Comparison of the Structure and Composition of Bacterioplankton Communities in the Ballast Water of Commercial Ships and the Duluth-Superior Harbor

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DEDICATION

This thesis is dedicated to my mother, Lorraine M. Beebe, and to the memory of my father, Emmett R. Beebe.

ABSTRACT

Ship ballast water is a potential vector for the introduction and spread of aquatic nonindigenous species (NIS). Although most studies focus on invasive plants and animals, there is an increased interest in the potential for invasive microbes. The Duluth-Superior Harbor (DSH), located in a freshwater estuary at the confluence of Lake Superior and the St. Louis River, receives more ballast water discharge than any other Great Lakes port yet little is known about the bacterioplankton communities in the harbor or the ballast water released into the harbor. The two most important factors affecting the successful establishment of an aquatic NIS through ballast water discharge are propagule pressure, and the abiotic and biotic characteristics of the new environment. Water was collected at six sites in Lake Superior, the Duluth-Superior Harbor, and the lower St. Louis River to characterize bacterioplankton communities. Other water samples were collected from the ballast tanks of ten commercial ships visiting the Duluth-Superior Harbor and the Western Lake Superior Sanitary District (WLSSD) because these sources may influence the genetic structure and composition of natural bacterioplankton communities in the harbor. Three distinct bacterioplankton communities representative of Lake Superior, the Duluth-Superior Harbor, and the St. Louis River habitats were detected in water samples collected on one day in early September 2009 (ANOSIM, $p < 0.05$) using a DNA fingerprint analysis (T-RFLP). These results were consistent with findings of other investigations that found distinct bacterioplankton communities in estuarine mixing zones. Water temperature, dissolved organic carbon (DOC), ammonium (NH_4^+), and nitrate (NO_3^-) were more highly correlated with differences in the genetic structure of bacterioplankton communities along a transect from the river through the harbor than other environmental variables.

DNA fingerprint analyses also indicated that bacterioplankton communities discharged into the DSH with ballast water and treated wastewater effluent were different from bacterioplankton communities found within the harbor. The most common bacterial phyla found in freshwater habitats (i.e., *Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Bacteroidetes*) were also seen in 16S rRNA gene clone libraries of

freshwater ballast and the Duluth-Superior Harbor. The genetic structure and species composition of the seawater ballast bacterioplankton community from an ocean-going ship was fundamentally different than the bacterioplankton communities in freshwater ballast, and was uncharacteristically dominated by bacteria from the *Epsilonproteobacteria*. Interestingly, three environmental DNA sequences from the seawater ballast of this ship were similar to *Tennacibaculum soleae*, a marine fish pathogen (phylum *Bacteroidetes*, Class Flavobacteria). DNA from this pathogenic marine bacterium, if not its intake cells, survived in the ballast tank of a transoceanic ship for at least two weeks before the ballast water arrived at the Duluth-Superior Harbor. This example illustrates the potential for transporting potentially harmful bacteria over long distances in the ballast water of commercial ships to harbors in the Laurentian Great Lakes.

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CHAPTER I

Introduction and Overview

Over 180 nonindigenous species (NIS) have been established in the Great Lakes basin since 1840 (Ricciardi 2006). Biological invasions can result in dramatic environmental impacts (Mills et al. 1994, Ricciardi and MacIsaac 2011), substantial economic costs (Rothlisberger et al. 2012), and affect human health (Ricciardi et al. 2011). Hecky et al. (2004) describes the virtual re-engineering of the nutrient and energy flow in nearshore zones of Lake Erie by invasive dreissenid mussels (zebra mussel, *Dreissena polymorpha*; quagga mussel, *Dreissena bugensis*) increasing the potential for eutrophication and algal fouling of area shorelines. Rothlisberger et al. (2012) reports a conservative estimate of damage costs attributed to ship-borne NIS in the Great Lakes region of $138 million per year and the potential for annual economic losses exceeding $800 million for sportfishing alone. Ballast water transport has also been connected with the spread of paralytic shellfish poisoning (PSP) in humans caused by the ingestion of shellfish contaminated with toxic dinoflagellates, such as the invasive *Gymnodinium catenatum*, that can cause outbreaks leading to fatalities and hospitalizations (McMinn et al. 1997, Anderson et al. 1989, Rodrigues et al. 2012).

Ballast water discharged from transoceanic commercial ships has been implicated in the majority of the aquatic NIS introductions in the Great Lakes since the opening of the St. Lawrence Seaway in 1959 (Mills et al. 1994, Grigorovich et al. 2003, Ricciardi 2006). Ballast water is taken on-board ships for the purpose of stability during a voyage and is then discharged in port when loading cargo and often en route to reduce a vessel's draft in shallow waters (Carlton et al. 1995, Grigorovich et al. 2003).

Most of the studies about ballast water-mediated discharge of aquatic NIS have been focused on macroorganisms such as the zebra mussel (*Dreissena polymorpha*; Hebert et al. 1989), the sea lamprey (*Petromyzon marinus*; Smith et al. 1980), and the spiny water flea (*Bythotrephes longimanus*; Bur et al. 1986). However, only recently have microorganisms been added to the list of potential aquatic nonindigenous species.

In the emerging field of microbial invasion ecology, both pathogenic and non-pathogenic microbes in aquatic and terrestrial ecosystems are now being studied as potential invasive microbes (Litchman 2010). Bain et al. (2010) reported on the invasive pathogenic fish virus, Viral Hemorrhagic Septicemia (VHS), and its widespread distribution throughout the Great Lakes since it was first detected in 2005. Briand et al. (2004) examined the physiological characteristics of the toxic algal-bloom forming tropical cyanobacterium, *Cylindrospermopsis raciborskii*, to explain its invasive spread at temperate mid-latitudes. Van der Putten et al. (2007) proposed that pathogenic and symbiotic invasive soil microbes would have a strong effect on individual plant species, community diversity, and ecosystem function.

Prokaryotes are ubiquitous, highly abundant, and play a critical role in biogeochemical cycles in both aquatic and terrestrial ecosystems (Whitman et al. 1998, Azam and Worden 2004, Furhman et al. 1989, DeLong and Karl 2005, van der Heijden et al. 2008) yet, historically, there was little concern for the possibility of microbial invasive species. A long-held view in microbiology was that free-living microorganisms can be found essentially everywhere and was summed up in the saying, "everything is everywhere, but the environment selects" (Beijerinck 1913, Bass-Becking 1934). Widely distributed bacterial groups have been identified at the phylum level (Lemke et al. 2009, Glöckner et al. 2000) and at the species level (Nold and Zwart 1998, Roberts et al. 1995). However, some microbial species exhibit distinct geographic patterns (Whitaker et al. 2003, Cho and Tiedje 2000). The restricted range of microorganisms may be the result of different environmental conditions (Horner-Devine et al., 2003, Crump et al. 2007, Martiny et al. 2006; Nemergut et al. 2011) and limitations on dispersal (Martiny et al. 2006). For aquatic microorganisms transported in the estimated 3 to 5 billion metric tons of ballast water transferred globally each year (IMO 2008), some of these limitations to dispersal have been removed (Ruiz et al. 2000, Drake et al. 2001, Seiden et al. 2010, Sun et al. 2010).

The concern for ballast water-mediated transport of bacteria and viruses is due to their high abundance in aquatic habitats, potential toxicity and pathogenicity, dispersal capabilities due to their small size, and an ability to form resting stages (Ruiz et al. 2000,

Drake et al. 2007, Dolan 2005). Asexual reproduction, high growth rates, efficient resource utilization, and competitive capabilities may also contribute to the potential success of microbial invasive species (Litchman 2010, Ruiz et al. 2000).

Bacteria can survive in the ballast water of ships in free-living populations (Joachimsthal et al. 2004) or associated with particles and biota (Ruiz et al. 2000). In addition, bacteria can be found in residual sediments at the bottom of ballast tanks or in biofilms on internal surfaces (Drake et al. 2005, Drake et al. 2007). Bacterial abundance in ballast water can range from 10^7 cells to as much as 10^{10} cells per liter (Ruiz et al. 2000, Drake et al. 2002, Joachimsthal et al. 2004, Burkholder et al. 2007). Drake et al. (2007) estimated that as many as 10^{20} bacteria and viruses (3.9×10^{18} bacteria cells and 6.8×10^{19} viruses) can be discharged into the lower Chesapeake Bay region annually. Of these microorganisms, it was estimated that 56 % could survive in the Bay after discharge.

Pathogenic and indicator microorganisms (i.e., organisms associated with human and animal diseases) in ballast water are of particular concern (Joachimsthal et al. 2004; Burkholder et al. 2007). Knight et al. (1999) detected fecal coliforms, fecal streptococci, *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*, and *Vibrio cholerae* in residual ballast water collected from transoceanic vessels entering the St. Lawrence Seaway. In another study, Ruiz et al. (2000) detected pathogenic strains of *V. cholerae* (i.e., O1 and O139), the causative agents of human cholera, in zooplankton samples from 93% of the ships entering Chesapeake Bay. Though these studies simply detected the presence of harmful microorganisms, ballast water discharge was directly implicated as the vector for the introduction of a pathogenic strain of *V. cholera* into oyster beds off the coast of Alabama in Mobile Bay (McCarthy et al. 1992).

The only widespread method used to mitigate further introductions of NIS into the Great Lakes is ballast water exchange (BWE), which involves flushing out coastal organisms in ships' ballast water with mid-ocean water before the vessel enters the St. Lawrence Seaway (IMO 2004; Federal Register 1999). This BWE practice both dilutes organism abundance and changes the salinity in the ballast tank. Most coastal organisms would not be expected to survive in ocean water (Locke et al. 1993) and marine

organisms discharged into freshwater would be unlikely to survive and develop reproducing populations (Smith et al. 1999). Though studies have shown BWE is effective at reducing the abundance of planktonic organisms by up to 80 to 95 % (Ruiz and Reid 2007, Taylor et al. 2007), substantial numbers of organisms and their resting stages can remain in ballast tank residual water, sediments, and in internal surface biofilms (Hallegraeff and Bolch 1992, Galil and Hülsmann 1997, Drake et al. 2007). Although there is extensive development and research on mechanical, physical, and chemical methods to treat ballast water before or during discharge, currently no standard methods have been established. The final ruling from the United States Coast Guard (USCG) on regulations for ballast water management concluded that BWE will remain an interim protective measure until on-board ballast water treatment systems are available to meet ballast water discharge standards (BWDS) for the allowable concentrations of living organisms discharged by ballast water (Federal Register 2012). Similar BWDS have been established by a number of states in the Great Lakes region (i.e., Minnesota, Wisconsin, New York, and Michigan), and by the Environmental Protection Agency (EPA; Federal Register 2012; U.S. CRS Report 2012). These BWDS are in line with the ballast water management standards of the United Nations' International Maritime Organization (IMO) including benchmarks for the concentration of select indicator microorganisms (i.e., Toxigenic *V. cholerae* [serotypes O1 and O139], *Escherichia coli*, and intestinal enterococci; IMO 2008).

Current ballast water management practices on transoceanic ships entering the Great Lakes – St. Lawrence Seaway can potentially prevent future biological invasions (MacIsaac et al. 2002), but the further spread of an NIS within the Great Lakes after its initial discovery (secondary spread) will determine the final economic and environmental impacts (Lodge et al. 1998). Rup et al (2010) identified domestic ballast water transfers within the Great Lakes as the most important vector for the secondary spread of NIS. Domestic bulk carriers ('Lakers') transfer 95% of the ballast water transported within and between the Great Lakes. In the EPA's National Coastal Assessment Report IV (U.S. EPA 2012) based on water and sediment quality, benthic community condition, loss of coastal habitat, and fish tissue contaminants, the Great Lakes coasts received the lowest

overall condition score (2.2 = Fair to poor). Southeastern Alaska and American Samoa received the highest overall condition score (5 = Good). Since ballast water is typically taken up in coastal waters, domestic transfers of ballast water within and between the Great Lakes remain a concern. Another important finding by Rup et al. (2010) was the overall transport of ballast water from the lower to upper lakes. The majority of the initial discoveries of NIS within the Great Lakes occurred in the lower lakes (Grigorovich et al. 2003).

The two most important factors affecting the successful establishment of a NIS are propagule pressure and the abiotic and biotic characteristics of the new environment (Smith et al. 1999, Grigorovich et al. 2003, Lockwood et al. 2005, U.S. EPA 2008, Litchman 2010). Propagule pressure is defined as the number of non-native organisms introduced to a new environment including both the abundance of organisms and the frequency of introduction events (Lockwood et al. 2005, Wonham et al. 2000). Microbial abundance in ballast water can be used as a proxy for propagule pressure where ballast water discharge volume and shipping traffic patterns are used to estimate the frequency of inoculation (Drake et al. 2007). Invasion pressure includes the density, diversity, and species richness of organisms discharged in ballast water (Smith et al. 1999, Jousset et al. 2011).

The majority of the studies on prokaryotic organisms in ballast water estimated their abundance after a voyage, during a voyage, or before and after mid-ocean exchange (MOE; Drake et al. 2002, Drake et al. 2005, Sun et al. 2010, Seiden et al. 2011). Other studies detected and estimated the abundance of specific target organisms such as enteric bacteria (i.e., *E. coli*, *V. cholerae*, enterococci; Ruiz et al. 2000, Aquirre-Macedo et al. 2008, Joachimsthal et al. 2004, Ivanov 2006, Burkholder et al. 2007), human pathogenic bacteria (i.e., *Listeria monocytogenes*, *Mycobacterium* spp., *Pseudomonas aeruginosa*; Burkholder et al. 2007), toxic *Cyanobacteria* (Doblin et al. 2002), or bacteria associated with coral disease (i.e., *Serratia marcescens*, and *Sphingomona* spp.; Aquirre-Macedo et al. 2008). Yet, very few studies have explored the structure and composition of bacterial communities in ballast water (Tomaru et al. 2010) and compared these communities to those in the waters of the recipient port (Ma et al. 2009).

Biotic conditions in a new environment that can effect the establishment of microorganisms introduced via ballast water discharge include the community structure and composition of the resident bacterial communities (Jousset et al. 2011, Lichtman 2010), grazing pressure by zooplankton, and mortality due to viral lysis (Hahn and Höfle 2001, Muylaert et al. 2002, Berdjeb et al. 2011). Abiotic conditions of recipient aquatic environments, such as temperature, salinity, nutrient availability, water quality, and various anthropogenic disturbances, can also have a profound influence on whether newly introduced microorganisms survive and reproduce (Smith et al. 1999, Mack and D'Antonio 1998, Drake et al. 2007).

The international port in the Duluth-Superior Harbor (DSH), at the western end of Lake Superior, is the largest and busiest commercial port in the Great Lakes with almost 1000 vessel visits annually (DSPA 2012). The DSH, located at the confluence of Lake Superior and the St. Louis River in a freshwater estuary, received twice the total discharge volume and two times more ballast water discharge events than any other Great Lakes port during 2006 and 2007 (U.S. EPA 2008). In the EPA study, *Predicting Future Introductions of Nonindigenous Species to the Great Lakes* (U.S. EPA 2008), commercial ports in the Great Lakes identified to be at greatest risk of future invasions were Toledo, OH, Gary, IN, Duluth, MN, Milwaukee and Superior, WI, Chicago, Il, and Ashtabula and Sandusky, OH. These ports were identified based on the suitability of habitat and propagule pressure. Although this 2008 EPA study determined that the suitability of the habitat in the DSH was lower than for other ports in the Great Lakes, the potential for the transport of nonindigenous species to the DSH was determined to be high. The Duluth-Superior Harbor is located in one of four invasion "hotspots" within the Great Lakes based on initial NIS discoveries (Grigorovich et al. 2003).

It is crucial to monitor areas that are at most risk for invasions, such as the Duluth-Superior Harbor (Vander Zanden and Olden 2008). Ricciardi et al. (2011) likened biological invasions to natural disasters and emphasized the importance of a rapid response and assessment, which is intrinsically dependent on monitoring and early detection of alien organisms. Recently, there have been studies conducted within the Duluth-Superior Harbor to describe the abundance and distribution of native and

nonindigenous species of fish (Peterson et al. 2011), benthic invertebrates (Trebitz et al. 2010), and crustacean zooplankton (M. C. TenEyck and D. K. Branstrator unpublished data). However, there have been few studies to-date describing the bacterioplankton community structure and composition in the harbor and how this community compares to those found in the waters of Lake Superior and the St. Louis River. Similarly, there has been no research on the composition and diversity of bacterioplankton communities in ballast water discharged into the Duluth-Superior Harbor or other Great Lakes ports despite the potential risks to native organisms, human health, and the economies of communities within Great Lakes region.

In Chapter II, I present research findings on both the genetic structure of the bacterioplankton communities within the Duluth-Superior Harbor and various abiotic and biotic factors within the harbor that may influence these communities. In Chapter III, I compare bacterioplankton communities in ballast water and treated wastewater effluent entering the Duluth-Superior Harbor with the bacterioplankton communities within the harbor. The main research goals of this study were to: (1) determine if the structure of the bacterioplankton communities gradually transition along a transect from the St. Louis River, through the Duluth-Superior Harbor, and into Lake Superior, (2) determine whether the structure of the bacterioplankton community within the harbor simply reflects a mixture of bacterioplankton communities from Lake Superior and the St. Louis River, (3) evaluate abiotic and biotic factors that may influence the bacterioplankton community structure within the harbor, (4) compare the bacterioplankton community structure within the harbor to the structure of bacterioplankton communities in ballast water of commercial ships and treated wastewater effluent, (5) characterize the composition and diversity of bacterioplankton communities in the harbor and in both freshwater and seawater ballast, and (6) identify bacterial taxa in ballast water collected from ships entering the Duluth-Superior Harbor that may be of economic or environmental concern, or of threat to human health.

CHAPTER II

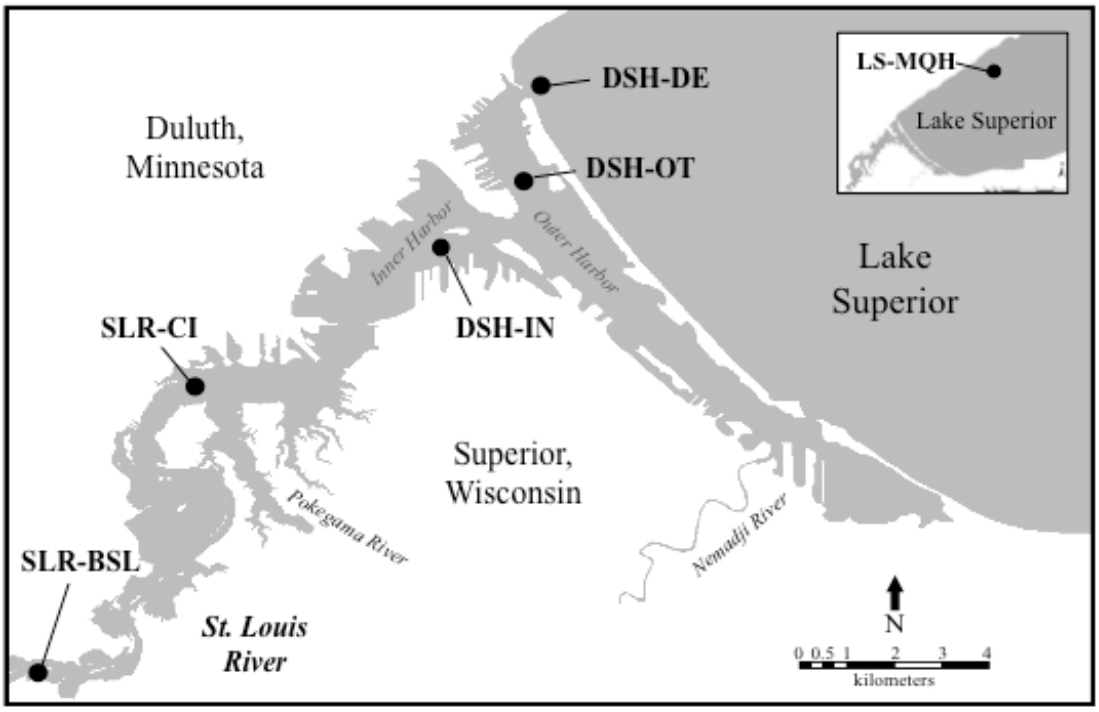
Changes in the Genetic Structure of Bacterioplankton Communities along a Transect through the Duluth-Superior Harbor

INTRODUCTION

Ballast water is a major vector in the introduction and spread of aquatic nonindigenous species (NIS) in the Great Lakes – St. Lawrence Seaway (Mills et al. 1994, Grigorovich et al. 2003). The Duluth-Superior Harbor (DSH), at the western end of Lake Superior, receives more ballast water discharge than any other Great Lakes port (MPCA 2008; Fig. II.1). Based on initial NIS discoveries, the Duluth-Superior Harbor is in one of four biological invasion "hotspots" in the Great Lakes (Grigorovich et al. 2003, Trebitz et al. 2010). It is important to monitor and assess areas most at risk for biological invasions (Vander Zanden and Olden 2008, Ricciardi et al. 2011). The potential for the successful introduction and spread of an invasive microbe via ballast water is affected by the abiotic and biotic characteristics of the recipient environment (Smith et al. 1999, Grigorovich et al. 2003, U.S. EPA 2008). Abiotic conditions that can influence the ability of potentially invasive microbes to survive and reproduce in the new environment include temperature, salinity, pH, nutrient availability, and anthropogenic disturbances (Smith et al. 1999, Fierer et al. 2007, Mack and D'Antonio 1998, Drake et al. 2007). Biotic conditions that may influence successful microbial invasion include the resident bacterial community structure and composition (Jousset et al. 2011, Lichtman 2010), grazing pressure by zooplankton, and mortality risks due to viral lysis (Hahn and Höfle 2001, Muylaert et al. 2002, Berdjeb et al. 2011).

The Duluth-Superior Harbor is located in a freshwater estuary at the confluence of the St. Louis River and Lake Superior, the freshwater lake with the largest surface area in the world. The hydrology of the Duluth-Superior Harbor is affected by the inflow of two rivers, two outflow channels, Lake Superior seiche oscillations, and a counter-clockwise current that flows along the Lake Superior shore (Stortz and Sydor 1980, Jordan et al. 1981, Beletsky et al. 1999; Fig. II.1).

Figure II.1. Map of the western end of Lake Superior (inset), the Duluth-Superior Harbor, and the Lower St. Louis River. Circles indicate sampling sites. Lake Superior-McQuade Harbor (LS-MQH); Duluth-Superior Harbor-Duluth Entry (DSH-DE); Duluth-Superior Harbor-Murphy Oil in the outer harbor (DSH-OT); Duluth-Superior Harbor-Midwest Energy in the inner harbor (DSH-IN); St. Louis River-Clough Island (SLR-CI); and St. Louis River-Boy Scout Landing (SLR-BSL).


LS-MQH
Lake Superior
DSH-DE
DSH-OT
Duluth,
Minnesota
Inner Harbor
Outer Harbor
Lake
Superior
SLR-CI
DSH-IN
Superior,
Wisconsin
Pokegama River
Nemadji River
SLR-BSL
St. Louis
River
N
0 0.5 1 2 3 4
kilometers

The St. Louis River (mean annual discharge of 56 $m^3 sec^{-1}$; Mitton et al. 1994) flows through the St. Louis River estuary entering the inner harbor in St. Louis Bay, continues through the outer harbor at the western end of Superior Bay, and finally discharges into Lake Superior primarily through the Duluth shipping channel (Duluth Entry). The Nemadji River (mean annual discharge of 11 $m^3 sec^{-1}$; MPCA/WDNR 1992) enters the outer harbor at the eastern end of Superior Bay adjacent to Allouez Bay and discharges into Lake Superior primarily through the Superior shipping channel (Superior Entry). The amplitude of the Lake Superior seiche entering the harbor typically ranges from 3 to 15 cm and has a fundamental oscillation period of 7.9 hours (Stortz and Sydor 1980) that can set up minor periodic oscillations every 2.1 hours within the harbor (Jordan et al. 1981). The mixing of river and lake water by these tide-like seiche oscillations help define the lower portion of the St. Louis River as a freshwater estuary (Herdendorf 1990).

The St. Louis River watershed (9,283 km^2) is primarily forested but includes wetlands, agriculture, urban, and open water sections (MDNR 2006). The presence of tannins and humic acids released from decaying organic material in swamps and bogs gives the St. Louis River water its characteristic 'tea-color' (Wagner 1976). In addition to high dissolved organic content, the St. Louis River and its tributaries are a major source of suspended solids, most of which are deposited within the harbor itself (Bahnick and Markee 1985). Other sources of suspended solids to the harbor include treated wastewater effluent, storm-water runoff, and resuspended sediments in ballast water discharge (Klarer and Millie 1994, Villac and Kaczmarska 2011, Bailey et al. 2003). The Western Lake Superior Sanitary District (WLSSD), a municipal and industrial wastewater treatment facility, discharges tertiary-treated effluent into the inner harbor of DSH at an annual rate of 40 million gallons per day (MGD). Treated wastewater effluent turbidity levels can vary and are affected by storm water and snow melt-water inflow. Storm water run-off can carry accumulated solids from streets, gutters, and other urban and industrial structures. A major secondary source of turbidity in the harbor is the resuspension of sediments due to seiche oscillations, ship traffic, and periodic dredging of shipping channels (Stortz and Sydor 1980). Resuspension of sediments can release

nutrients and increase bacterial cell abundance in the water column (Reddy et al. 1996, Holmroos et al. 2009, Pettibone et al. 1996).

A well-documented feature of river-dominated marine estuaries is an estuarine turbidity maxima (ETM) created by water density differences and the interaction of river flow and tidal forces. Particles of inorganic and organic origin are trapped in this zone of mixing extending the residence time of nutrients and particle-associated bacteria and setting up conditions for an estuarine ecotone (Crump et al. 1999, 2004). In Great Lakes estuaries, it is hypothesized that a vertical gradient may occur if cold lake water flows under much warmer river water, but this would be short-lived due to heat transfer (Dyer 1990). Residence times in Great Lakes estuaries may be more driven by hydrologic features (Morrice et al. 2004, McCarthy et al. 2007). In freshwater estuaries with restricted outflows, seiche activity can take the place of tides and induce temporary entrapment of lake plankton, suspended solids, and nutrients where lake and river waters mix (Klarer and Millie 1994). Like most Great Lakes estuaries, the St. Louis River estuary is considered a drowned river estuary, but it is also classified as a baymouth bar-type estuary due to the presence of Minnesota Point, a long narrow strip of sand influencing the mixing of lake and river water by protecting the estuary from high-energy wind and waves of Lake Superior.

In the transition zone of an estuary, a combination of physical, chemical, and biotic conditions influence the structure and composition of bacterial communities. In marine estuaries, salinity gradients define a unique estuarine ecotone (Bouvier et al. 2002, Crump et al. 2004, Bernhard et al. et al. 2005). In Great Lakes freshwater estuaries, nutrient rich river water mixes with phosphorus-deficient lake water to form an area with unique water chemistry (Lavrentyev et al. 2004, Mueller-Spitz et al. 2009). In the freshwater estuary, Old Woman Creek (Lake Erie), conductivity, turbidity and total suspended solids were highest at the confluence of lake and stream water (Herdendorf 1990). Fortunato and Crump (2011) demonstrated that physical conditions in the mixing zones of estuaries define the boundaries of distinct microbial habitats, but gradients affecting primary productivity (i.e., nutrients and light) and secondary productivity (i.e., grazing) influence the variability within microbial communities. In Old Woman Creek

estuary, Lavrentyev et al. (2004) found peak microbial biomass, peak plankton richness, and increased nutrient cycling in the mixing zone of Lake Erie and stream water. In addition, they estimated that microbial grazers consumed at least 50% of the daily production of bacteria and nanoplankton at the confluence of the lake and stream water. These findings are consistent with what has been established for nutrient cycling and aquatic food web dynamics in marine estuaries (Godhantaraman and Uye 2003, Iriarte et al. 2003, Lehrter et al. 1999, Roman et al. 2001).

Adequate residence time is a critical component in the influence of an estuarine mixing zone on nutrient cycling (Crump et al. 2004, Lavrentyev et al. 2004) and on communities of zooplankton (Morgan et al. 1997, Lavrentyev et al. 2004), phytoplankton (Ferreira et al. 2005, Wang et al. 2004), and bacterioplankton (Crump et al. 2004, Bernhard et al. 2005, Fortunato and Crump 2011). Crump et al. (2004) detected unique bacterial communities in the Columbia River estuary when the 17 to 18 day residence time of the estuarine water exceeded the average doubling times of the bacterioplankton communities measured in the summer and fall (1.1 and 1.9 days, respectively). Stortz and Sydor (1980) estimated the residence time in the Duluth-Superior Harbor to be on the order of 30 to 40 days based on a simulation study evaluating the transport of a dissolved pollutant entering the inner harbor at the Midwest Energy coal dock (Superior, WI). This long residence time may allow a unique bacterial community to develop within the Duluth-Superior Harbor.

To date, there have been few investigations of the structure or composition of bacterioplankton communities within the Duluth-Superior Harbor or the St. Louis River. This study was designed to examine the genetic structure of the bacterioplankton communities within the Duluth-Superior Harbor and evaluate a number of abiotic and biotic parameters that may influence the resident bacterioplankton communities. The first objective was to test the hypothesis that the genetic structure of bacterioplankton communities within the harbor is fundamentally different from the community structure of bacterioplankton populations in Lake Superior and the St. Louis River. Bray-Curtis ordination analysis was used to test whether the genetic structure of the bacterioplankton communities changed along a transect from the St. Louis River, through the Duluth-

Superior Harbor, and into Lake Superior. In addition, artificial mixtures of Lake Superior and St. Louis River water were used to determine if the bacterioplankton community structure within the harbor reflects a simple mixture of lake and river bacterioplankton. First, DOC concentrations in mixtures of lake and river water were used to identify locations in the harbor that may have had similar ratios of lake and river water. Next, the genetic structure of bacterioplankton communities within these mixtures, were compared to the genetic structure of bacterioplankton communities near the estimated locations within the harbor. The second objective of this study was to evaluate the potential influence of various abiotic and biotic factors on the structure of bacterioplankton communities in the Duluth-Superior Harbor.

METHODS

Study sites and sampling

Six sample sites were chosen along a 36 km transect from Lake Superior, through the Duluth-Superior Harbor into the Lower St. Louis River (Fig. II.1). The Lake Superior sample site (LS-MQH, 46°51.98' N; 91°55.42' W) was located 1.6 km offshore of Lake Superior approximately 15.5 km from the Duluth Entry. The Duluth Entry sample site (DSH-DE, 46°46.81'N; 92°04.95'W) was adjacent to the major discharge channel of the St. Louis River on the Lake Superior side. The outer harbor sample site (DSH-OT, 46°45.64' N; 92°05.59' W) was in the eastern end of Superior Bay near the Murphy Oil Marine Terminal Berth 3, and the inner harbor sample site (DSH-IN, 46°44.69' N; 92°06.96' W) was located in the St. Louis Bay near Superior Midwest Energy Terminal (St. Louis Bay). In the lower St. Louis River, sample site SLR-CI (46°42.95' N; 92°11.23' W) was located northeast of Clough Island within the boating channel. The upper St. Louis River sample site (SLR-BSL, 46°39.19' N; 92°13.68' W) was located upstream from the Oliver Bridge at Boy Scout Landing.

On September 3, 2009, three independent replicate water samples were collected at each sample site just below the water surface using 20-L plastic carboys. Carboys were rinsed with sample water prior to sample collection. No rain had occurred 48 hours

prior to sampling. Water samples were stored at 4°C within four hours of collection and filtered the same day.

Artificial mixtures of Lake Superior and St. Louis River water

Mixtures of Lake Superior and St. Louis River water were used to determine if the bacterioplankton community structure in the harbor simply reflected a mixture of bacterioplankton communities from the lake and river. Additional Lake Superior (LS-MQH) and St. Louis River (SLR-BSL) water was collected at the time of sampling and then combined in the laboratory into three different mixtures (i.e., 60% Lake: 40% River [60L/40R], 40 % Lake: 60% River [40L/60R], and 20% Lake: 80% River [20L/80R]). For each lake and river water mixture, total cells were collected on three membrane filters to extract microbial DNA and perform T-RFLP analysis (see details below). Minor et al. (2008) found DOC concentrations to be higher in the St. Louis River than those found in Lake Superior. A simple linear regression analysis was used to place the lake and river water mixtures along the sample transect through the harbor based on DOC concentrations (see analysis details below). Nonmetric multidimensional scaling (NMS) was used to place T-RFLP profiles of the lake and river water mixtures and field sample sites in ordination space.

