Bacterial Community Dynamics on Suspended Particle Microscopic Islands and Implications for the Theory of Island Biogeography

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DEDICATION

This dissertation is dedicated to my beloved husband and parents for their unconditional love and supporting.

ABSTRACT

Suspended particles and aggregates, which include marine or lake snow, organic or inorganic particles, detritus, and flocs, are numerically abundant and ubiquitous in most marine, lacustrine, and riverine environments. In general, aggregates have enriched nutrient and organic matter concentrations compared to equal volumes of surrounding water. Consequently, aggregates may provide a favorable microscopic habitat for microorganism in aquatic ecosystems, and they therefore can be considered as islands that could harbor a variety of bacterial species. So far, the abundance, chemical composition, and associated bacterial communities of aggregates have largely been studied in marine ecosystems, and very few studies have been done regarding aggregates in freshwater, especially limnetic environments. In this study, an Illumina next-generation sequencing technique was used to characterize bacterial communities on aggregates in waters from Lake Superior and the Duluth-Superior Harbor.

Spatial distributions of particle-associated (PA) and free-living (FL) bacterial communities were characterized and compared at five sites along a transect from the lower St. Louis River, through the Duluth-Superior harbor (DSH), to nearshore Lake Superior in July and October 2013. Several gradients of environmental parameters were found along this transect. For example, temperature, DOC and bacterial abundance increased from the lake to the river, while light transmission decreased. Chlorophyll *a* (Chl *a*), transparent exopolymer particle (TEP) and particulate organic carbon (POC) concentrations were higher in the estuary than the river or the lake. A total of 19,505 16S rRNA V6 hyper-variable region gene sequence reads were obtained for each community

sample, and then assigned to different bacterial phyla and orders to characterize the diversity of bacteria in particle-associated and free-living communities. The bacterial richness, Shannon-Wiener diversity and evenness indices of PA bacterial communities were all higher than for FL communities. The PA bacterial community in nearshore Lake Superior was significantly different from PA communities in the estuary (harbor and river). The PA bacterial community varied monthly, but no spatial or temporal differences were observed in FL bacterial communities along the transect. The PA bacterial communities had a significantly larger proportion of Cyanobacteria/Chloroplast than the FL bacterial communities, but smaller proportions of *Rhodospirillales* and *Burkholderiales*. The PA bacterial community in nearshore Lake Superior had a higher abundance of sequences from the *Flavobacteriales*, *Sphingobacteriales*, and *Verrucomicrobiales* compared to the estuary. Within the estuary, more sequences from Cyanobacteria/Chloroplast but fewer sequences from the *Rhizobiales* were observed in October than in July within the PA bacterial communities. Although the bacterial composition of PA communities was significantly different from FL communities, no environmental factors measured explained this difference.

Seasonal dynamics of environmental parameters and bacterial communities associated with sinking particles (SP), artificial aggregates (AA), and aggregate-free water (AFW) were investigated and compared from October 2012 to December 2013 at two sites in nearshore Lake Superior and the Duluth-Superior Harbor (DSH). Clear seasonal changes were observed on SP, AA and in AFW bacterial communities. But the seasonal dynamic in AFW bacterial communities was less pronounced than SP and AA

bacterial communities at the lake and harbor sites. The concentrations of all environmental parameters were higher in the DSH compared to nearshore Lake Superior, which indicate the two sites had very different environmental conditions. Bacterial communities on SP, AA and in AFW were different between the lake and harbor sites. For example, in both SP and AA communities, sequences from the Cyanobacteria/Chloroplast were more abundant at the harbor site, while sequences from *Sphingomonadaceae* and *Cytophagaceae* were more abundant at the lake site. More sequences from the *Comamonadaceae*, *Microbacteriaceae*, and *Acidimicrobiaceae* families, but less sequences from the *Burkholderiaceae* family were found in AA and AFW communities at the lake site compared to the harbor site. In addition, bacterial communities on SP and AA, as well as communities on AA and in AFW were significantly different from each other at both sites. Overall, AA bacterial communities had higher richness and larger Shannon-Wiener diversity and evenness indices compared to SP and AFW bacterial communities. There also were compositional differences between SP and AA, as well as between AA and AFW bacterial communities at both sites. SP bacterial communities had a higher abundance of *Comamonadaceae*, *Oxalobacteraceae*, *Caulobacteraceae*, and *Pseudomonadaceae* sequences compared to AA communities at both site. Cyanobacteria/chloroplast were more abundant on AA than on SP or in AFW communities. Sequences from the *Microbacteriaceae*, *Cryptosporangiaceae*, *Acidimicrobiaceae*, *Candidatus Pelagibacter*, *Comamonadaceae*, *Burkholderiaceae*, *Methylophilaceae*, and *Opitutaceae* were more common in AFW than AA bacterial communities. Although seasonality and geographic location can be

important, free-living and attached lifestyles may have even more influence on the composition of aquatic bacterial communities.

Flocculent organic aggregates were used to test whether the Theory of Island Biogeography applies at a microbial scale, and to examine how bacterial community develop and evolve. In this laboratory study, artificial aggregates were generated with water from nearshore Lake Superior (lake) and the Duluth-Superior Harbor (harbor). In the first experiment, changes in bacterial community richness were evaluated four times over 30 days after aggregates were mixed with different aggregate-free water samples which still contained their resident bacteria. The Theory of Island Biogeography predicts that the total number of species is depend upon the distance from sources of potential colonizers. Distance from a potential source of colonizers was simulated by diluting the aggregate-free water with sterile aggregate-free water. Bacterial communities on lake and harbor aggregates with different "distances" from potential bacterial colonizers showed different colonization patterns. Bacterial richness on lake aggregates increased with closer colonizer distances in harbor water, supporting Theory of Island Biogeography predictions. However, this trend was not observed on harbor aggregates in lake water. Bacterial communities on lake and harbor aggregates both showed some clear dynamics over time, and the variations between different sample times were greater than the variations between different dilution treatments. In the second experiment, it was found that bacterial communities on lake aggregates were significantly different from those formed on harbor aggregates, and bacterial community richness on lake aggregates was higher than those on harbor aggregates in general. When lake aggregates were mixed

with lake water, the aggregate bacterial community was different from those on lake aggregates incubated in harbor water. This difference was not observed when harbor aggregates were incubated in lake and harbor waters. Bacterial communities on different aggregate types had their own distinctive pattern of succession, which was relative similar when aggregates were mixed with their source water or the alternative water. These observations might indicate that aggregates generated with different water sources may have different nutrients and organic matter regimes that may favor different bacterial species to colonize and perform differently during the re-colonization process.

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CHAPTER ONE

Background and Overview

Bacteria are ubiquitous and diverse in aquatic ecosystems, and perform multiple critical functions throughout the water column. Autotrophic microbes, including algae and some bacteria, photosynthesize and fix carbon into organic matter and biomass. These organic materials flow into food webs, ultimately being stored within the sediment or exchanged with the atmosphere. From a conventional perspective, the carbon cycle in oceans is mainly attributed to algal primary production, which always stays within the particle phase. In this perspective, algae, especially diatoms and dinoflagellates, are grazed by herbivores within the "grazing food chain" (Steele, 1974), and during this process, very little dissolved organic matter is "spilled" for heterotrophic bacteria to utilize. Therefore, the importance of the role of bacteria and their metabolism were highly underestimated when studying fate of organic matter in oceans (Steele, 1974). However, Azam *et al.* (1983) proposed new terminology, the "microbial loop", to re-emphasize the importance of bacterial utilization of dissolved organic materials. They found a large amount of primary production was lost in the form of dissolved organic matter, instead of serving as a food source for zooplankton (Fogg, 1983). Bacteria are able to utilize these dissolved organic materials, and become a food source for small bacterivores, such as protozoa, and ultimately protozoa would re-enter the traditional grazing food chain by being consumed by zooplankton or larger creatures. According to several studies, the bacterial pathway of organic carbon flux in ocean is very important and about half of

primary production enters the microbial loop through bacterial utilization of DOM (Hagström *et al.*, 1979; Fuhrman and Azam, 1982; Azam *et al.*, 1983; Cole *et al.*, 1988).

Bacteria in the water column of aquatic habitats are not passively receiving homogeneous dissolved organic carbon leaking from grazing food chain. Some studies about the behavioral strategies of bacteria have revealed that besides dissolved organic material utilization, some bacteria also attach to all kinds of particulate organic matter, including living organisms, transparent polymer particles, and colloids (Azam *et al.*, 1994; Mitchell *et al.*, 1995; Azam *et al.*, 1998). Bacteria that attack this particulate organic matter with enzymes to liberate dissolved organic matter, play a very profound role in biogeochemical behavior of organic matter. Particulate organic materials are considered “hotspots” that support high bacterial diversity and intense bacterial metabolism (Hagström *et al.*, 1979; Fuhrman and Azam, 1982; Cole *et al.*, 1988).

Alldredge and Silver (1988) defined macroscopic aggregates of particulate organic matter, such as detritus, fecal pellets, living organisms, and inorganic matters, as marine snow. These flocculent particles range in size from a few microns to several centimeters. Generally, the particles with a size >500 μm are known as macroscopic organic aggregates, such as marine and lake snow, while micro-aggregates, which are <500 μm in size, include transparent exopolymer particles and Coomassie blue-stained particles (Simon *et al.*, 2002). Different sized aggregates have similar but different roles in aquatic ecosystems. The abundance and size of aggregates can show large variations at different sample locations, and at different depths within the water column even within individual sites (Alldredge and Silver, 1988). In general, the abundance of marine snow

in surface waters was considerably higher than in deep sea areas (Alldredge and Silver, 1988). Due to the limitation of sampling methods and the naturally fragile features of aggregates, however, information about the abundance of macroscopic aggregates is only available for larger size particles at nearshore environments.

Formation of marine snow is a complex process involving physical, chemical, and biological processes. Generally, marine or lake snow are generated through two main pathways. First, some aggregates are directly formed by living, dead or shed parts of algae or zooplankton, such as mucus feeding structures of some zooplankton species, molting of zooplankton, the gelatinous sheaths generated by algae, discarded Larvaceans houses, and detached filter nets of Pteropods. In addition, some flocculent fecal pellets can also directly form aggregates particles. Second, aggregates can be formed by biologically enhanced physical aggregation of micro-aggregates or small particles. The basic process of this pathway involves 1) collisions of suspended particles, which are controlled by several physical mechanisms, and 2) the probability of particles sticking together after collision, which depends on DOM concentrations on particles, as well as surface properties and bridging chemicals within the particles. Aggregate formation could also be a function of the combined processes mentioned above. Additional studies show that the activity of microorganisms (phytoplankton and bacteria) can directly influence and control the formation of flocculent aggregates like marine snow (Simon *et al.*, 2002).

Although macroscopic aggregates were first discovered and have mainly been studied in oceans (Suzuki and Kato, 1953; Fowler and Knauer, 1986; Alldredge and Silver, 1988; Alldredge *et al.*, 1990; Alldredge, 1992; Herndl, 1992; Larson and Shanks,

1996; Alldredge, 1998; Ploug *et al.*, 1999), some recent research has shown that aggregates are also found in other aquatic environments, such as lakes (Grossart and Simon, 1993; Grossart, 1995; Grossart *et al.*, 1997; Grossart and Simon, 1998a;b; Schweitzer *et al.*, 2001; Tang *et al.*, 2009), rivers (Berger *et al.*, 1996; Grossart and Ploug, 2000; Neu, 2000; Luef *et al.*, 2007), estuaries (Zimmermann, 1997; Crump *et al.*, 1999), and even the active sludge of wastewater (Schweitzer *et al.*, 2001). Multiple studies have demonstrated the significant ecological roles that aggregates perform in all aquatic environments. As food sources for zooplankton and fish, organic aggregates play an important role in the cycling and flux of nutrients and energy within food webs (Alldredge, 1972; Alldredge, 1976). Sinking particles in aquatic ecosystems transport nutrients, energy, carbon, and other materials from the surface water column to deep water and ultimately to benthic habitats (Fowler and Knauer, 1986; Kiørboe *et al.*, 2001), and therefore are one factor controlling sediment formation. The loosely organized and semi-open structure of these aggregates in oceans facilitates trapping dissolved molecules that attach to the surface of these aggregates (Witten and Cates, 1986). Thus, aggregates usually have elevated concentrations of biologically accessible nutrients and trace metals compared to surrounding waters. These nutrient-enriched microenvironments are always highly colonized with concentrated but very diverse microbial communities compared to an equal volume of surrounding seawater. These microhabitats are considered "hotspots" for POM solubilization, substrate hydrolysis and uptake, biomass production, respiration and substrate release during the process of aggregate decomposition and nutrient

recycling in the water column (Alldredge and Silver, 1988; Azam *et al.*, 1993; Grossart and Simon, 1993).

