study from the previous section, the survey of bacterial communities expanded from examining only the Galveston Bay system to including the entire Texas coast. Characterization of the endemic populations and understanding their dynamics is important to establish a study on invasive organisms. As stated previously, it is possible that transported species do not affect the native populations in a significant or invasive manner. The different ports located across the Gulf Coast provide potential hot spots for ballast-transport invaders. As with the Ports of Houston and Galveston section above, a goal of this last area of study is observing for drastic changes in bacterial communities in estuarine systems where major ports are found, and how the port

communities compare with those in the greater bay system. This section tested the final hypothesis of the study:

> *Hypothesis 4*: Bacterial communities will be similar in closely located port and bay systems, but will show greater variations in further systems.

In general, most systems clustered together based on geographic proximity, with some exceptions. The trend in community composition appeared to correlate less with geographic location and more with salinity of the bay system, which again confirms that salinity is the most influential abiotic factor on bacterial community composition. In both the spring and summer data sets, salinity and specific conductivity were highly variable, while temperature, pH and dissolved oxygen stayed relatively stable across systems. The systems were clustered into groups that seem most related to salinity, with the bays and ports having higher salinities more closely related to each other, and vice versa for the systems with low salinities. The separation occurs near the LaVaca Bay system, which is a small part of the larger Matagorda Bay, about the midpoint of the Texas Gulf Coast. Most of the major Texas River basins end around this system and the San Antonio Bay system to the immediate south (not included in this study). Beyond the Guadalupe and San Antonio River basins lies only the Nueces River until the Rio Grande at the border between Mexico and Texas. All the bay systems south from this point have limited fresh water inflow, accounting for the increase in salinity. The Nueces River empties into the Nueces Bay, which flows through the Corpus Christi Bay and into

the Gulf. However, in the spring data set, they were less than 80% similar, despite having almost identical temperatures and salinities only varying by 1ppt. The answer is not apparent, but could be related to site of water sample collection. The water was sampled off a pier into relatively deep waters in Corpus Christi Bay, while water was sampled off a boat ramp in shallow waters in Nueces Bay. This difference between littoral and pelagic zones could be the cause of the similarity discrepancy. These higher salinity systems have bacterial communities that would be acclimated to the higher salt content, versus the communities of the northern, more freshwater systems.

The major ports of commercial shipping in Texas are the Ports of Beaumont, Houston, LaVaca, Corpus Christi and Brownsville. There does not seem to be much evidence that the presence of the shipping industry has any effect on the bacterial population. However, the dataset is limited and additional sampling is necessary to verify trends. Only slight evidence lies in the spring data set in which Port LaVaca and Port of Brownsville were most similar to each other, sharing a 93.2% similarity. These two ports had very different salinities (13.88 and 36.78ppt, respectively), so salinity cannot account for the similarity, and this might be an indication of ship activity influencing bacterial communities. Again, other factors such as influx of dissolved organic carbon may have also been an influence, however this parameter was not measured.

None of the bacterial sequences derived from the spring data set matched the sequences from the ballast water clone library created in Chapter II. No members of the verrucomicrobia or the planctomycetes were found in any of the Texas waters, but

instead cyanobacteria and eukaryotic plastids were present, which the ballast water did not. In the ballast water, the predominant group was the gammaproteobacteria, followed by the alphaproteobacteria and betaproteobacteria. The bacteriodetes and actinobacteria made up a small percentage of the total population. In the Texas waters, the predominant group was the alphaproteobacteria, followed by actinobacteria, cyanobacteria, bacteriodetes, betaproteobacteria, then the gammaproteobacteria, and lastly, the plastids. The low number of gammaproteobacteria was surprising, as they are a ubiquitous, dominating group of bacteria.

The predominant bacterial groups found in the Texas bays and ports are discussed: The actinobacteria are high G+C gram positive bacteria that make up a very large phylum that contains over 30 families and are found in many diverse environments, including freshwater, estuarine and marine waters (Glockner et al., 2000, Ventura et al., 2007). They are primarily aerobic and are important to soil environments (Madigan et al., 2005). Of the 18 actinobacteria found in the study in this chapter, two were identified as *Cellulomonas* and *Agromyces*. *Cellulomonas* is a coryneform facultative aerobe that has a genetic G+C ratio of 71-73%. The GenBank match to the band excised from the Port Mansfield sample was obtained from a swab of a showerhead, indicating that is it most likely a freshwater-acclimated organism. *Agromyces*, found in Baffin Bay, is a filamentous facultative aerobe that lives in soil habitats (Gledhill and Casida, 1969) with an optimal growth temperature between 26-30^{oC} and a G+C ratio of 70-72% (Zgurskaya et al., 1992).