Physicochemical and biotic analyses

At each field sample site, vertical profiles of temperature, dissolved oxygen (DO), and specific conductivity were measured using a STD/CTD – model SD204 (SAIV A/S Environmental Sensors and Systems, Bergen, Norway). Water pH was measured using a YSI 63 meter (Yellow Springs Instruments, Yellow Springs, Ohio) and turbidity was measured using a Lamotte 2020e Portable Turbidity Meter (geo scientific ltd., Vancouver, BC, Canada). Duplicate water samples were collected near the surface at each site in acid-washed, brown, high-density polyethylene (HDPE), 1-L plastic bottles. Aliquots of water collected for UV-visible (UV-vis) spectroscopy were stored in amber glass bottles and processed within 4 h. For chlorophyll *a* (Chl *a*) analysis, water samples were filtered through glass-fiber filters (Whatman GF/F, 47 mm) under low light within 5

h of collection. The filters were wrapped in aluminum foil and stored at -20ºC until Chl *a* concentrations were measured. Aliquots of each glass fiber filtrate were used for UV-vis spectroscopy within 4 h. Two hundred ml portions of each glass fiber filtrate were stored frozen (-20ºC) in brown, HDPE bottles for nutrient analyses. A 40 ml portion of each glass fiber filtrate was acidified (pH 2) with 6N HCL for dissolved organic carbon (DOC) analysis and stored frozen (-20ºC).

DOC and $SUVA_{254}$ – DOC analyses were conducted on filtered water samples, using a high-temperature combustion technique in a TOC-$V_{CSH/SCN}$ Total Carbon Analyzer (Shimadzu, Columbia, MD). Prior to analysis, inorganic carbon was removed by acidifying (pH 2) and sparging leaving the non-purgeable organic carbon (NPOC) in the water samples. Potassium hydrogen pthalate (KHP) was used to calibrate the TOC analyzer and to evaluate performance. UV-vis spectroscopy was performed with a Genesys6 scanning spectrophotometer (Thermo Electron Corp.) scanning from 200 to 800 nm. UV-vis measurements at 254 nm were used to determine specific UV absorbance ($SUVA_{254}$) and spectral slope ($S_{250-400}$). $SUVA_{254}$ was calculated by dividing the UV absorbance at 254 nm (m^{-1}) by the DOC concentration (mg C/L^{-1}). When $SUVA_{254}$ is normalized for DOC concentrations, it strongly correlates with the aromatic portion of the DOC in aquatic systems (Weishaar et al., 2003). The spectral slope ($S_{250-400}$) values reflect the optical characteristics of a natural water sample and are calculated as described in Minor and Stephens (2008) using wavelengths from 250 to 400 nm (Obernosterer and Benner 2004, Minor et al. 2007).

Nutrient analyses – Dissolved inorganic nutrient concentrations were determined in filtered water sample using flow injection analysis (QuickChem®, Lachat Instruments, Loveland, CO). Nitrate was reduced to nitrite when passed through a copper-cadmium column and combined nitrite (reduced nitrate and original nitrite) was determined by the sulfanilamide method (Strickland and Parsons 1968). Henceforth, combined nitrite will be referred to as NO_3^-, since nitrate is more stable than nitrite and is in higher concentrations in aquatic ecosystems (Rouse et al. 1999). Dissolved orthophosphate (soluble reactive phosphorus, SRP) was determined by formation of reduced antimony-phosphomolybdate complex and measured colorimetrically (Strickland and Parsons

1968). The salicylate-hypochlorite method was used to measure dissolved ammonia-nitrogen (Reardon et al. 1966). The equilibrium between un-ionized ammonia (NH_3) and ionized ammonia (NH_4^+) in natural freshwater is primarily influenced by pH and temperature (Emerson et al. 1975; Soderberg and Meade 1991). Ammonium concentrations (NH_4^+ μg L^{-1}) at the time of sampling were estimated from dissolved ammonia-nitrogen (NH_3-N μg L^{-1}) concentrations by first converting NH_3-N to NH_3 (conversion factor 17/14; U.S. EPA 1979). The pH and temperature at each site were then used to determine the fraction of NH_4^+ at the time of collection based on equations from Emerson et al. (1975; U.S. EPA 1979).

Chlorophyll a – Chlorophyll was extracted from glass fiber filters in 90% acetone at 4ºC for 24 h. Extracts were analyzed with a Turner 10-AU fluorometer (Turner Designs, Sunnyvale, CA). The extracts were reanalyzed after acidification with 6N HCL. If a decline in relative fluorescence in acidified extracts was not detected, no correction for phaeopigment contribution was applied (Lorenzen 1967).

Prokaryotic cell abundance

A portion of each water sample was preserved with 37% formaldehyde (pre-filtered through 0.22 μm pore filter; 2% final concentration) and stored in the dark at 4°C for up to 2 weeks. A small portion of each preserved sample was placed on a black polycarbonate filter (25 mm dia., 0.22 μm pore; GE Water & Process Technologies, Trevose, PA). The DNA in cells was stained with 4'6-diamidino-2-phenylindole (DAPI; 10 μM final concentration), filtered onto the membrane filter, and prokaryotic cells (i.e., cells with no visible nucleus) were counted using a Nikon Eclipse 80i epifluorescence microscope (Porter and Feig 1980).

Bacterioplankton community analyses

Total DNA extraction – Microbial cells in water samples were collected onto Duropore® membrane filters (142 mm dia., 0.22 μm pore size; Millipore, Billerica, MA) by N_2 gas pressure filtration (< 40 psi) until the filters clogged (i.e., filtrate flow was substantially reduced). Membrane filters were stored in Whirl-Pak® bags (Nasco, Fort

Atkinson, WI) at -80°C. Frozen filters were crushed in the Whirl-Pak® bags before a one-eighth portion (by weight) was removed for DNA extraction. Since organic material, clay particles, and humic acids can co-extract with DNA and inhibit PCR amplification (Kirk et al. 2004), a MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) was used for all DNA extractions. Extracted DNA was stored in sterile elution buffer (10 mM Tris) at -80°C until further analysis.

Polymerase chain reaction (PCR) – For each sample, the bacterial 16S rRNA gene was amplified using 40 ng of extracted DNA as a template, Ilustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ), a bacterial-specific forward primer (27F-FAM; 5'-AGAGTTTGATCMTGGCTCAG-3'; Lane 1991), and a universal reverse primer (1492R; 5'-TACGGYTACCTTGTTACGACTT-3'; Lane 1991). The 27F-FAM primer was 5'- end labeled with 6-carboxyfluorescein (6-FAM). Integrated DNA Technologies (Coralvile, IA) synthesized all PCR primers. Each PCR reaction (25 µl) contained ~ 2.5 units of puReTaq DNA polymerase, 200 µM each dNTP in 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. PCR reaction tubes were placed in a preheated (94°C) thermal cycler block (DNA Engine® Peltier Thermal Cyclers; MJ Research). The PCR program included an initial denaturing step at 94°C for 3 min followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 90 s. After a final extension step at 72°C for 10 min, the PCR reaction tubes were stored at 4°C. The low number of cycles, excess primer, and low annealing temperature used in the PCR amplification procedure were to minimize PCR bias (Polz and Cavanaugh 1998, Acinas et al. 2005). PCR products of the expected fragment size (~1465bp), determined by agarose gel electrophoresis, were purified using an UltraClean™ PCR Clean-up™ Kit (MoBio Laboratories, Inc., Carlsbad, CA). Purified PCR products were eluded in nuclease-free water, stored at 4°C, and used within 24 hours.

Terminal restriction fragment length polymorphism (T-RFLP) analysis – Purified PCR products were digested at 37ºC for 3 hours using restriction enzymes *Hae*III, *Msp*I, and *Rsa*I (Promega, Fitchburg, WI). Digestion was terminated by incubation at 65ºC for 15 minutes. Digested PCR products were precipitated in ethanol, dried, and resuspended

in nuclease-free water. The fluorescent terminal restriction fragments (TRFs) of digested PCR products were separated by size using capillary electrophoresis on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA; BioMedical Genomics Center, University of Minnesota). Since unrelated bacterial species may produce the same terminal restriction fragment length for a particular primer-restriction enzyme combination, multiple restriction enzymes were used to more accurately resolve the natural diversity in bacterial communities (Liu et al. 1997, Marsh et al. 2000).

Sample electropherograms from GeneMapper® were directly imported into the BioNumerics® software package (Applied-Maths, Belgium) and converted to densitometric curves. To remove background noise in the densitometric curve profiles, a spectral analysis was performed for each sample set to determine the optimal settings for the Wiener Cut-off (least square filtering) and background scaling (background subtraction). In addition, a signal/noise ratio was calculated and used as an additional quality control parameter with the optimal value being greater than 50. Densitometric curve files were visualized as a gel image of bands and as a profile of corresponding peaks each representing one terminal restriction fragment (TRF). Each curve file represents a unique collection of peaks referred to as a DNA fingerprint for each bacterioplankton community. The BioNumerics® software estimates the TRF sizes in base pairs (bp) by referencing the internal standard (MapMarker® 1000; 23 standards ranging from 50 bp to 1000 bp) using the cubic spline fit algorithm. TRFs with estimated sizes outside of the range of the molecular weight standards were excluded from further analysis.

The fluorescence intensity of each peak was used to indicate the abundance of each TRF. Same-sized TRFs can represent more than one taxon that can be distinct from one another (Dunbar et al. 2001, Kaplan and Kitts 2003) and some organisms produce multiple TRF sizes (Nübel et al. 1996, Wintzingerode et al. 1997). As a result, TRFs can represent one bacterial group (taxon) or a number of bacterial groups (taxa). Individual peaks that were greater than 1% relative to the maximum fluorescence intensity of each lane were identified. This 1% minimum profiling was used to identify background noise in the peak profiles. In some samples, percent minimum profiling was adjusted upwards

to compensate for higher noise levels, but profiles containing a high level of noise (> 5 % of the maximum fluorescent intensity) were removed from further analysis. A shoulder sensitivity of 5 was used for profiles containing peaks without a local maximum or reported as close doublets. Across all T-RFLP profiles, for each restriction enzyme, peaks were aligned and placed into bin classes using a 1% position tolerance and 1% optimization. Peaks representing fragments differing by less than 0.5 bp were included in the same bin class. Dunbar et al. (2001) found reproducible peaks of less than 0.5 bp in the same DNA fingerprint indicating closely migrating fragments that differ in length or nucleotide sequence. For this reason, rounding the fragment size to the nearest integer is not recommended (Dunbar et al. 2001, Schütte et al. 2008). Peak assignments were visually inspected and manually adjusted when software placed peaks in inappropriate classes.

BioNumerics® numerical outputs representing peak heights were imported into Microsoft Excel. Utilizing relative peak heights (RPH) and presence-absence (PA, binary) data for the analysis of natural community T-RFLP profiles is recommended (Blackwood et al. 2003, Culman et al. 2008, Schütte et al. 2008). Fragments with peak heights less than 50 relative fluorescence units were removed from further analysis (Blackwood et al. 2003). Since the concentration of DNA injected into each lane in capillary electrophoresis can differ between samples and even replicates, T-RFLP peak profiles were relativized or standardized by dividing each peak height by the total sum of all peak heights for each sample (Liu et al. 1997, Dunbar et al. 2001, Osborn et al. 2000).

Statistical Analyses

Cluster analysis – Densitometric curves in each of the T-RFLP profiles were compared to explore the natural groupings of the sampling sites using similarity-based cluster analysis within BioNumerics®. Pairwise similarity matrices were calculated using Pearson correlation coefficients and dendrograms were constructed using the un-weighted pair group method with arithmetic mean (UPGMA) clustering algorithm. BioNumerics® estimates the uncertainty of the dendrogram branches using Cophenetic correlation comparing the matrix similarities and the dendrogram-derived similarities.

Ordination data analyses – Both relative peak height and presence-absence T-RFLP profiles were summarized and compared in Microsoft Excel. Dominant TRFs were identified in each restriction enzyme digest creating two TRF subsets, i.e., the most dominant TRFs and the least dominant TRFs. First, the relative peak heights for each TRF in a given sample were averaged (ignoring zeros). Then, dominant TRFs were identified in each restriction enzyme digest that had average peak heights representing greater than 5% of the total fluorescence in at least one sample profile.

Ordination techniques were performed using PC-ORD Version 4.0 software (McCune and Mefford 1999). PC-ORD was also used to determine the degree of sparsity (i.e., percent of empty cells in the data matrix or zero richness) and heterogeneity in all datasets used in ordination analyses. Heterogeneity was estimated by evaluating the average skewness, and coefficient of variation (CV) of totals for fragment data (Peck 2010). Following distance matrix calculations, all datasets were analyzed for the presence of outliers defined here as sample units or fragments with calculated distances > 2 standard deviations (SD) from the mean. Weak outliers have SDs ranging from 2.0 to 2.3 and moderate outliers have SDs ranging from 2.3 to 3. (McCune and Grace 2002). In ordination analyses, all groups should have similar within group dispersion to avoid false significant results (Legendre and Legendre 1998) and standard deviation is a widely used indicator of dispersion. All ordination analyses were run with and without outliers to determine if revealed patterns mainly reflected relationships associated with outliers. Ordinations were also run with and without rare fragments (Bernhard et al. 2005) defined here as fragments present in only one replicate of one sample. Deleting rare species is a common practice in community composition analyses to reduce noise (McCune and Grace 2002).

Bray-Curtis ordination – Bray-Curtis (polar) ordinations based on similarities of T-RFLP profiles were calculated to determine if the genetic structure of bacterioplankton communities gradually changed along a transect from Lake Superior, through the Duluth-Superior Harbor, and into the St. Louis River (Beals 1984, McCune and Grace 2002). In this study, Sorensen (Bray-Curtis) and Jaccard distance measures were used and the endpoints for both axes were objectively selected using variance regression. Sorensen

and Jaccard distance measures are recommended for abundance (relative peak height) and presence-absence datasets (respectively) since these distance measures do not consider TRF joint-absences as an indication of similarity between samples (Ramette 2007, Schütte et al. 2008). In addition, Sorensen and Jaccard distance measures are effective for zero-rich, heterogeneous datasets, and are not excessively sensitive to outliers (McCune and Grace 2002, Rees et al. 2004, Schütte et al. 2008, Peck 2010). The percent variance explained by each axis was calculated and the axes were presented in order of importance.

NMS ordination – Nonmetric multidimensional scaling (NMS) was performed with the Sorensen distance measure to explore relationships between environmental variables and the bacterioplankton communities in the harbor and river (Mather 1976, Kruskal 1964[b], McCune and Grace 2002). NMS ordinations are recommended for T-RFLP data since T-RFLP profiles rarely meet multivariate normality requirements of parametric ordination methods (Ramette 2007). In NMS plots, samples with greater similarity are in closer proximity in ordination space. In this study, the autopilot 'slow and steady' option was used to determine recommended number of axes. A final stress of 5-10 is considered a good ordination with minimal risk of false representation of redundancy patterns (Kruskal's Stress multiplied by 100; McCune and Grace 2002, Kruskal 1964[a], Clarke 1993). Although a final stress of 10-20 may provide a usable interpretation at the lower end, plots with stress values at the upper end may provide a misleading representation (Clarke 1993). To determine if the stress values were stronger than expected by chance, a Monte Carlo test was run 250 times on randomized data during each run. To verify consistency of the depicted patterns of T-RFLP profiles in ordination space, at least five solutions were determined for each analysis using the recommended number of dimensions. Presence-absence transformations may mask relationships with environmental factors so only relative peak height data was used in this analysis (Muylaert et al. 2002).

Relationships between environmental variables and the T-RFLP profiles of the harbor and river bacterioplankton communities reflected in the NMS ordination plots were explored using a joint-plot overlay of a second matrix containing the environmental

variables. It is recommended that environmental data with non-normal distribution should be transformed before use in ordination analysis (McCune and Grace 2002). The Shapiro-Wilk W test was used to test for normal distribution in environmental data (Shapiro and Wilk 1965) and was performed using Microsoft Excel Analyze-It Software, Ltd. Scatterplots of the ordination scores for each axis are examined in PC-ORD to identify non-linear relationships with environmental variables where the use of a nonparametric measure of correlation would be more appropriate than a parametric measure.

One-way analysis of similarity (ANOSIM) and similarity percentage (SIMPER) –The statistical differences between groups in the ordination plots were tested using ANOSIM with 999 permutations (Clarke 1993, Rees et al. 2004). In this analysis, an R statistic is computed (0 to 1). R-values > 0.75 indicate groups are well separated, R-values > 0.5 indicate separated groups with some overlap, and R-values < 0.25 indicate groups that are minimally separated (Ramette 2007). SIMPER analysis was used to estimate overall average dissimilarity in pairwise comparisons of separated groups as determined by ANOSIM analysis. In addition, SIMPER analysis was used to identify TRFs that contribute the most to the dissimilarity between groups in pairwise comparisons (Clarke 1993, Rees et al. 2004). In both ANOSIM and SIMPER analyses, Sorensen and Jaccard distance measures were used for abundance (RPH) and presence-absence (P-A) datasets, respectively. ANOSIM and SIMPER were performed using PAST Version 2.15 (Hammer et al. 2001).

Correlation analyses – To determine the relationships among ten environmental variables, the on-line software PAST Version 2.15 (Hammer et al. 2001) was used to calculate Pearson product-moment correlation coefficients (Pearson correlation coefficients). PAST software was also used to calculate Spearman's rank correlation coefficients (ρ) to identify significant correlations between environmental variables and the abundance of influential TRFs identified in SIMPER analysis. All correlation analyses were completed using average relative peak heights for TRFs calculated by averaging peak heights of the replicates from each sample site while ignoring zeros.

RESULTS

Physicochemical and biotic characteristics

Temperature profiles at most field sites showed a warmer upper water layer that extended to a depth of approximately 2 meters (Fig. II.2). At the inner harbor site (DSH-IN), the warmer upper layer extended 3 to 3.5 m below the surface. There was a distinct decrease in water temperature in the bottom 1.5 m at the Duluth Entry (DSH-DE) and outer harbor (DSH-OT) sites. Differences in specific conductivity were small within the water column at the inner harbor and St. Louis River sites (DSH-IN, SLR-CI and SLR-BSL). Specific conductivity, however, dropped in the last 1 to 1.5 meters at the Duluth Entry (DSH-DE) and the outer harbor (DSH-OT) sites.

Physicochemical and biotic characteristics of sites along the sample transect reflected both upstream and downstream gradients (Table II.1). The upper-most river site (SLR-BSL) had the highest temperature (21.6ºC) and DOC concentration (24.68 mg C L^{-1}) and both factors gradually declined through the harbor and out into the lake (14.1ºC, 1.72 mg C L^{-1}, respectively). In contrast, the highest levels of NO_3^- were in Lake Superior (327.1 µg L^{-1}) and gradually decreased through the harbor and upstream (SLR-BSL; 66.7 µg L^{-1}). Higher values of NH_4^+ concentration, turbidity, SRP, and specific conductivity were found within the harbor (158.2 µg L^{-1}, 4.6 NTU, 12.1 µg P L^{-1}, and 0.20 mS cm^{-1}, respectively) than in Lake Superior or the St. Louis River. Chlorophyll *a* concentration, an estimate of algal abundance (Bird and Kalff 1984), was highest at the furthest river site upstream (SLR-BSL; 5.87 µg L^{-1}). The highest Chl *a* concentration within the harbor was at the outer harbor site (DSH-OT; 4.22 µg L^{-1}). No correction for phaeopigment contribution was required for any sample. Filters for Chl *a* analysis were stored over 6 months before processing. Significant amounts of Chl *a* may be lost when filters are stored for over a month at -20ºC (Wasmund et al. 2006), so the reliability of these results was uncertain. The $SUVA_{254}$ value for the Lake Superior water sample (3.3) was lower than the $SUVA_{254}$ values for the harbor and river water samples (8.2 to 9.4 and 8.1 to 14.2, respectively) indicating less humified or aromatic DOM in Lake Superior compared to the harbor (Weishaar et al. 2003). The highest calculated $SUVA_{254}$ value (14.2) was at the SLR-CI river site in Pokegama Bay near the mouth of the Pokegama

Figure II.2. (A) Temperature and (B) specific conductivity profiles of the water column on September 3, 2009 at six field sites along a transect from Lake Superior (LS-MQH), through the Duluth-Superior Harbor (DSH-DE, DSH-OT, DSH-IN), and into the St. Louis River (SLR-CI, SLR-BSL). See text for site abbreviations.

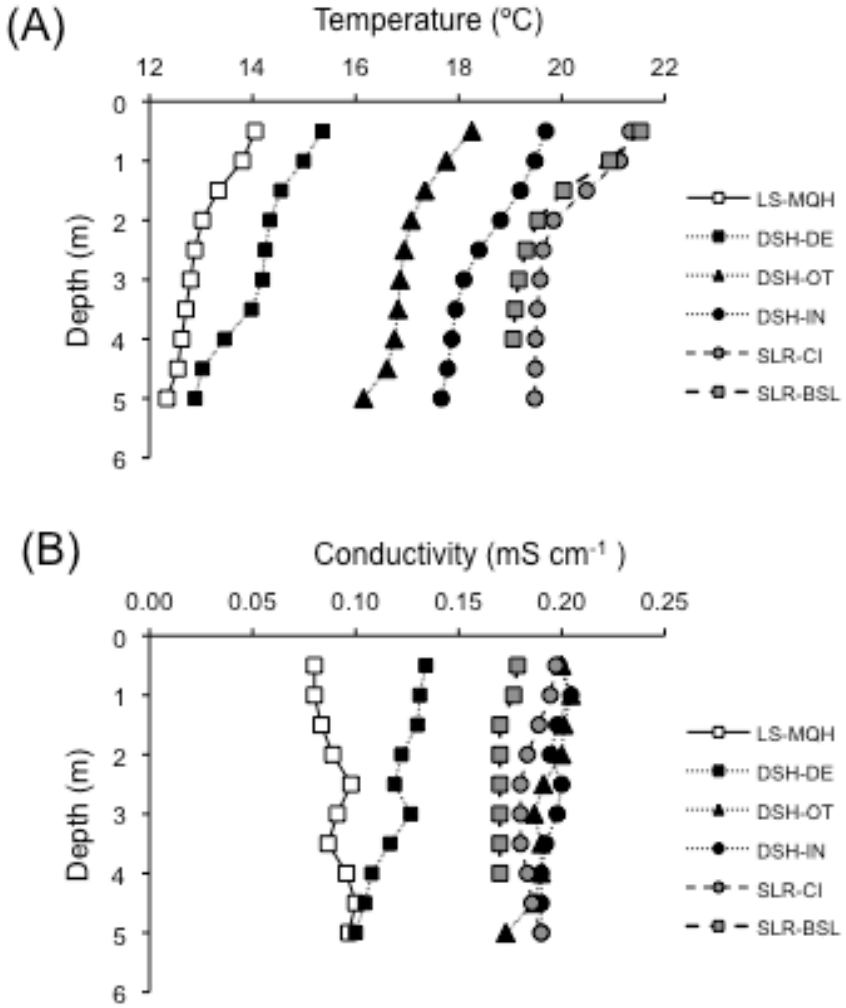

(A)
Temperature (°C)
12
14
16
18
20
22
0
1
2
3
4
5
6
Depth (m)
LS-MQH
DSH-DE
DSH-OT
DSH-IN
SLR-CI
SLR-BSL
(B)
Conductivity (mS cm-1)
0.00
0.05
0.10
0.15
0.20
0.25
0
1
2
3
4
5
6
Depth (m)
LS-MQH
DSH-DE
DSH-OT
DSH-IN
SLR-CI
SLR-BSL

Table II.1. Physicochemical and biotic conditions at six field sites along a transect from Lake Superior (LS), through the Duluth-Superior Harbor (DSH), and into the St. Louis River (SLR). Standard deviations are in parenthesis.

	LSH-MQH	DSH-DE	DSH-OT	DSH-IN	SLR-CI	SLR-BSL
Temperature (°C)	14.1	15.7	18.4	19.7	21.3	21.6
pH	7.12	7.00	6.88	6.84	6.94	6.78
Specific Conductivity (mS cm^{-1})	0.08	0.14	0.20	0.20	0.20	0.18
DO (mg L^{-1})	10.5	9.9	9.1	8.6	8.7	9.0
DOC (mg L^{-1})	1.72 (0.10)	6.98 (0.12)	9.70 (0.12)	13.83 (0.2)	19.90 (0.20)	24.68 (0.20)
$SUVA_{254}$	3.3	8.2	8.7	9.4	14.2	8.1
Spectral Slope (λ 250-400nm)	0.0180	0.0142	0.0139	0.0134	0.0130	0.0129
SRP (µg P L^{-1})	*	5.7 (0.2)	12.1 (0.2)	14.8 (0.6)	14.1 (0.3)	8.9 (0.3)
NH_4^+ (µg L^{-1})	9.7	90.9	158.2	61.5	23.2	4.9
NO3− (µg N L-1)	327.1 (1.5)	208.2 (0.7)	165.2 (0.6)	77.9 (0.4)	28.4 (0.1)	66.7 (0.7)
Turbidity (NTU)	0.5	2.7	3.3	4.6	3.5	4.6
Chl *a* (µg L^{-1})	0.37 (0.07)	3.49 (0.16)	4.22 (0.24)	3.76 (0.67)	3.63 (0.56)	5.87 (0.94)
Prokaryotic Cell Abundance (10^9 cells L^{-1})	0.74 (0.05)	2.03 (0.35)	2.44 (0.13)	2.56 (0.20)	3.39 (0.21)	2.99 (0.16)

Abbreviations: DO dissolved oxygen; DOC dissolved organic carbon; SRP soluble reactive phosphorus; Chl *a* total chlorophyll as Chl *a*

* indicates this value was below the SRP detection limit of 0.3 µg P L-1

River. The spectral slope ($S_{250-400}$) value was higher for the Lake Superior water sample (0.0180) than for the harbor and the river water samples (0.0130 to 0.0142 and 0.129 and 0.130, respectively).

Overall, the prokaryotic cell abundance decreased from the St. Louis River to Lake Superior (2.99 to 0.74 x 10^9 cells L^{-1}, respectively) but cell abundance was over 10% higher at the SLR-CI river site (3.39 x 10^9 cells L^{-1}) compared to the upper-most river site.

Several abiotic and biotic parameters were correlated ($p < 0.05$; Appendix Table A-1). Turbidity correlated positively with temperature, DOC, SRP, and prokaryotic cell abundance and negatively correlated with NO_3^-. There were no significant relationships between NH_4^+ and any other parameters. Environmental variables were normally distributed as determined by a Shapiro-Wilk W test (Shapiro and Wilk 1965) so transformations were not necessary (Appendix Table A-2).

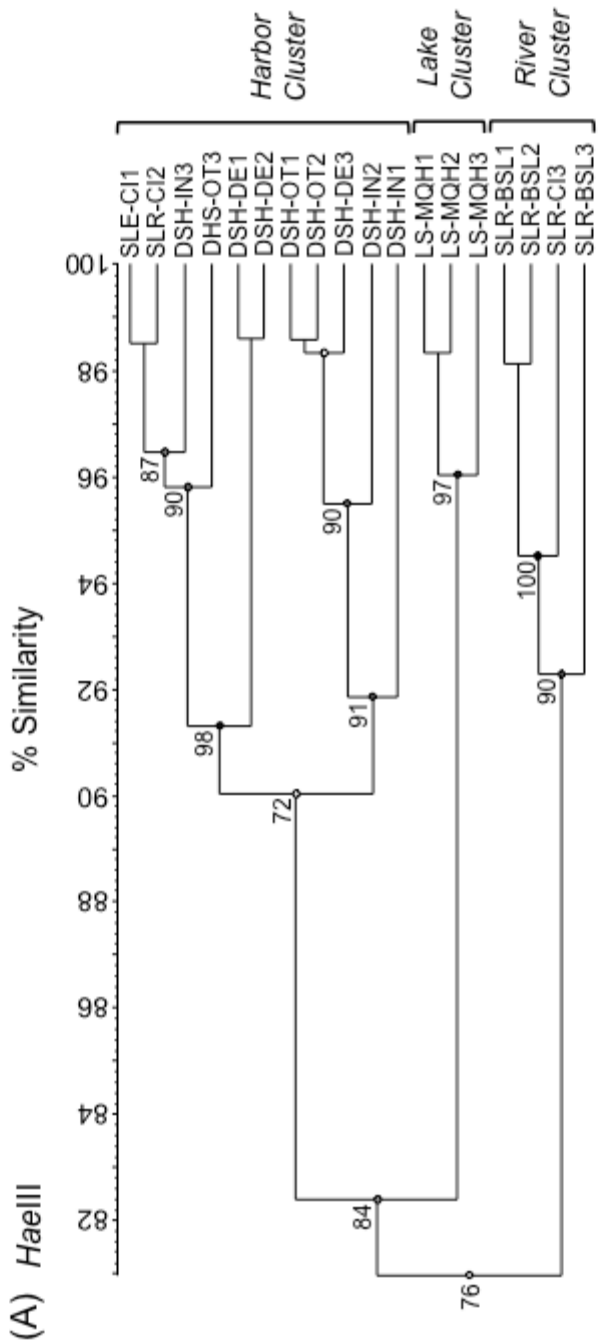
Analyses of the genetic structure of bacterioplankton communities

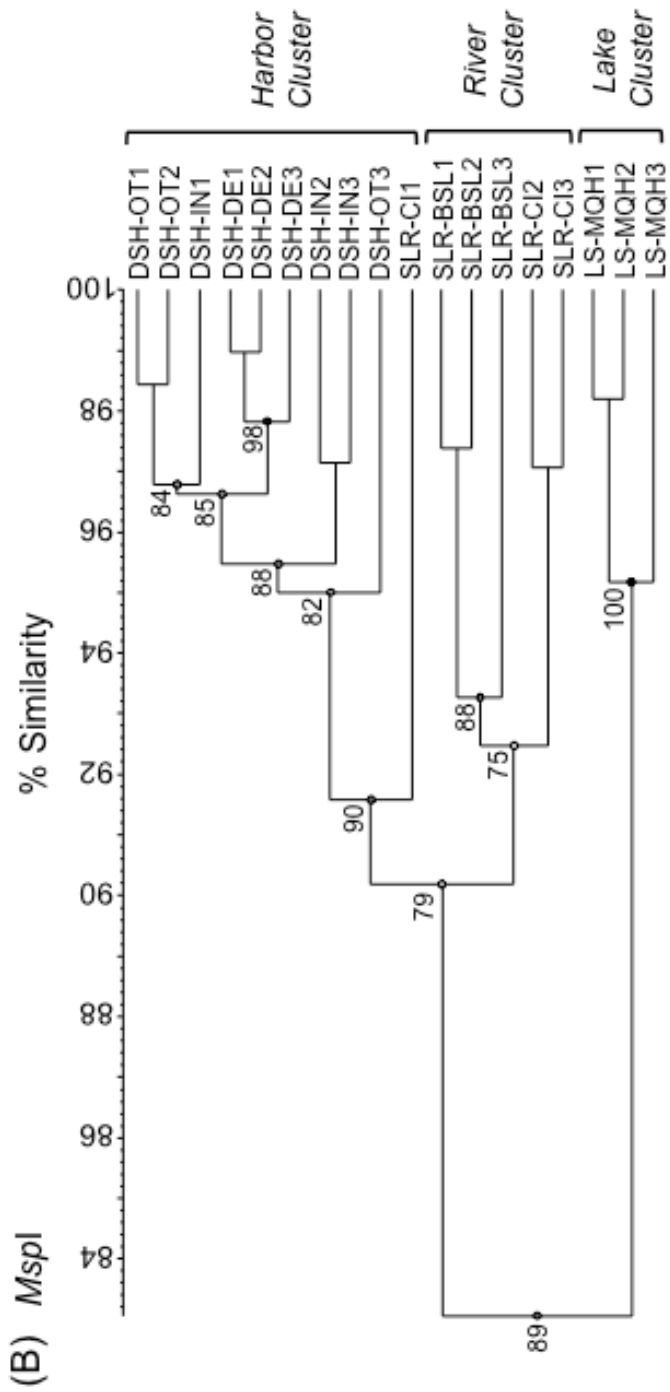
Comparison of the lake, harbor, and river bacterioplankton communities – Bacterioplankton communities formed at least 3 clusters as revealed by curve-based hierarchical clustering analysis with >75% similarity among all samples within each dataset (Fig. II.3). T-RLFP profiles of bacterioplankton communities from the lake samples formed distinct clusters in all three restriction enzyme digests of 16S rDNA PCR products. Replicate T-RFLP profiles from the SLR-CI river site were grouped in both the river and the harbor clusters in the *Hae*III and *Msp*I enzyme digests and only in the harbor cluster for the *Rsa*I enzyme digest. A typically high percent similarity between replicates reflects the high reproducibility of the T-RFLP analysis results.