Microbial groups colonizing aggregates are rather variable and dynamic, and include four major categories: algae, metazoans, protozoans, and prokaryotes. Very little is known about the roles that algae and metazoans play in decomposition and nutrient recycling on aggregates. However, some research has shown that these aggregates are actively consumed by larger organisms that cannot ingest smaller, freely suspended picoplankton cells. This pathway therefore links the microbial loop to higher trophic levels of food webs, and decreases the export of material and energy fluxes from the photic zone. Protozoans, including heterotrophic flagellates, ciliates, sarcodines and amoebae, are also found to highly colonize on aggregates with enriched numbers compared to the surrounding water (Silver *et al.*, 1978; Caron *et al.*, 1986; Herndl, 1988; Turley and Mackie, 1994; Silver *et al.*, 1998; Zimmermann-Timm *et al.*, 1998; Grossart *et al.*, 2003). In some studies, aggregates were found to be very favorable environments for the growth and survival of some protozoans by allowing them graze on aggregate-associated bacteria and algae (Caron *et al.*, 1982; Caron *et al.*, 1986). This grazing happened so intensively in some pelagic environments that it could actually reduce the loss of aggregates and POM due to sinking in the water column (Tiselius and Kiørboe, 1998; Simon *et al.*, 2002). However, the grazing rate of aggregate-associated protists on aggregate-associated bacteria was surprisingly low when compared to grazing of free-living bacteria in the surrounding water (Artolozaga *et al.*, 2002). This low grazing rate may be compensated by the higher biomass and nutrient content of aggregate-associated

bacterial cells (Grossart and Simon, 1998a). Bacteria have been found on all types of organic aggregates in a variety of ecosystems, including marine, lacustrine, riverine, and estuarine systems. Enriched bacterial populations and bigger bacterial cell sizes have been found on aggregates compared to the surrounding water, presumably because more favorable nutritional conditions are found on aggregates compared to the surrounding water.

Other studies have started to focus on the structure of bacterial communities on aggregates combined with their physiological properties to better understand the specific roles and functions that aggregate-associated bacterial communities play in aquatic ecosystems (Lee *et al.*, 1999; Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000; Grossart and Ploug, 2000). More detailed information regarding aggregate-associated bacterial community structure have become available as molecular techniques have advanced; these advances include community fingerprinting, fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes, clone libraries of the partial or complete 16S rRNA gene sequences, and next-generation sequencing of the 16S rRNA gene. Many studies compared bacterial community structure associated with aggregate or suspended particles to those free-living communities in surrounding water by separating aggregate-associated and free-living bacteria using serial filtration through different pore size filters to capture large size particles or aggregates and small size bacterial cells (Hollibaugh *et al.*, 2000; Riemann and Winding, 2001; LaMontagne and Holden, 2003; Rösel and Grossart, 2012; Mou *et al.*, 2013; Bižić-Ionescu *et al.*, 2015; Rieck *et al.*, 2015). In general, aggregate- or particle-associated bacterial community structure and

composition are very different from free-living communities in various environments, and the dominant phylotypes of each community differed accordingly. For example, several studies in marine ecosystems found the cluster of *Cytophaga/Flavobacteria* and *Sphingobacteria* (within the phylum of *Bacteroidetes*), or *Gammaproteobacteria* usually dominated bacterial communities on particles captured by filtration, aggregates made by marine diatom detritus, or naturally derived marine snows in variety of ocean environments (DeLong *et al.*, 1993; Rath *et al.*, 1998; Ploug *et al.*, 1999; Simon *et al.*, 1999; Bidle and Azam, 2001; Moeseneder *et al.*, 2001; Crespo *et al.*, 2013). Bacteria within the *Cytophaga/Flavobacteria* are able to decompose polymers such as complex polysaccharides, which is one of the major structure components of organic aggregates (Reichenbach, 2006).

Lake snow has very similar characteristics when compared to marine snow, including abundance, chemical composition, and setting velocity, microbial colonization, bacterial production and functional properties (Grossart and Simon, 1993). However, bacterial community structures on lake snow aggregates are very different from those on marine snow. The most predominant bacteria taxon found on natural lake snow in Lake Constance was *Betaproteobacteria*, constituting 27 to 42% of the total DAPI-stained cells (Weiss *et al.*, 1996). *Beta*- and *Alphaproteobacteria* were both rather dominant on lake snow in early stages of the experiments examining the colonization process of aged lake snow (Schweitzer *et al.* 2001). Then, a clear succession in bacterial community occurred after 3 days showing a dominance by *Betaproteobacteria* and a decreasing relative proportion of bacteria from the *Alphaproteobacteria*. Simultaneously, the relative

proportion of bacteria within the *Cytophaga/Flavobacteria* cluster also increased toward the end of the experiments (Schweitzer *et al.*, 2001). By using serial filtration through different pore size filters, particle-associated (PA) and free-living (FL) bacteria can be separated and their communities analyzed. Riemann and Winding (2001) found there was a significant "phylogenetic overlap" in both PA and FL assemblages in Lake Esrum (Denmark) during a phytoplankton bloom, and suggested not considering PA and FL bacterial communities as totally distinctive groups, but rather two fractions that have close interactions and connectivity. This result was confirmed by Bižić-Ionescu *et al.* (2014) by high throughput pyrosequencing in their study of PA and FL bacterial communities on limnic and coastal marine environments in Germany. Other studies still found PA bacteria communities were significantly different from FL bacterial communities (Mou *et al.*, 2013), and the temporal succession of bacterial community of structure for each life-style showed clearly different dynamic patterns that were associated with specific environmental parameters (Rösel and Grossart, 2012).

Estuaries are highly productive, dynamic, and turbid ecosystems characterized by the input of high loads of suspended particles or allochthonous materials. The hydrodynamics within the Columbia River estuary, including a strong tidal cycle and vertical stratification, lead to distinctive estuarine turbidity maxima (ETMs), which can increase the residence time of organic matter in the estuary, as well as the availability of this organic matter to estuarine bacteria and food webs (Crump and Baross, 1996). Estuaries are usually considered to be net-heterotrophic because of large amounts of particulate and dissolved organic matter present, and have very high heterotrophic

bacterial activity and production (Findlay *et al.*, 1991; Goosen *et al.*, 1997). Particle-associated bacteria have been found to dominate overall bacterial processes (Bent and Goulder, 1981; Bell and Albright, 1982; Schuchardt and Busch, 1991), and even account for 90% of total bacterial carbon production (Crump *et al.*, 1998). In addition, extracellular enzyme activity was correlated with high turbidity in the Columbia River estuary, and closely associated with particles (Crump *et al.*, 1998). In conclusion, bacteria associated with particles in estuaries may be responsible for most of the degradation of particulate organic materials in estuaries, and play key roles in transferring these materials into the active estuarine food webs by being consumed by detritivore (Crump and Baross, 1996).

Bacterial community composition in estuarine ecosystems is usually fairly diverse, which is not only caused by a high load of suspended particulate organic matter sediment resuspension and other allochthonous sources, but probably more importantly from the mixing of seawater and freshwater in estuaries. In the Elbe estuary (Germany) particle-associated bacterial communities in the brackish and marine sections were extremely different from those in the freshwater section (Zimmermann, 1997). In the limnetic section of this estuary, *Betaproteobacteria* and cells from the *Cytophaga/Flavobacteria* were in the highest proportions on particles; in the marine section, *Gammaproteobacteria* largely dominated while *Betaproteobacteria* constituted less than 12% of total DAPI-stainable cells, which was probably due to their intolerance of high salinity, and being replaced by certain marine species from the *Gammaproteobacteria* (Simon *et al.*, 2002). Similarly, *Cytophaga/Flavobacteria* were

also very dominant in the freshwater section in the Weser estuary (Germany), but decreased towards the marine section (Selje and Simon, 2003). Some studies have indicated that uniquely adapted estuarine bacterial communities could develop on suspended particle, and the free-living bacteria fraction would be more similar to bacterial communities in adjacent river or ocean environments. For example, members of the genus *Cytophaga*, *Alpha*-, *Gamma*- and *Deltaproteobacteria* were responsible for a large proportion of particle-associated bacteria in the Columbia River estuary (USA), but very few or none of these bacterial taxa were detected in particle-associated and free-living bacterial communities from both river and the coastal ocean (Crump *et al.*, 1999). Other studies have shown significant differences between particle-associated and free-living bacterial communities within different estuarine ecosystems by comparing band patterns derived from denaturing gradient gel electrophoresis (Bidle and Fletcher, 1995; Hollibaugh *et al.*, 2000).

Bacterial activity and community composition can be influenced by several biotic and abiotic factors. Usually the variability of these factors is caused by spatial or temporal dynamics, which can probably explain why bacterial community compositions are very different between marine and freshwater environments and show clear seasonal dynamics. Several chemical or biological gradients can occur in estuarine environments, such as salinity, turbidity, nutrient concentration, organic matter composition, phytoplankton biomass, and bacterivory zooplankton concentration. All of these gradients could influence the activity and composition of bacterial communities (Barcina *et al.*, 1997; Giovannoni and Rappe, 2000; Crump *et al.*, 2004). For example, Selje and

Simon (2003) found there was a clear transition of freshwater, brackish and marine bacterial communities along the salinity gradient in the Weser estuary, Germany. Similar results were also found in the Baltic Sea (Herlemann *et al.*, 2011), as well as Pocomoke River and Choptank River on the eastern shore of Maryland (Bouvier and del Giorgio, 2002). Besides estuarine environments, geography and spatial effects of different aquatic ecosystems or different areas within one individual system could also lead to correlations among biological activity and environmental variables. Several studies have suggested that regional and landscape characteristics among different Wisconsin lakes explain much variation in bacterial communities composition in addition to influences of seasonality, such as temperature, and climate (Lindström and Leskinen, 2002; Yannarell and Triplett, 2004; 2005). However, all these studies focused on overall or just free-living bacterial communities, while the influence of spatial dynamics on particle-associated bacteria was less studied.

The activity and diversity of bacterioplankton can not only be driven by site-specific environmental factors (intrinsic drivers), but also by the influences of seasonal changes (extrinsic drivers). These effects are often detected as synchronous changes of physical, chemical, and biological features over time (Magnuson *et al.*, 1990; Benson *et al.*, 2000; Kling *et al.*, 2000). Multiple studies from different aquatic environments have revealed there are recurring seasonal patterns in bacterial communities, and suggested those bacterial communities were highly correlated with some environmental parameters that change over time. For instance, water temperature, river flow rate, as well as concentrations of dissolved organic nitrogen and nitrate were the major factors that

influenced bacterial diversity in two non-intersecting temperate rivers in northeastern Massachusetts (Crump and Hobbie, 2005). Seasonal changes in bacterial productivity and community composition were driven by the shift of the source (terrestrial and phytoplankton) and lability of dissolved organic matter (DOM) in a tundra lake of Alaska (Crump *et al.*, 2003). In addition, several studies provided evidence for seasonal dynamics of phytoplankton or zooplankton influencing bacterioplankton communities in aquatic ecosystems due to their close interaction. For example, phytoplankton-derived detritus and exudates can be consumed by heterotrophic bacteria (Riemann and Winding, 2001; Worm *et al.*, 2001; Eiler and Bertilsson, 2004; Kent *et al.*, 2007; Paver and Kent, 2010), and bacterivorous flagellates can use bacteria as a food source (Šimek *et al.*, 2002; Matz and Jürgens, 2003; Matz and Kjelleberg, 2005; Pernthaler, 2005). Particle-associated and free-living bacteria can react differently to environmental variables within one site. Rösel *et al.* (2012) found seasonal environmental changes, such as temperature and nutrient contents, influenced members of particle-associated bacteria more than free-living bacterioplankton in Lake Tiefwaren (Germany). They also found a close association between particle-associated bacterial community composition and dynamics of zooplankton abundance, suggesting that the particle-associated bacterial community was coupled with organisms of high trophic levels in the food web, and contributes to their biomass (Rösel *et al.*, 2012).

Suspended particles or aggregates usually have enriched nutrients and organic materials, and high microbial biomass and productivity compared to an equal volume of surrounding water. Therefore, these particles or aggregates could be considered

microscopic “islands” that can be colonized by a variety of microorganisms. Such microscopic island might be used to test the applicability of the Theory of Island Biogeography at microscopic level. The Theory of Island Biogeography developed by MacArthur and Wilson (1967) can be used to estimate the number of species that would exist on islands based on the equilibrium of colonization of new species and extinction of native species on those islands. Overall, this theory relies upon immigration and extinction rates to explain differences in species richness or diversity on islands. More specifically, island size could affect the disappearance of native species that already exist on an island, and the distance of an island to a source of new species could affect the colonization rate of new species to that island.

Numerous studies have applied this theory to large scale islands. For example, Preston (1962) found a strong positive relationship between the number of land-plant species and island size in the Galápagos Islands. Similar species-island area relationships have been found for amphibians and reptiles in the West Indies (MacArthur and Wilson, 1967), ants within the Malaysian Archipelago (Wilson, 1959), bird species on islands in the Bismarck Archipelago (Diamond and Mayr, 1976), and vertebrates on islands in Lake Michigan (Hatt, 1948).