The proteobacteria are a large and diverse group of bacteria. The five classes of proteobacteria, named in sequential Greek letters (α, β, γ, δ, and ε) are grouped according to 16s rRNA similarities, with each group containing within itself a metabolically and ecologically diverse collection of bacteria.

The alphaproteobacteria –The alphaproteobacteria are a cosmopolitan group of gram negative, oligotrophic bacteria found in both freshwater and marine ecosystems (Nogales et al., 2007, Nold and Zwart, 1998). This class made up the majority of bacteria found in this study with 21 different species, with only one identified. The SAR11 clade, isolated from the Sargasso Sea (Giovannoni et al., 1990), has a world-wide distribution and includes *Pelagibacter ubique*, possibly the most numerous bacteria, comprising about one third of all prokaryotic cells in marine surface waters (Morris et al., 2002). *Pelagibacter ubique* was first identified from samples collected in the Sargasso Sea (Rappé et al., 2002), is distributed world-wide (Morris et al., 2002), has the smallest genome and cell size of any free living bacterium and generally exists as free living in the water column (Giovannoni et al., 2005). The *Pelagibacter ubique* genome contains a hypervariable region that allows for the abundance of the SAR11 clade in many diverse marine environments (Gilbert et al., 2008). Finding only one *P. ubique* in the Port of Galveston is a surprise, considering the natural abundance of the organism, but this is probably due to the limited sample size of sequenced bands.

The betaproteobacteria – The betaproteobacteria are a diverse group of aerobic or facultative aerobic gram negative bacteria that are versatile in degradation capabilities, with the majority being chemolithic and some phototrophs (Madigan et al., 2005).

Typically soil or freshwater inhabitants (Bouvier and del Giorgio, 2002), one clade of betaproteobacteria known as the OM43, as well as clones belonging to the *Burkholderiaceae* family have been found off the coast of Oregon (Rappé et al., 2000, Morris et al., 2006). Six of the seven betaproteobacteria were found in the Ports of Beaumont, Orange, Houston and Sabine Pass, which all have low salinities, and high levels of shipping traffic.

However, one betaproteobacteria was found in the least likely area, the highly saline Port of Brownsville. The *Hydrogenophaga* genus of bacteria are chemo-organotrophic, depending on organic chemicals as an energy and carbon source, or chemolithoautotrophic, using CO_2 as a carbon source and H_2 oxidation as an energy source (Kampfer et al., 2005). This group of bacteria is often found in association with oil-contaminated waters, activated sludge, or wastewater treatment plants (Amann et al., 1996). The species *H. flava* can degrade methyl *tert*-butyl ether (MTBE), an oxygenate additive to gasoline, into CO_2, acting as a bioaugmentation system in aquifers (Streger et al., 2002). While the species of *Hydrogenophaga* found in the Port of Brownsville has not been characterized, it probably possess the same capabilities as the others species, and probably is exhibiting bioaugmentation to some extent. The water in the Port of Brownsville was very contaminated with sludge and oil, and appeared reddish brown in color. The optimal salinity for *Hydrogenophaga* is not known, but generally assumed to be low, with most cultures grown on media with 0.5% (wt/vol) NaCl (equivalent of 5ppt) or less (Willems et al., 1989). The Port of Brownsville had a salinity of 36.8ppt; this discrepancy is a mystery, unless the organism found in this study is a new species of

Hydrogenophaga that exhibits a higher tolerance for salinity than other betaproteobacteria.

Five betaproteobacteria were found in the Sabine Lake system and two were identified: *Polynucleobacter necessarius subsp. asymbioticus* in the Port of Beaumont, and *Polynucleobacter necessarius subsp. necessarius* in Sabine Pass. Temperature and pH were slightly higher in Sabine Pass, but salinity was lower by 1.64ppt, which was expected, as the Port of Orange salinity was almost 0ppt, and Sabine Pass contains a mixture of all the ports in the Sabine Lake system. *Polynucleobacter necessarius* is an obligate intracellular endosymbiont of the hypotrichous, freshwater protozoan ciliate, *Euplotes aediculatus* (Springer et al., 1996). It was first describe in 1987 by Heckmann and Schmidt, and is characterized as being non-motile, mesophilic, aerobic, heterotrophic and inhabiting the cytoplasm of its host with anywhere from 900-1,000 cells. *Polynucleobacter necessarius* is a frequently-detected cosmopolitan freshwater bacterium and has been found in numerous climates such as temperate Central Europe, subtropical China, and tropical East Africa (Hahn, 2003). It is so abundant that it made up 60% of all bacterioplankton in one small, dystrophic freshwater habitat, and upon molecular analysis revealed extraordinarily low intraspecific diversity (Hahn et al., 2005). Although the salinity of both locations is a little brackish for *P. necessarius,* the overall Sabine Lake system is the only freshwater system of the Texas systems. This bacterium was not found in any of the other systems, which is expected based on its need for low salinities. It also was found twice in such a small sample size, an indication that it is very abundant in the Sabine system, matching the observations of previous studies.