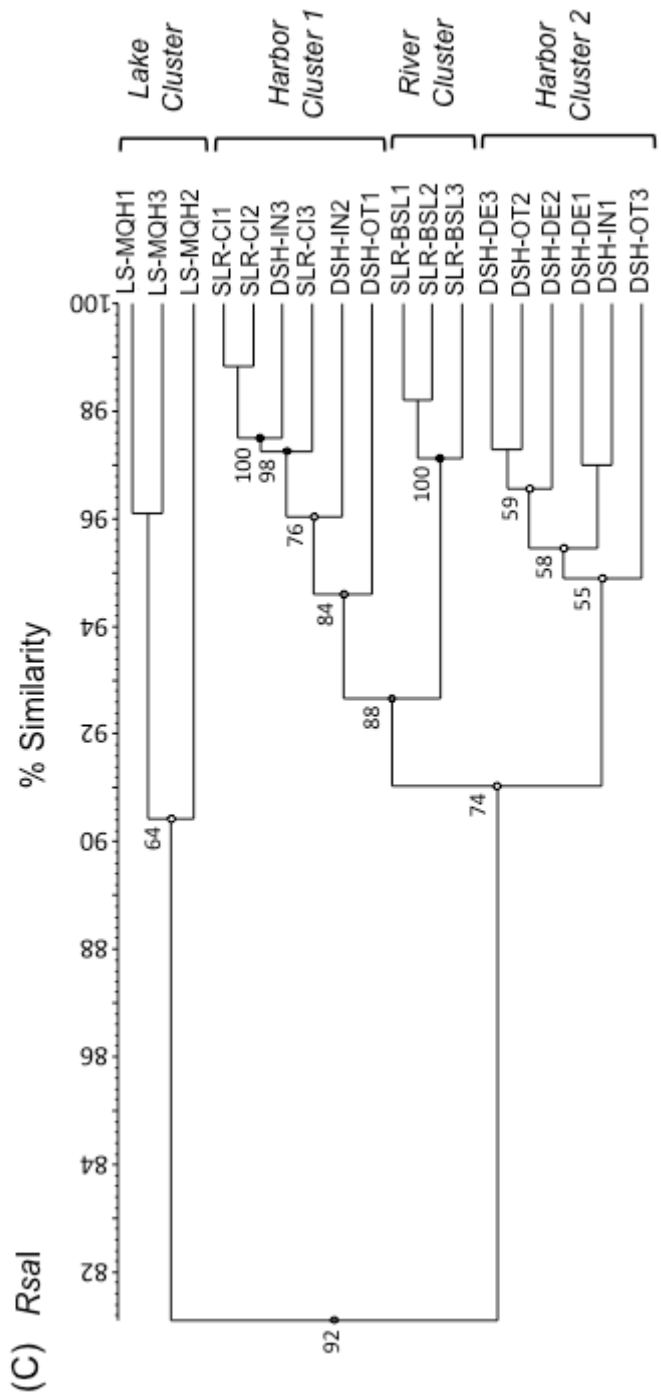
Data sets used in each ordination analysis were determined to be sparse and moderately heterogeneous (Appendix Tables A-3, A-4, and A-5). Overall consistency of sample site placement in ordination space when outliers or rare fragments were removed suggested robust patterns.

The Bray-Curtis ordinations of the bacterioplankton T-RFLP profiles constructed using relative peak height TRFs for samples along the transect through the Duluth-

Figure II.3. UPGMA dendrograms depicting hierarchical cluster analysis of T-RFLP profiles of *Hae*III (A), *Msp*I (B), and *RsaI* (C) restriction enzyme digests of bacterioplankton community 16S rDNA from field sites in Lake Superior, the Duluth-Superior Harbor, and the St. Louis River. Pairwise similarity matrices were calculated using the Pearson correlation coefficient. Numbers at branch nodes indicate the cophenetic correlation between the dendrogram-derived similarities and the matrix similarities. Site abbreviations are followed by replicate designation.


(A) HaeIII
% Similarity
100
98
96
94
92
90
88
86
84
82
SLE-Cl1
SLR-Cl2
DSH-IN3
DHS-OT3
DSH-DE1
DSH-DE2
DSH-OT1
DSH-OT2
DSH-DE3
DSH-IN2
DSH-IN1
LS-MQH1
LS-MQH2
LS-MQH3
SLR-BSL1
SLR-BSL2
SLR-Cl3
SLR-BSL3
87
90
98
72
90
91
97
84
100
90
76
Harbor Cluster
Lake Cluster
River Cluster

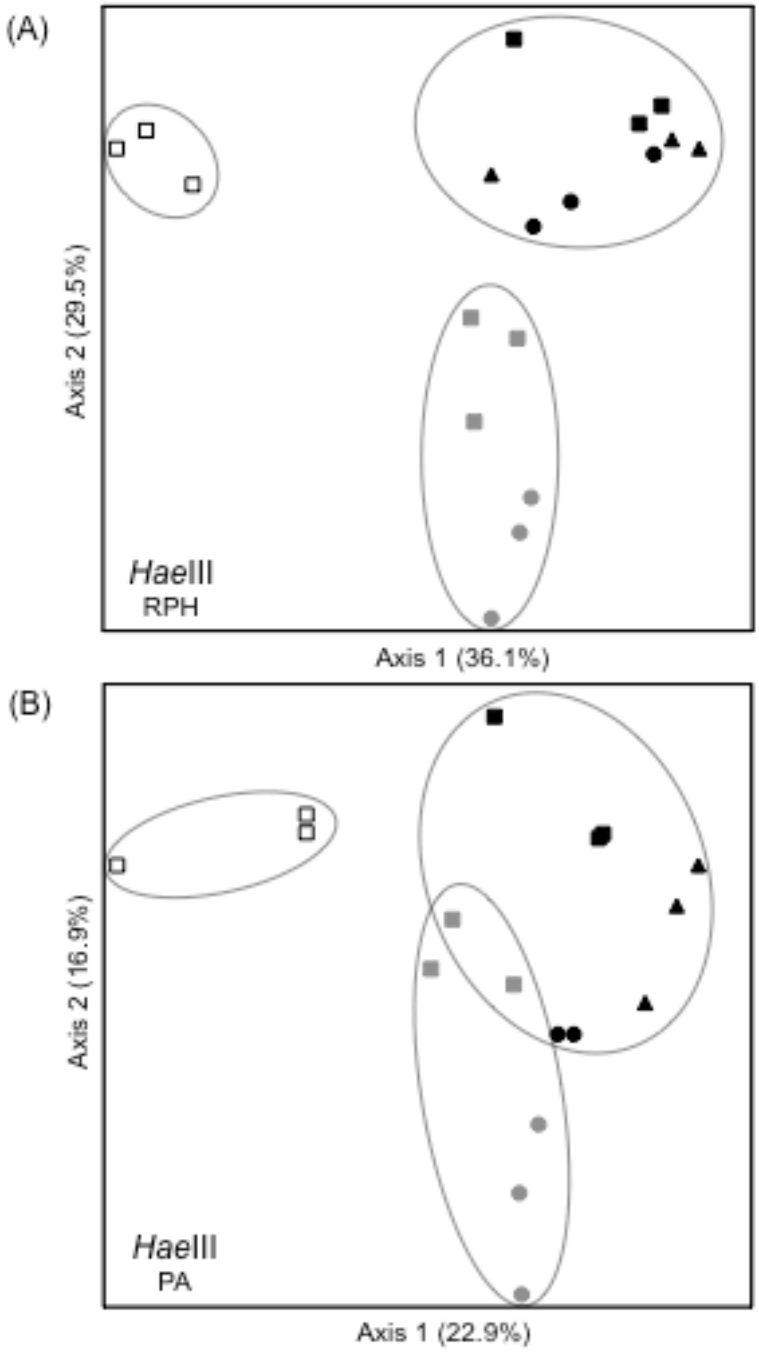

(B) MspI
% Similarity
100
98
96
94
92
90
88
86
84
DSH-OT1
DSH-OT2
DSH-IN1
DSH-DE1
DSH-DE2
DSH-DE3
DSH-IN2
DSH-IN3
DSH-OT3
SLR-CI1
SLR-BSL1
SLR-BSL2
SLR-BSL3
SLR-CI2
SLR-CI3
LS-MQH1
LS-MQH2
LS-MQH3
Harbor Cluster
River Cluster
Lake Cluster
84
85
98
88
82
90
79
88
75
100
89

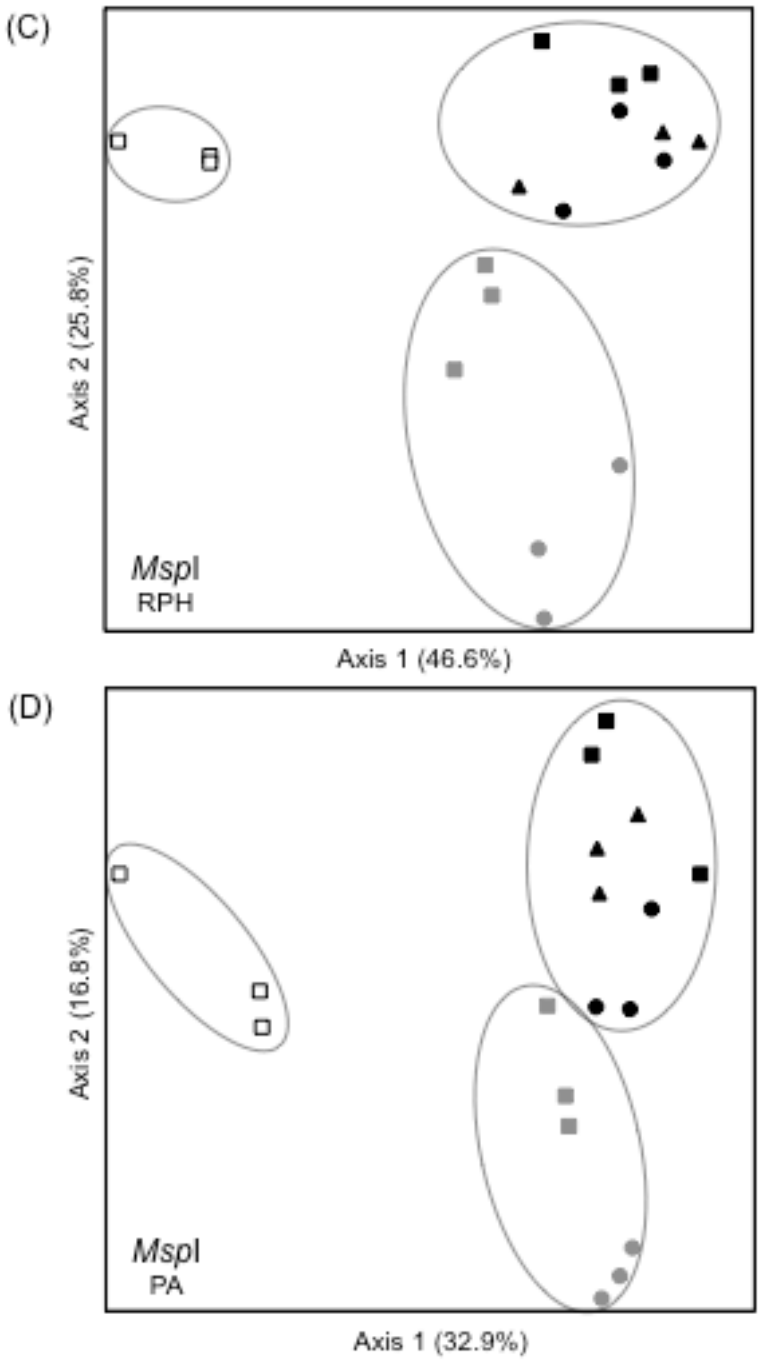

(C) Rsal
% Similarity
100
98
96
94
92
90
88
86
84
82
Lake Cluster
Harbor Cluster 1
River Cluster
Harbor Cluster 2
LS-MQH1
LS-MQH3
LS-MQH2
SLR-CI1
SLR-CI2
DSH-IN3
SLR-CI3
DSH-IN2
DSH-OT1
SLR-BSL1
SLR-BSL2
SLR-BSL3
DSH-DE3
DSH-OT2
DSH-DE2
DSH-DE1
DSH-IN1
DSH-OT3
64
92
74
88
84
76
100
98
100
59
58
55

Superior Harbor corroborated the findings of the curve-based analysis by separating bacterioplankton communities into three groups defined by their location (i.e., the lake, harbor, or river; Fig. II.4). Overall patterns were reproduced using TRF presence-absence (PA) data (Fig. II.4), but the river T-RFLP profiles were in closer proximity to the harbor T-RFLP profiles in ordination space. The T-RFLP profiles of bacterioplankton 16S rDNA from the Duluth Entry (DSH-DE) grouped with T-RFLP profiles from the outer harbor (DSH-OT) and the inner harbor (DSH-IN) samples, possibly due to the influence of river discharge at the Duluth Entry at the time of sampling. In five of the six ordination plots, bacterioplankton communities in the lake (LS-MQH) and in the outer harbor (DSH-OT) were the most dissimilar and were used as endpoints to define the first axis. The variance in the distance matrices explained by the first axes ranged from 22% to 55%. Adding a second axis to the ordinations distinguished bacterioplankton communities in the harbor from communities found in the St. Louis River and also increased the variance explained by the distance matrices by an additional 15 to 30%. Bacterioplankton communities from the upper-most river site (SLR-BSL) and the Duluth Entry (DHS-DE) were the most dissimilar along the second axis. Ordinations based on T-RFLP profiles of TRF abundance data explained more variance in the distance matrices (65 to 73%) than was explained when T-RFLP profiles were developed from TRF presence-absence data (40-56%).

Analysis of similarities (ANOSIM) indicated that the bacterioplankton communities in the lake, harbor, and river were well separated ($R > 0.75$, $p < 0.05$; Table II.2) when using T-RFLP profiles constructed from TRF abundance data. Ordination plots based on T-RFLP profiles constructed from TRF presence-absence data also grouped bacterioplankton communities in the same three groups (ANOSIM $p < 0.05$) with varying degrees of separation among the three restriction enzyme digests of the 16S rDNA PCR products (R-values from 0.30 to 1, $p < 0.05$; Table II.2). SIMPER analysis of TRF abundance data indicated the percent dissimilarity between bacterioplankton communities in Lake Superior and the Duluth-Superior Harbor water samples ranged from 39 to 44% when all three restriction enzyme digests were considered (Table II.3). The percent dissimilarity between bacterioplankton communities in the harbor and the

Figure II.4. Bray-Curtis ordinations based on Sorensen similarities of T-RFLP profiles of bacterioplankton community 16S rDNA from lake, harbor and river water samples. T-RFLP profiles consisted of relative peak height (RPH) of TRFs (A, C, and E) or the presence or absence (PA) of TRFs (B, D, and F). Open squares indicate Lake Superior samples (LS-MQH), solid black shapes indicate Duluth-Superior Harbor samples (DSH-DE, squares; DSH-OT, triangles; DSH-IN, circles), and solid grey shapes indicate St. Louis River samples (SLR-CI, squares; SLR-BSL, circles). For each plot, the endpoints of the two axes were objectively selected using variance regression. Ellipses were added subjectively following analysis. Ordinations of the *Hae*III [(A) and (B)] and *Msp*I [(C) and (D)] datasets were reflected vertically and horizontally for ease of viewing. Both ordinations of the *Rsa*I dataset [(C) and (D)] were only reflected vertically.


(A)
Axis 2 (29.5%)
HaeIII
RPH
Axis 1 (36.1%)
(B)
Axis 2 (16.9%)
HaeIII
PA
Axis 1 (22.9%)


(C)
Axis 2 (25.8%)
MspI
RPH
Axis 1 (46.6%)
(D)
Axis 2 (16.8%)
MspI
PA
Axis 1 (32.9%)

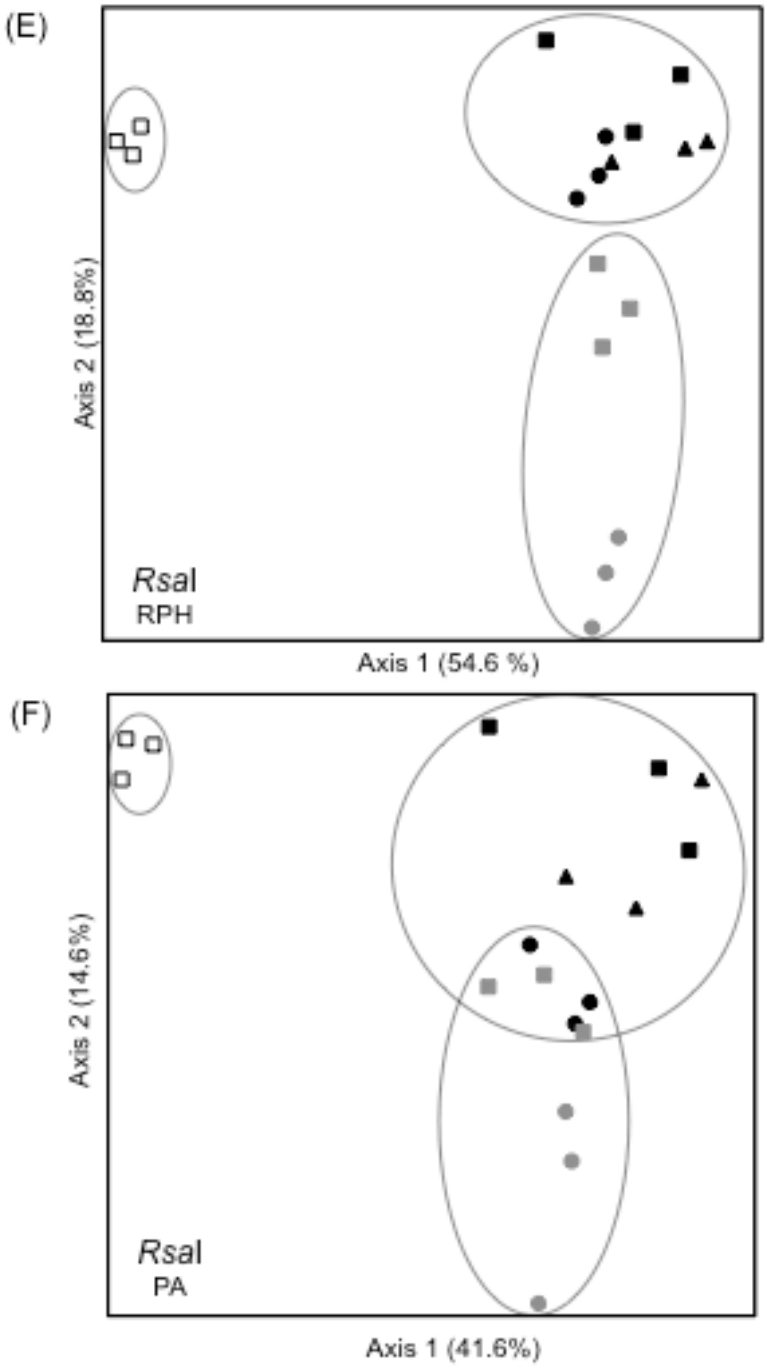

(E)
Axis 2 (18.8%)
RsaI
RPH
Axis 1 (54.6 %)
(F)
Axis 2 (14.6%)
RsaI
PA
Axis 1 (41.6%)

Table II.2. Values of the test statistic R for one-way analysis of similarities (ANOSIM) for pairwise comparisons of T-RFLP profiles of bacterioplankton communities in the lake, harbor, and river groups. Sorensen and Jaccard distance measures were used for abundance (RPH) and presence-absence (PA) data, respectively.

	HaeIII		*MspI*		*RsaI*	
	Abundance	Presence-absence	Abundance	Presence-absence	Abundance	Presence-absence
Lake Superior vs Duluth-Superior Harbor						
All TRFs	0.98**	0.68**	1.00**	1.00**	1.00**	1.00**
Most dominant TRFs [a]	0.86**	0.57**	0.99**	0	1.00**	0.69**
Least dominant TRFs [b]	0.95**	0.60*	1.00**	1.00**	1.00**	1.00**
Duluth-Superior Harbor vs St. Louis River						
All TRFs	0.75***	0.30**	0.84***	0.71***	0.77***	0.55***
Most dominant TRFs	0.82***	0	0.82**	0	0.73*	0
Least dominant TRFs	0.39**	0.31**	0.72***	0.69**	0.72**	0.57***
Lake Superior vs St. Louis River						
All TRFs	1.00*	0.75*	1.00*	1.00*	1.00*	1.00*
Most dominant TRFs	0.59*	0.56*	0.69*	0	1.00*	0.75*
Least dominant TRFs	1.00*	0.65*	1.00*	1.00**	1.00*	1.00*

Abbreviations: TRFs, terminal restriction fragments

a The most dominant fragments in each restriction enzyme digest are fragments with average peak heights representing greater than 5% of the total fluorescence in at least one sample profile. *HaeIII 10, MspI 8, RsaI 10*

b Least-dominant fragments are the fragments remaining in T-RFLP profiles after removal of the most dominant fragments.

*$p \leq 0.05$, **$p \leq 0.0l$, ***$p \leq 0.001$.

Table II.3. Similarities of percentages (SIMPER) for pairwise comparisons of T-RFLP profiles of bacterioplankton communities from the lake, harbor, and river groups. Sorensen and Jaccard distance measures were used for abundance (RPH) and presence-absence (PA) data, respectively.

	*Hae*III		*Msp*I		*Rsa*I	
Group comparisons	Abundance	Presence-absence	Abundance	Presence-absence	Abundance	Presence-absence
Lake Superior vs. Duluth-Superior Harbor	42.7%	42.5%	39.4%	44.0%	43.6%	40.9%
St. Louis River vs. Duluth-Superior Harbor	34.4%	34.8%	27.7%	25.9%	24.1%	23.2%
Lake Superior vs. St. Louis River	45.6%	43.0%	39.0%	44.1%	46.6%	41.9%

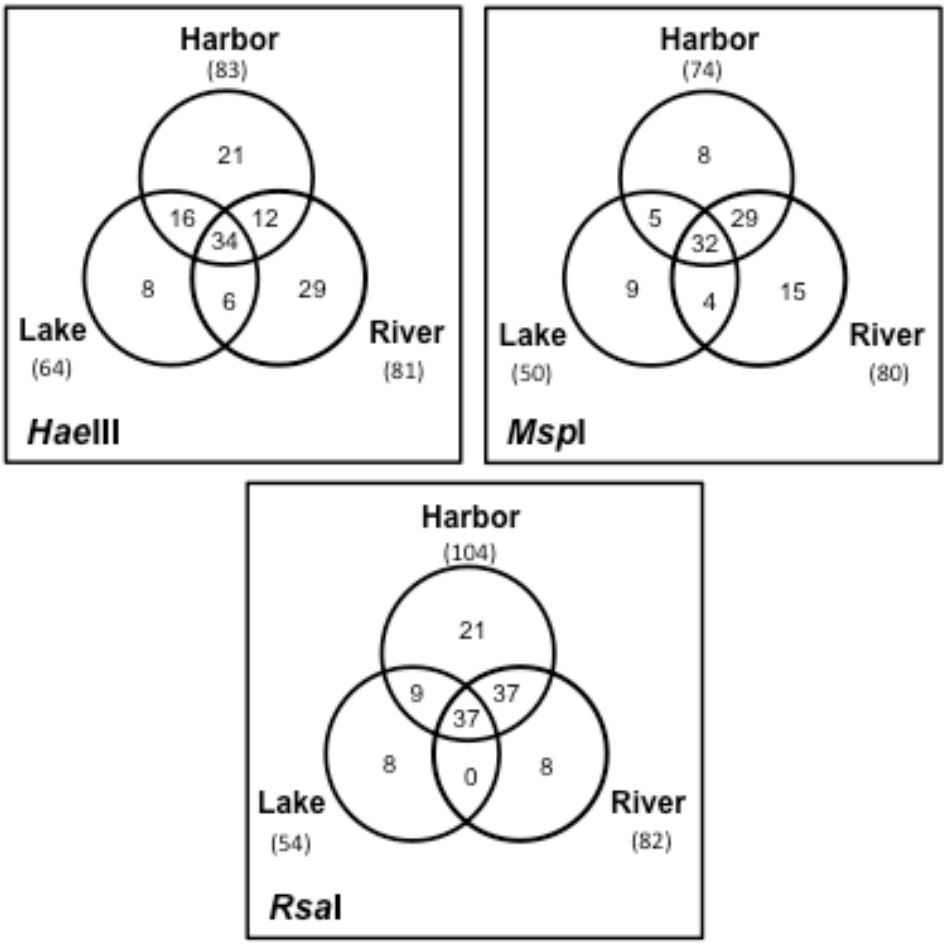
St. Louis River water samples ranged from 24 to 34%. SIMPER analysis of TRF presence or absence data produced similar percentages for the same pairwise comparisons (Table II.3).

The total number of terminal restriction fragments (TRFs) identified in *Hae*III, *Msp*I, and *Rsa*I enzyme digest datasets were 126, 103, and 121, respectively. Approximately 30% of the TRFs were present in bacterioplankton T-RFLP profiles in all three groups, i.e., Lake Superior, the Duluth-Superior Harbor, and the St. Louis River (Fig. II.5). T-RFLP profiles of harbor bacterioplankton communities shared more TRFs (75-90%) with communities in other regions of the sample transect than the lake or river groups (63 to 85% and 57 to 90%, respectively) when the *Hae*III and *Msp*I enzyme digests were evaluated. However, unique TRFs were detected in T-RFLP profiles of bacterioplankton communities from all three regions along the transect in all three restriction enzyme digests. Fewer unique fragments were detected in bacterioplankton community T-RFLP profiles from the Lake Superior samples than from the harbor or river samples. Approximately 17% of the TRFs from the *Hae*III restriction enzyme digest were only detected in bacterioplankton communities from the harbor samples. There were greater than 2.5 times more unique TRFs in bacterioplankton community T-RFLP profiles from the harbor region than in profiles from either the lake or the river areas, when *Rsa*I was used to digest the bacterioplankton 16S rDNA PCR products.

Dominant TRFs were identified in each restriction enzyme digest that had average peak heights representing greater than 5% of the total fluorescence in at least one sample profile. Ten dominant TRFs were identified in the *Hae*III restriction enzyme digest representing 69.5% of the total fluorescence in the dataset. Eight dominant fragments (86.2% total fluorescence) and 10 dominant fragments (80.1% total fluorescence) were identified in the *Msp*I and *Rsa*I restriction enzyme digests, respectively.

Additional ANOSIM analyses were conducted on T-RFLP profiles constructed using the most and least dominant TRFs to determine if the separation of bacterioplankton communities from different regions of the transect was primarily due to the most common TRFs in the T-RFLP profiles. Bacterioplankton communities were still observed to be in well separated groups in five of the six pairwise comparisons (R =

Figure II.5. Venn diagrams describing the number of terminal restriction fragments (TRFs) in T-RFLP profiles of bacterioplankton community 16S rDNA from the lake, harbor, and river groups using *Hae*III, *Msp*I, and *Rsa*I enzyme digests. Numbers in the overlapping portions of the circles are the number of fragments shared between groups. The total number of TRFs for each group is in parenthesis.


Harbor
(83)
21
16
12
34
8
6
29
Lake
(64)
River
(81)
HaeIII
Harbor
(74)
8
5
29
32
9
4
15
Lake
(50)
River
(80)
MspI
Harbor
(104)
21
9
37
37
8
0
8
Lake
(54)
River
(82)
RsaI

> 0.70, $p < 0.05$; Table II.2). Comparing the abundance of the most dominant TRFs resulted in a stronger separation between the harbor and river bacterioplankton communities in the *Hae*III and *Msp*I restriction enzyme digests ($R = 0.82$, $p < 0.001$; $R = 0.82$, $p < 0.01$, respectively) than when the least dominant TRF fragments were used ($R = 0.31$, $p < 0.01$; $R = 0.69$, $p < 0.01$, respectively). The abundance of the most and least dominant TRFs separated the bacterioplankton communities in the lake and harbor equally as well when the *Msp*I and *Rsa*I datasets were evaluated ($R = 0.99$, $p < 0.01$ and $R = 1$, $p < 0.01$; $R = 1$, $p < 0.01$ and $R = 1$, $p < 0.01$, respectively). The total percent contribution of the most dominant TRFs to the dissimilarity between the T-RFLP profiles of bacterioplankton communities from the lake, harbor, and river ranged from 41 to 63% (SIMPER). In the *Hae*III and *Msp*I restriction enzyme digests, the abundance of the most dominant TRFs had a greater influence on the dissimilarity between the T-RFLP profiles of bacterioplankton communities from the harbor and river (63.3% and 49.3%, respectively) than was found between with the communities in the harbor and lake (53.9% and 47.3%, respectively) or the communities in the river and lake (50.5% and 40.6%, respectively).

Comparison of lake and river water mixtures with field samples – The three mixtures of Lake Superior (L) and St. Louis River (R) water (i.e., 60L/40R, 40L/60R, and 20L/80R), had DOC concentrations of 10.69, 15.35, and 19.94 mg L^{-1}, respectively. A location along the sample transect was estimated for each lake and river water mixture based on its DOC concentration using a linear regression model ($y = 0.8707x + 7.4785$; $R^2 = 0.99$; $p < 0.05$; Fig. II.6). The distances from the Duluth Entry field site (DSH-DE) were 3.7 ± 3.8, 9.06 ± 3.7, and 14.3 ± 3.8 km for mixtures (i.e., 60L/40R, 40L/60R, and 20L/80R, respectively). This placed two of the mixtures (60L/40R and 40L/60R) within the harbor and one mixture 20L/80R between the two field sites in the St. Louis River.

NMS ordinations were performed to determine if the T-RFLP profiles of the artificial bacterioplankton communities in the lake and river water mixtures were most similar to the communities at field sites in closest proximity to the estimated locations of mixtures along the sample transect (Fig. II.7). Unfortunately, several T-RFLP profiles

Figure II.6. Dissolved organic carbon (DOC) concentrations along the sampling transect through the Duluth-Superior Harbor (solid black shapes: DSH-DE square, DSH-OT triangle, DSH-IN circle) and into the St. Louis River (solid grey shapes: SLR-CI square, SLR-BSL circle). The regression line indicates a significant decrease in DOC concentration from the river into the harbor. The dashed lines surrounding the regression line represent the 95% confidence intervals. Open diamonds represent corresponding distances from the Duluth Entry estimated for artificial mixtures using the equation: $y = 0.8707x + 7.4785$; $R^2 = 0.99$; $P < 0.05$. Proportions of Lake Superior (L) and St. Louis River (R) water in each mixture are depicted in boxes.