The relationship between species richness and island distance from the colonization source was found for birds on tropical islands of the Southwest Pacific, which showed a decreasing trend between species richness and island distance (Diamond, 1974). The recolonization process has been studied on the Krakatau islands following volcanic eruptions (Thornton, 1997; Whittaker, 2000) and on mangrove islands in the

Florida Keys after intentional depopulations (Simberloff and Wilson, 1970). More recently, the theory of island biogeography has been applied to ecosystems with high quality habitat surrounded by less-suitable habitats, including isolated mountain systems (Lomolino *et al.*, 1989; Kebede *et al.*, 2007), as well as natural habitats enclosed by urban areas (Fore and Guttman, 1999; Ohmura *et al.*, 2006).

Compared to the numerous studies that have applied the Theory of Island Biogeography to macroorganism populations and communities, very few studies have been completed considering microorganisms. A few studies have shown clear taxa-area relationships for natural microbial communities existing on "island" systems. The slope (z) of the log-log line relating taxa diversity and island or sample area ranges from 0.02 to 0.07 for contiguous habitat, and around 0.1 to 0.3 for "island" systems, which are generally smaller than the values found for macroorganisms including animals and plants (Bell *et al.*, 2005). For example, slopes of taxa-area relationship for ciliates and diatoms in the Polar Ocean and some adjoining regions of the North Atlantic were 0.066 and 0.077 respectively (Azovsky, 2002). Bacterial community diversity on mountain top lakes or water-filled "tree holes" had slopes of taxa-area relationship 0.161 and 0.26 respectively (Bell *et al.*, 2005; Reche *et al.*, 2005). Engineering-designed machine sump tanks and membrane bioreactors were also considered to be "island" systems, and bacterial diversity in them also showed clear taxa-volume relationships with slopes ranging from 0.246 to 0.294, and 0.206 respectively (van der Gast *et al.*, 2005; van der Gast *et al.*, 2006).

It would be very interesting to apply the Theory of Island Biogeography to suspended aggregates because they are well known as “hotspots” of bacterial activity with enriched bacterial populations. There are multiple advantages for using organic aggregates found in aquatic environments to test the Theory of Island Biogeography for bacterial communities (Lyons *et al.*, 2010). For example, large amounts of aggregates can easily be generated in the lab with a variety of water samples simply by rotating bottles of water on rolling tables. In addition, individual aggregates can be isolated or combined, and the bacterial communities on them can be analyzed in a relatively short period compared to studies of birds or big mammals on traditional islands. However, so far only a few studies have tested the Theory of Island Biogeography using organic aggregates and their associated bacterial communities. Lyons *et al.* (2010) first used lab-made organic aggregates to test the theory application in the context of disease. They found a significant species-area relationship between aggregate size and bacterial functional diversity (using Biolog EcoPlates). Kramer *et al.* (2013) revealed some relationships between colonization or extinction rates and background bacterial density or aggregate size by developing a model for the dynamics of bacterial pathogen species.

My dissertation research was designed to better understand the roles and functions that natural particle- or aggregate-associated bacterial communities play in freshwater ecosystems and their relationships with spatial or seasonal changes. The sample sites used in these studies were located in nearshore Lake Superior, the adjacent urban Duluth-Superior Harbor, and the St. Louis River estuary. Thus, my results should provide useful baseline information for understanding native bacterial communities and managing

healthy and sustainable nearshore lake and urban harbor ecosystems by evaluating the structure and composition of free-living and aggregate-associated bacterial communities in these sample areas. Additional information about the relationship between microscopic islands and bacterial communities, as well as the processes of re-colonization and succession of bacterial species, can be obtained by testing the applicability of the Theory of Island Biogeography to microscopic aggregate “islands”.

Two field studies and two lab experiments were completed to address following three objectives. (1) Identify the compositions of particle-attached and free-living bacterial communities along a transect from the lower St. Louis River Estuary through the Duluth-Superior Harbor to nearshore Lake Superior, and identify spatial environmental gradients along this transect that may influence bacterial community dynamics, (2) compare seasonal changes in the composition of bacterial communities associated with water, flocculent aggregates, and sinking aggregates from nearshore Lake Superior and the Duluth-Superior harbor, and assess whether any seasonal patterns in these three bacterial communities were correlated with certain environmental parameters, and (3) examine relationships between bacterial community richness or diversity on artificial particle aggregates and the distance to potential bacterial colonizers to test the Theory of Island Biogeography on a microscopic scale during the processes of re-colonization of freshwater aggregates.

CHAPTER TWO

Phylogenetic Analysis of Particle-Associated and Free-Living Bacterial Communities from the Lower St. Louis River to Nearshore Lake Superior

INTRODUCTION

Heterotrophic bacteria have significant biogeochemical importance in not only marine, but also freshwater ecosystem. The key role of bacteria in freshwater is more than simply being a principle degrader or decomposer of organic components, but also as biomass producers which can be readily utilized food sources for higher trophic levels. Among all bacteria, particle-associated cells are responsible for a high proportion of the total heterotrophic bacterial degradation of organic matter (Cammen and Walker 1982), and these bacteria can be 10-100 times more active than free-living bacteria (Crump *et al.*, 1998). The capability of bacteria to attach to the surface of particles and degrade the organic matter is due to their possession of certain specific adaptions (Bauer *et al.*, 2006). Therefore, particle-associated bacterial communities may be expected to be different from free-living communities. In addition, different compositions of organic compounds in particles may cause different bacterial communities to develop due to different particle-associated bacteria possessing various hydrolytic enzymatic activities (Karner and Herndl, 1992; Pinhassi *et al.*, 1999). Therefore, understanding the differences between particle-associated and free-living bacterial community compositions in aquatic environments could provide a more comprehensive understanding about bacterial life histories and their adaption in aquatic environments.

Estuaries are usually considered to be very productive and dynamic ecosystems, which are affected by ocean tidal movements and river discharge. In this type of environment, particle-associated bacteria are expected to account for most of the degradation of detrital organic matter, and play a significant role in the estuarine food web by being consumed by detritivores. Many studies have been completed in traditional brackish estuaries to study the particle-associated (PA) and free-living (FL) bacteria communities. However, only a few previous studies have focused on freshwater estuarine environments.

The Duluth-Superior Harbor is located in a freshwater estuary at the confluence of the St. Louis River and Lake Superior (Welch, 2012), and is the farthest-inland freshwater port and also the largest and busiest port on the Laurentian Great Lakes. Lake Superior is the largest freshwater lake in world by surface area (82,100 km^2), and is very unproductive and highly oligotrophic in terms of nutrient status compared with the other Laurentian Great Lakes (Munawar and Munawar, 2001). The St. Louis River discharges water into Lake Superior through the harbor, but water also enters the harbor from Lake Superior through seiche oscillations, with amplitudes ranging from 3 cm to 25 cm and exhibiting a 7.9-h seiche period in the lower St. Louis River which includes all the harbor waterway (Stortz and Sydor, 1980). Therefore, the lower portion of the St. Louis River is considered a freshwater estuary due to the mixing of tide-like lake water and river discharge (Herdendorf, 1990).

Although some studies found how freshwater and marine bacterioplankton communities mix along salinity gradients in brackish estuaries (Bidle and Fletcher, 1995;

Hollibaugh *et al.*, 2000; Troussellier *et al.*, 2002), Welch (2012) detected three distinct bacterioplankton communities from Lake Superior, the Duluth-Superior Harbor, and the St. Louis River habitats, and they did not show any transitional gradient from the river to lake. However, the answers to some questions are still unknown. For example, do particle-associated or free-living bacterial communities also follow this pattern? Does the diversity of bacterioplankton communities gradually change along the transition from river to lake, and are certain biochemical parameters related to these communities? Therefore, in this chapter, I evaluated possible links between particle-associated and free-living bacterial community diversity and composition to differences in environmental variables along the transition from the Duluth-Superior Harbor to nearshore Lake Superior.

Like brackish estuaries near oceans, the Duluth-Superior Harbor is characteristically very complex and a highly dynamic aquatic ecosystem, which receives several inputs that could bring in organic materials or nonindigenous bacteria. First, it receives large amounts of dissolved organic matter and suspended solids from St. Louis River (Bahnick and Markee, 1985). In addition to the high load of suspended matter from the river, particulate matter from iron ore docks, coal loading facilities, dredging, and most importantly, resuspension of bottom sediments by ship traffic may increase the concentration of suspended solids and increase turbidity in the Duluth-Superior Harbor. The Duluth-Superior Harbor is also subjected to a high degree of anthropomorphic change along its costal line and river channel (Rao and Schwab, 2007). This harbor receives an average of 40 million short tons of cargo and approximately 1,000 vessel

visits each year, as well as much more ballast water discharge than other Great Lakes ports (Welch, 2012). Introduction and spread of aquatic non-indigenous species (NIS) by ballast water has made the harbor one of four biological invasion "hotspots" in the Great Lakes (Grigorovich *et al.*, 2003; Trebitz *et al.*, 2010). In additional, about 40 million gallons of treated wastewater is discharged from the Western Lake Superior Sanitary District (WLSSD) each day (LaPara *et al.*, 2011). Storm water runoff carrying pollutants and clay originating from motor vehicles, roads, and lawns from developed areas may have dramatic impacts on the structure and function of the harbor ecosystem. Along with the discharge of ballast water, wastewater, as well as storm water, non-indigenous and even harmful bacterial species might be introduced into the Duluth-Superior Harbor, and then flow into Lake Superior.

So far, very little is known about the composition of bacterial communities in the St. Louis River estuary (SLRE), the Duluth-Superior Harbor, and the nearshore zone of Lake Superior. In this study, five sites were chosen along a transect through the SLRE and into Lake Superior, and sequencing of bacterial DNA was utilized to comprehensively investigated the compositions of particle-associated and free-living bacterial communities. This research provides important baseline knowledge about the diversity of natural bacterial communities in the SLRE, which should be very useful for managing healthy and sustainable the SLRE and nearshore lake ecosystems.

MATERIALS AND METHODS

Sample collection

Surface water samples (5 m below the surface) were collected from 5 sites in nearshore Lake Superior, the Duluth-Superior Harbor, and the lower St. Louis River on July 31 and October 2, 2013 (Figure 2-1, Table 2-1). At each site, a CTD cast was performed to determine the conductivity, temperature, depth, and some other physiochemical parameters of the water. In addition, water samples were collected with Niskin bottles on rosette sampler aboard the R/V Blue Heron. The water samples were stored at 4°C after collection. The first portion of water samples was preserved for bacterial abundance measurement within 1 h of collection. The second portion was processed for environmental parameters measurements within 1 day. The last portion was filtered to collect separate particle-associated and free-living bacterial communities (within one week).

Bacterial abundance

Triplicate 10 ml water samples from each site were preserved with 37% (w/v) formaldehyde solution (1.8% final concentration) within one hour after collection, and were stored in the dark at 4°C for up to 2 weeks. A small portion (0.5-1 ml) of each preserved sample was placed on a black polycarbonate filter (2.0 cm dia., 0.22 µm pore; GE Water & Process Technologies, Trevose, PA), and 200 µl of 4'6-diamidino-2-phenylindole (DAPI; 10 µM final concentration) was added to stain DNA in microbial cells for 5 minutes. The stained prokaryotic cells were filtered onto the membrane filter

under low pressure (15 cm Hg), and then counted using a Nikon Eclipse 80i epifluorescence microscope (Porter and Feig, 1980).

Bacterial community composition analysis

Serial filtration. Triplicate water samples from each site were serially filtered through 2.0 µm-pore Nuclepore polycarbonate (15 cm Hg vacuum) and then 0.22 µm-pore Duropore membrane filters (47 mm dia.; 50 cm Hg vacuum) to collect PA and FL bacterial cells. Membrane filters were stored in Whirl-Pak® bags (Nasco, Fort Atkinson, WI) at -80°C until DNA could be extracted.

DNA Extraction. Some organic material, clay particles, and humic acids could be captured along with bacterial cells on the membrane filters and inhibit PCR amplifications (Kirk *et al.*, 2004). Therefore, a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) was used for all DNA extractions. Each filter was cut into small pieces (c.a. 2mm × 20 mm) with sterilized scissors, and all filter pieces were used for DNA extraction following the manufacturer's protocol. Extracted DNA from all the samples was frozen at -80°C in separate microcentrifuge tubes until further analysis.