The gammaproteobacteria – The gammaproteobacteria is the largest, most diverse class of proteobacteria, containing many medically and scientifically important groups. Although they are known to be predominant in most ecosystems (Nold and Zwart, 1998), only two gammaproteobacteria were found in this study, one belonging to the halophilic genus *Halomonas*. *Halomonas* contains over 20 separate species and thrives in hypersaline (>30ppt) waters, usually cultured between 30-150ppt. However, many of the species can grow in salinities between 5-30ppt, although most have optimums with a high-end range of over 50ppt. This could allow some species of *Halomonas* adapted to lower salinities to survive in the brackish waters of the Texas coast. The one *Halomonas* found was in Aransas Bay, which had a salinity of 22.65ppt, reasonably within the allowable growth range for many species of *Halomonas*. The bacterial match on GenBank was collected from the Bay of Banyuls in France, off the northwest of the Mediterranean Sea (Larcher et al., unpublished) which has a very high average salinity over 30ppt (Charles et al., 2005). This presents a challenge: how could the bacterium found in Aransas Bay that matches the bacterium from the Bay of Banyuls with 100% similarity survive in a much lower salinity? It is likely that diversity based on 16s rRNA sequences fails to differentiate between bacteria acclimated to different climates, containing different enzyme and structural protein gene sequences not found in the ribosomal RNA regions.

The bacteroidetes – The bacteroidetes are one of the most abundant groups of aquatic bacteria, and have been found in coastal seawater, oceanic water, sea ice, and freshwater (O'Sullivan et al., 2004, DeLong et al., 1993). Of the eight that were found in

this study, only one was able to be identified. A member of the genus *Balneola* was found in Baffin Bay. Only two recognized species exist for the genus, *B. vulgaris* and *B. alkaliphila*. Both species were also found in the same surface waters of the Bay of Banyuls, France, as the *Halomonas* species discussed above. They are aerobic and motile, and can grow in relatively the same conditions (Urios et al., 2006; Urios et al., 2008). *Baneola vulgaris* has an optimal temperature of 30°C, salinity of 20ppt and pH of 8, while *B. alkaliphila* has an optimal temperature of 25°C, salinity of 30ppt and pH between 8-9. Baffin Bay had a temperature of 21.36°C, a pH of 7.47, and the highest salinity of 42.4ppt. While either of these two species could grow in the conditions of Baffin Bay, the bacterium found in this study shared only 96% similarity with the *Balneola* genus (Table III. 6). It is accepted among molecular biologists that any 16s rDNA similarity value below 97% between two organisms should be considered two different species (Madigan et al., 2005). Species whose 16s rDNA similarities are lower than 97% have DNA-DNA hybridization relatedness below 70%, the accepted threshold for species delineation (Rosselló-Mora and Amann, 2001). This will be discussed in detail in the next chapter.

The cyanobacteria – Although the cyanobacteria encompass many genera, the only representatives of this bacterial phylum found along the Texas gulf were the *Synechococcus* genus of tiny photosynthetic prokaryotes. *Synechococcus* is a very important contributor to primary productivity in aquatic habitats. It was first identified in 1979 by Waterbury et al., and has since been isolated and identified all over the world where, in combination with another genus, *Prochlorococcus*, they dominate global

primary productivity and are critical components at the base of the marine food web (Scanlan and West, 2002). Primary productivity percentages range from 5-10% in the coastal waters near Woods Hole, to 10-25% in the oligotrophic Sargasso Sea (Waterbury et al., 1986). *Synechococcus* can be found throughout all oceanic habitats, from the polar regions to the tropics, but are more abundant in warmer, more nutrient-rich surface waters (Waterbury et al., 1986). *Synechococcus* has been phylogenetically divided into 6 clusters: the *Cyanobacterium* (with G+C ratios of 39-43%), the *Synechococcus* (with G+C ratios of 47-56%), and the Cyanobium (with G+C ratios between 66-71%), and the Marine Clusters (MC): MC-A, MC-B, and MC-C (Chen et al., 2004; Waterbury et al., 1986). The MC-A cluster members contain the light-harvesting pigment phycoerythrin, while the MC-B and MC-C clusters use only phycocyanin as the major light harvesting pigment, and do not have phycoerythrin (Chen et al., 2004).