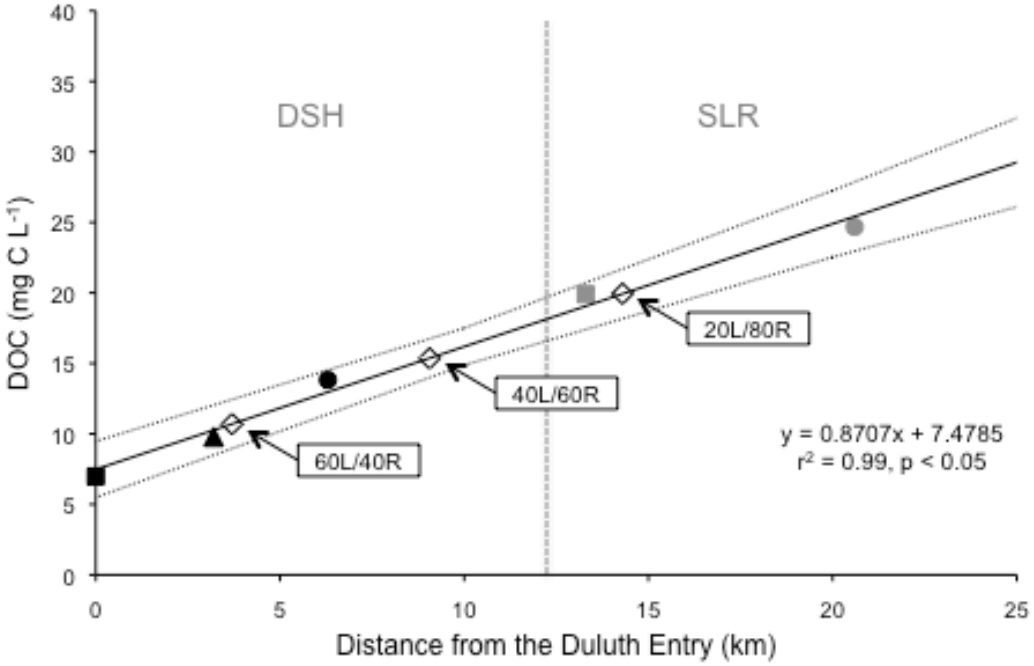
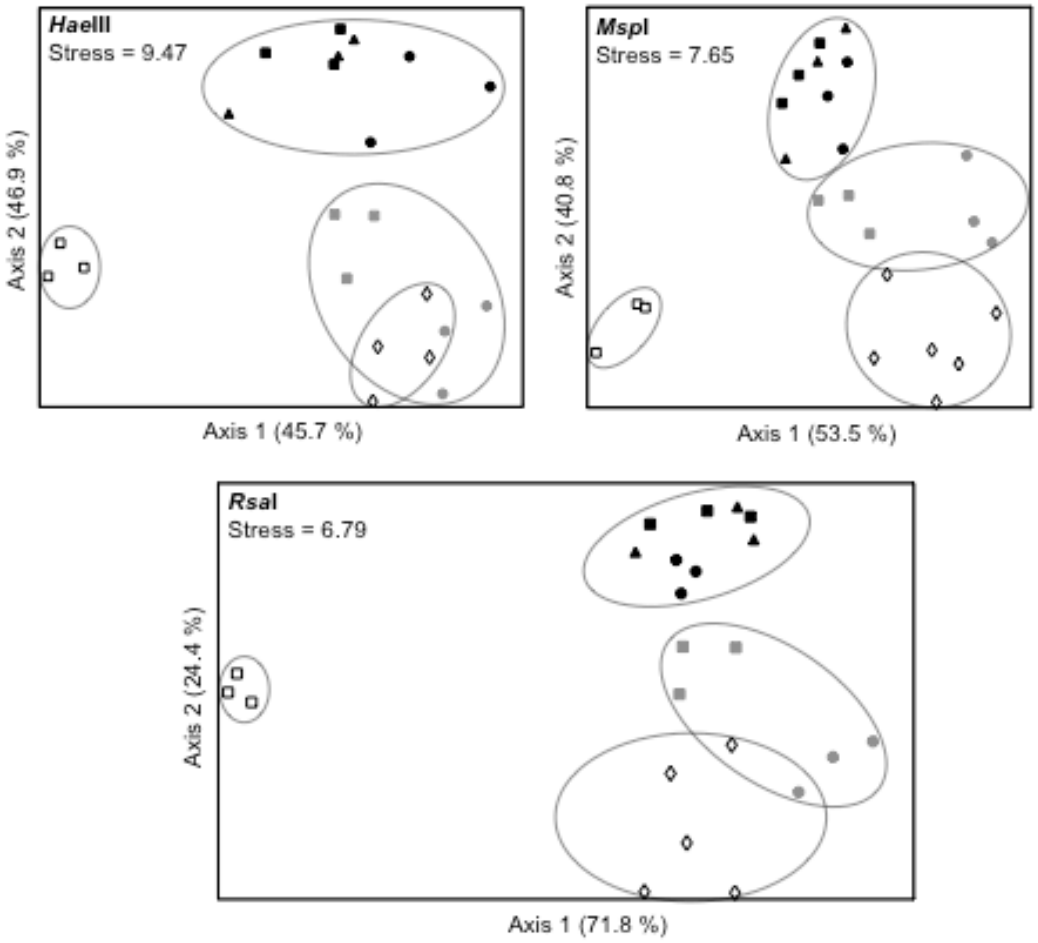

DSH
SLR
20L/80R
40L/60R
60L/40R
y = 0.8707x + 7.4785
r² = 0.99, p < 0.05
DOC (mg C L⁻¹)
Distance from the Duluth Entry (km)
0
5
10
15
20
25
30
35
40

Figure II.7. Nonmetric multidimensional scaling (NMS) ordinations based on Sorensen similarities of T-RFLP profiles of *Hae*III, *Msp*I, and *Rsa*I enzyme digests of bacterioplankton community 16S rDNA from artificial mixtures of lake and river water and samples from the lake, the harbor, and the lake. T-RFLP profiles consisted of relative peak height of TRFs. Open squares indicate Lake Superior samples (LS-MQH), solid black shapes indicate Duluth-Superior Harbor samples (DSH-DE, squares; DSH-OT, triangles; DSH-IN, circles), solid grey shapes indicate St. Louis River samples (SLR-CI, squares; SLR-BSL, circles), and open diamonds indicate artificial mixtures of lake and river water.


HaeIII
Stress = 9.47
Axis 2 (46.9 %)
Axis 1 (45.7 %)
MspI
Stress = 7.65
Axis 2 (40.8 %)
Axis 1 (53.5 %)
RsaI
Stress = 6.79
Axis 2 (24.4 %)
Axis 1 (71.8 %)

from the lake and river mixture samples were removed due to their poor quality. All the T-RFLP profiles for the 20L/80R mixtures, regardless of restriction enzyme used, had high noise signals and could not be used. One of the T-RFLP profile replicates of the 60L/40R mixture in each of the *Hae*III and *Rsa*I restriction enzyme digests were removed due to low total signal or very few peaks and two replicate T-RFLP profiles of the artificial community in the 60L/40R mixture for the *Hae*III digest were removed due to high noise signals. Only abundance data (RPH) was used for these ordination analyses since solutions computed using presence-absence (PA) data resulted in final stress values > 15.

The structure of the artificial bacterioplankton communities in the lake and river water mixtures was most similar to the structure of bacterioplankton communities in the St. Louis River in all three restriction enzyme digests as illustrated by the NMS ordination plots of T-RFLP profiles constructed from TRF peak abundance data (Fig. II.7). The first axis in all ordination plots separated bacterioplankton communities in Lake Superior from those in the harbor, the river, and the lake and river water mixtures. The second axis in all the ordination plots accounted for a significant percentage of the variance in the distance matrices (24% to 47%) and placed bacterioplankton communities from the lake and river water mixtures at the greatest distance from bacterioplankton communities in the harbor. In the *Hae*III dataset, the bacterioplankton communities in the lake and river water mixtures appeared to group with bacterioplankton communities from the St. Louis River, but in the *Msp*I and *Rsa*I datasets, the bacterioplankton communities from the mixtures and the river formed separate but overlapping groups. Ordination plots from all three restriction enzyme digests were determined to be 'good solutions' (Final stress < 10) and produced stronger axes than would be expected by chance (Monte Carlo stress test, 250 permutations, $p < 0.01$; Appendix Table A-6).

Relationships between the genetic structure of bacterioplankton communities and environmental variables

The relationship between a number of abiotic and biotic factors and the genetic structure of the bacterioplankton communities in the Duluth-Superior Harbor and the St.

Louis River was examined using nonmeteric multidimensional scaling (NMS) ordination of T-RFLP profiles of abundance data and a joint-plot overlay of a second matrix containing environmental variables (Figs. II.8 and II.9). Ordination plots from all three restriction enzyme digests were determined to be 'good solutions' (Final stress < 10) and produced stronger axes than would be expected by chance (Monte Carlo stress test, 250 permutations, $p < 0.01$; Appendix Table A-6). Temperature, DOC, and NH_4^+, were the most influential environmental factors contributing to the variability in the bacterioplankton community structure along the sample transect (Figs. II.8 and II.9). An additional variable, NO_3^-, was correlated with differences in bacterioplankton community structure when *Rsa*I enzyme digests of bacterial DNA was used to construct T-RFLP profiles. A number of relationships between environmental factors and ordination axes were non-linear, so non-parametric Kendall's tau rank correlation was used. In the *Hae*III and *Msp*I datasets, temperature and DOC were positively correlated (tau > 0.6) and NH_4^+ was negatively correlated (tau > 0.6) with axis 1 (Table II.4). In the *Rsa*I dataset, temperature and DOC were negatively correlated (tau > 0.9) and $NH4^+$ was positively correlated (tau > 0.7) with axis 2. In addition, NO_3^- was also positively correlated with axis 2 (tau > 0.7) in the *Rsa*I dataset. A complete table of correlations with ordination axes can be viewed in the Appendix (Table A-7).

The Spearman's rank correlation coefficient (ρ) was used to further examine the relationship between the environmental variables measured in this study and the bacterioplankton communities in the Duluth-Superior Harbor and the St. Louis River water samples. SIMPER analysis was performed on both abundance (RPH) and presence-absence (PA) datasets to identify TRFs that contributed the most to the dissimilarity between the T-RFLP profiles of the bacterioplankton communities in the harbor and river. When considering abundance data, many of the TRFs that contribute the most to the dissimilarity between the harbor and the river bacterioplankton communities were the most dominant TRFs in each restriction enzyme digest. The most influential TRF in the abundance data from the *Hae*III restriction enzyme digest (18.8% contribution to dissimilarity) was positively correlated with NH_4^+ ($\rho = 0.94$, $p < 0.01$) and was found in much greater abundance in the T-RFLP profiles of bacterioplankton

Figure II.8. (A) Nonmetric multidimensional scaling (NMS) ordinations based on Sorensen similarities of T-RFLP profiles of *Hae*III enzyme digests of bacterioplankton community 16S rDNA using relative peak height of TRFs. Black shapes indicate Duluth-Superior Harbor samples (DSH-DE, squares; DSH-OT, triangles; DSH-IN, circles) and grey shapes indicate St. Louis River samples (SLR-CI, squares; SLR-BSL, circles). Vectors represent environmental variables that correlated (Kendall's tau > 0.6) with T-RFLP profiles of bacterioplankton communities in the two habitat groups. Vector length is proportional to the strength of the association with ordination axes. (B) Distribution of temperature and DOC and NH_4^+ concentrations along the sampling transect from the river through the harbor. Black shapes indicate Duluth-Superior Harbor samples and grey shapes indicate St. Louis River samples.

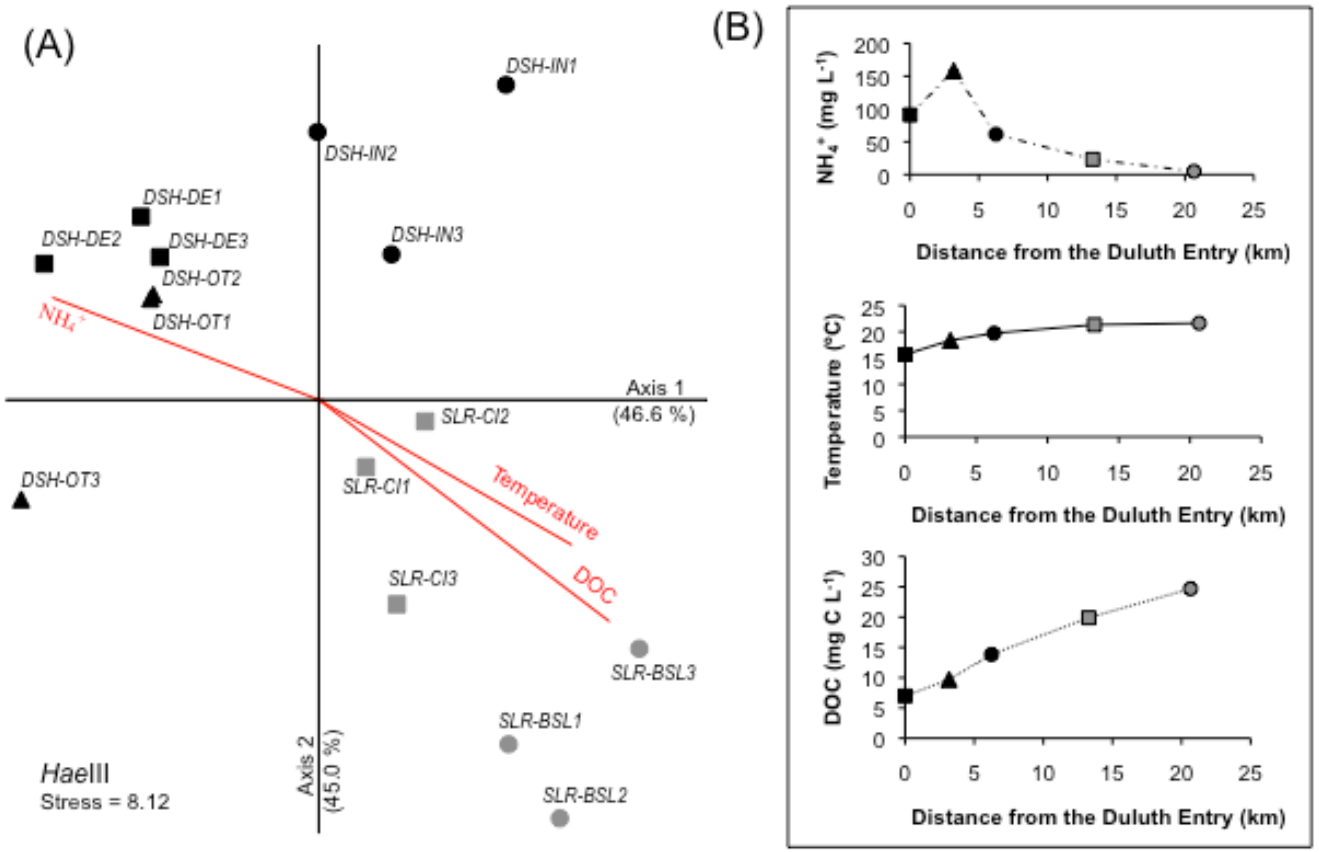
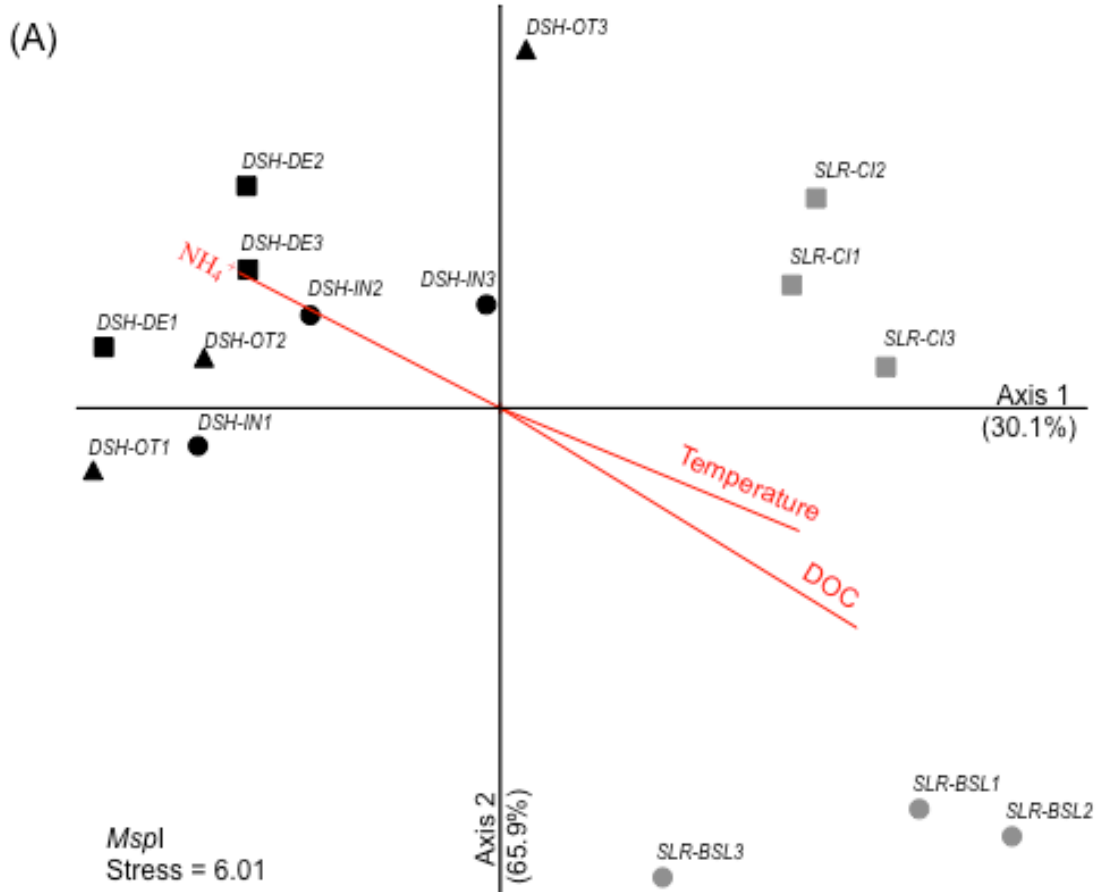

(A)
DSH-IN1
DSH-IN2
DSH-DE1
DSH-DE2
DSH-DE3
DSH-IN3
DSH-OT2
DSH-OT1
NH4+
Axis 1
(46.6 %)
SLR-CI2
DSH-OT3
SLR-CI1
Temperature
DOC
SLR-CI3
SLR-BSL3
SLR-BSL1
HaeIII
Stress = 8.12
Axis 2
(45.0 %)
SLR-BSL2
(B)
NH4+ (mg L-1)
Distance from the Duluth Entry (km)
Temperature (°C)
Distance from the Duluth Entry (km)
DOC (mg C L-1)
Distance from the Duluth Entry (km)

Figure II.9. Nonmetric multidimensional scaling (NMS) ordinations based on Sorensen similarities of T-RFLP profiles of *Msp*I (A) and *Rsa*I (B) enzyme digests of bacterioplankton community 16S rDNA using relative peak height of TRFs. Black shapes indicate Duluth-Superior Harbor samples (DSH-DE, squares; DSH-OT, triangles; DSH-IN, circles) and grey shapes indicate St. Louis River samples (SLR-CI, squares; SLR-BSL, circles). Vectors represent environmental variables that correlated (Kendall's tau > 0.6) with T-RFLP profiles of bacterioplankton communities in the two habitat groups. Vector length is proportional to the strength of the association with ordination axes.


(A)
DSH-OT3
DSH-DE2
SLR-CI2
DSH-DE3
NH_4^+
SLR-CI1
DSH-IN2
DSH-IN3
DSH-DE1
DSH-OT2
SLR-CI3
Axis 1
(30.1%)
DSH-IN1
DSH-OT1
Temperature
DOC
SLR-BSL1
SLR-BSL2
Axis 2
(65.9%)
SLR-BSL3
MspI
Stress = 6.01

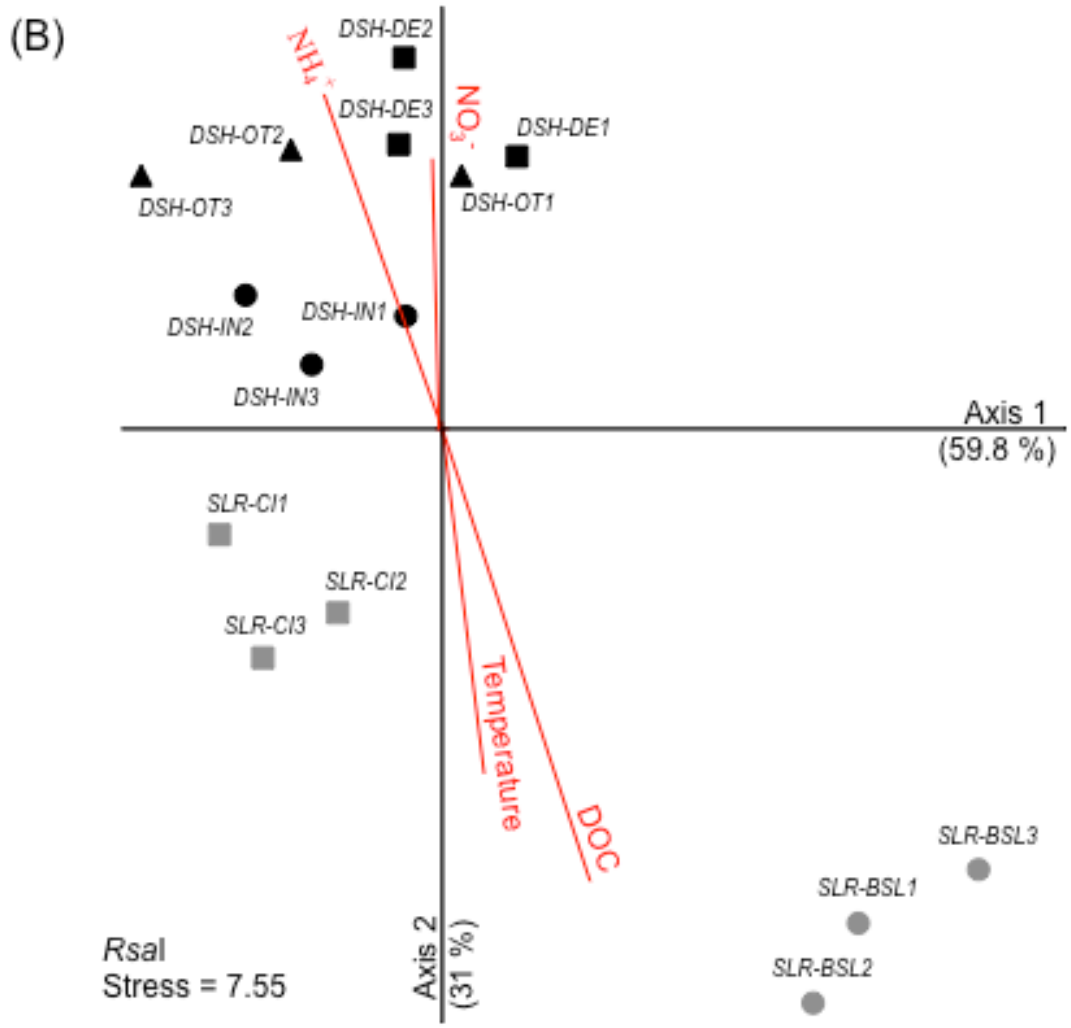

(B)
DSH-DE2
DSH-DE3
DSH-DE1
DSH-OT2
DSH-OT3
DSH-OT1
DSH-IN2
DSH-IN1
DSH-IN3
SLR-CI1
SLR-CI2
SLR-CI3
SLR-BSL3
SLR-BSL1
SLR-BSL2
NH4+
NO3-
Temperature
DOC
Axis 1
(59.8 %)
Axis 2
(31 %)
Rsal
Stress = 7.55

Table II.4. Kendall's tau rank correlations (tau > 0.6) between physiochemical and biotic parameters and axes 1 and 2 for NMS ordination analyses of bacterioplankton T-RFLP profiles.

	Axis 1	Axis 2
*Hae*III		
Temperature	0.741	(0.535)
DOC	0.741	(0.535)
NH_4^+	(0.761)	0.350
*Msp*I		
Temperature	0.638	(0.411)
DOC	0.638	(0.411)
NH_4^+	(0.658)	0.350
*Rsa*I		
Temperature	0.144	(0.905)
DOC	0.144	(0.905)
NH_4^+	(0.247)	0.761
NO_3^-	0.041	0.720

Brackets ( ) around the coefficient signifies negative correlations. No p-values are given in PC-ORD.

communities within the harbor than in either the lake or river habitats. Five of the ten most influential TRFs in the same restriction enzyme digest were positively correlated with temperature and DOC ($\rho > 0.82$, $p < 0.05$) and were responsible for 23.7% of the dissimilarity between the harbor and river bacterioplankton community T-RFLP profiles. One TRF in each of the HaeIII and RsaI restriction enzyme digests was positively correlated with SRP ($\rho > 0.82$, $p < 0.05$). When considering presence-absence data, the most influential TRFs in the T-RFLP profiles in the HaeIII, MspI, and RsaI restriction enzyme digests were positively correlated with NH_4^+ ($\rho = 0.88$, $p < 0.05$; $\rho = 0.88$, $p < 0.05$; $\rho = 0.94$, $p < 0.05$; respectively) and these TRFs were only detected in the bacterioplankton communities in the harbor samples.

DISCUSSION

Analysis of the genetic structure of bacterioplankton communities along the transect from the St. Louis River through the Duluth-Superior Harbor, and into Lake Superior revealed three well-separated types of communities that were defined by their location (Figs. II.3 and II.4). Bacterioplankton communities from the lake, harbor, and river were comparably separated when either the abundance (relative peak height) or the presence or absence of terminal restriction fragments (TRFs) were used to construct community profiles. Abundance data typically reflects the more dominant members of a community while presence-absence data enhances the effect of more rare members. When either the most dominant or less dominant bacterial taxa were considered, the bacterioplankton communities from the lake, harbor, and river habitats were determined to be in separate groups (Table II.2). These comparisons indicate that the more abundant bacterial taxa did not exclusively determine the separation of bacterioplankton communities.

LaPara et al. (2011) reported comparable ordination patterns in a comparison of bacterial community structure along a similar sample transect through the Duluth-Superior Harbor using NMS ordination analysis of automatic ribosomal intergenic spacer

analysis (ARISA) profiles. However, it was not determined in this study whether the ARISA profiles of the bacterial communities in Lake Superior, the Duluth-Superior Harbor, and the St. Louis River formed separate, statistically significant groups.

The most abundant bacterial taxa in the bacterioplankton communities from the lake, harbor, and river habitats were also widely distributed. This finding is consistent with other studies that relate bacterial taxa distribution and abundance (Pommer et al. 2007, Nemergut et al. 2011). The higher abundance of a few bacterial groups may lead to their more widespread dispersal (Fenchel and Finlay 2004, Nemergut et al. 2011), whereas more rare bacterial groups may even be dispersal limited (Bell 2010). However, an important point to consider is the potential bias of many commonly used molecular techniques. Molecular community profiling methods, such as T-RFLP, that are based on PCR amplification using universal primers may preferentially detect more dominant groups (i.e., $\geqq$ 1% of the total cell number) while the more rare groups (i.e., < 1%) remain undetected (Pedrós-Alió 2006, Blackwood et al. 2007). Populations of rare microbes can be major contributors to the overall diversity in microbial communities. Nevertheless, other researchers have proposed that bacterial groups most important in energy flow and carbon and nutrient cycling (e.g. ammonia oxidizers) may be the more abundant groups (Pedrós-Alió 2006, Ward and O'Mullan 2002).

Nearly 40% of the bacterial taxa detected in the Duluth-Superior Harbor bacterioplankton communities were also detected in both Lake Superior and the St. Louis River communities (Fig. II.5). This pattern of overlapping bacterial communities across spatial gradients has been previously observed. The mixing zone at the confluence of a river and a lake or ocean creates a unique environment where bacterial communities will contain members of both source waters (Crump et al. 1999, 2004; Alonso et al. 2010). In the Columbia River estuary, Crump et al. (1999) detected both marine groups (SAR11 in *Alphaproteobacteria*) and freshwater groups (*Betaproteobacteria* and *Verrucomicrobium* spp.) in free-living bacterioplankton. In the Milwaukee harbor, bacterial communities reflected a mixture of bacterioplankton groups from both Lake Michigan and a number its tributaries (Mueller-Spitz et al. 2009).

However, nearly one-third of the bacterial taxa detected in the harbor bacterioplankton communities were not detected in either the lake or river communities (Fig. II.5). These unique bacterial taxa in the harbor may have been introduced into the harbor from sources other than the lake or river or they may have been present in the bacterioplankton communities of the lake or the river samples in very low numbers, but had increased in abundance when exposed to the abiotic or biotic conditions in the Duluth-Superior Harbor. Overall, the abundance of bacterial taxa contributed more to the differences between the structure of the harbor and river bacterioplankton communities than between the structure of either the lake and harbor or the lake and river communities (Fig. II.4, Table II.2). Stortz and Sydor (1980) estimated the residence time of a dissolved pollutant entering the inner harbor over the course of 8 hours to be between 30 and 40 days. This long residence time combined with normal bacterial growth rates may allow different bacterioplankton populations to proliferate within the harbor.

The St. Louis River and Lake Superior are the major natural sources of bacteria influencing the genetic structure of bacterioplankton communities detected in the Duluth-Superior Harbor. Overall, the prokaryotic cell abundance increased from Lake Superior to the St. Louis River (0.74 to 2.99 x 10^9 cells L^{-1}, respectively). The low prokaryotic cell abundance in Lake Superior determined in this study was similar to findings of previous studies (Auer and Powell 2004, Hicks et al. 2004). Other natural sources of bacteria that may influence the bacterioplankton community structure in the harbor include soil bacteria from surface water run-off, sloughing of benthic biofilms, and hyporheic sediments in the mixing zone of shallow groundwater and surface water (Crump et al. 2007, Febria et al. 2010). The $SUVA_{254}$ values for Lake Superior (3.3) and the St. Louis River (8.9) were similar to those reported by Minor and Stephens (2008; 1.3 and 8.4 respectively) but the highest $SUVA_{254}$ value was observed at the river site in Pokegama Bay (SLR-CI, Fig. II.1). The Pokegama River watershed is predominantly forested and has its headwaters in an extensive system of red clay wetlands. Ågren et al. (2008) detected higher $SUVA_{254}$ levels from wetland sources than forest runoff alone, which may account for the elevated $SUVA_{254}$ value at the SLR-CI site. The high $SUVA_{254}$ value at this site suggests that wetland dissolved organic matter (DOM) could

be reaching the harbor from this St. Louis River tributary. In addition, the Pokegama River can be turbid and contain suspended clays transported from the Pokegama wetlands (Johnston et al. 2001). Irvine et al. (2002) found a strong positive relationship between bacteria and suspended solids. Similarly, prokaryotic cell abundance and turbidity were positively correlated in this study (Appendix Table A-1). So, bacteria from wetlands in this watershed may have entered the harbor and contributed to the differences in the bacterioplankton communities observed in the harbor samples when compared to the Lake Superior and the upper St. Louis River samples.

The two principal anthropogenic sources of bacteria to the Duluth-Superior Harbor are treated wastewater effluent discharged into the inner harbor and ballast water discharged into both the inner and outer harbors. Inflow and infiltration of water into the sanitary sewer system during heavy rainstorms or snowmelt can cause sanitary sewer overflows resulting in increased nutrients, bacteria, and suspended solids in storm water run-off. Ballast water can contain freshwater and marine bacterioplankton and bacteria associated with suspended sediments. Residual water and sediments in a ships' ballast tank can also be a source of bacteria to the harbor. In a ship's ballast tank, a portion of the ballast water and sediments (residuals) remain after ballast water discharge and is considered 'unpumpable.' These sediments can then be resuspended and partially discharged during subsequent ballast water management practices (Bailey et al. 2003). Drake et al. (2005) reported an estimated 6.8×10^{14} bacterial cells detected in the residuals of a ships' ballast tank. In 2005, an estimated 5 billion gallons of ballast water was discharged into the Duluth-Superior Harbor (MPCA 2008). Using the estimated range of bacterial cells in ballast water reported by Sun et al. (2010; 2.5×10^{8} to 2.1×10^{9} cells L^{-1}), 4.7×10^{18} to 4.0×10^{19} bacterial cells could potentially be discharged into the DSH annually.

In the artificial community experiment, the T-RFLP profiles of bacterioplankton assemblages in all the lake and river water mixtures were most similar to the T-RFLP profiles of bacterioplankton communities from the St. Louis River (Fig. II.7), but the estimated location of two of the lake and river water mixtures, based on the DOC concentrations, were in the harbor (Fig. II.6). These results may indicate that the

bacterioplankton communities in the harbor are not simple mixtures of bacterioplankton from Lake Superior and the St. Louis River, but they may also be due to an artifact of the T-RFLP analysis or the effect of photodegradation on DOC concentrations. During the PCR procedure on the artificial bacterioplankton assemblages, a greater number of 16S rDNA sequences from the river water than from the lake water may have been amplified due to the higher prokaryotic cell abundance in the river water sample when compared to the lake water sample (Table II.1). As a result, a larger fraction of the PCR products used in the T-RFLP analysis of the lake and river water mixtures were of river origin allowing more TRFs from the river water sample to be raised to detectable levels. Consequently, the genetic structure of the artificial bacterioplankton assemblages would be most similar to the structure of the bacterioplankton communities from the St. Louis River.

When exposed to UV radiation, dissolved organic matter (DOM) is subject to photodegradation resulting in decreased DOC concentration and changes in optical characteristics such as reduced aromaticity, i.e., lower $SUVA_{254}$ values, and increased spectral slope (De Haan 1993, Brinkmann et al. 2003, Biddanda and Cotner 2003, Minor and Stephens 2008). In this study, a loss of DOC due to photodegradation in the surface water along the sample transect would cause an underestimation of the distance from the Duluth Entry that was determined for each of the artificial lake and river water mixtures. The $SUVA_{254}$ values and spectral slope ($S_{250-400}$) values calculated for this study were consistent with those previously described for Lake Superior and a number of its tributaries (Minor and Stephens 2008). The $SUVA_{254}$ value was lower in Lake Superior than in the harbor or river and the spectral slope ($S_{250-400}$) value was higher in Lake Superior than in the harbor or the river. The decrease in $SUVA_{254}$ values and increase in spectral slope ($S_{250-400}$) values along the sample transect through the harbor is consistent with changes due to photodegradation. However, it could not be determined by data collected in this study whether the changes in optical characteristics in the DOC along the sample transect was due to photodegradation or simple dilution from seiche activity, the effects of microbial activity, or other abiotic or biotic conditions.

The potential influence of various environmental variables on the genetic structure of bacterioplankton communities in the Duluth-Superior Harbor and the St.

Louis River was examined in this study. The structure and composition of aquatic bacterioplankton communities can be influenced by temperature (Yannerall and Triplett 2005), pH (Fierer et al. 2007), DOC concentrations (Methe and Zehr 1999, Judd et al. 2006), nutrient concentrations (Bell et al. 1982, Pace and Funk 1991, Carlsson and Caron 2001), and primary productivity (Horner-Devine et al. 2003). The results from NMS ordination analyses indicated temperature, dissolved organic carbon (DOC), ammonium (NH_4^+), and nitrate (NO_3^-) were more highly correlated with differences in the genetic structure of bacterioplankton communities along the transect from the river through the harbor than the other environmental variables measured in this study. The structure of the bacterioplankton communities from the river is associated with high water temperature and DOC concentration while bacterioplankton communities in the harbor were related to the higher NH_4^+ and NO_3^- concentrations detected within the harbor (Table II.1, Figs. II.8 and II.9).