Polymerase Chain Reaction (PCR). The bacterial V6 hyper-variable region of the 16S rRNA gene was amplified in triplicate with tagged PCR primers (967F and 1046R). 967F was in the form of a cocktail of five modified primers (5'-CNACGCGAAGAACCTTANC, 5'- CAACGCGAAAAACCTTA, 5'-CAACGCGCAGAACCTTACC, 5'-ATACGCGARGAACCTTACC, and 5'-CTAACCGANGAACCTYACC), which was described to increase the number of taxa

that matched primer sequences (Teske and Sorensen, 2008). The 1046R reverse primer (5'-ID-CGACRRCCATGCANCACCT) had a barcoded Illumina adapter sequence including a six-base multiplexing identification barcode which was unique to each sample (Bartram *et al.* 2001). The PCR amplification mixture (20 µl/reaction) contained: 1 µl 200 µM dNTPs (Promega, Madison, WI); 5 µl 10× PCR buffer (Denville Scientific, South Plainfield, NJ); 1 µl 5.0 units of Choice Taq DNA polymerase (Denville Scientific, South Plainfield, NJ); 2 µl 0.2 µM concentration of each PCR primer; and 11 µl Nuclease-Free water. Fifty ng of bacterial DNA were added to amplification mixtures as the DNA template at the last step. PCR amplification was conducted in a BioRad DNA Engine Thermal Cycler using the calculated control method. After an initial denaturation at 95°C for 5 min, 25 PCR cycles were completed each consisting of a 95°C denaturation step for 30 sec, 55°C annealing step for 30 sec, and a 72°C extension step for 30 sec. Finally, a 2 min extension at 72°C ended each PCR reaction run. PCR products were cleaned using an Ultraclean PCR Clean-up DNA Purification Kit (MoBio Laboratories Inc., Carlsbad, CA). The concentration and quality of the PCR products were assessed on a Nanodrop 2000c spectrophotometer (Wilmington, DE).

Illumina Sequencing and Analysis. The triplicate PCR products from each sample with unique tags were pooled for each bacterial community sample, and then all PCR products were sent to the University of Minnesota Genomics Center for running paired-end multiplex sequencing on an Illumina MiSeq platform. Raw sequences were obtained as .fastq files and submitted to the National Center for Biotechnology Information Sequence Read Archive. Sequencing results are available through GenBank

BioProject PRJNA357109. Sequence reads were analyzed on the Minnesota Supercomputing Institute server with the mother software program, version 1.27.0 (Schloss *et al.*, 2009). The uninformative and primer sequences were trimmed off from the original sequence reads, and different samples were sorted based on the barcodes specific to each sample. Sequences were aligned to the SILVA database (Pruesse *et al.*, 2007), and then chimeras were also removed by using UCHIME (Edgar *et al.*, 2011). The reasonable sequence reads from each sample were randomly subsampled to normalize any difference in the numbers of sequence reads obtained from each sample while still capturing as much genetic diversity as possible. The processed sequences were then taxonomically classified into operational taxonomic units (OTUs) at the ≥ 97% sequence similarity level using the Wang method of furthest neighbor clustering and the Ribosomal Database Project (RDP) taxonomic database release 9 (Cole *et al.*, 2009). All sequences with abundances < 2 were removed before further analysis.

Environmental parameters measurements

Chlorophyll *a*. Triplicate portions of water from each site were filtered onto GF/F glass filter filters, and photosynthetic pigments were extracted from the filters with 90% (v/v) acetone at -20°C for 18-24 h. 90% Acetone (v/v) was made by mixing 900 ml of acetone with 100ml of Milli-Q water. Pigment extracts were analyzed using a Turner 10-AU fluorometer (Turner Designs, Sunnyvale, CA) before and after acidification with 6N HCl (v/v). Acidification was used to correct for interference by pheophytin *a*, which is the common degradation product of chlorophyll *a*. Relative fluorescence units (RFU)

were used to calculate chlorophyll *a* (µg/L) concentrations in the water samples (Lorenzen, 1967).

Particulate organic carbon (POC) and dissolved organic carbon (DOC). One POC sample from each site was collected on a GF/C glass-fiber filter (Whatman, 47mm, 1.2 µm nominal pore size, pre-combusted and pre-weighed) by filtering water until the filter clogged. GF/C glass fiber filters are usually used for collection of suspended solids in natural waters (Aas and Hicks, 1993). These filters were stored at -20°C until POC could be analyzed using a Costech ESC 4010 elemental analyzer as described in Zigah *et al.* (2011). In addition, a 40 ml portion of each filtrate was collected in an acid-washed and combusted brown glass vial for DOC analysis, and acidified to pH 2 with 40 µl 6N HCL to remove inorganic carbon and leave the non-purgeable organic carbon (NPOC) in the sample. All processed samples were stored at 4°C for up to 2 weeks until DOC was measured using a TOC-VCSH/SCN Total Carbon Analyzer (Shimadzu, Columbia, MD) as described by Zigah *et al.* (2011).

Transparent exopolymer particles (TEP). TEP concentrations in water were determined by the Alcian blue staining spectrophotometric method (Passow and Alldredge, 1995). Triplicate fresh water samples from each site were gently filtered through polycarbonate filters (0.4 µm pore size, 47 mm dia., GE Water & Process Technologies, Trevose, PA) at constant 150 mm Hg vacuum. Particles on filters were stained for less than 2 sec with 0.5 ml of 0.02% Alcian blue solution in 0.06% acetic acid (pH = 2.5) and then rinsed with Milli-Q water. The filters were placed into vials containing 6 ml of 80% (v/v) sulfuric acid, and the vials were swirled for at least 2 hours.

Absorption of the solution were measured using a Spectrophotometer (GENESYS 20, Thermo Spectronic) at 787 nm. TEP concentrations were reported in units of "μg/L Xeq", which relates the amount of absorbed Alcian blue to the equivalent weight of the xanthan gum (8GX, Sigma-Aldrich).

Statistical analyses

Ordination analysis. The distance matrices between samples were calculated using the Bray-Curtis measure of dissimilarity (Bray and Curtis, 1957) and imported into PC-ORD version 6.08 software for nonmetric multidimensional scaling (NMS) analysis. Five environmental variables of each sample site were analyzed with distance matrices in PC-ORD version 6.08 to examine the relationships between bacterial community structures (determined with the partial 16S rRNA gene sequences from each community sample) and environmental variables. In NMS plots, samples with greater similarity are closer in ordination space, and relationships between environmental variables and bacterial communities were performed using a joint-plot overlay of a second matrix containing the environmental variables.

Correlation analysis. To determine the relationship between 5 environmental variables and bacterial community structures, the Mantel test was used to calculate Pearson product-moment correlation coefficients (r) and the corresponding p values using PC-ORD version 6.08. The Mantel test is usually used to evaluate statistical correlations between environmental variables and ordination scores. Spearman rank correlations were calculated to examine relationships between environmental parameters and the

abundance of specific lineages, which were performed using online software PAST (Hammer *et al.*, 2001). All statistical analyses used $p < 0.05$ as the cutoff for statistical significance.

Bacterial community richness, diversity and evenness. Bacterial community richness (total number of unique OTUs), Shannon-Wiener diversity, and Shannon-index based evenness were calculated for all samples based on following equations using mothur version 1.27.0 (Schloss *et al.*, 2009).

$$H' = -\sum_{i=1}^{S} \frac{n_i}{N} \times \ln \frac{n_i}{N}$$

$$J' = \frac{H'}{H'_{max}} = \frac{H'}{\ln S}$$

where *H'* and *J'* are Shannon-Wiener diversity and evenness indices respectively. *S* is the number of observed OTUs, n_i is the number of individuals in OTU *i*, and *N* is the total number of individuals in the community.

Analysis of molecular variance (AMOVA) and similarity percentage (SIMPER). AMOVA is a method to detect whether the centers of two groups are more separated than the variation among samples in same group by utilizing molecular markers (Excoffier *et al.*, 1992), which was performed using mothur version 1.27.0 (Schloss *et al.*, 2009). SIMPER analysis was used to evaluate the overall average dissimilarity percentage in separated groups determined by AMOVA analysis. The dissimilarity percentage was measured by using the Bray-Curtis measure, which was performed using the online software program PAST (Hammer *et al.*, 2001). SIMPER analysis was also

used to identify the contribution of each bacterial taxon to the dissimilarity between groups.

Detection of differential abundant features (Metastats). Significant differences in abundance of bacterial phyla or orders between different assemblages were detected using the online software Metastats (White *et al.*, 2009), with 1000 permutations and $p < 0.05$ as the cutoff for statistical significance.

RESULTS

Overview of bacterial communities along the transect

For all samples collected in July and October, 19,505 Illumina sequence reads per site were subsampled for further analysis to avoid any site-specific difference in read numbers. The coverage of each sample was estimated at 96 ± 1.4%, which indicated in this study our sampling was very thorough and only few OTUs remained unsampled (Table 2-5). A mean of 1,638 ± 323 OTUs were identified among all PA samples, while 1,001 ± 284 mean OTUs were observed among all FL samples. Considering all samples, 99.9% of OTUs were classified as *Bacteria*, and 0.1% as *Archaea*, and all classified OTUs could be assigned to 18 ± 3 phyla among all sites. Among all observed bacterial phyla, the *Proteobacteria* was most dominant, and accounted for 64.9 ± 12.5% of total sequences. Several other abundant bacterial phyla (at least 1% of total sequences) included *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria*, *Verrucomicrobia*, *Planctomycetes*, and *Firmicutes* (Figure 2-2). These seven phyla accounted for 97.2 ± 1.1% of sequences among all samples. A total of 6 less abundant phyla (abundance

ranging from 0.1% to 1% of total sequences) accounted for $2.5 \pm 1.0\%$ of total sequences reads among all samples, and included the *Acidobacteria*, *Synergistetes*, *Gemmatimonadetes*, *Chloroflexi*, *Deinococcus-Thermus*, and *Nitrospira* bacterial phyla (Figure 2-3).

Comparison of PA and FL bacterial community structure

A nonmetric multidimensional scaling (NMS) ordination was built based on distance matrices between samples followed by AMOVA to detect differences between PA and FL bacterial communities in July and October, 2013 (Figure 2-4, Table 2-2). The FL communities formed a distinct group that was different from the composition of the PA bacterial communities ($p < 0.001$). Regardless of the sample month, the PA bacterial communities from nearshore Lake Superior (site 1) were different ($p = 0.03$, Table 2-2) from the PA bacterial communities from the estuary (sites 2 to 5), while the FL fraction did not show any difference between nearshore Lake Superior and estuary sample sites ($p = 0.11$, Table 2-2). In addition, the PA bacterial communities in the estuary (sites 2 to 5) were different in July and October, 2013 ($p = 0.018$, Table 2-2), while FL bacterial communities were not different in July and October ($p = 0.216$, Table 2-2).

Bacterial community and environmental parameters

Seven environmental parameters (water temperature, light transmission, conductivity, chlorophyll *a* (Chl *a*), dissolved organic carbon (DOC), particulate organic carbon (POC), and transparent exopolymer particles (TEP) as well as bacterial abundance

in water) were measured at each site in July and October, 2013 (Table 2-1). Relationships between environmental parameters and bacterial community structure were explored by using a bioplot overlapping a second matrix including all the environmental parameters in NMS analyses (Figure 2-4). Overall, no single environmental parameter measured clearly explained the difference in the composition of PA and FL bacterial communities (Figure 2-4). However, some of these parameters were correlated with PA and FL bacterial community structures (Table 2-3). Temperature, light transmission, conductivity, DOC and POC concentrations were correlated with the PA bacterial community structure along the sample transect from the St. Louis River to Lake Superior (all $p's < 0.05$, Table 2-3), while temperature and TEP concentration were correlated with the FL community structure (both $p's < 0.05$, Table 2-3) along the sample transect.

Correlations between 7 environmental parameters and abundance of the seven most abundant phyla identified from all sites (*Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria*, *Verrucomicrobia*, *Planctomycetes*, and *Firmicutes*) were calculated. Temperature was correlated with the abundance of PA *Proteobacteria* (r = 0.758, p = 0.011), the total (PA and FL) *Bacteroidetes* (r = -0.939, p < 0.001) and *Verrucomicrobia* (r = -0.818, p = 0.004). Light transmission was correlated with total *Bacteroidetes* (r = 0.818, p = 0.004), *Verrucomicrobia* (r = 0.697, p = 0.025), and total *Planctomycetes* (r = -0.745, p = 0.013). TEP concentrations increased with FL *Proteobacteria* (r = 0.733, p=0.016), but decreased with FL *Actinobacteria*, (r = -0.661, p = 0.038), and FL *Cyanobacteria* (r = -0.855, p = 0.002). DOC and POC concentrations increased with the total (PA and FL) *Planctomycetes* (r = 0.733, p = 0.016; r = 0.661, p =

0.038; respectively). Conductivity and chlorophyll *a* were not related to the abundance of any bacterial phyla in the PA or FL bacterial communities.

Several environmental parameters were significantly correlated with each other (*p's* < 0.05, Table 2-4). For example, light transmission along the transect decreased with every environmental parameter measured except water conductivity and Chl *a* concentration. Water conductivity increased with all measures of organic materials (Chl *a*, TEP, DOC and POC concentrations), and all the measures of organic materials increased with each other along the sample transect (Table 2-4).