Synechococcus also plays major roles in coastal estuaries. Estuarine *Synechococcus* belong to the MC-B cluster, while the marine members belong to the MC-A cluster (Chen et al., 2006; Wang and Chen, 2008), and all the estuarine, coastal and marine *Synechococcus*, grouped together with the *Prochlorococcus* form a monophyletic group based on members containing Form I RuBisCO, while all freshwater cyanobacteria and marine filamentous cyanobacteria cluster into the Form II RuBisCO group (Chen et al., 2004). So the estuarine *Synechococcus* are more closely related to the marine members of the genus than to the freshwater. *Synechococcus* has been found in many estuarine systems, including Chesapeake Bay and Florida Bay, as well as dominating regions of the Mississippi River plume, which is the major

contributor of nutrients to the Louisiana and Texas coast (Wawrik and Paul, 2004). The *Synechococcus* genotypes changed with salinity, and were more abundant in regions with higher salinity and temperature (Chen et al., 2004; Phlips et al., 1999).

All nine cyanobacterial DGGE bands were identified by GenBank as members of the genus *Synechococcus*. Four were identified as the same organism, *Synechococcus sp. KUAC 3041*, isolated from Kuwaiti seawater (accession number EF152371), and were found in Aransas and Copano Bay, the Port of Corpus Christi and Port Mansfield, all systems in the lower half of the Texas Gulf Coast, where temperature and salinity were between 20-23°C and 22-33ppt, respectively. Two bands identified as *Synechococcus* was found in the Port of Galveston. Band 5C only displayed 94% similarity with the GenBank entry, but this was the closest match for the band in total, indicating a possible new bacterium not described previously. The other three bands were found in the LaVaca Bay system, one in the bay itself, two in the port, where salinity is slightly lower than the bay systems to the south. The uncultured *Synechococcus* clones closest to these bands were found in the Chesapeake Bay, so these bands are most likely included in the MC-B group. Because all these bacteria were found in brackish estuarine systems, they are all most likely in the MC-B and Form I RuBisCO groups, differentiating them from the freshwater cyanobacteria. These groups are very important to the primary productivity of the estuarine ecosystem, and were expected to be a very abundant group.

However, the sampling of bacterial sequences from all the systems is minimal, and many of the bands failed to yield readable sequences, so the sequencing data provide just a small snapshot of the larger population. The percentages of bacteria found do not

necessarily signify predominant bacteria in the Texas systems. There are several points at which bias can enter. First, PCR bias might preferentially over amplify certain bacterial sequences, making it appear as though they were more abundant. Secondly, band excision was a purely subjective decision, and not all bands were excised and sequenced. Lastly, not all excised bands sequenced successfully. This leaves huge gaps in the total picture of Texas bacterial communities.

Future studies are needed to fill in these gaps. Sampling trips need to hit all estuarine systems in the state of Texas, and collect water from the middle of the system as well as by the shore. Comparisons between seasons would show how the communities varied temporally, but to do this, sampling sites need to remain consistent. Further studies would involve looking at seasonal patterns over the course of many years to see if the same observations concerning salinity and diversity are made. Additionally, sequencing of more bands from this study will add community characterization for these systems, providing the framework for a catalog of the endemic species of the Texas Gulf Coast. Only with an initial dataset can we assess the impact of invasive bacteria. This study is the first characterization of bacterial diversity in the ports and bays of Texas. The dataset provides an important benchmark for evaluation of invasive species via ballast water in the years to come as the shipping industry in Texas increases.

CHAPTER IV

CONCLUSIONS

Conclusions

Bacterial Communities in Ballast Water

Although previous studies have counted total bacterial abundance via cell counts, the diversity of bacteria in ballast tanks was unknown prior to this study. Construction of a 16S rRNA clone library and rarefaction analysis revealed high bacterial diversity in the ballast tank examined in this thesis. Short travel distances and introduced nutrients upon ballast water exchange could account for the highly diverse community in an otherwise closed system. Bacterial phyla found in the ballast tank were typical of coastal and estuarine marine habitats. Phylogenetic analysis of nearest 16S rRNA relatives indicated that the bacterial groups found in the ballast water most likely originated from a fresh or slightly brackish, habitat. The most scrutinized bacteria in invasive studies of ballast water have been pathogens like *Vibrio cholerae* and *Escherichia coli*, but one of the specific aims of this study was to look beyond pathogenic bacteria in ballast communities to examine the total bacterial diversity. Surprisingly, not a single human pathogen was detected by this clone library. PCR detection may not be adequate to detect single cells and incubation of ballast water in selective media is recommended for future screening for pathogens. Previous studies of pathogens in ballast water have isolated these bacteria using selective media (Burkholder et al., 2007; Ruiz et al., 2000).

DGGE analysis of the bacterial community compositions between ships that had exchanged ballast water in the Pacific Ocean were on average at about 50% similar (Chapter III). This indicates that there are shared parallel selection pressures within the tank environment, possibly the 'ballast water bacterial community', however a high level of diversity between ships still exists. The 16S rRNA clone library analysis of ballast water purportedly exchanged in the EEZ off the west coast of Africa (Chapter II) appeared to have either a unique 'ballast water' community composition or a mixture of marine pelagic as well as coastal/estuarine communities. Additional analyses of ballast tanks are necessary to determine if these trends persist.