Surface water temperatures along the sample transect gradually decreased as the warm water of the St. Louis River mixed with the cool water of Lake Superior (Fig. II.2). The further extension of the warmer upper layer into the water column at the inner harbor site (DSH-IN) when compared to the other field sites may have been due to the additional heat load from the treated wastewater effluent discharged near the site. The average temperature for WLSSD wastewater effluent in September 2009 was 34.9ºC (Tuominen, personal comm.). Even with a warmer layer of water in the upper portions of the water column at all sites within the harbor and river, differences in the specific conductivity at various depths were minimal indicating sufficient mixing at the field sites. Lake water influence was more evident at the Duluth Entry (DSH-DE) and in the outer harbor (DSH-OT) where reduction in specific conductivity recorded at a depth of four to five meters coincided with the decrease in water temperatures. Schroeder and Collier (1966) describe a similar phenomenon at the mouth of the Cuyahoga River at Cleveland, Ohio where warmer river waters with higher conductivity flowed over cooler lake water with lower conductivity. Numerous studies report the broad effects of temperature on bacterial communities including effects on abundance, growth rates, and productivity in

addition to community structure and composition (Shiah and Ducklow 1994, Yannarell and Triplett 2004, Kan et al. 2006, Crump and Hobbie 2005).

The low DOC concentration in Lake Superior and the higher DOC concentrations measured in the St. Louis River (Table II.1) were similar to those previously reported for Lake Superior and some of its tributaries (Hicks et al. 2004, Minor and Stephens 2008). Changes in the composition or supply of DOM, for example during low flow conditions or storm events, can affect bacterial productivity, growth rates, and influence taxonomic shifts in bacterial communities (Judd et al. 2006, Cottrell and Kirchman 2000, Kritzberg et al. 2006, Jones et al. 2009, Fierer et al. 2007, Kirchman and Rich 1997).

The abundance of a number of the dominant bacterial taxa of bacterial communities in the harbor and river were strongly influenced by gradients in temperature and DOC. In the *Hae*III restriction enzyme digest, both the abundance and presence of one dominant fragment (TRF69) positively correlated with temperature and DOC and contributed to differences between the structure of the bacterial communities in the harbor and river (SIMPER). The TRF69 fragment was two times more abundant in the inner harbor (DSH-IN) than in either of the river sites and in very low abundance in the other harbor sites. The higher abundance of TRF69 in the inner harbor than in the river suggests that there was adequate residence time within the inner harbor for the TRF69 bacterial population to respond to the different conditions found in the harbor.

The concentration of NO_3^- was highest in Lake Superior and gradually declined through the Duluth-Superior Harbor and into the St. Louis River (Table II.1). Lake Superior is considered to be an oligotrophic lake (Biddanda et al. 2001) and is characterized by high nitrate and low phosphorus concentrations (Sterner 2011). Lake Superior may have been the source of elevated NO_3^- concentrations at the Duluth Entry (DSH-DE) and outer harbor (DSH-OT). During the summer months, increased levels of NO_3^-, at the lower portions of Lost Creek Wetlands (Wisconsin), was attributed to Lake Superior seiche activity (Morrice et al. 2004).

Ammonium (NH_4^+) and soluble reactive phosphorus (SRP) concentrations were higher within the harbor when compared with concentrations in the lake or the upper river (Table II.1). Concentrations of NH_4^+ and SRP at the lower river site (SLR-CI) may

have been influenced by the Pokegama River discharge. Johnston et al. (2001) detected higher NH_4^+ and SRP concentrations in fall surface water samples at the Pokegama Wetland, on the Pokegama River, than at the Fon du Lac Wetland, on the main stem of the St. Louis River. The concentration of NH_4^+ in the outer harbor (DSH-OT) was 30 times higher than the concentration detected at the upper-most river site (SLR-BSL). Although mechanisms that caused differences in bacterioplankton community structure between the river, harbor, and lake could not be determined, bacterioplankton abundance and growth rates are known to increase in response to elevated inorganic nutrient concentrations from other studies (Pace and Funk 1991, Carlsson and Caron 2001).

The abundance of the bacterial taxa whose presence or absence contributed the most to the dissimilarity between the harbor and river bacterioplankton communities, in each of the three restriction enzyme digests, positively correlated with NH_4^+ and was only detected in the harbor samples. The most dominant TRF in the T-RFLP profiles from the *Hae*III restriction enzyme digest positively correlated with NH_4^+ and was over two-fold more abundant in the harbor than in either the river or lake. It is unknown whether specific bacterial groups were responding to the high concentrations of NH_4^+ in the harbor, but Bell et al. (1982) found NH_4^+ to be the most influential physicochemical parameter measured in two Canadian rivers that correlated with bacterial diversity.

Sediment resuspension may have been a source of NH_4^+ and SRP to the water column in the Duluth-Superior Harbor. Sediments are resuspended in the harbor due to lake induced seiche oscillations, ship traffic, and the periodic maintenance of shipping channels by dredging (Stortz and Sydor 1980). Erdmann et al. (1994) found a 47-fold increase in suspended solids in the water column immediately following the passage of a ship. During re-suspension of sediments, NH_4^+ and SRP can be released to the water column (Simon 1989, Reddy et al. 1996). Once in the water column, NH_4^+ is desorbed and rapidly oxidized to NO_3^- through nitrification or assimilated by microorganisms. In a similar manner, following re-suspension of sediments, SRP is released into the water column where it is readily taken-up by microorganisms or re-adsorbs to particles (House et al. 1998, Lijklema 1980, Zhang and Huang 2007). Retention of nutrients in sediments

is a function of both residence time and biotic demand (Morrice et al. 2004, Brookshire et al. 2005).

A major natural source of NH_4^+ in aquatic sediments is from microbial degradation of organic matter (Blackburn and Henriksen 1983). Another source is the reduction of NO_3^- to NH_4^+ or dissimilatory nitrate reduction to ammonium (DNRA) that may be significant in some aquatic systems (Gardner et al. 2006). Ammonium (NH_4^+) readily adsorbs to particles and is the dominant nitrogen form in anoxic sediments (Forsberg and Ryding 1979). In a river system, only small amounts of phosphorus are from natural sources such as riparian vegetation (Meyer and Likens, 1979), the weathering of soil parent material, and the atmosphere (Holton et al. 1988). Anthropogenic sources such as agriculture and urban surface run-off, and other point sources often contribute a greater proportion of the phosphorus-load to an aquatic system (Withers and Jarvie 2008). Treated wastewater effluent is considered to be a major anthropogenic source of nutrients to aquatic systems (USGS 2006, Millier and Hooda 2011). The WLSSD treated wastewater effluent is discharged into the inner harbor in close proximity to the inner harbor site (DSH-IN) and just upstream from the outer harbor site (DSH-OT).

The turbidity levels were highest at the upper-most river site (SLR-BSL) and at the inner harbor site (DSH-IN). During a two-year study, TenEyck and Branstrator (unpublished data) detected higher turbidity levels in the inner and outer portions of the Duluth-Superior Harbor compared to turbidity levels in the St. Louis River. The St. Louis River and its tributaries are a major source of suspended solids including eroded soils (e.g. red clay), particulate organic matter, and re-suspended sediments most of which is deposited within the harbor itself (Bahnick and Markee 1985). Other sources of suspended solids into the harbor include treated wastewater effluent, storm-water runoff, and sediments released when ballast water is discharged (Klarer and Millie 1994, Villac and Kaczmarska 2011).

In addition to releasing nutrients, resuspended sediments can release bacteria (Pettibone 1996). Pettibone et al. (1996) reported a ten-fold increase in bacteria cell counts in the water column following the passage of a 195.7-m bulk cargo vessel in the

Buffalo River (New York). Suspended particulate matter can provide surface areas for colonization by bacteria, and particle-associated bacterial populations can be fundamentally different from communities of free-living bacterioplankton (Crump et al. 1999, DeLong 1993).

Methodological considerations

There are a number of pitfalls and biases associated with molecular profiling techniques based on PCR amplification that should be mentioned at this point. Preferential cell disruption (Wintzingerode et al. 1997) and variable results between different DNA extraction procedures (Luna et al. 2006) can affect the original DNA template used in PCR methods. Biases associated with the PCR procedures include PCR inhibition due to organic matter, clay particles, and humic acids (Kirk et al. 2004, Tebbe and Vahjen 1993), primer hybridization efficiency and specificity (Brunk et al. 1996, Rainey et al. 1994), preferential amplification (Reysenbach et al. 1992), random events occurring in early PCR cycles (e.g. PCR drift; Polz and Cavanaugh 1998), amplification of contaminant DNA (Wintzingerode et al. 1997), and formation of chimeric DNA due to competition for primer or as a result of DNA damage during rigorous cell lysis (Pääbo et al. 1990, Wintzingerode et al. 1997). In the T-RFLP method, erroneous results can also occur due to incomplete or non-specific restriction (Marsh 2000). Other factors affecting T-RFLP analyses include effects on electrophoretic mobility by different dye labels, the purine content of PCR amplicons, and interference of shorter fragments by larger fragments in capillary electrophoresis (Kaplan and Kitts 2003, Schütte 2008, Danovaro et al. 2006). Despite these pitfalls and biases, the size and fluorescence signal of individual TRFs are highly reproducible making T-RFLP a widely used, reliable and robust tool for rapid comparisons of microbial communities (Osborn et al. 2000, Blackwood et al. 2003, Schütte 2008). The similar results obtained for replicates in this study supports these findings.

Conclusion

Three distinct bacterioplankton communities representative of Lake Superior, the Duluth-Superior Harbor, and the St. Louis River habitats were detected in water samples collected on one day in early September 2009. The genetic structure of the bacterioplankton community did not transition gradually from the St. Louis River, through the Duluth-Superior Harbor, and into Lake Superior as it might be expected to if the harbor communities were merely mixtures of bacterioplankton from the river and lake. Differences in the abundance of bacterial groups played a prominent role in the dissimilarity between the harbor and the river bacterioplankton communities. Although the exact mechanism(s) responsible for the differences detected in the genetic structure of the bacterioplankton communities in the Duluth-Superior Harbor and the St. Louis River could not be determined with the data collected in this study, it is clear that the structure of these communities was related to differences in the chemical and physical parameters found in these habitats. It is important to realize that a small number of sites were sampled on one late summer day in this study, and so it does not constitute a thorough analysis of bacterioplankton communities in Lake Superior, the Duluth-Superior Harbor, or the St. Louis River. However, the results from this study were consistent with findings of other investigations that distinct bacterioplankton communities can be found in estuarine mixing zones.

CHAPTER III

Comparison of Bacterioplankton Communities in the Ballast Water of Commercial Ships, Treated Wastewater Effluent, and the Duluth-Superior Harbor

INTRODUCTION

The spread and establishment of aquatic nonindigenous species (NIS) via ballast water transport is of concern worldwide (IMO 2008). Ballast water is taken on-board ships for the purpose of stability during a voyage and discharged in port when loading cargo and possibly en route to reduce a vessels draft in shallow waters (Carlton et al. 1995, Grigorovich et al. 2003). In addition, commercial ships exchange ballast water taken up in coastal waters with mid-ocean water before entering the Great Lakes-St. Lawrence Seaway to fulfill ballast water management requirements designed to mitigate the spread of aquatic NIS (IMO 2004; Federal Register 1999). An estimated 3 to 5 billion metric tons (Mt) of ballast water is transported globally each year (IMO 2008). The concern for the transport of potentially invasive microbes in ballast water is due to their high abundance in aquatic habitats, their potential for pathogenicity and toxicity, and the ability of some bacteria to enter a reversible dormant state in stress environments (Ruiz et al. 2000, Drake et al. 2007, Dolan 2005). Dormant pathogens may be resistant to antibiotics since antimicrobial agents usually target actively growing cells (Jones and Lennon 2010). High growth rates, efficient resource utilization, and small size facilitating dispersal are additional traits that can contribute to the survival and establishment of microorganisms in a new environment (Litchman 2010, Ruiz et al. 2000).

An important factor determining the successful establishment of an invasive species is propagule pressure, that is, the abundance of non-native organisms introduced to a new environment and the frequency of introduction events (Lockwood et al. 2005, Wonham et al. 2000). Microbial abundance in ballast water can be used as a proxy for propagule pressure while ballast water discharge volume and shipping traffic patterns are used to estimate the frequency of inoculation (Drake et al. 2007). In addition to the

abundance of organisms being introduced, invasion pressure includes the diversity and species richness of organisms discharged in ballast water (Smith et al. 1999, Jousset et al. 2011).

The majority of the studies on prokaryotic organisms in ballast water have focused on abundance. Burkholder et al. (2007) found bacterial abundance was unrelated to vessel type, exchange status, or age of water. A number of studies also reported no differences in bacterial abundance between exchanged and un-exchanged ballast water at the end of a voyage though some studies detected changes in bacterial abundance during the voyage (Drake et al. 2007, Drake et al. 2002, Seiden et al. 2010). Tomaru et al. (2010) detected a significant decrease in bacterial cell abundance following mid-ocean exchange (MOE). Bacterial cell abundance has also been compared among habitats within the ballast tank, i.e., ballast water, residual water and sediments, and biofilms on internal structures of ballast tanks (Drake et al. 2007). Other studies have estimated the abundance of specific target organisms such as enteric bacteria (i.e., *E. coli*, *V. cholerae*, enterococci; Ruiz et al. 2000, Aquirre-Macedo et al. 2008, Joachimsthal et al. 2004, Ivanov 2006, Burkholder et al. 2007), human pathogenic bacteria (i.e., *Listeria monocytogenes*, *Mycobacterium* spp., *Pseudomonas aeruginosa*; Burkholder et al. 2007), toxic *Cyanobacteria* (Doblin et al. 2002), or bacteria associated with coral disease (i.e., *Serratia marcescens*, and *Sphingomona* spp.; Aquirre-Macedo et al. 2008).

Only a few studies have explored the structure and composition of bacterial communities in ballast water and compared these communities to those in the waters of recipient ports. For example, Tomaru et al. (2010) used denaturing gradient gel electrophoresis (DGGE) to detect changes in bacterial community structure before and after mid-ocean exchange and Ma et al. (2009) used restriction fragment length polymorphism (RFLP) and 16S rDNA sequencing to compare 65 clones developed from bacterioplankton communities in ballast water collected from one ship and bacterial communities in local seawater.

The international port in the Duluth-Superior Harbor receives more ballast water discharge from commercial ships than any other port in the Great Lakes (U.S. EPA 2008) and is considered to be in one of four invasion ‘hotspots’ within the Laurentian Great

Lakes (Grigorovich et al. 2003). Bacterial populations from treated wastewater are also discharged into the harbor by the Western Lake Superior Sanitary District (WLSSD) tertiary treatment plant at a rate of approximately 40 million gallons a day (MGD). Bacteria introduced with wastewater effluent can have a significant influence on the bacterial community structure in the receiving waters (Wakelin et al. 2008).

The overall goal of this study was to compare the genetic structure of bacterioplankton communities from ballast water and treated wastewater with natural communities in the Duluth-Superior Harbor. First, the community structure of bacterioplankton collected from these water sources was compared using terminal restriction fragment length polymorphism (T-RFLP) analysis, a genetic fingerprinting technique. Then, four 16S rRNA gene clone libraries were created for three ballast water samples and one harbor water sample to compare the composition of the bacterioplankton communities. Finally, the DNA sequences from the four clone libraries were examined to identify DNA sequences associated with known human or animal pathogens.

METHODS

Study location

The Duluth-Superior Harbor (DSH) is located on the western arm of Lake Superior and is a major industrial shipping port with an estimated 27 km of maintained shipping channels. The hydrology of the DSH is affected by the inflow of two rivers, two outflow channels, Lake Superior seiche oscillations, and a counter-clockwise current that flows along the Lake Superior shore. The St. Louis River (mean annual discharge of 56 $m^3 sec^{-1}$; Mitton et al., 1994) flows through the St. Louis River estuary, enters the harbor at St. Louis Bay, and then discharges into Lake Superior primarily through the Duluth shipping channel (Duluth Entry). The Nemadji River (mean annual discharge of 11 $m^3 sec^{-1}$; MPCA /WDNR, 1992) enters the harbor at the eastern end of Superior Bay adjacent to Allouez Bay and then discharges into Lake Superior primarily through the Superior shipping channel (Superior Entry). Lake Superior seiche amplitudes entering the DSH typically range from 3.0 to 15 cm with fundamental and minor periodic

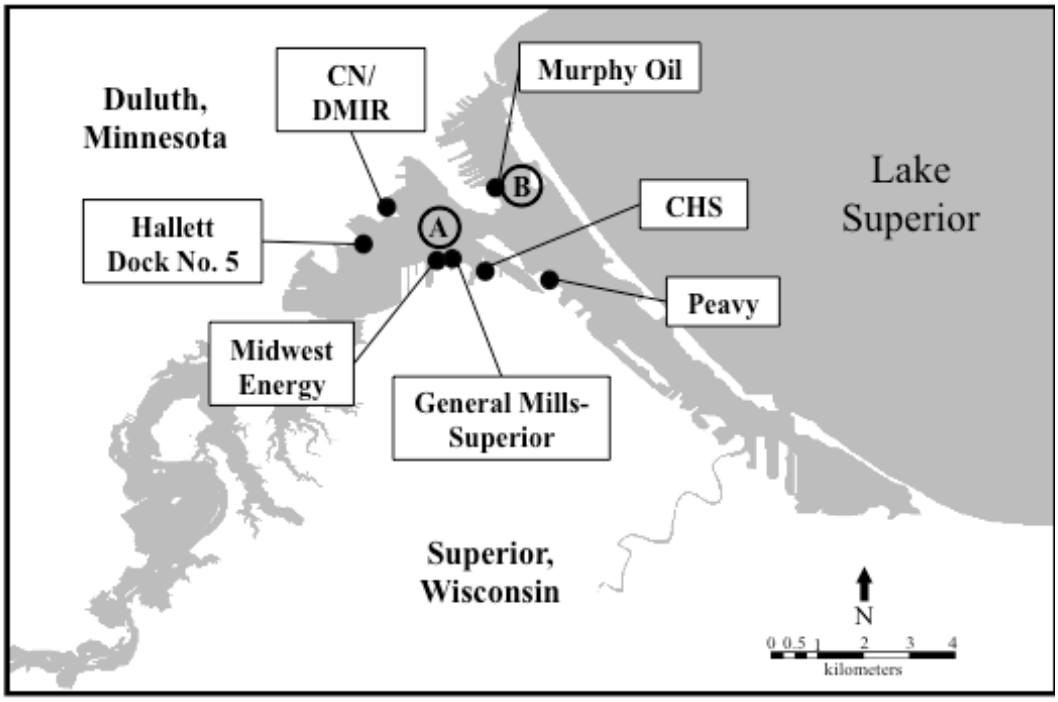
oscillations every 7.9 and 2.1 hours, respectively (Sydor and Stortz, 1980, Jordan et al. 1981).

Water samples for bacterioplankton community analyses

Between July 2009 and November 2009, ballast water samples were collected from ten commercial vessels at seven different dock locations in the DSH in cooperation with the Minnesota Pollution Control Agency (MPCA) during routine activities including loading cargo, unloading cargo, deballasting or refueling (Fig. III.1). Ballast water was collected from a ballast tank of six transoceanic vessels and four lake freighters (Table III.1). Only two seawater ballast samples were collected from the transoceanic vessels. These vessels had each undergone mid-ocean exchange (MOE) of their ballast water approximately two weeks before entering the DSH. Ballast tanks that were sampled were chosen based on their proximity to the dock where the ship was berthed, depth of ballast water within the tank, ease of access to the tank, and the origin of the ballast water. Three replicate water samples were collected in pre-rinsed, 20-L plastic carboys from the ballast tank by using a length of 0.5" polyethylene siphon tubing lowered through a sounding tube located on the deck of the ship. Ballast water was collected from Ship 6 (FWB6) with a ballast pump during active ballast water discharge because of the low volume of water in the ballast tank. Harbor surface water adjacent to the dock (dockside) was collected using a pre-rinsed 3-gallon plastic container either before or immediately after ballast water was sampled. On October 30, 2009, three replicate treated wastewater effluent samples were collected in 20-L plastic carboys at the Western Lake Superior Sanitary District wastewater treatment plant (Duluth, MN) from an outgoing pipe spigot.

Water was collected from two regions, in the DSH, near dock areas where ballast water is routinely discharged by commercial ships. On September 3, 2009, three replicate near-surface water samples were collected in 20-L plastic carboys in the outer harbor (DSH-OT, 46°45.64' N; 92°05.59' W) at the eastern end of Superior Bay near the Murphy Oil Marine Terminal Berth 3 and in the inner harbor (DSH-IN, 46°44.69' N; 92°06.96' W) in St. Louis Bay near Superior Midwest Energy Terminal (St. Louis Bay; Fig. III.1). All carboys were rinsed with harbor water prior to collecting samples. No

Figure III.1. Map of Duluth-Superior Harbor. Letters indicate locations of harbor sample sites. Encircled 'A 'indicates the inner harbor site (DSH-IN) and encircled 'B' indicates the outer harbor site (DSH-OT). Black circles indicate docks where ballast water samples were collected. *Murphy Oil*, Terminal Berth 3-Murphy Oil Marine Terminal; *CHS*, CHS No. 1; *Peavey*, Peavey Conner's Point Elevator; *General Mills*-Superior, General Mills Superior Elevators S & X; *Midwest Energy*, Midwest Energy Resources Co.-Superior Midwest Energy Terminal; *CN/DMIR*, CN Dock 6.


Duluth,
Minnesota
CN/
DMIR
Murphy Oil
Lake
Superior
B
CHS
A
Hallett
Dock No. 5
Peavy
Midwest
Energy
General Mills-
Superior
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N
0 0.5 1 2 3 4
kilometers

Table III.1. Transit information and ballast water data from commercial vessels that were sampled in the Duluth-Superior Harbor from July to November 2009.

Ballast water sample	Date (mm/yy)	Ship type [a]	Last port of call	Ballast water origin [b]	Water type	Age of ballast water (days) [c]	Prokaryotic cells - ballast water (10^9 cells L^{-1})	Prokaryotic cells - dockside (10^9 cells L^{-1})
1	Jul-09	TO-GC	Chicago, Illinois; USA	Cleveland Harbor, Ohio; USA	FW	9	ND	2.27
2	Aug-09	LF-BC	Gary Harbor, Indiana; USA	Gary Harbor, Indiana; USA	FW	3	1.01	1.72
3	Aug-09	TO-GC	Burns Harbor, Indiana; USA	Burns Harbor, Indiana; USA	FW	4	1.00	2.60
4	Aug-09	LF-BC	Thunder Bay, Ontario; Canada	Thunder Bay, Ontario; Canada	FW	1	1.68	2.42
5	Aug-09	LF-BC	Hamilton, Ontario; Canada	Hamilton, Ontario; Canada	FW	4	1.88	2.68
6 [d]	Aug-09	LF-BC	St. Clair, Michigan; USA	St. Clair, Michigan; USA	FW	3	1.04	2.54
7	Oct-09	TO-GC	Aratu, Brazil	Southwest Atlantic Ocean (23°56' N; 47°33' W)	SW	13	0.27	1.67
8	Oct-09	TO-BC	Milwaukee Harbor, Wisconsin, USA	Milwaukee Harbor, Wisconsin; USA	FW	3	1.50	1.34
9	Nov-09	TO-BC	Bilbao, Spain	Northeast Atlantic Ocean (47°46' N; 30°06' W)	SW	16	0.79	1.95
10	Nov-09	TO-GC	Montreal, Canada	Hudson Bay, Canada (63°19' N; 90°38' W)	BrW	59	0.47	0.93

Abbreviations: FWB, freshwater ballast; SWB, seawater ballast; BrWB, Brackish water ballast; ND, not determined

[a] TO-GC, transoceanic-general cargo; TO-BC, transoceanic-bulk carrier; LF-GC, lake freighter-general cargo

[b] ocean regions classified by United Nation's Food and Agriculture Organization (FAO) as described in Smith et al. 1999

[c] age of ballast water calculated as time difference between ballast water uptake and date of ballast water sample collection

[d] collected from ballast pump during active ballast water discharge

rain occurred within 48 hours prior to sampling. All ballast water, treated wastewater, and harbor water samples were transported to the lab within hours of collection, stored at 4°C and processed the same day.

Prokaryotic cell abundance

A portion of each water sample was preserved with 37% formaldehyde (pre-filtered through 0.22 μm pore filter; 2% final concentration) and stored in the dark at 4°C for up to 2 weeks. A small portion of each preserved sample was placed on a black polycarbonate filter (25 mm dia., 0.22 μm pore; GE Water & Process Technologies, Trevose, PA). The DNA in cells was stained with 4'6-diamidino-2-phenylindole (DAPI; 10 μM final concentration), filtered onto the membrane filter, and prokaryotic cells (i.e., cells with no visible nucleus) were counted using a Nikon Eclipse 80i epifluorescence microscope (Porter and Feig 1980).

Bacterioplankton community analyses

Total DNA extraction – Microbial cells in water samples were collected onto Duropore® membrane filters (142 mm dia., 0.22 μm pore; Millipore, Billerica, MA) by N_2 gas pressure filtration at <40 psi until the filters clogged (i.e., filtrate flow was substantially reduced). Individual membrane filters were folded and stored in Whirl-Pak® bags (Nasco, Fort Atkinson, WI) at -80°C. Frozen filters were crushed in the Whirl-Pak® bags before a one-eighth portion (by weight) was removed for DNA extraction. A MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) was used for all DNA extractions. Extracted DNA was stored in sterile elution buffer (10 mM Tris) at -80°C until used for molecular analyses.

Polymerase chain reaction (PCR) – The bacterial 16S rRNA gene was amplified from 40 ng of extracted DNA as a template with illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ) using the bacterial specific primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'; Lane 1991), and the universal primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'; Lane 1991; Integrated DNA Technologies, Coralville, IA). The 27F primer was 5' end labeled with 6-carboxyfluorescein (6-FAM).

When reconstituted to a 25 µl volume, each PCR bead tube contained ~ 2.5 units of puReTaq DNA polymerase, 200 µM each dNTP in 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. PCR bead tubes with DNA templates and 1.5 µl of 10 µM solutions of each primer were placed in a preheated (94°C) thermal cycler block (DNA Engine® Peltier Thermal Cyclers; MJ Research). The amplification protocol included an initial denaturing step at 94°C for 3 min followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 90s. After a final extension step at 72°C for 10 min, the PCR bead tubes were stored at 4°C. PCR products of the expected fragment size (~1465bp), as determined by agarose gel electrophoresis, were purified using an UltraClean™ PCR Clean-up™ Kit (MoBio Laboratories, Inc., Carlsbad, CA). Purified PCR products were eluded in nuclease-free water, stored at 4°C, and used within 24 hours.

Terminal restriction fragment length polymorphism (T-RFLP) analysis – Differences in the overall structure of bacterioplankton communities in all ballast water, treated wastewater, and harbor water samples were evaluated using T-RFLP analysis. Purified PCR products were digested at 37ºC for 3 hours using restriction enzymes *Hae*III, *Msp*I, and *Rsa*I (Promega, Fitchburg, WI). Digestion was terminated by incubation at 65ºC for 15 minutes. Digested PCR products were precipitated in ethanol, dried, and resuspended in nuclease-free water. Fluorescently labeled and purified PCR products were digested in separate reactions using *HaeIII*, *MspI*, and *Rsa*I restriction enzymes (Promega, Fitchburg, WI). The fluorescent terminal-restriction fragments (TRFs) of digested PCR products were separated by size on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA; BioMedical Genomics Center, University of Minnesota). Sample electropherograms from GeneMapper® software were directly imported into the BioNumerics® software package (Applied-Maths, Belgium) and converted to densitometric curves. The BioNumerics® software estimated the TRF sizes in base pairs (bp) by comparison to the MapMarker® 1000 molecular weight standard (i.e., 23 DNA fragments ranging from 50 bp to 1000 bp in size) using a cubic spline fit algorithm. TRFs with estimated sizes outside of the range of the molecular weight standard were excluded from further analysis. Individual peaks that were greater than 1%

relative of the maximum fluorescence intensity of each lane were defined as peaks (bands). In some samples, this percentage was adjusted upwards to compensate for higher noise levels, but profiles containing a high level of noise (> 5 % of the maximum fluorescent intensity) were removed from further analysis. For each restriction enzyme, TRF peaks in all T-RFLP profiles were aligned and placed into bin classes using a 1% position tolerance and 1% optimization. Assignments were visually inspected to detect erroneous assignments.

Statistical analyses of bacterioplankton T-RFLP profiles

Cluster analysis – Natural groupings among bacterioplankton communities from different sources were illustrated using hierarchical clustering. For each restriction enzyme digest, densitometric curves of each T-RFLP profile were compared using similarity-based cluster analysis within BioNumerics®. Pairwise similarity matrices were calculated using Pearson correlation coefficients and dendrograms were constructed using the un-weighted pair group method using arithmetic averages (UPGMA) clustering algorithm. The uncertainty of dendrogram branches was estimated using Cophenetic correlation comparing the matrix similarities and the dendrogram-derived similarities.

NMS ordination – Nonmetric multidimensional scaling (NMS) was performed using PC-ORD Version 4.0 (McCune and Mefford 1999) with the Sorensen distance measure to explore relationships between freshwater ballast samples and harbor water samples (dockside and pelagic). TRF peak heights were imported into Microsoft Excel and fragments with peak heights < 50 relative fluorescence units were removed before further analysis (Blackwood et al. 2003). T-RFLP peak profiles were standardized by dividing each peak height, by the total sum of all peak heights for each sample since the concentration of DNA injected into each lane in capillary electrophoresis can differ between samples and even replicates (Liu et al. 1997, Dunbar et al. 2001, Osborn et al. 2000). NMS is a non-parametric multivariate analysis method that uses an iterative algorithm to extract the strongest patterns of community structure from a dataset and summarizes these patterns on a minimum number of ordination axes (Mather 1976, Kruskal 1964[b], McCune and Grace 2002). Ordination plots with a final stress of 5-10

were considered good ordinations with minimal risk of false representation of redundancy patterns (Kruskal's Stress multiplied by 100; McCune and Grace 2002, Kruskal 1964[a], Clarke 1993), although a final stress of 10-20 may provide a usable interpretation at the lower end (Clarke 1993). A Monte Carlo test was run 250 times on randomized data during each run to determine if the stress values were stronger than expected by chance. In this study, the autopilot 'slow and steady' option was used to determine recommended number of axes.

One-way analysis of similarity (ANOSIM) – The statistical difference between groups in ordination plots was tested using ANOSIM with 999 permutations (Clarke 1993, Rees et al. 2004). In this analysis, an R statistic is computed (0 to 1). R-values > 0.75 indicate groups are well separated, R-values > 0.5 indicate separated groups with some overlap, and R-values < 0.25 indicate groups that are minimally separated (Ramette 2007). ANOSIM was performed on T-RFLP profiles consisting of relative peak heights of TRFs in PAST Version 2.15 software (Hammer et al. 2001) using a Sorensen distance measure.