PA and FL bacterial community diversity and composition

Overall, PA bacterial communities had a higher richness (*sobs*), a higher Shannon-Wiener diversity index (*H'*) and a higher Shannon-index based evenness (*J'*) than FL bacterial communities (Table 2-5), and all the indices were significantly different from PA and FL bacterial communities (all *p's* < 0.01). Within the PA fraction, there was no clear trend of diversity and evenness in bacterial communities along the transect, but the FL bacterial communities had the lowest diversity and evenness index at site 3 (within harbor). As mentioned before, seven bacterial phyla could account for more than 95% of total sequences among all samples. The abundance of several phyla were significantly different between PA and FL bacterial communities (all *p's* < 0.01). For example, members of the *Proteobacteria* and *Actinobacteria* were both more abundant in FL communities (72.6 ± 11.6% and 9.1 ± 3.8% respectively) than in PA communities (57.2 ± 7.8% and 4.7 ± 2.4% respectively), while members of the

Cyanobacteria/Chloroplast and *Firmicutes* were more abundant in PA communities (11.3 ± 8.3% and 2.4 ± 0.7% respectively) than FL communities (0.9 ± 0.6% and 1.4 ± 0.8% respectively). For the 6 less abundant phyla, members of the *Acidobacteria*, *Gemmatimonadetes*, and *Nitrospira* were all more abundant in PA communities (1.3 ± 0.6%, 0.8 ± 0.5%, and 0.2 ± 0.1% respectively) than in FL communities (0.4 ± 0.2%, 0.3 ± 0.2%, and 0% respectively).

Overall, the composition of PA bacterial communities was different than FL bacterial communities. To obtain more detailed information regarding the phylogenetic composition of PA and FL bacterial communities, all the partial 16S rRNA gene sequences obtained by Illumina sequencing were also assigned to bacterial orders (137 ± 34) for all samples (Figure 2-5). Sequences from the 17 most abundant bacterial orders accounted for 81.1 ± 4.6% and 89.6 ± 3.1% of all sequences found in the PA and FL bacterial communities, respectively. DNA sequences from members of the *Burkholderiales*, an order in the *Proteobacteria* phylum, were most commonly encountered in both PA and FL communities (20.7 ± 3.0% and 29.3 ± 5.0% of all sequences, respectively). The other four most abundant bacterial orders in PA communities were in three bacterial phyla and were the Cyanobacteria/Chloroplast (11.3 ± 8.3%), *Sphingobacteriales* (7.9 ± 2.9%, *Bacteroidetes* phylum), *Rhodospirillales* (5.9 ± 2.0%, *Proteobacteria* phylum), and *Rhizobiales* (5.6 ± 2.4%, *Proteobacteria* phylum). The most commonly encountered bacterial orders in FL communities were the *Rhodospirillales* (22.1 ± 11.1%, *Proteobacteria* phylum), *Actinomycetales* (8.8 ± 3.6%, *Actinobacteria* phylum), *Sphingobacteriales* (7.7 ± 5.1%, *Bacteroidetes* phylum), and

Methylophilales (5.7 ± 2.7%, *Proteobacteria* phylum). SIMPER analysis was performed to evaluate the dissimilarity between PA and FL bacterial communities at the taxonomic level of bacterial orders, and Metastats was used to detect the significantly differences in the abundance of these orders. *Rhodospirillales*, *Cyanobacteria*, *Burkholderiales*, *Actinomycetales*, and *Methylophilales* were the top 5 bacterial orders and accounted for more than 50% of the average dissimilarity between PA and FL communities, and these 5 orders were all significantly different in abundance between the PA and FL communities (all $p's<0.01$, Figure 2-5). Members of the *Rhodospirillales*, *Burkholderiales*, *Actinomycetales*, and *Methylophilales* were more abundant in FL communities than in PA communities, while Cyanobacteria/Chloroplast were more common in the PA communities. Members from less abundant bacterial orders, *Flavobacteriales* (4.7 ± 3.2%), *Caulobacterales* (3.9 ± 2.1%), *Neisseriales* (2.6 ± 1.7%), *Planctomycetales* (3.0 ± 0.6%), *Hydrogenophilales* (1.1 ± 0.9%), and Subdivision 3 unidentified order (1.0 ± 0.4%) were all more abundant in PA communities than in FL bacterial communities (Figure 2-5).

There was no difference in the composition of free-living bacterial communities at the different sites in July or October, 2013 (Figure 2-4). However, particle-associated bacterial communities were different at the nearshore Lake Superior (site 1) and estuarine sites (sites 2 to 5). Therefore, we utilized Metastats and SIMPER to analyze the differences between PA bacterial communities collected from nearshore Lake Superior (site 1) and the inner estuary (sites 2 to 5; Figure 2-6). Although no differences were detected between the Shannon-Wiener diversity or evenness indices of PA-Lake and PA-

Estuary groups, there were some differences between the number of sequences contributed by several bacterial orders in these groups (Figure 2-6). Together, the 17 most abundant bacterial orders accounted for 84.4 ± 2.9% and 83.5 ± 2.3% of total sequences read of PA-Lake and PA-Estuary groups, respectively. Among the 17 most abundant orders, members of the Cyanobacteria/Chloroplast, *Flavobacteriales*, *Sphingobacteriales*, *Verrucomicrobiales*, *Caulobacterales*, *Sphingomonadales*, *Burkholderiales*, and *Rhodospirillales* were the top 8 bacterial orders that contributed more than 50% of the average dissimilarity between the PA-Lake and PA-Estuary groups, and the relative abundance of DNA sequences from these 8 bacterial orders was greater in the PA-Lake community, except for the Cyanobacteria/Chloroplast and *Burkholderiales* (all $p's < 0.05$). The PA-Lake group had more bacterial sequences from members of the *Flavobacteriales* (8.7 ± 4.6%), *Sphingobacteriales* (11.6 ± 1.2%), *Verrucomicrobiales* (5.4 ± 3.7%), *Caulobacterales* (6.4 ± 1.9%), and *Sphingomonadales* (4.9 ± 1.8%), but fewer sequences *Rhodospirillales* (3.9 ± 0.1%) compared to PA-Estuary group (3.7 ± 2.1%, 7.0 ± 2.3%, 0.9 ± 0.6%, 3.3 ± 1.8%, 1.7 ± 0.9%, and 6.3 ± 1.9% respectively; Figure 2-6). In addition, sequences from the *Neisseriales* (3.1 ± 1.6%), *Actinomycetales* (4.7 ± 2.6%), *Hydrogenophilales* (1.4 ± 0.9%), and *Xanthomonadales* (1.2 ± 0.3%) were also found to be more abundant in the PA-Estuary group than in the PA-Lake group (0.9 ± 0.2%, 3.2 ± 1.0%, 0.2 ± 0.1%, and 0.2 ± 0.01% respectively).

In addition to the difference in PA-Lake and PA-Estuary bacterial communities, there was also a difference in the composition of PA-Estuary bacterial communities in July and October (Figure 2-7). The bacterial community Shannon-Wiener diversity index

for the PA-Estuary group was higher in July (5.61 ± 0.22) than in October (5.13 ± 0.16, $p < 0.05$). Among the17 most abundant bacterial orders, sequences from the Cyanobacteria/Chloroplast, *Burkholderiales*, *Rhizobiales*, *Rhodocyclales*, *Planctomycetales*, *Rhodobacterales*, and *Sphingobacteriales* (top 7 bacterial orders) contributed more than 50% to the dissimilarity between PA-Estuary bacterial communities in July and October. Bacterial sequences from the *Rhizobiales* (7.8 ± 1.3%), *Neisseriales* (4.3 ± 1.3%), and *Hydrogenophilales* (2.1 ± 0.5%) were more abundant in particle-associated bacterial communities from the estuary in July (all p's < 0.05), while sequences from the Cyanobacteria/Chloroplast (18.1 ± 9.2%) and *Verrucomicrobiales* (1.3 ± 0.5%) were more abundant in October (both p's < 0.05).

DISCUSSION

Bacterial community structure and environmental parameters along the transect

Along a similar transect from the river to the nearshore lake, Welch (2012) reported total bacterial communities of the lake, the harbor, and the river were well separated using T-RFLP DNA fingerprint analysis, and LaPara (2011) reported similar results using NMS ordination analysis based on automatic ribosomal intergenic spacer analysis (ARISA). In this study, PA communities but not FL communities were different in the lake and the estuary, which could be seen in the NMS ordination analysis (Figure 2-2). One of the differences between this study and the previous studies mentioned above is the techniques that were used for bacterial community analysis. Welch and LaPara used T-RFLP and ARISA analysis, which are both community fingerprints analysis,

while Illumina sequencing of the 16S rRNA gene of community members was used in this study to thoroughly analyze the bacterial composition. The big disadvantage of community fingerprints analyses are that they provide qualitative but not quantitative data (Osborn *et al.*, 2000), and are unable of detecting the presence of rare taxa (Bent *et al.*, 2007). The abundance coverage of community fingerprints is very low. For example, the total number of terminal restriction fragments (TRFs) identified by Welch (2012) ranged from 103 to 126. In this study, the large number of partial bacterial sequences (i.e. >19,000) obtained for each individual sample provided for more than 95% coverage of estimated OTUs in these communities. In addition, one terminal restriction fragment (TRF) may indicate more than one distinct bacterial species, and one bacterial species may contribute to multiple fragments (Kent *et al.*, 2003). Therefore, using TRFs to evaluate differences between communities could inaccurately estimate the number of taxa (or OTUs) in communities.

There were clear gradients along the transect from the lower St. Louis River through the Duluth-Superior Harbor to nearshore of Lake Superior in five environmental parameters. For example, values of temperature, conductivity, chlorophyll *a*, DOC, POC, and TEP were higher but light transmission was lower at the harbor and river sites than in the nearshore lake (Table 2-1). A well-studied feature, the estuarine turbidity maxima (ETM) forms in river dominated oceanic estuaries due to the interaction between river flow and ocean tidal movement (Berner and Berner, 2012). Instead of ocean tidal forces, seiche activity in freshwater estuaries like those in the Great Lakes could also interact with river outflow and influence the amount of suspended solids, inorganic or organic

particles, and nutrients (Klarer and Milli, 1994). In this study, there was lower light transmission and higher POC in the harbor and river (sites 3, 4, and 5) than in nearshore Lake Superior. There was also higher DOC at sites 4 and 5 in the St. Louis River, which was in accordance with Welch's study (Welch, 2012).

Temperature is an important environmental factor that can affect bacterial abundance (Kan *et al.*, 2006) and production (Crump and Hobbie, 2005), and one of the most important seasonal drivers regulating bacterial community structure (Heidelberg *et al.*, 2002; Rösel *et al.*, 2012). PA bacterial community structure showed a strong correlation with temperature, conductivity, DOC and POC, while FL community structure significantly correlated with temperature and TEP along the transect from the river through the harbor to the lake (all $p's < 0.05$) (Table 2-3). In this study, both PA and FL bacterial community structures in the estuary were associated with higher water temperatures, and *Proteobacteria* in the PA bacterial communities, showed a positive correlation with temperature.

The light transmission was highest in nearshore Lake Superior (site 1), and dramatically decreased through the harbor and reached its lowest level in the lower St. Louis River (site 5) (Table 2-1). Higher light transmission can indicate lower amounts of suspended solids and turbidity so it was not surprising that light transmission was negatively correlated with chlorophyll *a*, TEP, DOC and POC concentrations along this transect (Table 2-4). The St. Louis River and its tributaries have large inputs of suspend solids from eroded soils (e.g. red clay), particulate organic matter, colored dissolved organic matter (CDOM) and re-suspended sediment (Bahnick and Markee, 1985), treated

wastewater effluent, storm-water runoff, and sediment released by ballast water discharging (Klarer and Millie, 1994; Villac and Kaczmarska, 2011).

Transparent exopolymer particles (TEP) are one type of EPS and made up of acid polysaccharides. Usually, TEP formation starts with dissolved precursor substances (Passow, 2000), which are released by aquatic organisms, such as phytoplankton (e.g. diatoms and *Cyanobacteria*). TEP concentrations are usually closely associated with phytoplankton blooms and increased Chlorophyll *a* concentrations (Passow, 2002a), as also observed in this study (Table 2-4). Interestingly, only the structure of FL bacterial communities (not PA communities) and the abundance of FL proteobacterial sequences, were strongly correlated with the concentration of TEP along the transect.

Although some physical and chemical parameters demonstrated clear gradients along the transect (Table 2-1), none of these parameters alone could explain the observed differences between PA and FL bacterial communities. Therefore, factors that affect the separation of PA and FL communities might be internal but not external, which means that some groups of bacteria are more naturally prone to attach to particles while some others are not.