Ballast water exchange is the most common form of invasive control in the shipping industry, and it is mandated by the International Maritime Organization until water treatment technology is required onboard (S. 1578, The Ballast Water management Act of 2007, Hawkes, 2007). Many studies have sought to examine the efficacy of this practice, including measuring abundance of organisms before and after exchange to developing a model (Locke et al., 1993, Burkholder et al., 2007, Costello et al., 2007). The studies that measured exact numbers of organisms focused on eukaryotes, and studies on bacteria only measured cell abundance. This study was the first to characterize bacterial diversity in the ballast tank and use this data as a means of evaluating the efficacy of ballast water exchange.

The 16S rRNA clone library analysis in this study indicated that ballast water exchange did not completely replace the port water and its inhabitants, and that this was not a 100% efficient form of invasive species control. A model proposed to calculate the

efficiency of ballast water exchange failed to reject the null hypotheses that the exchange had been 100% effective and it had been completely ineffective, and were unable to draw a definitive conclusion (Costello et al., 2007). The results of this study support this finding that while not completely efficient at eliminating all the original species picked up in a port, ballast water exchange does help to mitigate the problem.

Many factors may contribute to lack of full exchange of ballast water, including inclement weather, time, and the individual structural design of the ballast tanks allowing inoculates to remain within the tank, either attached to algae or picoeukaryotes, or within biofilms. Biofilms are organic matrices that can form on any surface within the tank submerged in water, providing a protective refuge from stress factors. While containing fewer organisms than the ballast water, biofilms have been shown to harbor bacteria, acting as potential seed banks releasing bacteria into new waters pumped onboard (Drake et al., 2005). This study points to the undeniable fact that ballast water is still a vector for microbial transport and a viable invasive threat.

Bacterial Communities in Texas Estuaries

The bacterial communities in the Texas estuaries are relatively unknown. In order to assess the impact of ballast water on the endemics, it was important to characterize them. While this study is mainly preliminary in nature, with only a few sample seasons, it does shine a first light onto the dynamics of Texas estuarine microbes. Both the temporal study of the Ports of Houston and Galveston as well as the spatial study of the other Texas ports and bays showed delineations that coincided with changes

in salinity. It is evident by the response to changes in salinity that the organism that inhabit the Port of Houston are acclimated to lower salinities, and probably consist of bacterial phyla found in lower salinity waters. The community of the Port of Galveston is opposite in salinity tolerance, being more acclimated to higher, more saline conditions.

To assess the effect of deballasting in the Ports of Houston and Galveston, I was looking for radical changes in the community of each month. As stated earlier, the Port of Houston alone receives over 720,000 metric tons of ballast discharge in a year. If bacteria released from this ballast water were able to survive and proliferate, this influence should have been visible in the DGGE gels. While main constituents of the endemic population would have been present, new bands would be detected each month. If the ballast water bacteria were able to out-compete the endemics, I would have seen a complete disruption of the communities over time. It is possible, however, that simply not enough time passed to observe the total effect of the species introduced by deballasting. It was observed, however, that the relatively stable communities varied according to the season and changes in salinity.

The analysis of Texas ports and bays revealed a similar pattern. The systems north of the Guadalupe River had more freshwater input, resulting in lower overall salinities, while the southern systems had much higher salinities. The only evidence for an effect of the shipping industry on port communities was evident in similarities between the Port of Brownsville and Port LaVaca in the spring 2007 data set. However, the reasons for this similarity are unknown as they did not share any sequences nor do the bands appear to be in the same location, and could be an artifact in the algorithm

used to calculate percent similarity. Rapid changes in salinity produced disruptions in community composition. If deballasting of estuarine or even marine bacteria in the ballast water is having an adverse effect on the native populations, it is not a large enough impact to be observable yet.

To perform the ecological studies, the 16s rRNA gene was used. The 16s rRNA gene is commonly used in phylogenetics and microbial ecology because it has the necessary characteristics for a good genetic marker: it is ubiquitous and conserved (for universal analysis), but also has variable regions for more fine-scaled analysis (family and genus levels) (Case et al., 2007). However, it also displays multiple operon copy number and intragenomic heterogeneity, which can cause the appearance of multiple organisms in molecular fingerprints when only one is actually present (Crosby and Criddle, 2003; Acinas et al., 2004). This leads to biases in diversity and decreased resolution in phylogenetic constructions (Case et al., 2007). One way to solve this problem is to use another house-keeping gene of single copy number that also contains the needed characteristics of a good phylogenetic marker, such as the RNA polymerase β subunit (rpoB), found in all bacteria (Case et al., 2007).