Construction of bacterioplankton 16S rRNA gene clone libraries

Three ballast water samples and one harbor water sample were selected to construct 16S rRNA gene clone libraries to identify the types of bacteria present in these samples. The inner harbor sample (DSH-IN) was chosen because it was taken from a part of the DSH where the most ballast water is discharged by commercial ships. Seawater ballast from Ship 9 was chosen based on quality of T-RFLP profiles as compared to the sample from Ship 7. The two freshwater ballast samples were selected from the larger set of ballast samples based on whether they came from ports that contribute a large volume of ballast water to the Duluth-Superior Harbor and from locations associated with current or predicted future NIS invasions (Grigorovich et al. 2003, U.S. EPA 2008). Information on ballast activity was obtained for commercial ships arriving in the DSH from the National Ballast Information Clearinghouse (NBIC; http://invasions.si.edu/nbic/) database, which includes arrival port, arrival date, last port, ballast water source, and discharge volume. Patterns of ballast water discharge were

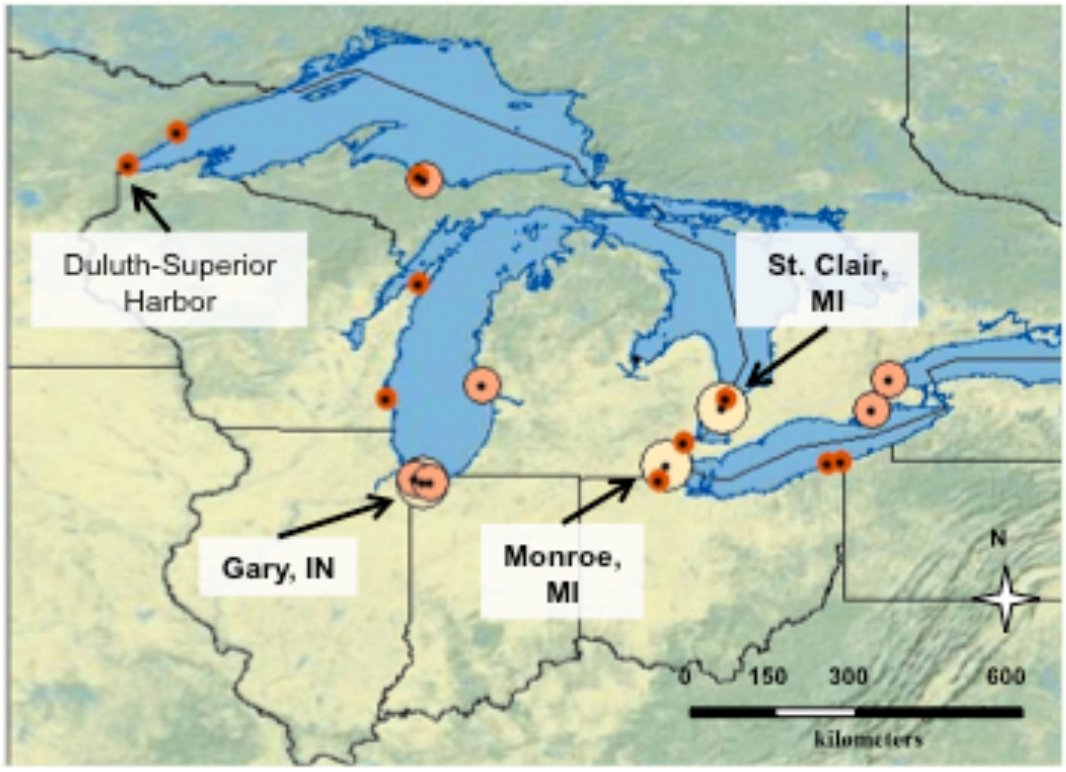
analyzed using Microsoft Excel software and ArcGIS version 9.1 (ESRI, Relands California).

Information from ballast water management (BWM) logs submitted to NCIB for arrival to Duluth, MN and Superior, WI were downloaded and combined in Microsoft Excel. Entries from ocean-going vessels (approximately 49,000 Mt), from unknown sources (approximately 300,000 Mt), and from vessels that did not discharge into the harbor were removed from the dataset. The 490 remaining entries including 74 different vessels and 64 different ballast water source harbors and were sorted by greatest to least discharge volume. The top 20 source ports constituted nearly 90% of the total discharge (Fig. III.2; Appendix Fig. A-1). The top three sites each contributed over 1 million metric tons of ballast water including St. Clair, MI (25.46%), Gary, IN (9.38%), and Monroe, MI (8.65%). A total of 15.1 million metric tons (MMt; 3.7 billion gallons) were discharged into the Duluth-Superior Harbor in 2009.

One freshwater ballast sample chosen for creating a clone library was from Ship 6 (Table III.1). Ballast water from this ship originated from St. Clair, MI located in the Lake Huron-Lake Erie corridor, an aquatic invasion 'hotspot' (Grigorovich et al. 2003). The second freshwater ballast sample was chosen from a transoceanic ship (Ship 3; Table III.1) that had taken up freshwater ballast in Burns Harbor in Lake Michigan. Burns Harbor was the fourth largest contributor of ballast water discharged into the Duluth-Superior Harbor (0.97 MMt) during the 2009-shipping season and is located within 15 miles of Gary, IN. Gary Harbor is the second most high-risk port for future invasions of nonindigenous species (NIS) as determined by the EPA (2008).

Four 16S rRNA gene clone libraries were constructed for the DSH-IN harbor water sample and the three ballast water samples (FWB3, FWB6, SWB9). The same PCR procedure used for T-RFLP was followed but a non-labeled 27F forward primer was used. For each clone library, amplicons from duplicate PCR reactions were pooled to minimize PCR bias (Blackwood et al. 2003). The combined amplicons were cloned using a TA Cloning® Kit with One Shot® INVαF' Chemically Competent *E. coli* (Invitrogen™). *Escherichia coli* cells containing clonal inserts were spread on Luria-Bertani (LB) agar containing 100 μg ml^{-1} ampicillin and grown overnight at 37ºC.

Figure III.2. Volume of ballast water discharged into the Duluth-Superior Harbor by ships arriving from various ports in the Laurentian Great Lakes during the 2009-shipping season. Black circles depict source port locations. The diameter of the circles and the type of color fill indicate the millions of metric tons (MMt) of ballast water discharged from each source port. The three largest contributors to the total ballast water discharged during 2009 (15.1 MMt) into the Duluth-Superior Harbor were St. Clair, MI (3.75 MMt), Gary, IN (1.38 MMt), and Monroe, MI (1.27 MMt).


Duluth-Superior Harbor
St. Clair, MI
Gary, IN
Monroe, MI
N
0
150
300
600
kilometers
0.1 – 0.5
0.5 -1
1 - 4
Ballast water (MMt)

Randomly selected clones were picked with sterile toothpicks and grown overnight at 37°C in LB broth containing 100 μg ml^{-1} ampicillin in 96-well microplates. Sterile glycerol was added (final concentration 10 %) to each well and the microplates were stored at -80°C.

For DNA sequence analyses, clones were grown overnight in LB broth containing 100 μg ml^{-1} ampicillin at 37°C. Plasmids were isolated using an UltraClean® Standard Mini Plasmid Prep Kit (MoBio Laboratories, Inc) and sequenced on an ABI Prism 3730xl DNA Analyzer (Applied Biosystems) at the University of Minnesota BioMedical Genomics Center. The 16S rRNA gene sequences from clonal inserts were edited and evaluated for quality using 4Peaks version 1.7.2 software (4Peaks by A. Griekspoor and T. Groothuis, mekentosj.com). The sequences were evaluated with VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) to identify possible vector-contaminated clones. Quality sequences of approximately 500 base pairs (bp) in length were retained for further analysis. Each of these high quality sequences was assigned to phylogenetic groups based on nearest-neighbor sequences identified in GenBank (> 90% identity) using the BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Analyses of bacterioplankton clone libraries

The LIBSHUFF computer program (http://libshuff.mib.uga.edu) was used to determine if the clone libraries were derived from statistically different bacterioplankton communities. Similarity percentages between selected sequences were calculated by creating a similarity matrix in *dnadist* in PHYLIP (http://hpc.icrisat.cgiar.org/Pise/5.a/phylogeny/dnadist.html). Sequences in each clone library were grouped into operational taxonomic units (OTUs) at the ≧ 97% sequence similarity level by using the web-based bioinformatics tool FastGroupII (Yu et al. 2006; http://biome.sdsu.edu/fastgroup/fg_tools.htm). Rarefaction analyses were performed in FastGroupII to compare the richness among samples that have been un-equally sampled. However, rarefaction curve analysis does not reflect richness or evenness of the original microbial communities (Hughes et al. 2001) because it does not correct for unseen taxa

(Kan et al. 2007). Therefore, the richness of the original community was estimated using the nonparametric richness estimator, Chao1 (Chao 1984). Chao1 is useful for datasets with a disproportionate number of low-abundance classes, but can underestimate richness if coverage is low (Chao 1984, Hughes et al. 2001). The percent coverage was calculated to determine the completeness of the each clone library (Good 1953).

Two indices were used to measure diversity, the Shannon-Wiener index and Simpson's index. The Shannon-Wiener index (H') correlates with species richness and evenness and is more heavily weighted to rare species (Shannon and Weaver 1949, Wiener 1948, Krebs 1989, Hill et al. 2003). Simpson's index gives the probability that two randomly chosen individuals would belong to the same species and is more heavily weighted to dominant species. Simpson's index values range from 0 to 1, with the greater value representing no diversity, so the Simpson's Reciprocal index was used here for ease of interpretation. The Simpson's index is not as sensitive to low coverage as the Shannon-Wiener index (Hill et al. 2003). Calculations for Chao1, coverage, and the diversity indices were performed using SPADE (Species Prediction and Diversity Estimation; Chao and Shen 2005).

Phylogenetic analysis – The partial 16S rRNA gene sequences within each clone library were aligned using the multiple sequence alignment software program, CLUSTALW implemented in MEGA version 4.0 (Tamura et al. 2007). Aligned sequences were evaluated in MALLARD (http://www.bioinformatics-toolkit.org/Mallard/index.html) to check for chimeric or other anomalous sequences. Phylogenetic groups of clone sequences, their nearest neighbors defined by BLAST, and a collection of taxonomically related sequences from the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) were aligned using CLUSTALW. Consensus phylogenetic trees were constructed from 1,000 bootstrap replicate trees with MEGA using the neighbor-joining algorithm and pairwise distance estimates calculated using the Kimura 2 parameter evolutionary model.

RESULTS

Prokaryotic cell abundance

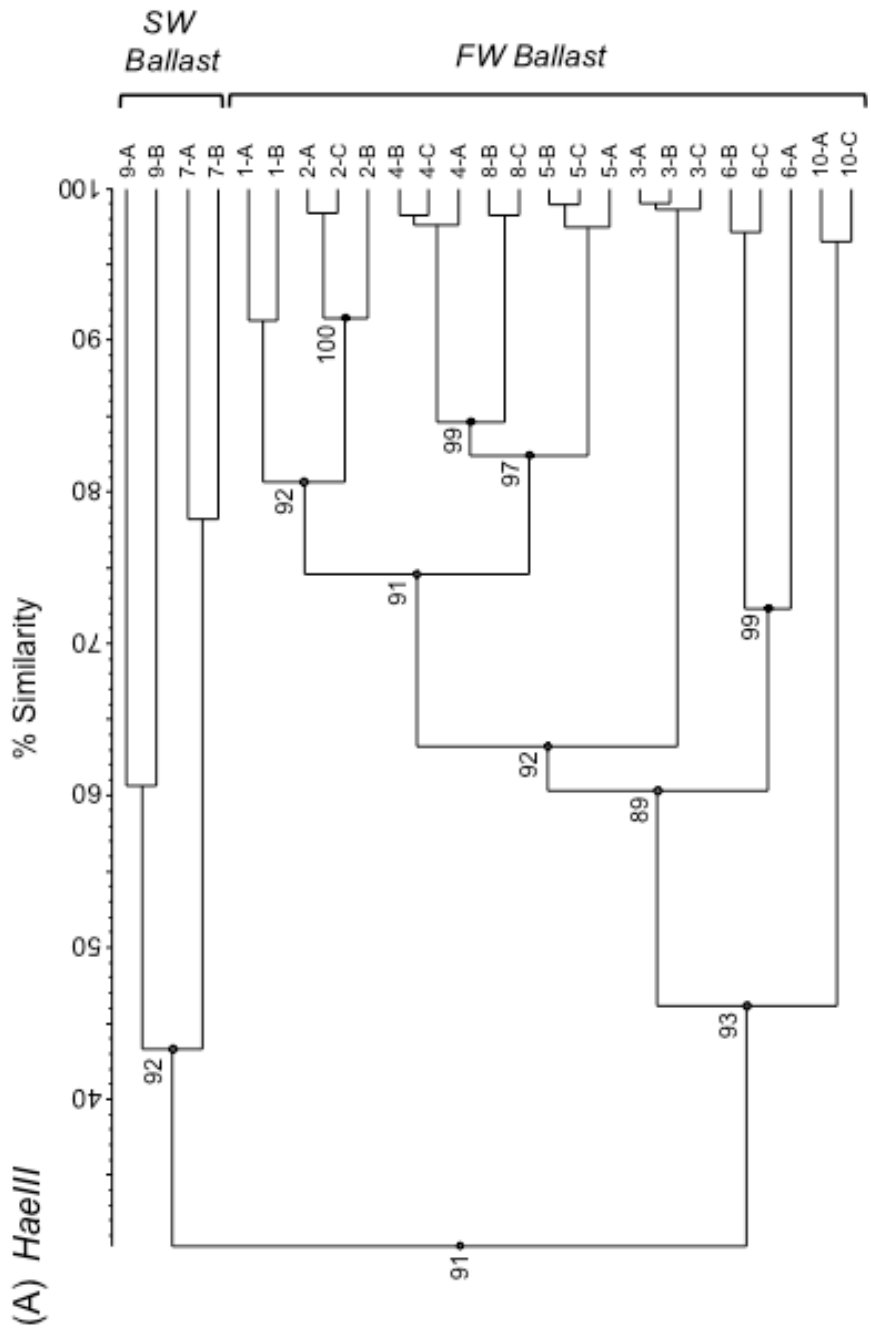
The abundance of prokaryotic cells in ballast water ranged from 0.27 to 1.88 x 10^9 cells L^{-1} and from 0.93 to 2.68 x 10^9 cells L^{-1} in dockside water samples (Table III.1). Prokaryotic cell abundance was higher in freshwater ballast water than in seawater and brackish water ballast samples (Student's *t* test; $p < 0.05$). Prokaryotic cells were less abundant in ballast water than in DSH water adjacent to the dock when all ballast and dockside water samples were considered (Student's *t* test; $p < 0.05$). Prokaryotic cell abundances were similar in the two field sites sampled in the inner (DSH-IN; 2.56 x 10^9 cells L^{-1}) and outer (DSH-OT; 2.44 x 10^9 cells L^{-1}) harbor of the DSH. Prokaryotic cells could not be counted in WLSSD wastewater effluent because of the very high amount of particulate material in these samples.

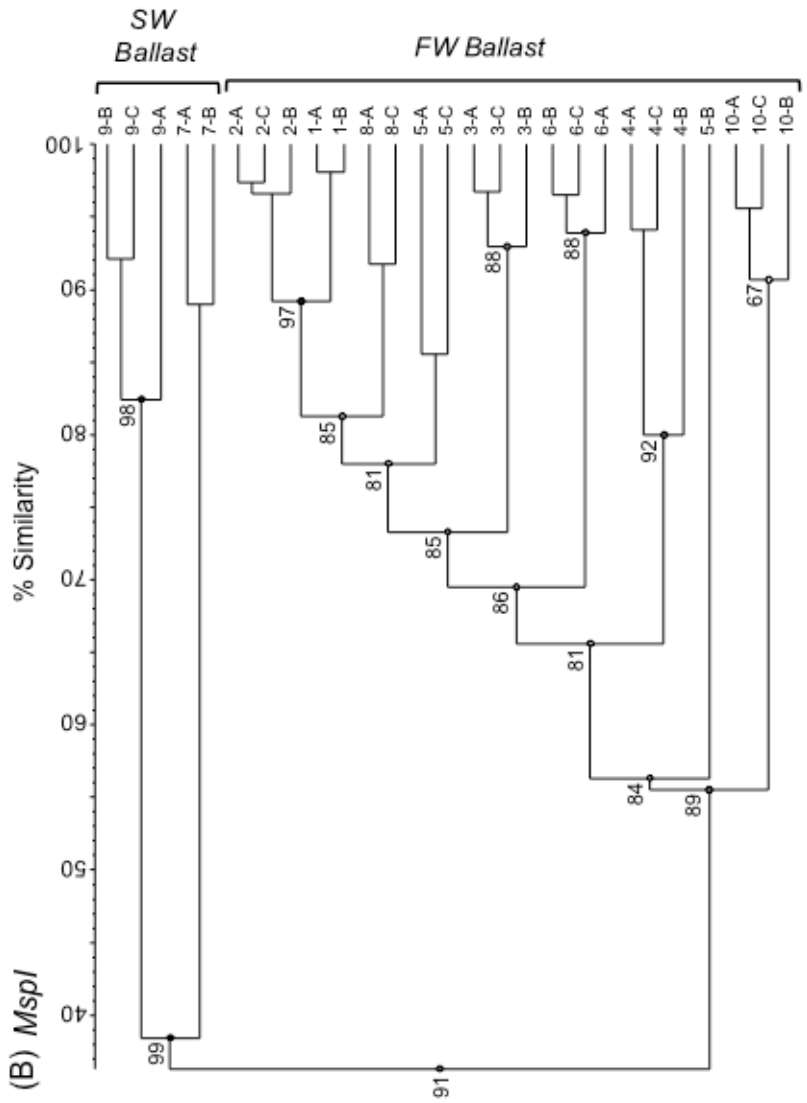
T-RFLP profiles of bacterioplankton communities

The T-RFLP profiles of bacterioplankton communities in freshwater ballast were well separated from seawater ballast communities when densitometric curves were used to construct T-RFLP profiles of these communities, regardless of the enzyme used to digest the PCR fragments (Fig. III.3). Two well-separated clusters were also observed in peak-based analyses (ANOSIM: $R = 0.924$ to 0.988, $p < 0.01$). The bacterioplankton community T-RFLP profiles of the two seawater ballast samples were less than 35% similar to those of the other ballast water samples. The community profiles showed a high degree of reproducibility because replicates of samples often formed clusters of > 90% similarity.

The bacterioplankton communities in freshwater ballast and harbor water depicted in NMS ordination plots were minimally separated ($R = 0.288$-0.499, $p < 0.001$; Fig. III.4). Bacterioplankton communities from the inner (DSH-IN) and outer (DSH-OT) harbor always grouped with bacterioplankton communities in the dockside samples when any of the three restriction enzymes was used to construct T-RFLP profiles. Final stress values ranged from 9.17 to 11.41, indicating that the ordination axes were stronger than would be expected by chance (Monte Carlo Stress test, 250 permutations, $p < 0.01$).

Figure III.3. UPGMA dendrograms showing hierarchical clustering of T-RFLP profiles of bacterioplankton communities in ballast water from commercial ships sampled in the Duluth-Superior Harbor during 2009. T-RFLP profiles were generated using three different restriction enzyme digests of the same samples – *Hae*III (A), *Msp*I (B), and *Rsa*I (C). Letters after the ship numbers indicate replicates of the same sample. Numbers at branch nodes are Cophenetic correlation coefficients that estimate the uncertainty of dendrogram branches.


SW Ballast
FW Ballast
9-A
9-B
7-A
7-B
1-A
1-B
2-A
2-C
2-B
4-B
4-C
4-A
8-B
8-C
5-B
5-C
5-A
3-A
3-B
3-C
6-B
6-C
6-A
10-A
10-C
100
90
80
70
60
50
40
% Similarity
(A) HaeIII
92
100
99
97
92
91
99
92
89
93
91


SW Ballast
FW Ballast
9-B
9-C
9-A
7-A
7-B
2-A
2-C
2-B
1-A
1-B
8-A
8-C
5-A
5-C
3-A
3-C
3-B
6-B
6-C
6-A
4-A
4-C
4-B
5-B
10-A
10-C
10-B
100
90
80
70
60
50
40
% Similarity
(B) Mspl
98
99
97
85
81
85
86
88
88
81
92
84
89
91
67

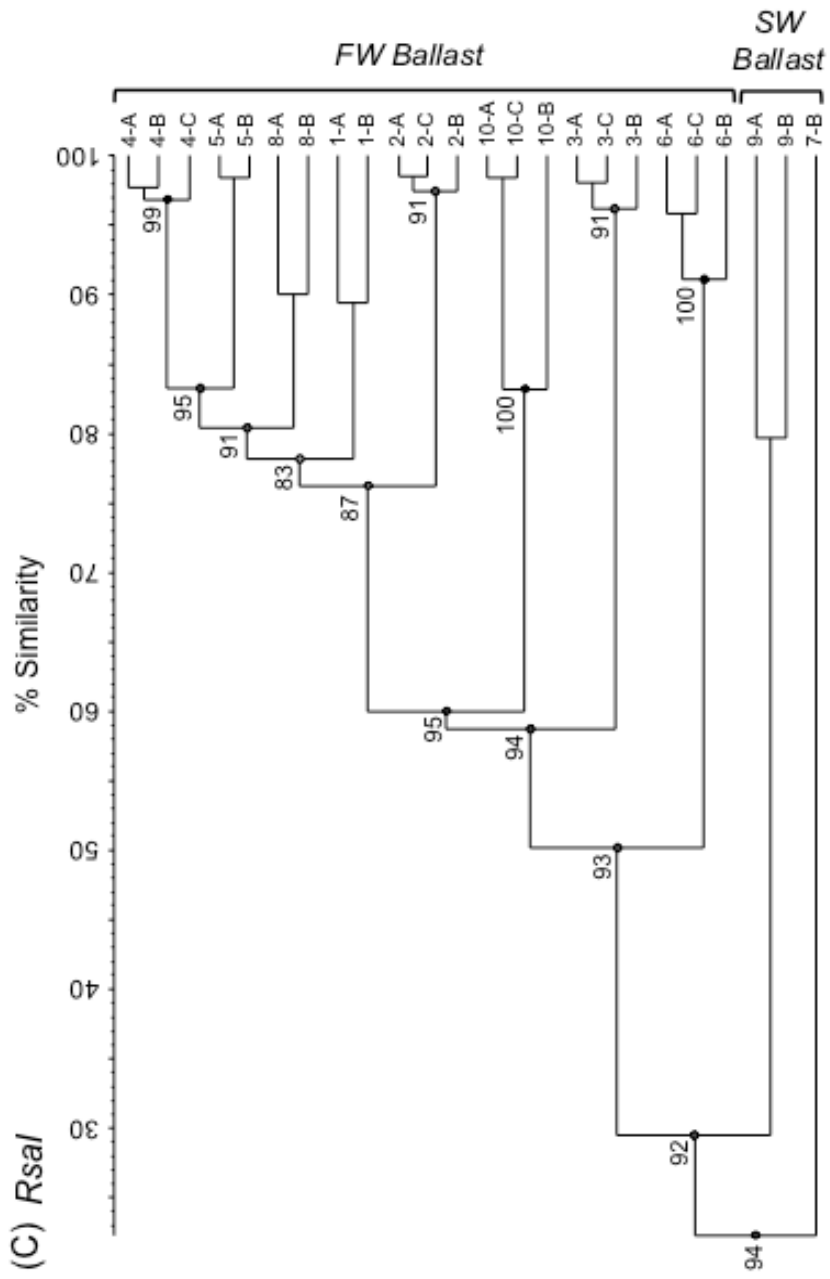
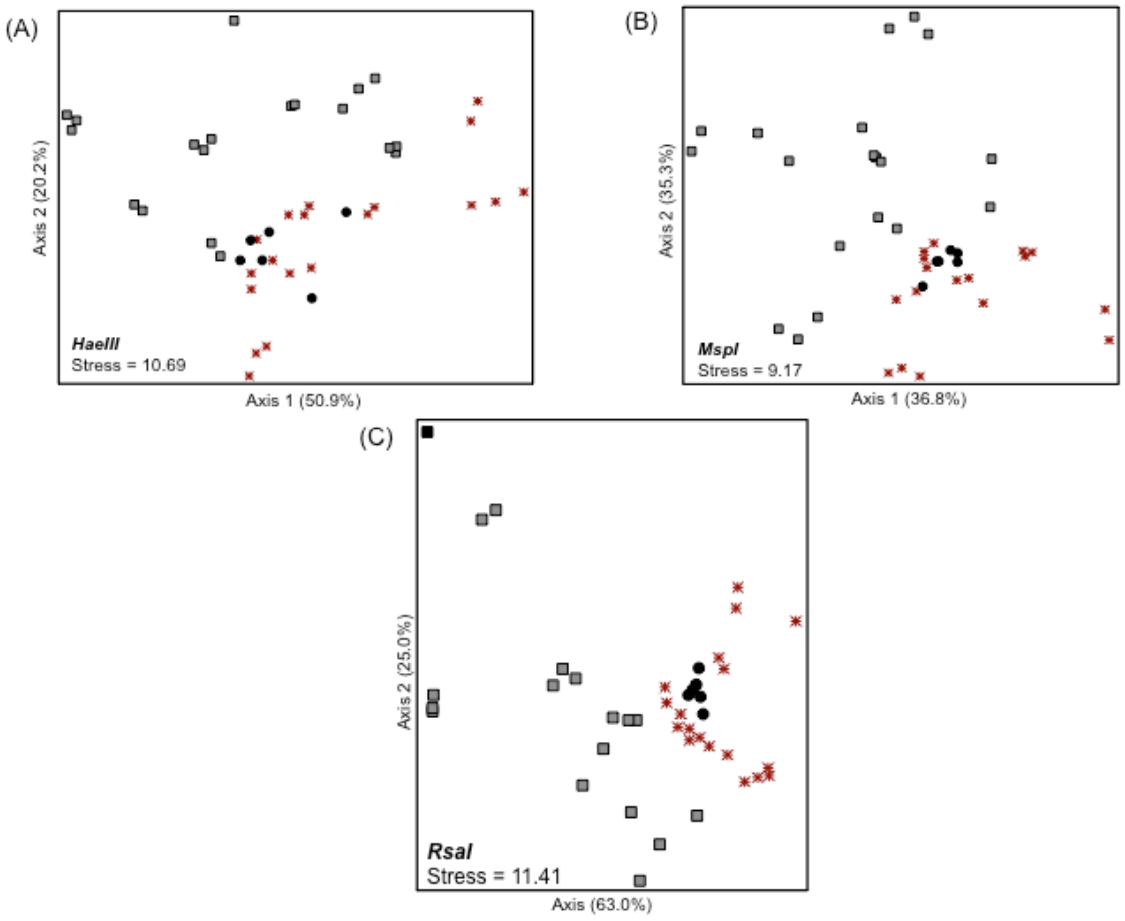

FW Ballast
SW Ballast
4-A
4-B
4-C
5-A
5-B
8-A
8-B
1-A
1-B
2-A
2-C
2-B
10-A
10-C
10-B
3-A
3-C
3-B
6-A
6-C
6-B
9-A
9-B
7-B
100
90
80
70
60
50
40
30
% Similarity
(C) Rsal
99
95
91
83
87
91
100
91
95
94
93
100
92
94

Figure III.4. NMS ordinations of T-RFLP profiles of bacterioplankton communities in freshwater ballast, dockside water samples, and at two Duluth-Superior Harbor sites. T-RFLP profiles were generated using three different restriction enzyme digests of the same samples – *Hae*III (A), *Msp*I (B), and *Rsa*I (C). Grey squares indicate ballast water samples and asterisks indicate dockside water samples. Black circles indicate harbor water samples collected at outer (DSH-OT) and inner (DSH-IN) harbor sites. Stress values for each ordination are reported on each graph.


(A)
Axis 2 (20.2%)
HaeIII
Stress = 10.69
Axis 1 (50.9%)
(B)
Axis 2 (35.3%)
MspI
Stress = 9.17
Axis 1 (36.8%)
(C)
Axis 2 (25.0%)
RsaI
Stress = 11.41
Axis (63.0%)

Densitometric curves of the T-RFLP profiles of the bacterioplankton communities in the WLSSD and the harbor water samples formed two distinct clusters in all three restriction enzyme digest (Fig. III.5). Percent similarities between clusters ranged from 22.8 to 33%. These two clusters were supported in peak-based analyses. T-RFLP profiles of bacterioplankton communities of treated wastewater effluent and harbor water were well separated when the relative peak heights of terminal restriction fragments (TRFs) were used to construct profiles (ANOSIM: $R = 1$, $p < 0.05$).

Bacterioplankton 16S rRNA gene clone libraries

To further characterize the bacterioplankton communities, 16S rRNA gene clone libraries were developed for three ballast and one harbor water sample. Initially, partial 16S rDNA fragments from 753 clones were sequenced. Clones removed from analysis included three clones with possible vector contamination, four sequences determined to be anomalous by MALLARD, and 123 clones with poor quality (i.e., low or absent signal, possible DNA contamination, background noise). No chimeric sequences were identified. The FWB3, FWB6, and SWB9 clone libraries had 16 to 21 poor quality clones removed whereas DSH-IN had 67 poor quality clones removed. Partial 16S rDNA sequences (approximately 500bp) from 623 clones were used in the final analysis (Table III.2).

Results from LIBSHUFF indicated that the four clone libraries were derived from distinctly different bacterial communities ($p < 0.01$). Percent coverage estimates for OTUs ($\geqq$ 97% sequence identity) ranged from 60 to 72% indicating incomplete sampling of the original bacterial communities (Table III.2). Shannon-Wiener indices and Chao1 richness estimates were not significantly different among the four clone libraries. However, both the Shannon-Wiener index and Chao1 can be affected by low coverage. Thus, the Simpson's Reciprocal Index (SRI), which is less affected by coverage was also calculated (Table III.2). The FWB6 clone library of the freshwater ballast from St. Clair had the highest Simpson's diversity (SRI = 54.7) and the seawater ballast for Ship 9 (SWB9) had the lowest diversity (SRI = 10.4). Although the Simpson's diversity of the

Figure III.5. UPGMA dendrograms showing hierarchical clustering of T-RFLP profiles of bacterioplankton communities in the Duluth-Superior Harbor and treated wastewater effluent collected from the Western Lake Superior Sanitary District (WLSSD). T-RFLP profiles were generated using three different restriction enzyme digests of the same samples – *Hae*III (A), *Msp*I (B), and *Rsa*I (C). Letters after the site names indicate replicates of the same sample. Numbers at branch nodes are Cophenetic correlation coefficients that estimate the uncertainty of dendrogram branches.

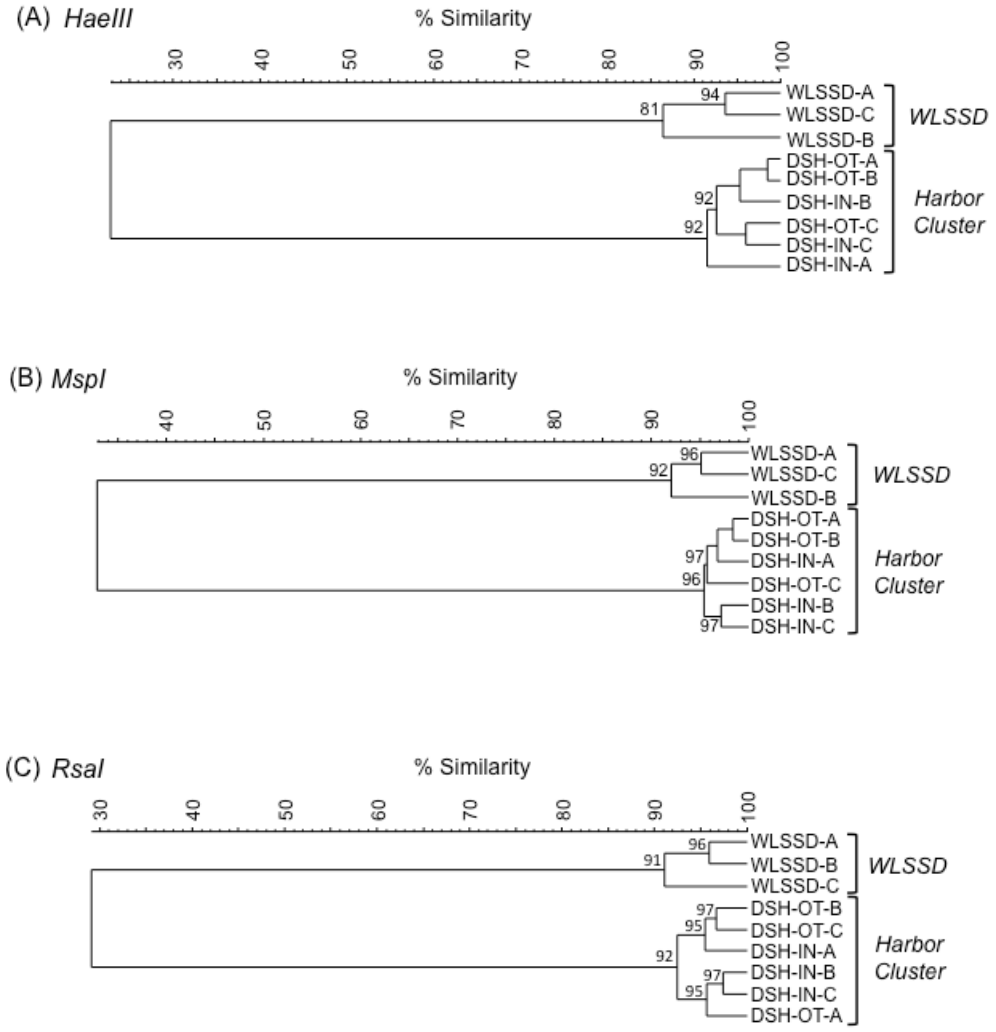

(A) HaeIII
% Similarity
30
40
50
60
70
80
90
100
94
81
92
92
WLSSD-A
WLSSD-C
WLSSD-B
DSH-OT-A
DSH-OT-B
DSH-IN-B
DSH-OT-C
DSH-IN-C
DSH-IN-A
WLSSD
Harbor
Cluster
(B) MspI
% Similarity
40
50
60
70
80
90
100
96
92
97
96
97
WLSSD-A
WLSSD-C
WLSSD-B
DSH-OT-A
DSH-OT-B
DSH-IN-A
DSH-OT-C
DSH-IN-B
DSH-IN-C
WLSSD
Harbor
Cluster
(C) RsaI
% Similarity
30
40
50
60
70
80
90
100
96
91
97
95
92
97
95
WLSSD-A
WLSSD-B
WLSSD-C
DSH-OT-B
DSH-OT-C
DSH-IN-A
DSH-IN-B
DSH-IN-C
DSH-OT-A
WLSSD
Harbor
Cluster

Table III.2. Information about the number of clones sequenced and the diversity of bacterial 16S rRNA gene clone libraries developed for three ballast water samples and the inner harbor site of the Duluth-Superior Harbor (DSH-IN).