PA and FL bacterial community diversity and composition

Particle-associated bacterial communities were different from FL bacterial communities along the transect from the lower St. Louis River to nearshore Lake Superior. The PA bacterial community had a larger number of OTUs, higher Shannon-Wiener diversity and evenness indices. Similar results have been reported in previous

studies about PA and FL bacterial communities from different aquatic environments, including a coastal lagoon area (LaMontagne and Holden, 2003), the Mediterranean Sea area (Acinas *et al.*, 1999; Ghiglione *et al.*, 2007; Crespo *et al.*, 2013), but not in the western basin of Lake Erie (Mou *et al.*, 2013) and some marine ecosystems (Hollibaugh *et al.*, 2000; Kellogg and Deming, 2009). The high coverages (96 ± 1.4%) of sequenced samples in this study indicated additional sampling would not necessarily detect many more unique OTUs. Bacterial richness detected in this study (1320 ± 441 OTUs and 134 ± 37 orders among all samples), however, was much higher than in several previous studies including a study of bacterial communities in the western basin of Lake Erie (only 35-66 unique orders were detected; Mou *et al.*, 2013), in a coastal lagoon area, where only 19-39 normalized replicated peaks were detected (LaMontagne and Holden, 2003), and in the northwest Mediterranean Sea, where between 354-978 unique bacterial OTUs were detected (Crespo *et al.*, 2013).

More than 95% of the total sequences from all samples in this study were assigned to seven bacterial phyla. Among these phyla, the abundance of *Bacteroidetes*, *Verrucomicrobia*, and *Planctomycetes* sequences were not significantly different in the PA and FL bacterial communities. *Proteobacteria* was the most dominant phylum among both PA (57.2 ± 7.8%) and FL fractions (72.6 ± 11.6%), but the abundance of proteobacterial sequences was different between these groups and contributed the most (38.2%) to their difference. Within the *Proteobacteria* phylum, *Rhodospirillales* (*Alphaproteobacteria*) and *Burkholderiales* (*Betaproteobacteria*) were the two most abundant bacterial orders, and the sequences from both orders were more common in FL

communities. *Rhodospirillales* species are commonly found in freshwater environments, and most members in this order are known as photoheterotrophic bacteria but their photosynthesis occurs only under anoxic conditions. Their free-living life history strategies may help them harvest enough sunlight for photosynthesis and degrade large number of organic compounds within the harbor. Members of the *Burkholderiales* within the *Betaproteobacteria* class were also very abundant in both PA and FL bacterial communities (20.7 ± 3.0%; 29.3 ± 5.0%; respectively). Previous studies have indicated that members of *Betaproteobacteria* are often associated with particles (Weiss *et al.*, 1996; Šimek *et al.*, 1999; Lemarchand *et al.*, 2006) and *Cyanobacteria* (Eiler *et al.*, 2006). In this study, sequences from the *Burkholderiales* order were very abundant in PA communities but even more abundant in FL communities indicating that some members within this order may have free-living life history strategies. Actually, some previous studies found that several species within the *Burkholderiales* are described to be free-living planktonic freshwater bacteria, such as the new *Polynucleobacter* species within the family *Burkholderiaceae* inhabiting freshwater systems such as lakes, ponds, rivers, and streams (Hahn *et al.*, 2010; Hahn *et al.*, 2011a; Hahn *et al.*, 2011b; Hahn *et al.*, 2012), as well as two species belonging to the genus *Limnohabitans* (*Comamonadaceae*), which could account for up to 30% of free-living bacteria in a broad range of freshwater systems (Glöckner *et al.*, 2000; Zwart *et al.*, 2002; Page *et al.*, 2004; Šimek *et al.*, 2010).

In addition to *Proteobacteria*, sequences from the *Cyanobacteria* and *Actinobacteria* bacterial phyla were also different between PA and FL bacterial communities, and contributed more than 10% to this differences. *Cyanobacteria* were

also detected to be more abundant in the PA (11.3 ± 8.3%) than the FL (0.9 ± 0.6%) fraction, which could be caused by larger cell size or colony-forming *Cyanobacteria* being captured in PA fractions. Similar results were reported in several previous studies, such as a long term investigation of PA and FL bacterial communities in Lake Tiefwaren (Germany) (Rösel *et al.*, 2012), and in four lakes located in the Mecklenburg Lake District (northwestern Germany) (Allgaier and Grossart, 2006). *Actinomycetales* was the most abundant order within the phylum *Actinobacteria* (94.2 ± 3.8%) in this study, and was significantly higher in the FL bacterial community (8.8 ± 3.6%) than the PA community (4.4 ± 2.4%). *Actinobacteria*, one phylum of Gram-positive bacteria, were historically thought to be found primarily in soil environments (Goodfellow and Williams, 1983), but additional studies have found that the members of *Actinobacteria* were common and numerically abundant in freshwater ecosystems. Some of these studies found that members of the *Actinomycetales* were more numerically abundant in FL bacterial assemblages in both oligotrophic and eutrophic freshwater lakes (Allgaier and Grossart, 2006; Li *et al.*, 2011; Rösel *et al.*, 2012; Mou *et al.*, 2013). The differences between *Actinomycetales* in attached and free-living communities may indicate different phylotypes have different abilities to adapt to their respective microenvironments (Allgaier and Grossart, 2006). For example, most members within the family *Mycobacteriaceae* (*Actinomycetales*) were associated with eukaryotic hosts, which caused bacteria in this family to be almost completely associated with PA communities (Cirillo *et al.*, 1997; Bland *et al.*, 2005). Members of the acI lineage within the *Actinomycetales* were more abundant in FL assemblages (Allgaier *et al.*, 2007; Tang *et*

al., 2009), and their free-living lifestyle in freshwater surface waters may be due to their potential ability to harvest light to produce energy (Newton *et al.*, 2011).

Other bacterial orders, such as *Methylophilales*, *Flavobacteriales*, *Caulobacterales*, *Neisseriales*, *Planctomycetales*, and *Hydrogenophilales*, were at relatively lower abundance, but still showed significant differences between the PA and FL bacterial communities. Some of these orders are still largely understudied.

Differences between PA bacterial community composition in the lake and estuarine environments

Some of environmental parameters were different in nearshore Lake Superior compared with the St. Louis River estuary. For example, temperature, conductivity, DOC and POC were all higher in the estuary, while light transmission was significantly higher at the nearshore Lake Superior site (all $p's<0.05$). Welch (2012) also found nearshore Lake Superior had a high level of NO_3^-, but lower values of NH_4^+ and SRP (soluble reactive phosphorus) than the estuary. Therefore, nearshore Lake Superior and the St. Louis River estuary are different aquatic habitats that may influence bacterial community compositions.

Particle-associated bacterial communities (at the order level) were different in nearshore Lake Superior and the estuary while FL bacterial communities were not different. Although the PA bacterial community richness, diversity and evenness were not significantly different between the lake and estuary (all $p's > 0.05$), the composition of PA bacterial communities in the lake and the estuary were highly different from each

other. Sequences from the *Flavobacteriales* (8.2%), *Sphingobacteriales* (7.2%), and *Verrucomicrobiales* (6.9%) were more abundant in the PA bacterial community from nearshore Lake Superior compared to the St. Louis River estuary and contributed to the difference between these PA communities. Bacteria in both the *Flavobacteriales* and *Sphingobacteriales* (*Bacteroidetes* phylum) are chemoorganotrophic and possess the ability to take up and degrade dissolved organic matter (DOM) and various biopolymers (such as cellulose and chitin) in aquatic environments. Therefore, it might be expected that higher abundances of *Flavobacteria* may occur in locations or at times when DOM concentration are high. Only a few studies have observed this trend. Eilers *et al.* (2001) found the maximum abundance of *Flavobacteria* during short-living phytoplankton blooms in the North Sea, and Battin *et al.* (2001) found that the abundance of *Flavobacteria* increased dramatically after a storm when a large amount of allochthonous organic material washed into a glacial stream. In this study, sequences from both the *Flavobacteriales* and *Sphingobacteriales* were more abundant in PA bacterial communities in nearshore Lake Superior rather than the St. Louis River estuary where higher chlorophyll *a*, TEP, DOC and POC concentrations were observed. Thus, factors besides organic matter concentrations must also influence the growth and abundance of bacteria in these two orders.

Sequences from members of the *Verrucomicrobiales* were also more abundant in PA communities at the nearshore Lake Superior site (5.4 ± 3.7%) than in the estuary (0.9 ± 0.6%), and this order accounted for 70.2 ± 12.9% and 26.8% ± 12.0% of total *Verrucomicrobia* in lake and estuary respectively. In this study, the abundance of the

Verrucomicrobiales order decreased with temperature (r = -0.915, p < 0.001), and increased with light transmission (r = 0.624, p = 0.043), and members from this order were more abundant in PA communities at the nearshore Lake Superior site with lower concentration of Chlorophyll *a*, TEP, DOC and POC. The clade CL120-10 *Verrucomicrobia* were found to be more abundant in the euphotic water columns of Crater Lake, which may have been due to their tolerance to UV light and their ability of recycling carbon and nutrient that coupled with phytoplankton growth and primary production (Urbach *et al.*, 2001). However, very little is known about the function and preference of habitats of the *Verrucomicrobia* phylum.

Caulobacterales (*Alphaproteobacteria*) and *Sphingomonadales* (*Alphaproteobacteria*) were also more abundant in PA communities at the nearshore Lake Superior site. Some members of the *Caulobacterales* are stalked cells that use this appendage to attach to surfaces. So, it was not surprising to find members of this order in PA bacterial communities but it is unknown why they were more abundant in nearshore Lake Superior. Worth mentioning, some members of the order *Sphingomonadales* are considered to be microcystin-degrading bacteria (Ho *et al.*, 2007; Ho *et al.*, 2010), indicating that some members of this order may associate with *Cyanobacteria*.

Differences between the PA bacterial community composition in summer and fall

PA bacterial communities from the St. Louis River estuary were different in July and October 2013 but FL communities were not. While the total number of OTUs in PA bacterial communities were not different in July and October, bacterial community

diversity and evenness were both higher in July than in October (both *p's* < 0.05). Temperature is a very influential environmental factor that can affect cells, populations, and communities. Water temperature was higher in the St. Louis River estuary when measured in late July while Chlorophyll *a* concentrations were higher in early October 2013 (Table 2-1, both *p's* < 0.001). Similarly, the abundance of cyanobacterial sequences in PA communities from the St. Louis River estuary were also higher in October (18.1 ± 9.2%) compared to July (5.5 ± 1.7%). There are several reasons *Cyanobacteria* can become dominant in aquatic environments. The abundance of *Cyanobacteria* can increase with elevated temperature due to higher temperature optima compared to other phytoplankton (Martin and Katrin, 2000). However, this apparently was not the case in this study. Nutrients, such as phosphorus and nitrogen (NH_4^+), have been indicated to play a central role in regulating the formation of harmful cyanobacterial bloom, especially for Microcystic and other non-N-fixing cyanobacteria (Kappers, 1980; Blomqvist *et al.*, 1994; Paerl, 1988, 2008). However, in this study, we did not measure dissolved inorganic nutrient concentrations, so it cannot be concluded that increased-nutrient concentrations or different nutrient regimes led to the higher abundance of cyanobacterial sequences in October than in July.

Conclusions

In summary, particle-associated (PA) and free-living (FL) bacteria communities were significantly different from each other along the transect from the St. Louis River estuary to nearshore Lake Superior in both summer and fall. PA bacterial communities

had a larger number of OTUs (sobs), a higher Shannon-Wiener diversity index (H') and a higher Shannon-index based evenness (J') than FL bacterial communities. Members of the Rhodospirillales, Burkholderiales, Actinomycetales, and Methylophilales were more abundant in FL communities than in PA communities, while Cyanobacteria/Chloroplast were more common in the PA communities. Although 7 environmental parameters were measured along the transect during both sampling seasons, none of them could clearly explain the difference in the composition of PA and FL bacterial communities. Therefore, we could speculate that the factors that affect the separation of PA and FL communities might be internal but not external, which means that some groups of bacteria are more naturally prone to attach to particles while some others are not.

There were clear gradients in several environmental parameters along the transect from the lower St. Louis River estuary to nearshore Lake Superior. For example, temperature, conductivity, Chlorophyll *a*, DOC, POC, and TEP were greater at the harbor and river sites, but light transmission was higher in nearshore lake site. Therefore, the environmental conditions at the nearshore lake site were very different from these at the harbor or river sites. Only PA bacterial communities were different in nearshore Lake Superior and the estuary while FL bacterial communities were not different. Specifically, sequences from the *Flavobacteriales*, *Sphingobacteriales*, *Verrucomicrobiales*, *Caulobacterales* and *Sphingomonadales* were more abundant in the PA bacterial community from nearshore Lake Superior compared to the St. Louis River estuary.

The difference between sampling seasons can only be seen in PA bacterial communities in estuary sites. Although the water temperature was higher in summer,

more Cyanobacteria/Chloroplast sequences and higher concentration of chlorophyll *a* were found in October.

TABLES

Table 2-1. Environmental parameters measured in water at five sites sampled in the St. Louis River Estuary (Minnesota) on July 31 and October 2, 2013. Standard deviations are shown in parenthesis if applicable.