It is accepted among molecular biologists that any 16s rDNA similarity value below 97% between two organisms should be considered two different species (Madigan et al., 2005). Species whose 16s rDNA similarities are lower than 97% have DNA-DNA hybridization relatedness below 70%, the accepted threshold for species delineation (Rosselló-Mora and Amann, 2001). However, molecular data cannot resolve the species concept for bacteria. It requires an approach that combines DNA sequence data with

numerical analyses of independently co-varying characters that may not be consistently found within the taxon. Rosselló-Mora and Amann (2001) define this phylo-phenetic species concept as "a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by discriminative phenotypic property." This is important, because we can no longer just sequence individual bacteria, compare them to other known sequences, and automatically label them as a novel species when the similarity percentage falls below 97. When studying bacteria through cultivation-independent methods, as was done is this study, it is important to keep sequences in a phylogenetic framework, keeping in mind the high reproduction rates and horizontal gene transfer that occurs in prokaryotes. This is useful for distinguishing speciation events in bacteria, as discussed below.

Invasive Bacteria Concerns

In 1999, Executive Order 13112 defined an invasive species as "an alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health." (Federal Register, 1999). This definition is extremely anthropocentric and does not take slighter ecological nuances into account. The result of this mindset is that invasive bacteria have long been considered to only be pathogens, like *Vibrio cholerae*, and studies on ballast water bacteria transport have focused only on finding pathogenic bacteria, instead of examining all the different species that undergo intercontinental translocation. In 2000, Richardson et al. proposed that the term

"invasive" should not concern environmental and economic impact, but rather the extent to which an introduced population can survive and proliferate in novel environments, regardless of effect on native species.

To be successfully invasive, a population must overcome many barriers and survive through several life stages (Richardson et al., 2000; Colautti and MacIsaac, 2004; Kolar and Lodge, 2001). The first barrier is geographical, which usually requires human interaction to surpass and includes the timing of introduction to a new environment and survival during transport. The next two barriers are local environmental factors, such as abiotic conditions and native community dynamics, and reproductive success. Overcoming these barriers leads to establishment or naturalization, in which a species must be able to survive and proliferate in the new environment. A species becomes invasive if it can become wide-spread by overcoming the last three barriers: dispersal, environmental factors in the human-modified or alien-dominated area, and environmental factors presented by the native communities in any new environment that is reached.

The success of the would-be invaders is mainly determined by three factors: propagule pressure, physicochemical requirements and community interactions (Colautti and MacIsaac, 2004). Propagule pressure is the number of introduced bacteria at a given event in conjunction with the frequency of introduction events. In the case of ballast water, the total number of bacteria was shown in this study to be very high as well as the number of ships that arrive and deballast in the Port of Houston on a daily basis. This suggests that ballast water transport results in high propagule pressure. The probability

of a species becoming established increases as the numbers of individuals and introduction events increase (Kolar and Lodge, 2001). The physicochemical requirements determine whether or not the introduced species can survive in the receiving environment. While it is true that bacteria possess broad tolerances to physical and chemical conditions, aquatic bacteria in particular seem to be most sensitive to salinity, according to this study, and are adapted for a specific range of salt concentrations. Community interactions determine if the new organisms can successfully compete and survive with the native populations. Nutrient uptake mechanisms as well as usable sources of carbon and energy vary between bacteria, and may limit the efficiency and extent of nutrient intake, decreases competitiveness. All three of these factors can work for or against the new population at almost any stage of the invasion process.

A model was proposed by Colautti and MacIsaac (2004) to illustrate the different stages of invasion by plants. This model was modified specifically for ballast water transport of bacteria (Fig. IV. 1). There are 5 stages of invasion. Organisms are in stage 0 when the propagules are residing in the donor region. In our case, the donor region is the port of origin, where the original ballast water was obtained, or in the surface region of oceanic waters where ballast water exchange is about to occur. Stage one begins when the bacteria are in transit inside of the ballast tank. If they survive the voyage and are released into the receiving port, they have overcome the geographical barrier and now enter stage 2, the introduction, where they must overcome the novel biotic and abiotic factors. This is the stage in which the bacteria in Chapter II were intercepted, and their potential to survive in the new environment unknown. In stage 3

the bacteria are established, but localized and rare. This seems to be the case with the majority of bacteria found in the community profiles along the Texas Gulf Coast.