Clone library[a]	Number of clones	Number of unique OTUs	% coverage	Chao1 (95% CI)	Shannon-Wiener index (95% CI)	Simpson's Reciprocal index (95% CI)
DSH-IN	116	63	60.3	178 (113-326)	4.28 (3.74-4.83)	27.3 (26.5-28.1)
FWB3	168	82	67.3	169 (125-260)	4.44 (4.01-4.81)	37.6 (37.2-38.0)
FWB6	173	88	65.9	220 (152-360)	4.57 (4.27-4.87)	54.7 (54.3-55.1)
SWB9	166	62	71.7	216 (127-430)	3.66 (2.99-4.44)	10.4 (9.90-10.8)

[a] DSH-IN = inner harbor site; FWB3 = freshwater ballast from ship 3; FWB6 = freshwater ballast from ship 6; SWB9 = seawater ballast from ship 9

Abbreviations: OTUs, operational taxonomic units defined at 97% sequence identity; CI, confidence interval

harbor water clone library (DSH-IN) was lower than the diversity indices for the two freshwater ballast libraries, only the diversity indices derived from similar sample sizes should be directly compared (Hill et al. 2003). The harbor clone library (DSH-IN) had at least 50 fewer clones than the three ballast water libraries.

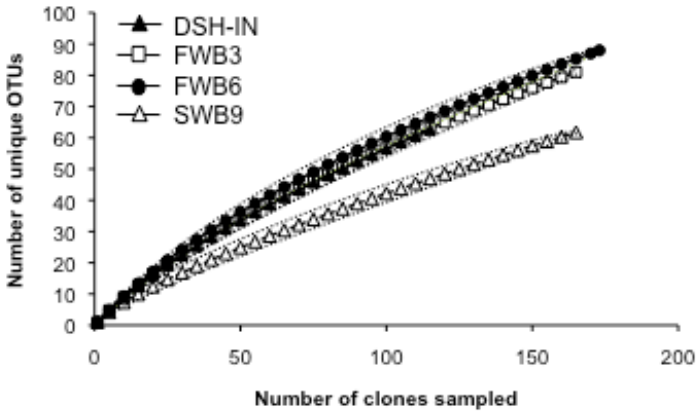
Rarefaction analysis gives an indication of sample richness within each clone library and a rough estimate of diversity. The rarefaction curves at high sequence similarity (≧97%) for all three freshwater samples (i.e., FWB3, FWB6, DSH-IN) overlapped (Fig. III.6.A), suggesting similar levels of richness within these clone libraries compared to the seawater ballast clone library (SWB9). At this high level of sequence similarity, none of the rarefaction curves reached an asymptote indicating incomplete coverage of species richness in all four samples. A sequence similarity of 80% has been used to indicate bacteria belonging to the same phylum (Hong et al. 2006). The distinct decline in the rate of detected OTUs in rarefaction curves calculated for the SWB9 and FWB6 libraries at 80% sequence similarity indicated that most of the dominant bacterial phyla were detected in these samples (Fig. III.6.B). The trajectory of the rarefaction curves of the FWB3 and DSH-IN clone libraries indicated that some dominant bacterial phyla that may have been present in these samples were not detected, but the FWB3 library showed greater phylum-level richness than the DSH-IN library. A large percentage of OTUs in each of the clone libraries occurred only once (singletons = 28 to 40%).

Taxonomic assignments of clones made by comparing clonal sequences to sequences in the GenBank library usually reflected the freshwater or marine habitat of the water samples used to create the 16S rRNA gene clone libraries (Table III.3). In the three freshwater clone libraries, the majority of the nearest GenBank database sequences with > 97% maximum identity were isolated from lakes. In the seawater ballast clone library, the nearest sequences were isolated from marine sources. All but one of the clones used in this analysis had ≥ 90% max identity with a 16S rRNA gene sequence in GenBank (Appendix Fig. A-3).

For the DSH-IN clone library, the bacterial phylogenetic groups assigned the most

Figure III.6. Rarefaction curves of OTUs for 16S rRNA gene clone libraries from the inner harbor site (DSH-IN), and ships carrying freshwater ballast (FWB3, FWB6) and seawater ballast (SWB9). The numbers in the ballast water clone library names correspond to the ballast water samples in Table III.1. Rarefaction curves are shown for OTUs with 97% sequence similarity (A) and OTUs with 80% sequence similarity (B).

(A)


DSH-IN
FWB3
FWB6
SWB9
Number of unique OTUs
Number of clones sampled
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200

(B)

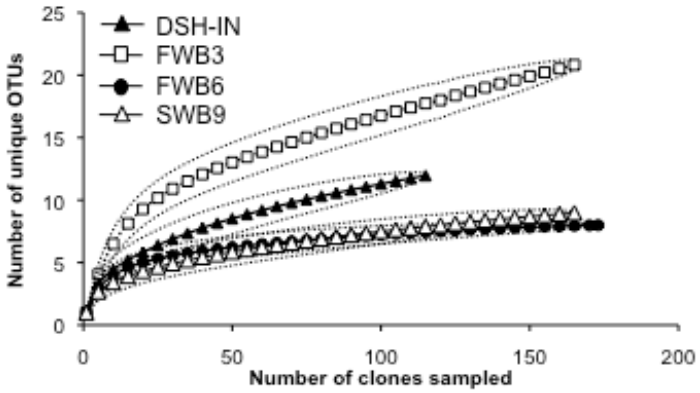

DSH-IN
FWB3
FWB6
SWB9
Number of unique OTUs
Number of clones sampled
0
5
10
15
20
25
0
50
100
150
200

Table III.3. Environmental habitats of sequences in the BLAST database with >97% maximum identity with16S rRNA gene clones in the FWB3, FWB6, SWB9 and DSH-IN libraries. For each clone library, the number of sequence matches are shown for each habitat type and the percentage of the total matches are shown in parentheses.

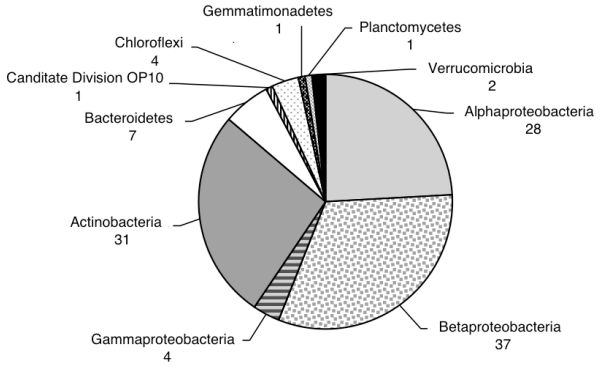
	Bacterial 16S rRNA Gene Clone Library			
Habitat of Closest Sequence Match	DSH-IN	FWB3	FWB6	SWB9
Freshwater habitats				
Lake	79 (73.1%)	71 (57.3%)	101 (68.0%)	1 (0.7%)
Harbor		10 (8.1%)	1 (0.7%)	
reservoir, pond	11 (10.2%)	10 (8.1%)	5 (3.4%)	1 (0.7%)
river, stream, riverine rock	5 (4.6%)	12 (9.7%)	22 (15.0%)	
Wetland		6 (4.8%)	3 (2.0%)	
aqua culture, water filtration, microcosm, drinking water	1 (0.9%)	3 2.4%)	1 (0.7%)	
Snow	1 (0.9%)		1 (0.7%)	
Unspecified-freshwater	1 (0.9%)	1 (0.8%)	3 (2.0%)	
Marine habitats				
Coast water, seawater-inlet, Loch, reef	1 (0.9%)	1 (0.8%)		16 (10.9%)
Gastropod symbiont, associated with marine organisms				55 (37.4%)
Harbor (salinity 26.5 ppt)				6 (4.1%)
Sediment-deep sea, methane-seep				2 (1.4%)
Sediment-salt marsh				1 (0.7%)
Deep Sea Hydrothermal vents, vent chimney structures				30 (20.4%)
Seawater-surface water, 2-m depth				18 (12.2%)
Deep sea				2 (1.4%)
Seawater-bioflim				1 (0.7%)
Unspecified-marine				10 (6.8%)
Other habitats				
Estuary	3 (2.8%)	4 (3.2%)		3 (2.0%)
Plastid; chloroplast			2 (1.4%)	
Particle-attached bacteria			2 (1.4%)	
Anaerobic digester, activated sludge, cellulosic waste	3 (2.8%)			
Soil	3 (2.8%)	5 (4.0%)	4 (2.7%)	
Unspecified		1 (0.8%)	2 (1.4%)	1 (0.7%)
Total	108 (100%)	124 (100%)	147 (100%)	146 (100%)

sequences were *Betaproteobacteria* (32%), *Actinobacteria* (27%), and *Alphaproteobacteria* (24%; Fig. III.7). Bacterial clones attributed to the *Actinobacteria* (32%) were the most abundant in the FWB3 clone library followed by clones assigned to the *Bacteroidetes* (20%), *Alphaproteobacteria* (14%), and *Verrucomicrobia* (14%) bacterial phylogenetic groups. In the FWB6 clone library, the *Bacteroidetes* (44%) phylum had the most representatives followed by the *Alphaproteobacteria* (18%) and *Actinobacteria* (15%). In the seawater ballast SWB9 clone library, 48% of the bacterial clones were assigned to the *Epsilonproteobacteria* followed by *Alphaproteobacteria* (28%) and *Bacteroidetes* (11%). Only 4% of the clones were affiliated with the *Gammaproteobacteria*. More clones were assigned to the *Betaproteobacteria* in the Duluth-Superior Harbor water clone library, as might be expected for a freshwater habitat. Clonal sequences affiliated with the *Alphaproteobacteria*, however, were more common in the two freshwater ballast clone libraries than sequences from sequences the *Betaproteobacteria*. In the seawater ballast library, clones affiliated with the *Alphaproteobacteria* were much more common than those affiliated with the *Betaproteobacteria*, as is expected for a marine environment. Phylogenetic trees were constructed for one bacterial phylum (Actinobacteria) and two bacterial classes (*Alphaproteobacteria*, phylum *Proteobacteria*; *Flavobacteria*, phylum *Bacteroidetes*) by comparing bacterial clone sequences from this study with some of their nearest neighbors obtained from GenBank and sequences selected from scientific literature (Figs. III.8 and III.9). While bacterial clones from the freshwater and seawater ballast samples were distributed throughout the class *Alphaproteobacteria*, large numbers of clones were found within taxonomic groups of the order *Rickettsiales* (Fig. III.8). In the freshwater libraries, most alphaproteobacterial clones were found in the LD12 clade (FWB3,14; FWB6,10, DSH- IN, 20) while most alphaproteobacterial clones (28) were placed in SAR11 cluster in the marine ballast library (SWB9; Fig. III.8).

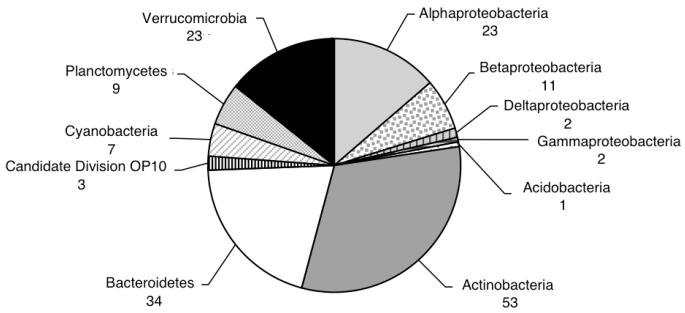
Bacterial clones affiliated with the *Actinobacteria* were common in the freshwater ballast and harbor water clone libraries (Fig. III.7). Only seven clones from the SWB9

Figure III. 7. Percentage of 16S rRNA clones assigned to different bacterial phyla and *Proteobacteria* class levels for each of the clone libraries developed for the inner harbor site (DSH-IN), freshwater ballast (FWB3, FWB6), and seawater ballast (SWB9). Numbers below the taxonomic labels indicate the number of clones assigned to each taxon.

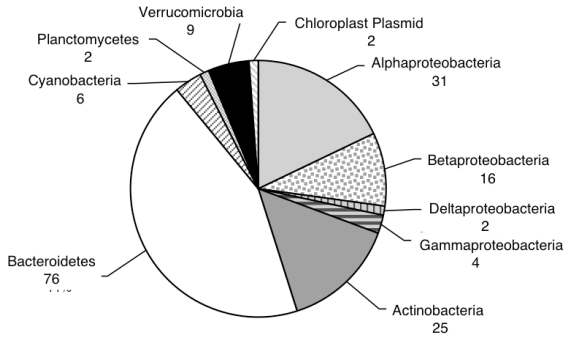
Duluth-Superior Harbor - Site 4 (DSH4)



Freshwater Ballast - Ship 3 (FWB3)



Freshwater Ballast - Ship 6 (FWB6)



Seawater Ballast - Ship 9 (SWB9)

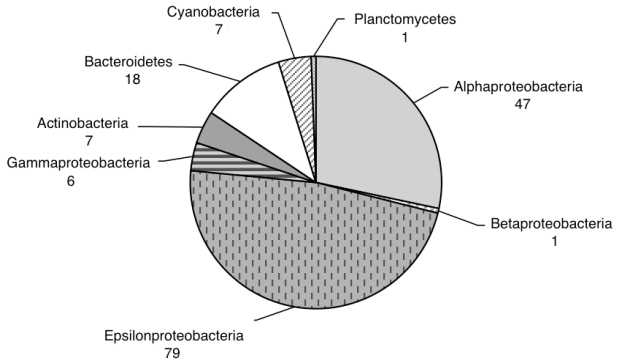


Figure III.8. Phylogenetic relationship of clones in the four 16S rRNA gene clone libraries constructed for ballast and harbor water to 16S rDNA sequences from cultured bacteria and uncultured clones from the alpha-Proteobacterial class. Clone names from this study are shown in bold and include information abut the water source (DSH, Duluth-Superior Harbor; FWB, freshwater ballast; and SWB, seawater ballast), ship number, clone designation, and a unique symbol for the water source (i.e., DSH4-square, FWB3-triangle, FWB6-inverted triangle, and SWB9-circle). Closely related clonal sequences are grouped together and the number of clones from each clone library are shown in parentheses. The accession numbers of sequences selected from GenBank are shown in parenthesis. Evolutionary distances were calculated using the Kimura 2-parameter. Numbers at nodes indicate the percentage of bootstrap iterations (of 1,000 replicate trees) that support each branching point. Scale bar indicates substitutions per nucleotide position. *Aquifex pyrophilus* (M83548) was used as an outgroup.

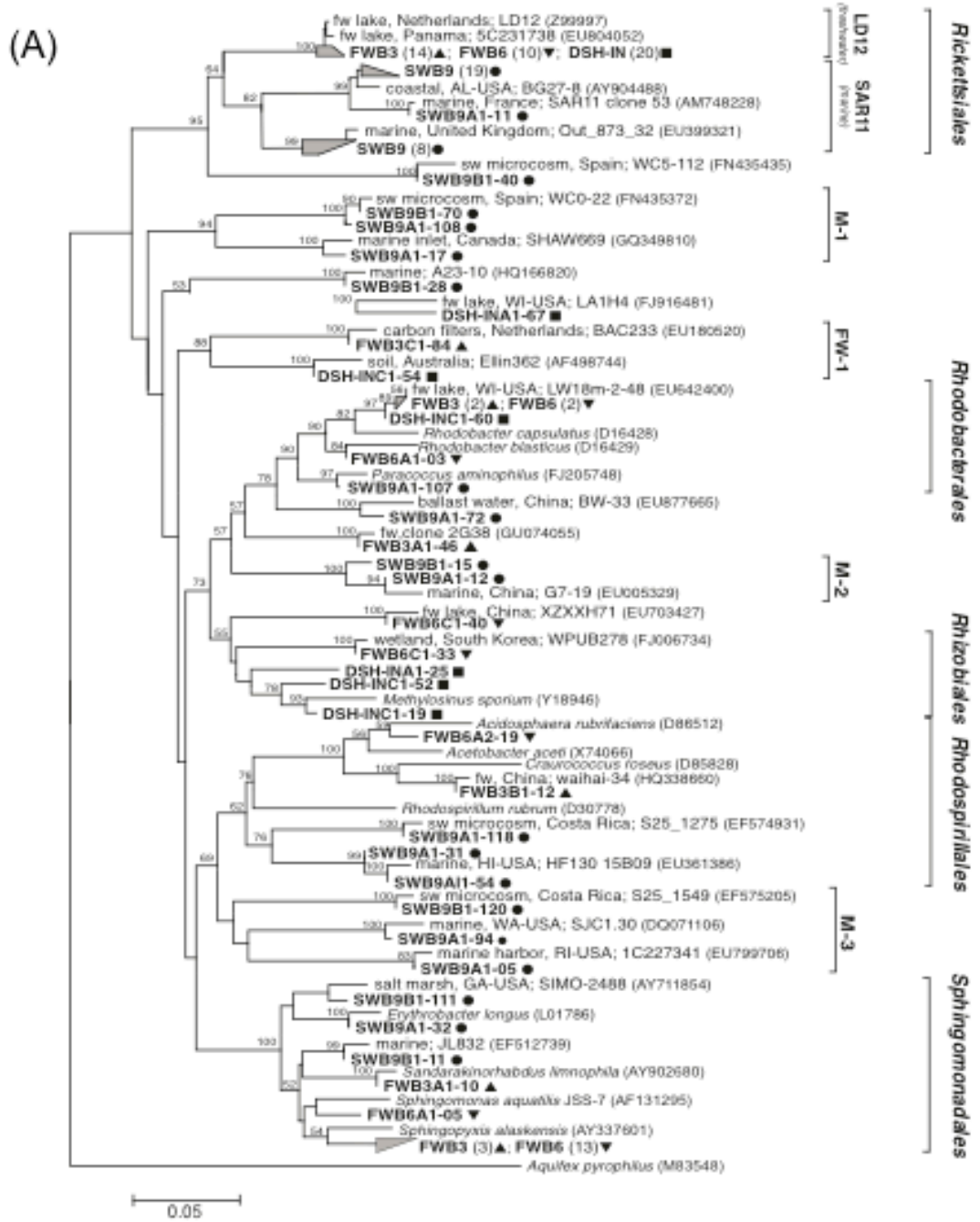
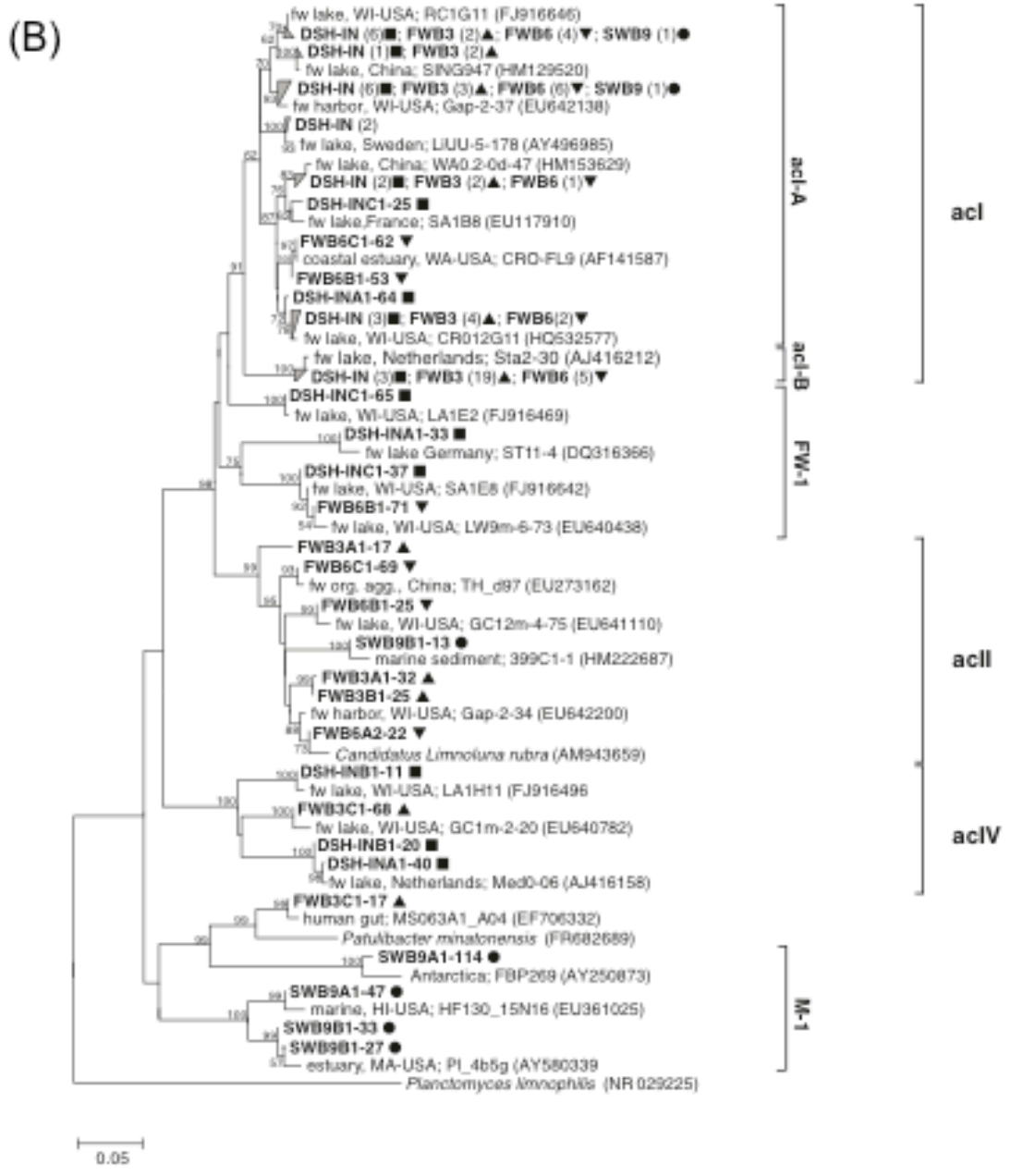

(A)
fw lake, Netherlands; LD12 (Z99997)
fw lake, Panama; 5C231738 (EU804052)
FWB3 (14)▲; FWB6 (10)▼; DSH-IN (20)■
SWB9 (19)●
coastal, AL-USA; BG27-8 (AY904488)
marine, France; SAR11 clone 53 (AM748228)
SWB9A1-11 ●
marine, United Kingdom; Out_873_32 (EU399321)
SWB9 (8)●
sw microcosm, Spain; WC5-112 (FN435435)
SWB9B1-40 ●
sw microcosm, Spain; WC0-22 (FN435372)
SWB9B1-70 ●
SWB9A1-108 ●
marine inlet, Canada; SHAW669 (GQ349810)
SWB9A1-17 ●
marine; A23-10 (HQ166820)
SWB9B1-28 ●
fw lake, WI-USA; LA1H4 (FJ916481)
DSH-INA1-67 ■
carbon filters, Netherlands; BAC233 (EU180620)
FWB3C1-84 ▲
soil, Australia; Elin362 (AF498744)
DSH-INC1-54 ■
fw lake, WI-USA; LW18m-2-48 (EU642400)
FWB3 (2)▲; FWB6 (2)▼
DSH-INC1-60 ■
Rhodobacter capsulatus (D16428)
Rhodobacter blasticus (D16429)
FWB6A1-03 ▼
Paracoccus aminophilus (FJ205748)
SWB9A1-107 ●
ballast water, China; BW-33 (EU877666)
SWB9A1-72 ●
fw clone 2G38 (GU074055)
FWB3A1-46 ▲
SWB9B1-15 ●
SWB9A1-12 ●
marine, China; G7-19 (EU005329)
fw lake, China; XZXXH71 (EU703427)
FWB6C1-40 ▼
wetland, South Korea; WPUB278 (FJ006734)
FWB6C1-33 ▼
DSH-INA1-25 ■
DSH-INC1-52 ■
Methylosinus sporium (Y18946)
DSH-INC1-19 ■
Acidosphaera rubrifaciens (D86512)
FWB6A2-19 ▼
Acetobacter aceti (X74066)
Craurococcus roseus (D85828)
fw, China; waihai-34 (HQ338860)
FWB3B1-12 ▲
Rhodospirillum rubrum (D30778)
sw microcosm, Costa Rica; S25_1275 (EF574931)
SWB9A1-118 ●
SWB9A1-31 ●
marine, HI-USA; HF130 15B09 (EU361386)
SWB9AI1-54 ●
sw microcosm, Costa Rica; S25_1549 (EF575205)
SWB9B1-120 ●
marine, WA-USA; SJC1.30 (DQ071106)
SWB9A1-94 ●
marine harbor, RI-USA; 1C227341 (EU799706)
SWB9A1-05 ●
salt marsh, GA-USA; SIMO-2488 (AY711854)
SWB9B1-111 ●
Erythrobacter longus (L01786)
SWB9A1-32 ●
marine; JL832 (EF512739)
SWB9B1-11 ●
Sandarakinorhabdus limnophila (AY902680)
FWB3A1-10 ▲
Sphingomonas aquatilis JSS-7 (AF131295)
FWB6A1-05 ▼
Sphingopyxis alaskensis (AY337601)
FWB3 (3)▲; FWB6 (13)▼
Aquifex pyrophilus (M83548)
0.05
LD12
SAR11
(freshwater)
(marine)
M-1
FW-1
M-2
M-3
Rickettsiales
Rhodobacterales
Rhizobiales
Rhodospirillales
Sphingomonadales

Figure III.9. Phylogenetic relationship of clones in the four 16S rRNA gene clone libraries constructed for ballast and harbor water to 16S rDNA sequences from cultured bacteria and uncultivated clones from the *Actinobacteria.* Clone names from this study are shown in bold are from this study and are the same as in Fig. III.9. Closely related clonal sequences are grouped together and the number of clones from each clone library are shown in parentheses. The numbers of grouped clones are in italicized parenthesis next to clone library designation. The accession numbers of sequences from GenBank are shown in parenthesis. Evolutionary distances were calculated using the Kimura 2-parameter. Numbers at nodes indicate the percentage of bootstrap iterations (of 1,000 replicate trees) that support each branching point. Scale bar indicates substitutions per nucleotide position. *Planctomyces limnophilis* (NR029225) was used as an outgroup.


(B)
fw lake, WI-USA; RC1G11 (FJ916646)
DSH-IN (6)■; FWB3 (2)▲; FWB6 (4)▼; SWB9 (1)●
DSH-IN (1)■; FWB3 (2)▲
fw lake, China; SING947 (HM129520)
DSH-IN (6)■; FWB3 (3)▲; FWB6 (6)▼; SWB9 (1)●
fw harbor, WI-USA; Gap-2-37 (EU642138)
DSH-IN (2)
fw lake, Sweden; LIUU-5-178 (AY496985)
fw lake, China; WA0.2-0d-47 (HM153629)
DSH-IN (2)■; FWB3 (2)▲; FWB6 (1)▼
DSH-INC1-25 ■
fw lake,France; SA1B8 (EU117910)
FWB6C1-62 ▼
coastal estuary, WA-USA; CRO-FL9 (AF141587)
FWB6B1-53 ▼
DSH-INA1-64 ■
DSH-IN (3)■; FWB3 (4)▲; FWB6(2)▼
fw lake, WI-USA; CR012G11 (HQ532577)
fw lake, Netherlands; Sta2-30 (AJ416212)
DSH-IN (3)■; FWB3 (19)▲; FWB6 (5)▼
DSH-INC1-65 ■
fw lake, WI-USA; LA1E2 (FJ916469)
DSH-INA1-33 ■
fw lake Germany; ST11-4 (DQ316366)
DSH-INC1-37 ■
fw lake, WI-USA; SA1E8 (FJ916642)
FWB6B1-71 ▼
fw lake, WI-USA; LW9m-6-73 (EU640438)
FWB3A1-17 ▲
FWB6C1-69 ▼
fw org. agg., China; TH_d97 (EU273162)
FWB6B1-25 ▼
fw lake, WI-USA; GC12m-4-75 (EU641110)
SWB9B1-13 ●
marine sediment; 399C1-1 (HM222687)
FWB3A1-32 ▲
FWB3B1-25 ▲
fw harbor, WI-USA; Gap-2-34 (EU642200)
FWB6A2-22 ▼
Candidatus Limnoluna rubra (AM943659)
DSH-INB1-11 ■
fw lake, WI-USA; LA1H11 (FJ916496
FWB3C1-68 ▲
fw lake, WI-USA; GC1m-2-20 (EU640782)
DSH-INB1-20 ■
DSH-INA1-40 ■
fw lake, Netherlands; Med0-06 (AJ416158)
FWB3C1-17 ▲
human gut; MS063A1_A04 (EF706332)
Parulibacter minatonensis (FR682689)
SWB9A1-114 ●
Antarctica; FBP269 (AY250873)
SWB9A1-47 ●
marine, HI-USA; HF130_15N16 (EU361025)
SWB9B1-33 ●
SWB9B1-27 ●
estuary, MA-USA; PI_4b5g (AY580339
Planctomyces limnophilis (NR 029225)
0.05
acI-A
acI-B
FW-1
acI
acII
acIV
M-1

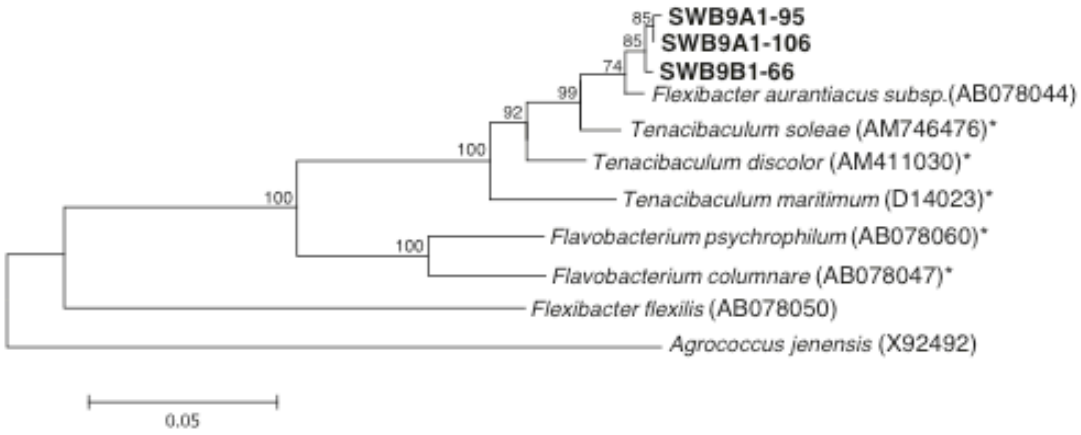
clone library were affiliated with the *Actinobacteria* and four of these clones were in the distinct marine clade (M-1) of this phylum (Fig. III.9). Bacterial clones affiliated with the *Bacteroidetes* phylum were present in all the clone libraries, but more commonly found in the freshwater ballast libraries than in the clone libraries constructed for harbor water or seawater ballast (Fig. III.7). Interestingly, three bacterial clones (SWB9A1-95, SWB9A1-106, and SWB9B1-66) in the seawater ballast clone library clustered with the genus *Tenacibaculum* (Fig. III.10) – a genus within the class *Flavobacteria* (phylum *Bacteroidetes*). The partial 16S rDNA sequences of these clones were 97% similar to the sequences for *Tenacibaculum soleae*, a fish pathogen.

DISCUSSION

Prokaryotic cell counts in ballast water samples were similar to bacterial abundances reported in a review of bacterial abundance in ballast water (Sun et al., 2010). Bacterial abundances were usually twice as high in freshwater ballast than either seawater or brackish ballast water (Table III.1). However, prokaryotic cells in ballast water were usually half as abundant as prokaryotic cells in harbor water samples taken adjacent to the docked ships. These results were consistent with previous studies that reported lower bacterial abundances in ballast water as compared to the receiving port water (Joachimsthal et al. 2004, Sun et al. 2010).