Site	Latitude	Longitude	Temperature (°C)	Light Transmission (%)	Conductivity (µS/cm)	Chl a (µg/L)	TEP (µg/L Xeq)	DOC (mg/L)	POC (mg/L)	Bacterial Abundance ($\times 10^6$ cell/ml)
Site					Date: July 31					
1	46° 47.070' N	92° 03.415' W	14.0	73.6	0.051	0.27	34.3 (3.9)	2.8	0.12	0.91
2	46° 46.408' N	92° 05.977' W	18.7	24.6	0.086	1.13	219.7 (20.8)	14.2	0.31	4.33
3	46° 44.747' N	92° 07.579' W	20.0	16.9	0.085	0.60	507.5 (64.6)	22.3	0.47	6.10
4	46° 43.403' N	92° 08.598' W	19.9	18.1	0.080	0.59	371.9 (191.9)	25.3	0.37	6.06
5	46° 43.020' N	92° 10.182' W	19.5	14.7	0.076	0.46	173.1 (3.0)	25.0	0.45	5.60
Site					Date: October 2					
1	46° 47.070' N	92° 03.415' W	14.1	69.8	0.050	0.27	95.0 (2.2)	5.5	0.09	1.12
2	46° 46.408' N	92° 05.977' W	14.7	50.1	0.069	1.27	110.4 (5.4)	12.3	0.35	2.21
3	46° 44.747' N	92° 07.579' W	16.3	19.4	0.115	1.70	215.8 (8.9)	30.9	0.74	4.04
4	46° 43.403' N	92° 08.598' W	16.3	15.1	0.113	2.22	188.6 (59.6)	39.2	0.71	4.62
5	46° 43.020' N	92° 10.182' W	16.1	19.4	0.113	1.46	229.2 (42.1)	39.7	0.53	5.48

Table 2-2. Analysis of molecular variance (AMOVA) was used to compare the distance matrix between different types of bacterial communities. Statistical p values were obtained based on AMOVA. Similarity percentages (D) are shown for bacterial orders in the different types of bacterial communities and were calculated with SIMPER.

Group Comparison	*p* value	D (%)
Overall PA vs. FL	<0.001	41.8
PA-Lake vs. PA-Estuary	0.03	32.6
FL-Lake vs. FL-Estuary	0.11	-
PA Estuary July vs. October	0.018	31.3
FL Overall July vs. October	0.216	-

Table 2-3. Mantel test analysis showing the relationship between 7 environmental parameters and particle-associated (PA) or free-living (FL) bacterial communities. Values printed in bold italic typeface indicate significant correlations ($p < 0.05$). The r value is the Pearson's correlation coefficient.

	PA		FL	
	r	*p*	*r*	*p*
Temperature	***0.572***	***0.002***	***0.398***	***0.018***
Light Transmission	***-0.378***	***0.043***	-0.377	0.079
Conductivity	***0.481***	***0.004***	0.264	0.074
Chlorophyll *a* (Chl *a*)	0.1508	0.196	-0.022	0.482
Transparent Expolymer Particles (TEP)	-0.121	0.718	***0.587***	***0.005***
Dissolved Organic Carbon (DOC)	***0.412***	***0.014***	0.066	0.329
Particulate Organic Carbon (POC)	***0.400***	***0.013***	0.152	0.202

Table 2-4. Spearman rank correlations coefficients between environmental variable.

	Temperature	Light transmission	Conductivity	Chl *a*	TEP	DOC	POC
Temperature							
Light transmission	**-0.806****						
Conductivity	0.383	-0.492					
Chl *a*	0.152	-0.358	**0.845****				
TEP	0.612	**-0.685***	**0.815****	**0.697***			
DOC	0.418	**-0.673***	**0.839****	**0.733***	**0.733***		
POC	0.406	**-0.697***	**0.863****	**0.782****	**0.806****	**0.879****	

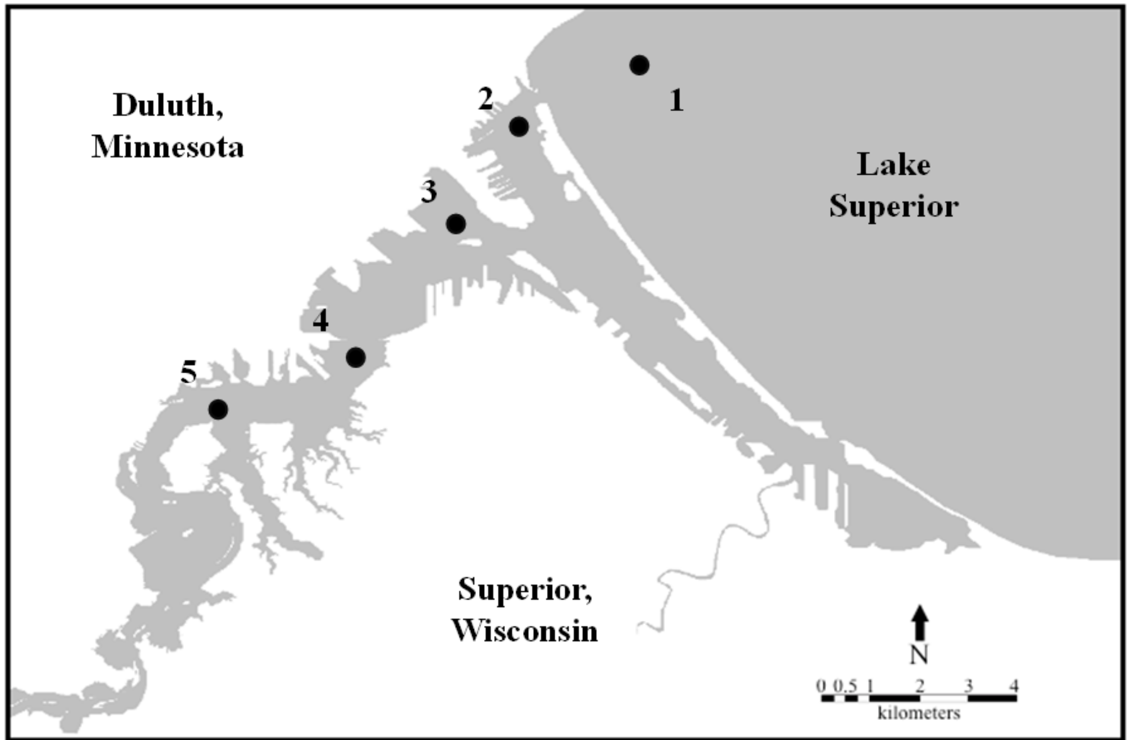
Abbreviations: Chl *a* chlorophyll *a*; TEP transparent exopolymer particles; DOC dissolved organic carbon; POC particulate organic carbon
*Bold are significant at $p < 0.05$; **Bold are significant at $p < 0.01$.

Table 2-5. Sequencing coverage, bacterial richness (*sobs*), Shannon-Wiener diversity (*H'*) and evenness (*J'*) indices were compared between PA and FL bacterial communities at different sites along the transect.

Site		Coverage		*sobs*		*H'*		J'	
		Jul	Oct	Jul	Oct	Jul	Oct	Jul	Oct
1	PA	95%	95%	1708	1688	5.22	5.43	0.70	0.73
	FL	97%	98%	1261	689	4.91	4.00	0.69	0.61
2	PA	95%	98%	1839	747	5.84	4.94	0.78	0.68
	FL	98%	96%	806	1361	3.82	4.51	0.68	0.68
3	PA	95%	95%	1728	1650	5.58	5.33	0.75	0.72
	FL	98%	97%	715	924	3.04	4.32	0.72	0.63
4	PA	95%	96%	1859	1608	5.69	5.13	0.76	0.70
	FL	96%	98%	1269	759	4.51	3.96	0.63	0.60
5	PA	94%	95%	1773	1781	5.31	5.12	0.71	0.68
	FL	96%	98%	1386	842	4.84	3.96	0.67	0.59

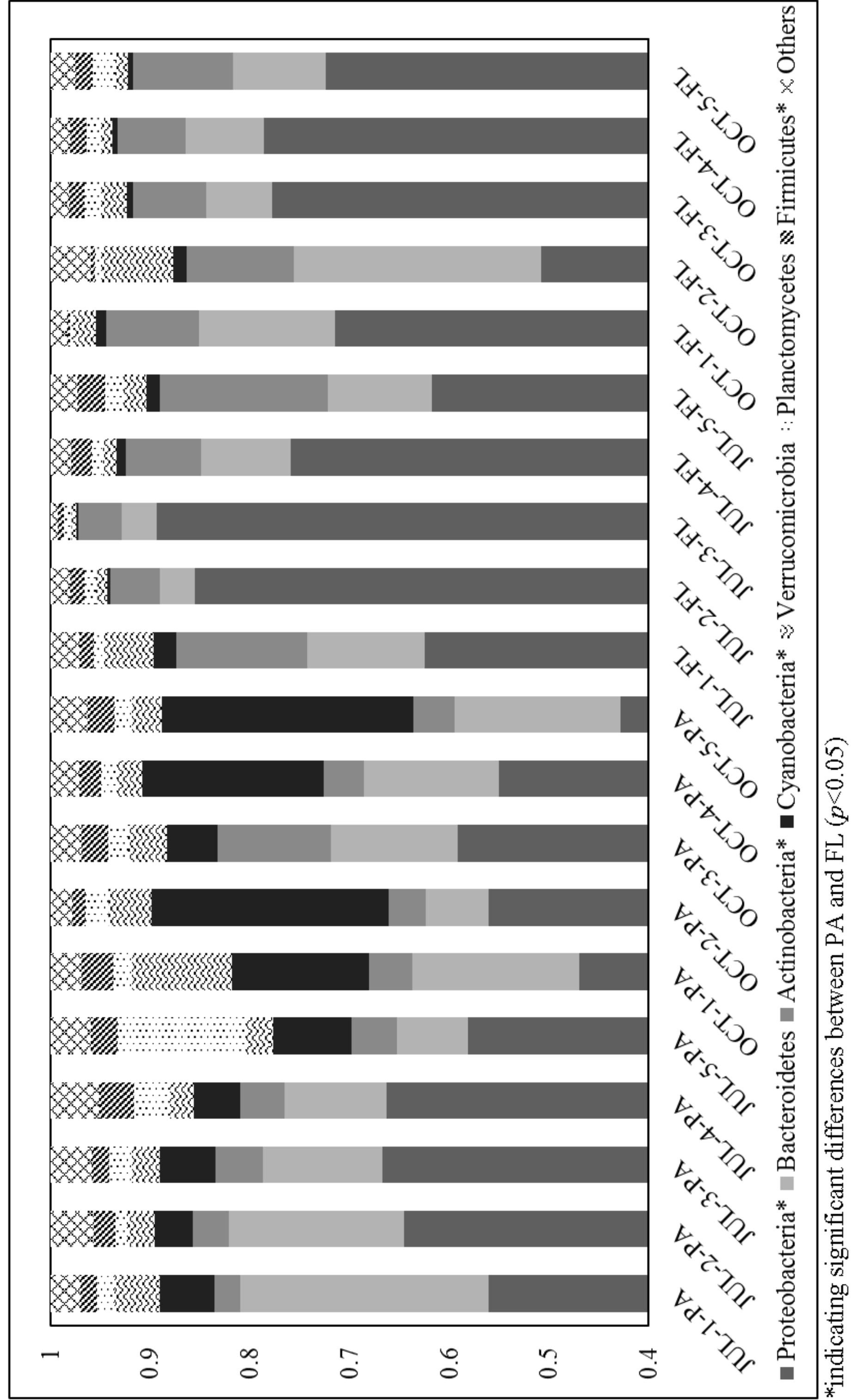
FIGURES

Figure 2-1. Map of sampling station locations along the transect from nearshore Lake Superior (site 1), through the Duluth-Superior Harbor (site 2 and 3), to lower St. Louis River (site 4 and 5). The coordinates of each sample site are given as Table 2-1.