Propagule pressure and responses to physicochemical requirements and community interactions will determine if the bacteria enter stage 4a, where the bacteria are established in wide-spread systems yet not dominant, or 4b where the bacteria remain localized, but dominant the community. In stage 4a, high propagule pressure allows the bacteria to quickly spread to surrounding environments throughout the port and into the nearby bay system. However, limitations due to non-optimal physicochemical factors and inefficiency in community interactions prevent the bacteria from becoming dominant. In stage 4b, physicochemical requirements are met and community interactions are successful, allowing the bacteria to dominate the community, but propagule pressure is low, keeping them locally distributed. Finally, at stage 5, the bacteria are both wide-spread among the bay systems as well as dominant in the community. Propagule pressure will determine how wide-spread a population becomes, that is, which stage 3 bacterial populations will reach stage 4, and which stage

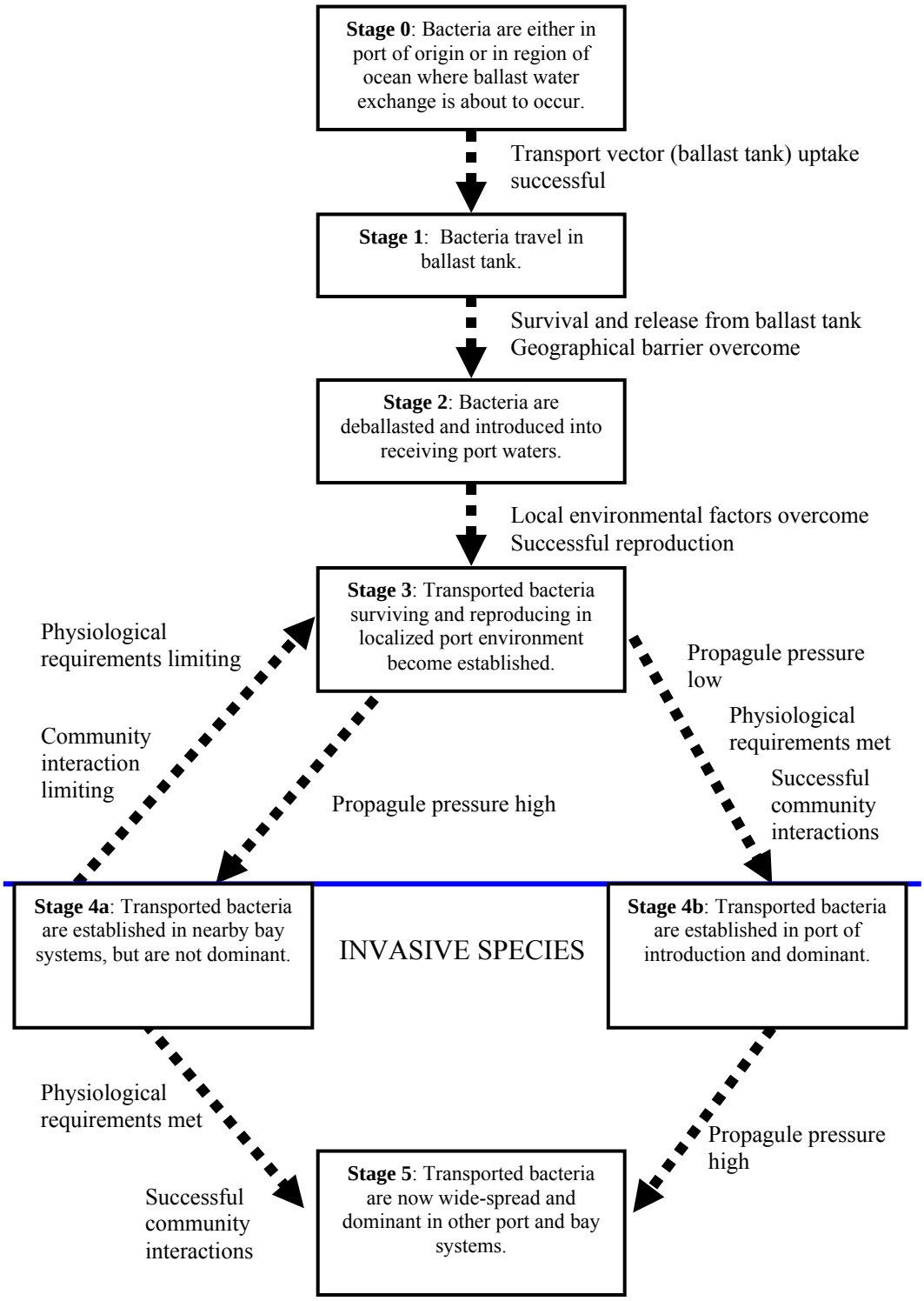

Fig. IV. 1. Invasive progression model for ballast water-transported bacteria. Modified from Colautti and MacIsaac, 2004.

4b populations will achieve stage 5, while physicochemical and community factors will determine how dominant the populations become within the community, that is, which stage 3 bacterial populations become stage 4b, and which 4a populations will reach stage 5.

At stage between 1 and 5, once the bacteria have left their original environment, they are thought to be introduced or transported, while any populations reaching stages 4a or 4b and 5 are said to be invasive (Colautti and MacIsaac, 2004). Using this model, we turn the focus away from whether or not the bacterium is pathogenic, and to how prevalent and dominant the population has become in the new environment. This is an important step to take in the consideration of bacterial invasive species. Previously, only pathogens were targets for invasive investigations. This study is the first to remove the 'pathogen-colored glasses' from the field of invasive bacteria, and focus on the ecology and movement of populations. Identifying the stage that each bacterial population has reached following ballast water transport needs will vary between the different species that exist in the population and is dependant on a spatial scale. The rate at which the bacteria will spread from the terminal water, where the deballasting occurs, to the port water and out to the bay water will vary according to the three factors discussed above: propagule pressure, physicochemical requirements and community interactions; each will be unique to the individual bacterial species.