Both the frequency of introduction events and the abundance of organisms in ballast water contribute to propagule pressure. Ballast water was primarily transported to the DSH during the 2009-shipping season from the lower Great Lakes, with over 30% of the ballast water discharged originating from within the Lake Huron-Lake Erie corridor—one of four invasion 'hotspots' in the Laurentian Great Lakes (Grigorovich et al. 2003). In 2010, Rup et al. reported a similar pattern of ballast water that was transported from the lower lakes to the upper Great Lakes. Using the average prokaryotic cell abundance in ballast water determined in this study ($1.07 \pm 0.5 \times 10^{8}$ cells L^{-1}) and the estimated ballast water discharge volume of during the 2009-shipping season (3.7 billion gallons), the Duluth-Superior Harbor received approximately $2.05 \pm 1.0 \times 10^{17}$

Figure III.10. Phylogenetic relationship of three 16S rRNA gene clones from ship 9 (shown in bold typeface) that were affiliated with the family *Flavobacteriaceae* (phylum *Bacteroidetes*). The phylogenetic identity of these clones was most similar to several marine fish pathogens (designated with an asterisk). The accession numbers of sequences from GenBank are shown in parenthesis. Evolutionary distances were calculated using the Kimura-2-parameter. Numbers at nodes indicate the percentage of bootstrap iterations (of 1,000 replicate trees) that support each branching point. Scale bar indicates substitutions per nucleotide position. *Agrococcus jenensis* (X92492) and *Flexibacter flexilis* (AB078050) are used as outgroups.


85
85
74
99
92
100
100
100
SWB9A1-95
SWB9A1-106
SWB9B1-66
Flexibacter aurantiacus subsp.(AB078044)
Tenacibaculum soleae (AM746476)*
Tenacibaculum discolor (AM411030)*
Tenacibaculum maritimum (D14023)*
Flavobacterium psychrophilum (AB078060)*
Flavobacterium columnare (AB078047)*
Flexibacter flexilis (AB078050)
Agrococcus jenensis (X92492)
0.05

prokaryotic cells from ballast water in 2009. MacIsaac et al. (2002) found propagule pressure from bacteria in ballast water was 7 to 8 orders of magnitude higher than the propagule pressure for small zooplankton (rotifers, copepods, and cladocerans).

The genetic structure of bacterioplankton communities in freshwater and seawater ballast from commercial ships was distinctly different, as indicated by the T-RFLP analysis (Fig. III.). This result was not unexpected because pelagic marine bacterial communities have previously been shown to be fundamentally different than those found in freshwater (Glöckner et al. 2000). However, there also appeared to be differences between bacterioplankton communities in freshwater ballast originating from different ports. The genetic structure of bacterioplankton communities in the Duluth-Superior Harbor and freshwater ballast water formed two separate, yet overlapping groups (Fig. III.4). It is interesting that despite the fact that the ballast and dockside water samples were collected from seven dock locations in the DSH during five months of the ten month shipping season in Lake Superior, the genetic structure of bacterioplankton communities in these two types of samples were so well separated. In addition, the inner harbor (DSH-IN) and outer harbor (DSH-OT) samples collected on September 3, 2009 consistently clustered with the DSH dockside samples. This pattern suggests that either there are differences between the structure of bacterioplankton communities in the DSH and other ports in the Laurentian Great Lakes, or that the structure of bacterioplankton communities in freshwater ballast may be altered by contamination from residual sediment or by environmental conditions within the ballast tank before the ballast water is discharged.

Another source of bacterioplankton and possibly nonindigenous species to the DSH is treated wastewater from the WLSSD wastewater treatment plant in this harbor. Treated wastewater effluent is a major source of bacteria, such as fecal coliforms and potential pathogens, to freshwater and coastal waters (Cabral 2010). The genetic structure of the bacterioplankton community discharged with treated wastewater from the WLSSD plant in the Duluth-Superior Harbor was unique compared to bacterioplankton communities from the inner and outer parts of the DSH ($R = 1$, $p < 0.01$; Fig. III.5). The difference, however, might have been observed because the treated wastewater sample

was collected two months later in the fall (Oct. 30) than the harbor water samples (Sept. 3). The WLSSD tertiary treatment plant mean annual discharge of 40 MGD was approximately four times greater than the mean annual ballast water discharge (~10 MGD) to the DSH in 2009. LaPara et al. (2011) determined that treated wastewater effluent entering the Duluth-Superior Harbor was a major source of *Bacteroides* bacteria (phylum *Bacteroidetes*) and tetracycline resistant genes to water in this harbor. Effects of bacterial populations entering a new environment may not be immediately detected since a portion of the bacterial communities detected in environmental samples may be dormant (metabolically inactive; Cole 1999). These dormant microbes can then serve as seed banks affecting bacterial community structure and composition in future generations (Jones and Lennon 2010).

It is impossible to sample microbial communities exhaustively, but the LIBSHUFF analysis indicated that the 16S rDNA clone libraries from the three ballast waters (FWB3, FWB6, and SWB9) and the DSH water (DSH-IN) were distinctly different. Comparing the diversity between bacterial communities can also provide information on their relative differences (Hughes et al. 2001). The 16S rDNA clone libraries constructed for freshwater sources (DHS-IN, FWB3, and FWB6) were more diverse than the clone library derived from the seawater ballast (SWB9). The most diverse clone library (FWB6) was created from the freshwater ballast transported from St. Clair, MI and discharged into the DSH.

It was not unexpected to see that the taxonomic assignments of the bacterioplankton clones reflected the freshwater or marine habitat of the water samples used to create the clone libraries (Table III.3). Similarly, it was not surprising that the majority (>57%) of the 16S rDNA clones from the freshwater ballast libraries were affiliated with bacterial DNA sequences isolated from lakes because all these ballast waters were taken aboard ships in the Laurentian Great Lakes. Bacterial phyla typically found in freshwater aquatic habitats are *Alphaproteobacteria, Betaproteobacteria, Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* (Zwart et al. 2002, Glöckner et al. 2000), and this was typical of the freshwater ballast and harbor water clone libraries in this study (Fig. III.7). Marine bacterioplankton communities can be

fundamentally different from communities found in freshwater (Glöckner et al. 1999, Crump et al. 1999). *Betaproteobacteria* are not typically found in saline environments, however, Bouvier and del Giorgio (2007) found a dramatic increase in *Betaproteobacteria* when marine communities were incubated in virus-depleted ambient water. This finding indicates that betaproteobacteria are present in some marine environments but only one 16S rDNA clone affiliated with the *Betaproteobacteria* was found in the seawater ballast clone library (Fig. III.7).

Alphaproteobacteria, *Gammaproteobacteria* and *Bacteroidetes* are most common bacterial taxonomic groups found in marine ecosystems (Pommier et al. 2006) but this was not typical of the seawater ballast clone library (SWB9). The SWB9 clone library was dominated by bacterial clones (48%) from the *Epsilonproteobacteria* phylum (Fig. II.7). A majority of bacterioplankton clones (58%) in the seawater ballast were affiliated with bacterial 16S rDNA sequences collected from marine animals and deep sea hydrothermal vent areas (Table III.3). *Epsilonproteobacteria* and their environmental DNA sequences are commonly found in hydrothermal vent ecosystems, marine sediments, and pelagic marine redoxclines (Nakagawa et al. 2005, Labrenz et al. 2007). Redoxclines form between oxic and anoxic or sulfidic waters. The high percentage of *Epsilonproteobacteria* in the seawater ballast clone library may indicate these conditions were present within the ballast tank of the ocean-going Ship 9. Is it possible that ocean-going ships exchanging ballast water in mid-ocean environments are not only picking up and transporting bacteria associated with deep-sea hydrothermal vents but also providing the required conditions for them to survive?

It has been proposed that ballast tanks may act as incubators causing increases in bacterial abundance over time (Drake et al. 2002), but many studies have indicated that bacterial abundance in ballast tank waters decreases by the end of the voyage (Drake et al. 2007, Burkholder et al. 2007, Tomaru et al. 2010). Although bacterial abundance and diversity in ballast tanks might be expected to decrease over time, some microorganisms may have the ability to survive the harsh conditions within the tank (Seiden et al. 2011). As a result, the structure of bacterial communities within ballast water could change during voyages in the ballast tanks of ships. The work of Tomaru et al. (2010) confirms

this possibility by finding changes in the composition of bacterial communities in ballast water over time using denaturing gradient gel electrophoresis (DGGE). Joachimsthal et al. (2003) indicated changes in the relative numbers of aerobic, microaerophilic, and facultative anaerobic microorganisms in a ballast tank were affected by conditions in the ballast tank. For example, the transition to anaerobic conditions in the ballast tank during a voyage may increase the abundance of facultative anaerobic microorganisms.

Alternatively, the large number of *Epsilonproteobacteria* clones in the seawater ballast may indicate residual sediment in the ballast tank was resuspended during the transit of this ship from the Atlantic Ocean or afterwards. The seawater ballast sample used to create the SWB9 16S rRNA gene clone library was collected during active ballast discharge, which may have disturbed residual sediment in the ballast tank. Finally, the high abundance of *Epsilonproteobacteria* clones in the seawater ballast may be an artifact of the molecular techniques used in this study such as preferential disruption of cells during DNA extraction (Wintzingerode et al. 1997) or preferential amplification (Reysenbach et al. 1992) during PCR procedure. Although the phylogenetic affiliation of the *Epsilonproteobacteria* in the SWB9 clone library could be identified, the marine habitat that was the source or the reason why epsilonproteobacterial clones were so abundant in the seawater ballast library could not be reconciled.

Almost 17% of the clones in the SWB9 clone library were assigned to the SAR11 clade in *Alphaproteobacteria* (Fig. III.8). First identified in the North Atlantic Ocean, the SAR11 clade is now considered to be the dominant type of heterotrophic bacterioplankton in many marine ecosystems (Morris et al. 2002). Similarly, 17% of the clones in the DSH-IN harbor clone library were in the *Alphaproteobacteria* LD12 clade (Fig. III.8), also referred to as the freshwater SAR11 cluster (Morris et al. 2012). Sequences from the LD12 clade are commonly found in freshwater lakes (Zwart et al. 2002, Simonato et al. 2010). Ma et al. (2009) found the majority of the *Alphaproteobacteria* clones in ballast and seaport water were affiliated with *Rhodobacteraceae*, a common marine family. Only one clone from the SWB9 clone library, however, was assigned to the *Rhodobacteraceae* family.

Historically, the *Actinobacteria* were primarily thought to be associated with soils and were previously known as the high G+C gram-positive bacteria (Goodfellow and Williams 1983). However, studies using molecular techniques have shown that members of the *Actinobacteria* are common in many freshwater aquatic habitats including lakes and rivers (Glöckner et al. 2000, Crump and Hobbie 2005), and it is now considered one of the most common freshwater bacterioplankton groups (Glöckner et al. 2000). In this study, the clones affiliated with the *Actinobacteria* phylum were much more common in the freshwater clone libraries than in the seawater ballast clone library. Almost 84% of the freshwater clones in the *Actinobacteria* phylum were assigned to the *acI* cluster of the *Actinobacteria* (Fig. III.9). Predominance of bacteria from the *acI* cluster has been previously documented in freshwater ecosystems (Glöckner et al. 2000, Warnecke et al. 2004) and it is an exclusively freshwater clade. Members of the *Actinobacteria* have also been found in a variety of marine environments such as the deep sea and the Sargasso Sea (Sogin et al. 2006, Venter et al. 2004). Morris and Vergin (2005) found marine *Actinobacteria* may play a role in dissolved organic carbon dynamics and they are known to be tolerant of conditions where organic carbon in low. However, only 4 of 166 clones in the seawater ballast library (SWB9) were affiliated with the *Actinobacteria* but all these were found in a marine cluster (Fig. III.9).

One of the most interesting outcomes of the phylogenetic analysis of the ballast water clone libraries was finding specific bacteria within the *Bacteroidetes* phylum. Members of the *Bacteroidetes* are very abundant in aquatic environments (Glöckner et al. 2000, Cottrell and Kirchman 2000, O'Sullivan et al. 2002). Of particular interest, though, were three bacterial clones from the seawater ballast clone library (SWB9) that clustered within the genus *Tenacibaculum* in this phylum (Fig. III.10). The sequences of these clones were 97% similar to *Tenacibaculum soleae*, a causative agent of a marine fish disease (Tenacibaculosis) considered an important threat to marine finfish aquaculture worldwide (Lopez et al. 2010). The *Tenacibaculum* genus also contains other fish pathogens such as *T. maritimum* (Suzuki et al. 2001) and *T. discolor* (Piñeiro-Vidal et al. 2008[b]).

Tenacibaculum soleae was first isolated from diseased sole *(Solea senegalensis* Kaup) reared at a fish aquaculture facility in Galicia in northwestern Spain, a region adjacent to the Bay of Biscay (Piñeiro-Vidal et al. 2008[a]). A majority of fish farms pass seawater through net pens. The last port of call for the transoceanic vessel carrying the ballast water used to create the SWB9 clone library was Bilbao, in north-central Spain about 19 km from the Bay of Biscay. According to ballasting records obtained from the chief officer of the ship and submitted to the Minnesota Pollution Control Agency, Ship 9 picked up ballast water 220 km off the coast of Spain in the Bay of Biscay, exchanged the ballast water in the mid-Atlantic Ocean five days later, and arrived in Duluth-Superior Harbor 23 days after leaving the coast of Spain. Although Ruiz and Reid (2007) estimated that the abundance of planktonic organisms could be reduced up to 95% following ballast water exchange (BWE), conditions within ballast tanks are dynamic and organisms are not evenly distributed (Carlton 1995, Zhang and Dickman 1999). The efficiency of BWE can depend on conditions at the site of exchange and the designs of the ballast tanks and ship (Dickman and Zhang 1999). Low abundances of coastal organisms can be retained even after successful BWE due to incomplete flushing of small volumes of unpumpable residual water in the ballast tanks of ships (Seiden et al. 2011).

If the DNA was from viable and potentially pathogenic *Tenacibaculum* bacteria in this study, it is unlikely these cells could survive long after being discharged into the Duluth-Superior Harbor because of the osmotic stress these marine bacteria would encounter in this freshwater environment (Smith et al. 1999). Although some *Tenacibaculum* spp. can survive and grow in 10% seawater, the salinity tolerance range of *Tenacibaculum soleae* is between 55 and 100% seawater (Piñeiro-Vidal et al. 2008[a]). Nevertheless, the detection of DNA from a pathogenic bacterial species in ballast water discharged into the Duluth-Superior Harbor demonstrates the potential for the rapid, trans-oceanic transport of harmful bacteria in ballast water.

Conclusion

In summary, the genetic structure of bacterioplankton communities in ballast water and treated wastewater collected during 2009 was different from bacterioplankton communities in the Duluth-Superior Harbor. These findings indicate there may be a cause for concern about the potential introduction of pathogenic and potentially invasive bacteria into the harbor. Bacterial propagule pressure from ship ballast water may be high, even though prokaryotic cells in ballast water were typically one-half as abundant as prokaryotic cells in the harbor. The most common bacterial phyla found in freshwater habitats were also seen in the clone libraries of freshwater ballast and the Duluth-Superior Harbor. The genetic structure and species composition of the seawater ballast bacterioplankton community from an ocean-going ship was fundamentally different than the communities in freshwater ballast, and uncharacteristically dominated by bacteria from the *Epsilonproteobacteria* instead of the more commonly found bacterial phyla in marine ecosystems. DNA from the LD-12 clade of *Alphaproteobacteria* was common in freshwater ballast, while alphaproteobacterial DNA from the SAR11 cluster was common in the seawater ballast of the one ocean-going ship examined. Although there should be greater concern for introducing potentially harmful bacteria into the Duluth-Superior Harbor from other freshwater harbors rather than marine sources, this study demonstrated that DNA from a pathogenic marine bacterium, if not its intact cells, survived in the ballast tank of a transoceanic ship for at least two weeks before the ballast water was discharged into Duluth-Superior Harbor. This result demonstrates the potential for transporting potentially harmful bacteria over long distances in the ballast water of commercial ships to harbors in the Laurentian Great Lakes.

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APPENDIX

Table A-1. Significant ($p < 0.05$) Pearson correlation coefficients among ten environmental variables.

	Temperature	DO	DOC	$SUVA_{254}$	SRP	NH_4^+	NO_3^-	Chl a	pH	Turbidity
DO	(0.92)**									
DOC	0.96**	–								
$SUVA_{254}$	–	(0.82)*	–							
SRP	–	(0.97)**	–	0.84*						
NH_4^+	–	–	–	–	–					
NO_3^-	(0.97)**	0.95**	(0.91)*	(0.89)*	(0.88)*	–				
Chl a	–	–	0.82*	–	–	–	–			
pH	(0.85)*	0.84*	–	–	–	–	–	(0.92)**		
Turbidity	0.87*	(0.91)*	0.82*	–	0.83*	–	(0.90)*	0.89*	(0.96)***	
Prokaryotic Cell Abundance	0.94**	(0.90)*	0.89*	0.93**	0.85*	–	(0.97)**	0.82*	–	0.86*

Abbreviations: –, no significant correlation; DO, dissolved oxygen; DOC, dissolved organic carbon; SRP, soluble reactive phosphorus; Chl a, total chlorophyll as Chl a *$p \leq 0.05$; **$p \leq 0.01$ ***$p \leq 0.001$

Table A-2. Results from the Shapiro-Wilk W test for normality for environmental variables used in joint-plot overlays in NMS ordination analysis.

	W Statistic [a]	p-value
Temperature	0.92	0.499
DO	0.88	0.286
DOC	0.98	0.952
SRP	0.92	0.502
NH_4^+	0.89	0.314
NO_3^-	0.93	0.566
Chl a	0.88	0.271
pH	0.97	0.884
Turbidity	0.88	0.263

[a] A significant W statistic suggests the null hypothesis of non-normal distribution is not rejected. ($\alpha = 0.05$)

Table A-3. Data summary and outlier analysis for datasets used in Bray-Curtis ordination of T-RFLP profiles from all sampling sites. Standard deviations in parenthesis.

	Percent empty cells in matrix [a]	Average skewness of fragments [b]	Percent CV of fragment totals [c]	Sample outliers [d]	Fragment outliers	Comments on removal of outliers
*Hae*III						
RPH	67.6%	2.48	275.1%	None	TRF100 (2.04) TRF121 (2.11) TRF60 (2.51)	No significant pattern change
P-A[e]	67.6%	1.77	103.6%	DSH-IN1 (2.30) LS-MQH1 (2.30)	None	Only solution when DSH-IN1 was removed
*Msp*I						
RPH	49.9%	1.44	265.1%	LS-MQH3 (2.57)	None	No significant pattern change
P-A	49.9%	0.56	68.8%	LS-MQH3 (2.57)	None	
*Rsa*I						
RPH	50.1%	1.62	211.0%	LS-MQH2 (2.08) LS-MQH3 (2.03)	None	No significant pattern change
P-A	50.1%	0.9	71.8%	LS-MQH2 (2.31)	None	No significant pattern change

Abbreviations: RPH, relative peak height; P-A, presence-absence; CV, coefficient of variation

[a] In this study, zeros do not necessarily mean zero.

[b] Skewness is a measure of the asymmetry of the dataset and is equal to zero for normally distributed data.

[c] Coefficient of variation is a measure of variation in total abundance among fragments. CV>100 indicates the dominance of some fragments and/or the rarity of others.

[d] Outliers are defined as fragments with distances greater than 2 standard deviations (SD) from the mean distance based on the distance matrix. A weak outlier has SD from 2 to 2.3 and a moderate outlier has SD from 2.3 to 3.

[e] The outlier DSH-IN1 was chosen as an endpoint by variance regression in Bray-Curtis ordinations grouping all other samples in very close proximity. Removing DSH-IN1 resulted in similar patterns in ordination space as was found in other Bray-Curtis ordinations and was left out of this analysis.

Table A-4. Data summary and outlier analysis for datasets used in NMS ordination analysis of T-RFLP profiles from the lake/river mixtures and all sampling sites.

	Percent empty cells in matrix [a]	Average skewness of fragments [b]	Percent CV of fragment totals [c]	Sample outliers [d]	Fragment outliers	Comments on removal of outliers
*Hae*III	69.2%	2.71	276.7%	LS-MQH3 (2.22)	TRF24 (2.04) TRF53 (2.09) TRF121 (2.14) TRF 60 (2.42)	No overall pattern change
*Msp*I	59.0%	1.98	274.1%	LS-MQH3 (2.83)	None	No overall pattern change
*Rsa*I	57.5%	1.98	224.0%	LS-MQH1 (2.43) LS-MQH2 (2.38) LS-MQH3 (2.30)	None	No overall pattern change

Abbreviation: CV, coefficient of variation

[a] In this study, zeros do not necessarily mean zeros

[b] Skewness is a measure of the asymmetry of the dataset and is equal to zero for normally distributed data.

[c] Coefficient of variation is a measure of variation in total abundance among fragments. CV>100 indicates the dominance of some fragments and/or the rarity of others.

[d] Outliers are defined as fragments with distances greater than 2 standard deviations (SD) from the mean distance based on the distance matrix. A weak outlier has SD from 2 to 2.3 and a moderate outlier has SD from 2.3 to 3 (McCune and Grace 2002).

Table A-5. Data summary and outlier analysis for datasets used in NMS ordination analysis of T-RFLP profiles from the harbor and river water samples. Standard deviations in parenthesis.

	Percent empty cells in matrix [a]	Minimum skewness of sites [b]	Minimum kurtosis of sites [c]	Average skewness of fragments	Percent CV of fragment totals [d]	Sample outliers [e]	Fragment outliers	Comments on removal of outliers
*Hae*III	66.5%	3.21	10.13	2.38	272.9%	SLR-BSL2 (2.06)	TRF20 (2.00) TRF43 (2.01) TRF121(2.04) TRF37 (2.09) TRF60 (2.48)	No overall pattern change
*Msp*I	47.8%	3.82	16.34	1.17	249.9%	SLR-BSL2 (2.18)	TRF190 (2.04) TRF64 (2.00)	No overall pattern change
*Rsa*I	43.9%	3.14	9.59	1.43	201.8%	None	None	

Abbreviations: CV, coefficient of variation

[a] In this study, zeros do not necessarily mean zeros

[b] Skewness is a measure of the asymmetry of the dataset and is equal to zero for normally distributed data.

[c] Kurtosis indicates the 'peakedness' of the dataset and is equal to zero for normally distributed data.

[d] Coefficient of variation is a measure of variation in total abundance among fragments. CV>100 indicates the dominance of some fragments and/or the rarity of others.

[e] Outliers are defined as fragments with distances greater than 2 standard deviations (SD) from the mean distance based on the distance matrix. A weak outlier has SD from 2 to 2.3 and a moderate outlier has SD from 2.3 to 3 (McCune and Grace 2002).

Table A-6. Summary of the NMS ordination analysis results for final solutions. Two-dimensions were used in all NMS ordination procedures upon recommendation following the auto-pilot 'slow and steady' option in PC-ORD.

Data set		Final stress [a]	Final instability	Number of iterations	Monte Carlo [b] stress	Variance in distance matrix		Plot reflection	Seed number
						Axis 1	Axis 2		
Sites only									
	***Hae*III**	8.12	0.00123	55	11.7	46.6%	45.0%	V	3038
	***Msp*I**	6.01	0.00403	52	12.06	65.9%	30.1%	none	1174
	***Rsa*I**	7.55	0.00473	40	15.39	59.8%	31.0%	H	3250
Sites + artificial mixtures									
	***Hae*III**	9.46	0.00204	27	11.7	46.9%	45.7%	none	3917
	***Msp*I**	7.65	0.00034	45	10.54	40.8%	53.5%	none	5336
	***Rsa*I**	6.79	0.00461	24	15.39	71.8%	24.4%	V/H	1651

Abbreviations: V, vertical; H, horizontal; V/H, vertical and horizontal

[a] Final stress: 5-10 indicates a good ordination with minimal risk of false inferences.

[b] Monte Carlo permutation test was performed 250 times on randomized data. A final stress value less than the Monte Carlo minimum stress value indicates stronger axes extracted from the dataset then would be expected by chance. For all Monte Carlo tests $p < 0.01$.

Table A-7. Correlations between physicochemical parameters and NMS ordination axes 1 and 2.

	Axis 1			Axis 2		
	r	r^2	tau	r	r^2	tau
*Hae*III						
Temperature	0.831	0.690	**0.741**	(0.628)	0.394	(0.535)
DOC	0.889	0.790	**0.741**	(0.778)	0.605	(0.535)
SRP	0.278	0.077	0.165	0.147	0.022	0.206
NH_4^+	(0.851)	0.724	**(0.761)**	0.526	0.277	0.350
NO_3^-	(0.819)	0.671	(0.555)	0.442	0.195	0.350
pH	(0.669)	0.448	(0.535)	0.444	0.197	0.247
Turbidity	0.807	0.651	0.535	(0.264)	0.070	(0.167)
Chl *a*	0.580	0.336	0.350	(0.768)	0.589	(0.432)
*Msp*I						
Temperature	0.808	0.652	**0.638**	(0.519)	0.270	(0.411)
DOC	0.882	0.777	**0.638**	(0.694)	0.482	(0.411)
SRP	0.187	0.035	0.021	0.219	0.048	0.041
NH_4^+	(0.755)	0.571	**(0.658)**	0.542	0.293	0.350
NO_3^-	(0.762)	0.580	(0.576)	0.340	0.116	0.226
pH	(0.384)	0.148	(0.267)	0.718	0.516	0.535
Turbidity	0.405	0.164	0.267	(0.616)	0.379	(0.329)
Chl *a*	0.516	0.266	0.247	(0.878)	0.771	(0.555)
*Rsa*I						
Temperature	0.295	0.087	0.144	(0.854)	0.730	**(0.905)**
DOC	0.558	0.312	0.144	(0.978)	0.956	**(0.905)**
SRP	(0.504)	0.255	(0.350)	(0.088)	0.008	(0.165)
NH_4^+	(0.499)	0.249	(0.247)	0.840	0.941	**0.761**
NO_3^-	(0.120)	0.014	0.041	0.760	0.489	**0.720**
pH	(0.539)	0.290	(0.226)	0.624	0.390	0.535
Turbidity	0.425	0.180	0.021	(0.629)	0.396	(0.535)
Chl *a*	0.841	0.707	0.206	(0.751)	0.564	(0.350)

Abbreviations: DO, dissolved oxygen; DOC, dissolved organic carbon; SRP, soluble reactive phosphorus; Chl *a*, total chlorophyll as Chl *a*
Correlations recorded from NMS ordinations with joint-plot overlay using environmental variables in a second matrix. No p-values are given in PC-ORD. Brackets ( ) around the coefficient signifies negative correlations. Boldface type indicate variables with strong correlations (Kendall's tau > 0.6).

Figure A.1. Histogram showing the volume and source of ballast water (in metric tons, MT) that was discharged into the Duluth-Superior Harbor by commercial ships during the 2009-shipping season.

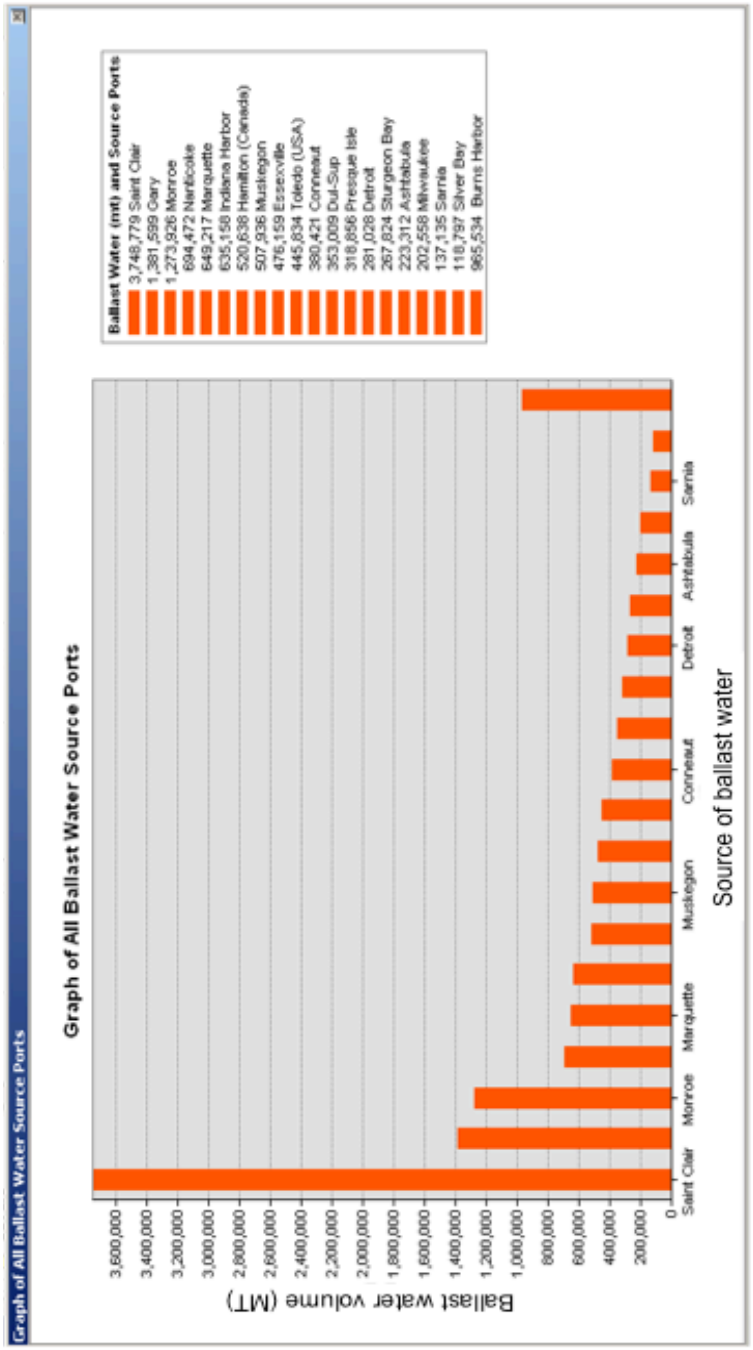
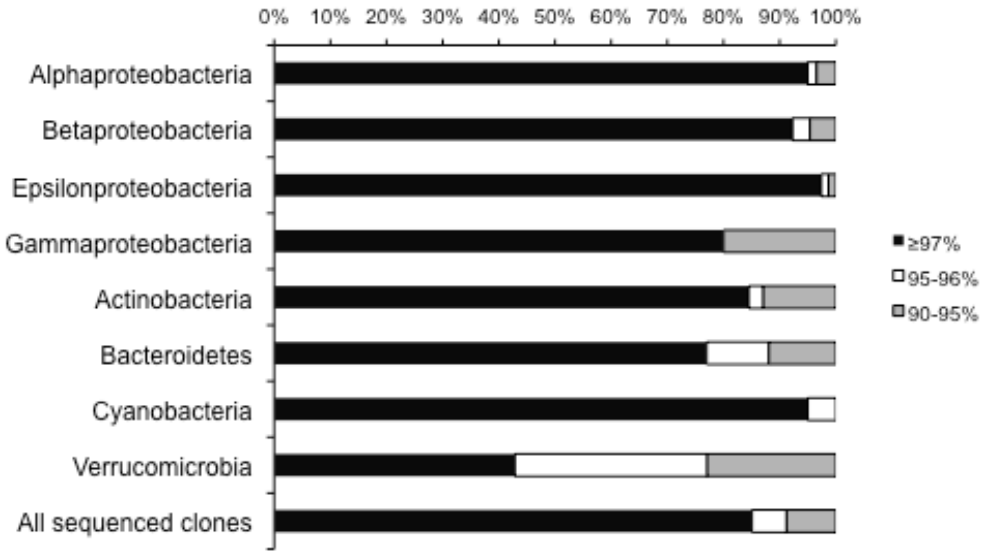

Graph of All Ballast Water Source Ports
Graph of All Ballast Water Source Ports
Ballast Water (mt) and Source Ports
3,748,779 Saint Clair
1,381,599 Gary
1,273,926 Monroe
694,472 Nanticoke
649,217 Marquette
635,158 Indiana Harbor
520,638 Hamilton (Canada)
507,936 Muskegon
476,159 Essexville
445,834 Toledo (USA)
380,421 Conneaut
353,009 Dul-Sup
318,856 Presque Isle
281,028 Detroit
267,824 Sturgeon Bay
223,312 Ashtabula
202,558 Milwaukee
137,135 Sarnia
118,797 Silver Bay
985,534 Burns Harbor
Ballast water volume (MT)
3,600,000
3,400,000
3,200,000
3,000,000
2,800,000
2,600,000
2,400,000
2,200,000
2,000,000
1,800,000
1,600,000
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
Saint Clair
Monroe
Marquette
Muskegon
Conneaut
Detroit
Ashtabula
Sarnia
Source of ballast water

Figure A.2. Identity of the bacterial phylum for the BLAST library sequences that was the best match to clones in the four 16S rRNA gene clone libraries constructed for the three ballast water samples and the inner harbor of the Duluth-Superior Harbor.


0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Alphaproteobacteria
Betaproteobacteria
Epsilonproteobacteria
Gammaproteobacteria
Actinobacteria
Bacteroidetes
Cyanobacteria
Verrucomicrobia
All sequenced clones
≥97%
95-96%
90-95%