It is impossible to divorce the concept of invasive species with the concept of a natural biogeography and endemic members. If an organism does not have a native environment, it cannot invade another. Biogeography of bacteria is still debated in the

microbial ecology scientific community. Having a biogeography entails a distribution in specific geographical areas, where dispersion is limited and local extinctions are possible. It has been argued that the high propagule pressure of bacteria lead to such large populations that local extinction would not occur and dispersal of bacteria or endospores would be very high (Fenchel, 2003; Fenchel and Finlay, 2004). Bacteria can be transported for great distances by many vectors (wind, water, attachment to organisms or motile substrates) across the globe, making the occurrence of allopatric speciation low. Observations like these led to the hypothesis of the Dutch biologist Bass-Becking that "Everything is everywhere, but the environment selects." According to this hypothesis, only stochastic processes control the distribution of bacteria in a ubiquitous manner, and environmental conditions affect growth conditions and proliferation. This severs historical explanations for the current population distributions (Fenchel, 2003).

However, recent cases have been made indicating a biogeography for bacteria, especially for marine populations. A latitudinal diversity gradient was found (similar to that of eukaryotes) in which bacterial species richness was strongly and inversely correlated with latitude, and positively correlated with sea surface water temperature (both at the time of sampling and annual averages), as well as salinity, but to a weaker extent (Furhman et al., 2008). Species richness was not correlated to variables associated with productivity, such as chlorophyll concentrations, annual primary productivity, distance from shore and bacterial abundance. Another study found a similar trend in latitude correlations with species richness evidencing a latitudinal gradient tin marine bacterial diversity, where only 10% of sampled bacterial ribotypes showed ubiquitous distribution between polar, temperate and tropical regions (Pommier et al., 2005). Falcón et al. (2008) found evidence for biogeography in bacterial assemblages in open ocean and coastal waters that was related to distance from shore as well as environmental factors such temperature, salinity and chlorophyll *a*. Extremophile bacteria, such as *Sulfolobus solfataricus*, which thrives in hot, acidic environments, have been found to be more closely related between areas of closer geographical proximity than to those further away, providing evidence of a natural and historical biogeography (Whitaker et al., 2003).

One of the largest obstacles in deciding if a bacterium has a biogeography is separating the effects of environmental selection pressures, both abiotic and biotic from species divergences caused by dispersal to a new geographic area (Whitfield, 2005). The changes in bacterial communities along the Texas Gulf Coast observed in this study are

probably due to environmental heterogeneity, but the introduction of novel species by ballast water is a dispersal mechanism that could allow for species divergence.

Perhaps the best way to examine these hypotheses is to continually monitor the progress of the ballast-transported bacterial populations using DGGE of 16S rRNA, and perform subsequent phylogenetic analyses to observe if and how much the new generation diverges from the parent population. Analysis of functional genes may also detect these changes sooner.

Overall Conclusions

This study shows that the level of bacteria present in the ballast tanks of commercial cargo ships is high and extremely diverse. The most important factor concerning the survival of an introduced species is the habitat from which it originated (Kolar and Lodge, 2001). In the ballast tank analyzed in this study, more than 50% of the community appeared to originate from a port-like habitat, rendering them very likely to survive introduction and become established in novel port environments, such as the Port of Houston. However, if there is an effect of bacterial dispersal during deballasting, it was not evident on the community analyses of the Port of Houston, either temporally or spatially, at least for the limited dataset examined in this study. There did not appear to be any disruption in an of the Texas estuarine systems by port activity; rather the bacteria experienced population turnover related to salinity and geographical distance. In order to truly examine long term effects, these systems will need to be monitored continuously for many years.

In order to see if deballasted bacterial populations survive in stage 3 or progress to stages 4 and 5, community fingerprints need to be made for ballast tanks arriving into the Port of Houston and compared to continual community fingerprints of the surround port and bay areas. This will allow us to see if they become widespread, dominant, or both. Any of these scenarios would label the bacteria as invasive. Clone libraries that of almost complete sequences from ballast bacteria should be made to continue characterizing the different species that can be transported via this vector. Perhaps certain species have better adaptations to the particular environment of the ballast tank, and are found more often than others. These key species can be used as indicator bacteria in a port water sample of deballasting activity. Much work still needs to be done to determine the true invasive potential of these microscopic stowaways. However, this study lays down the framework and takes the first steps towards this ecological goal.

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