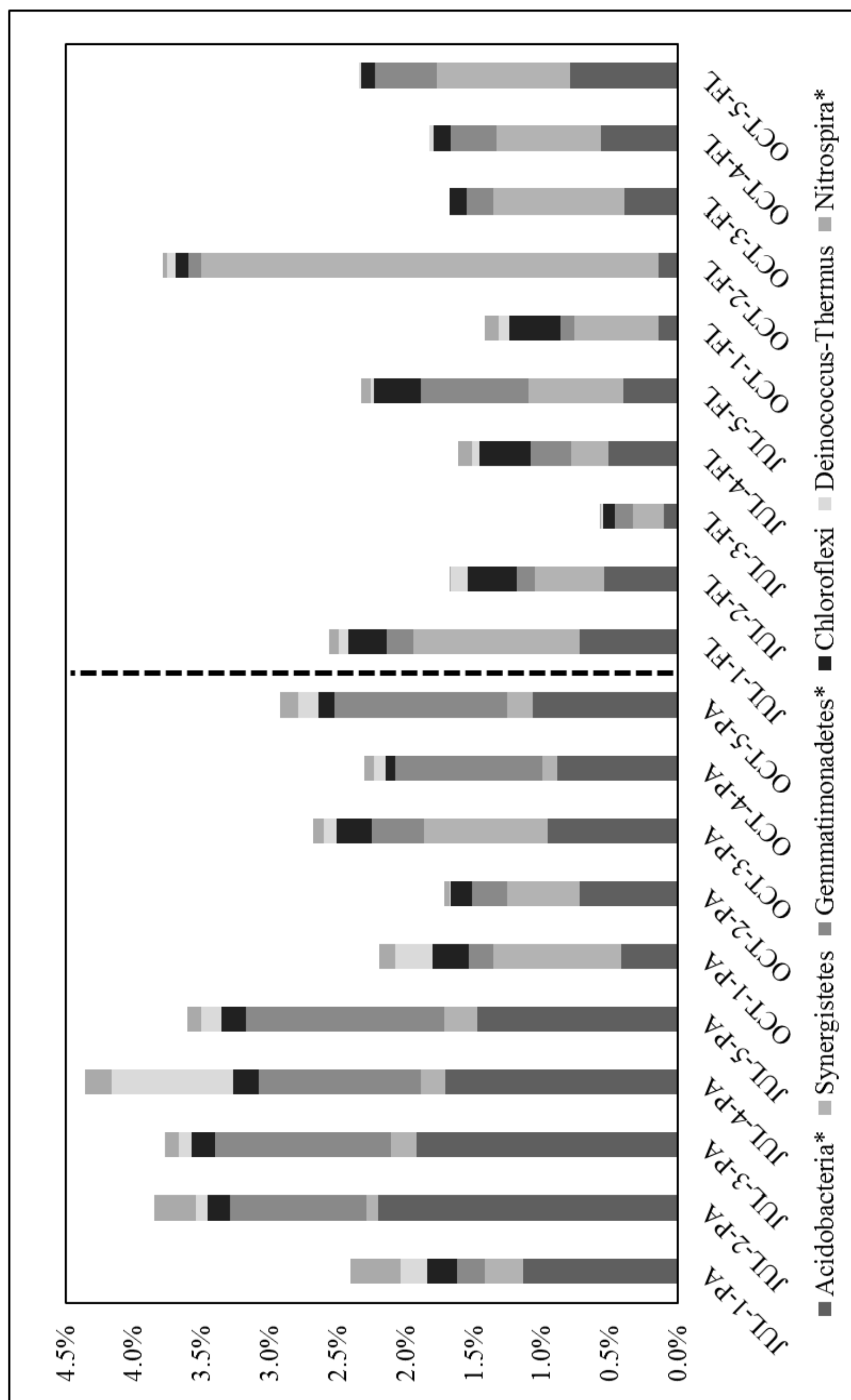
Duluth,
Minnesota
Lake
Superior
Superior,
Wisconsin
1
2
3
4
5
N
0 0.5 1 2 3 4
kilometers

Figure 2-2. Distribution of the 7 most abundant bacterial phyla (>1% of total sequences) among all PA and FL samples in July and October 2013.



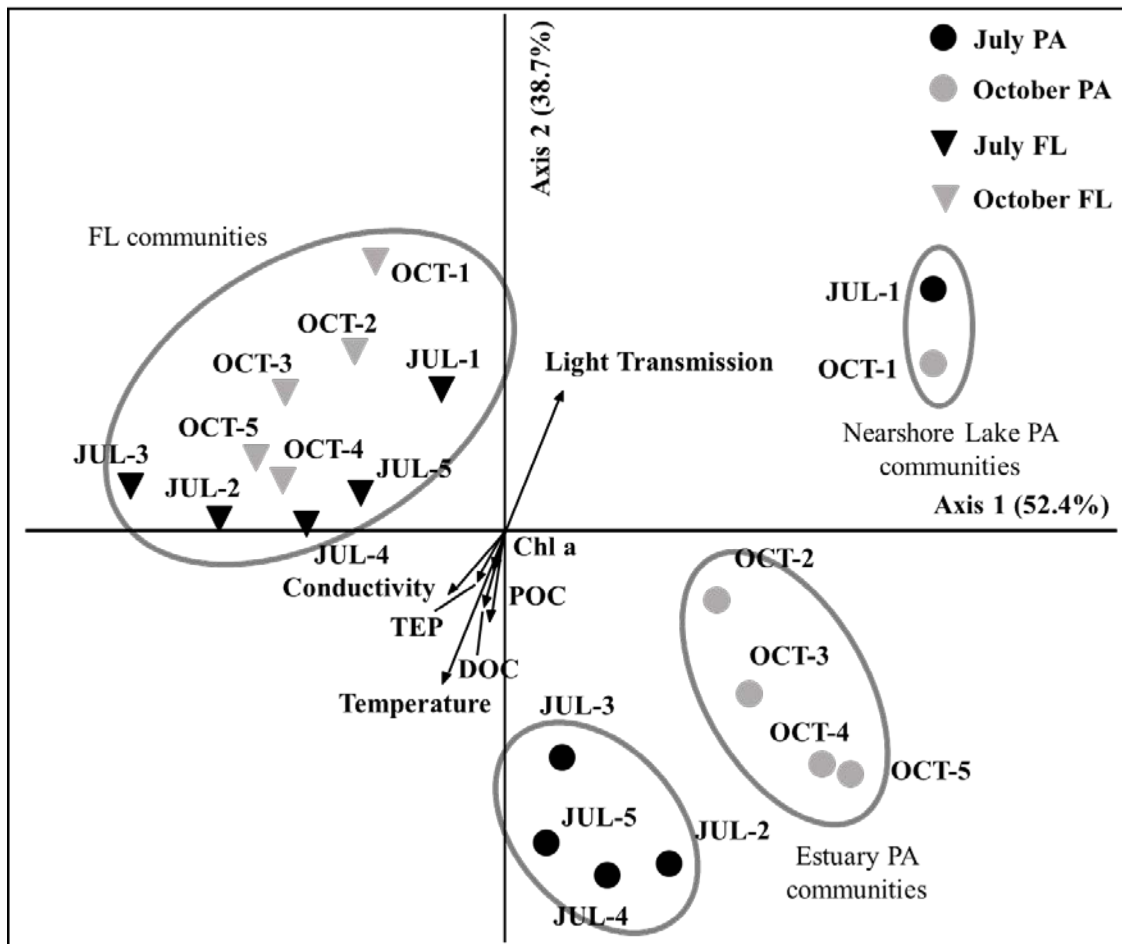
*indicating significant differences between PA and FL (p<0.05)

Figure 2-3. Distribution of 6 less abundant bacterial phyla (0.1-1% of sequences) among all PA and FL samples in July and October 2013.



*indicating significant differences between PA and FL ($p<0.05$)

Figure 2-4. Nonmetric multidimensional scaling (NMS) analysis of particle-associated (PA) and free-living (FL) bacterial communities structure calculated from the distance matrix derived from a comparison of OTUs from each water sample collected in July and October, 2013 for the St. Louis River Estuary. Environmental variables are shown as black vectors. Samples were collected from a nearshore area of Lake Superior (1), within the Duluth-Superior Harbor (2 and 3), and lower St. Louis River (4 and 5); the detailed locations of each sample being collected are shown in Figure 2-1.



July PA
October PA
July FL
October FL
Axis 2 (38.7%)
Axis 1 (52.4%)
FL communities
OCT-1
OCT-2
OCT-3
JUL-1
OCT-5
OCT-4
JUL-3
JUL-2
JUL-5
JUL-4
Light Transmission
JUL-1
OCT-1
Nearshore Lake PA communities
Chl a
Conductivity
POC
TEP
DOC
Temperature
OCT-2
OCT-3
OCT-4
OCT-5
JUL-3
JUL-5
JUL-2
JUL-4
Estuary PA communities

Figure 2-5. Distribution of the 17 most abundant bacterial orders found in PA and FL bacterial communities at all sties combined with 19,505 sequence reads per sample. Differences in abundance at the bacterial order level between PA and FL bacterial communities were determined using Metastats and are indicated with * ($p < 0.05$), ** ($p < 0.01$).

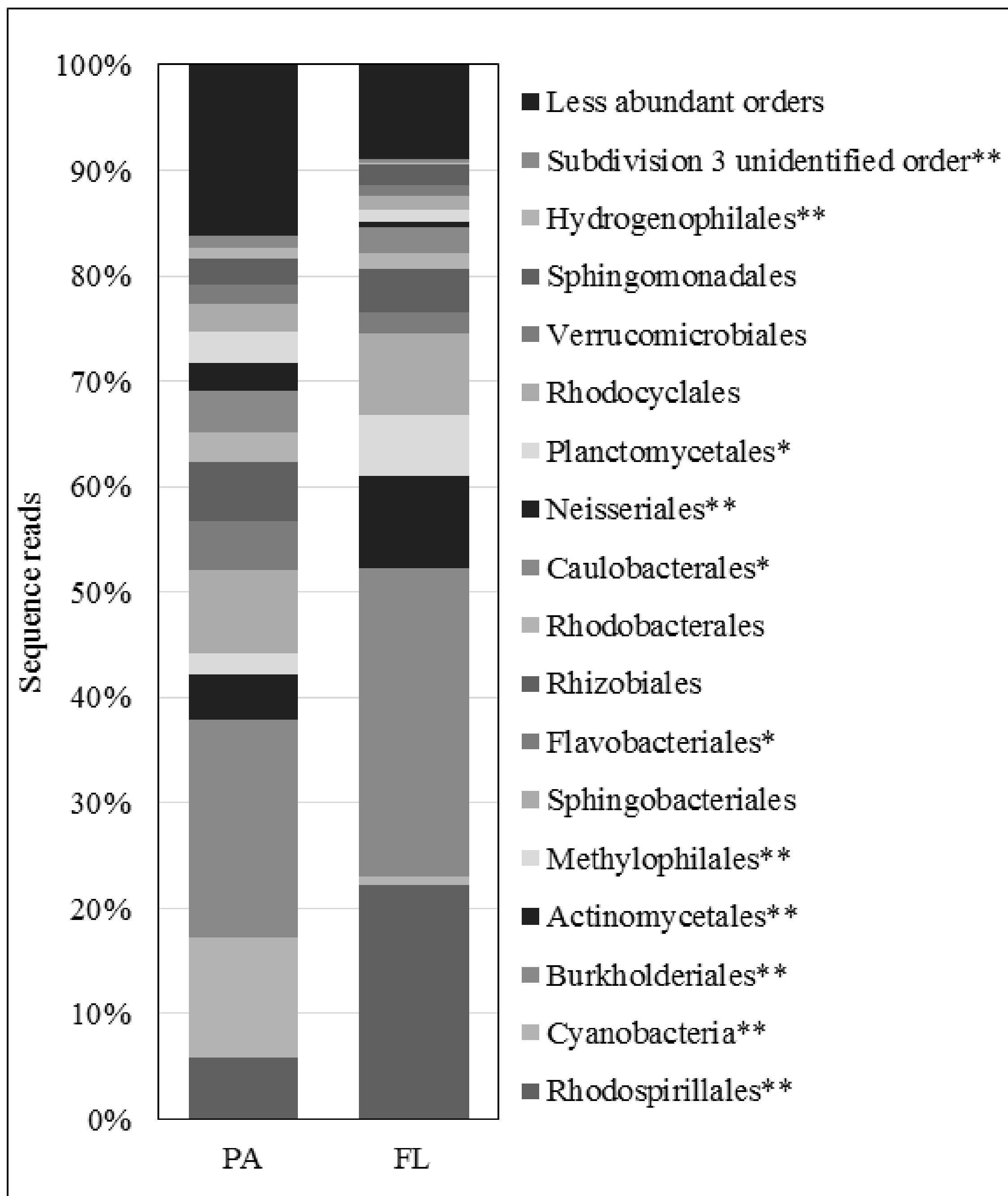

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sequence reads
PA
FL
Less abundant orders
Subdivision 3 unidentified order**
Hydrogenophilales**
Sphingomonadales
Verrucomicrobiales
Rhodocyclales
Planctomycetales*
Neisseriales**
Caulobacterales*
Rhodobacterales
Rhizobiales
Flavobacteriales*
Sphingobacteriales
Methylophilales**
Actinomycetales**
Burkholderiales**
Cyanobacteria**
Rhodospirillales**

Figure 2-6. Distribution of the 17 most abundant bacterial orders found in particle-associated (PA) bacterial communities from nearshore Lake Superior (PA-Lake) and the Duluth-Superior Harbor (PA-Estuary; combined sample of all estuary sites) with 19,505 sequence reads per sample. Differences in the abundance of bacterial orders between the two types of particle-associated bacterial communities were determined by Metastats and are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

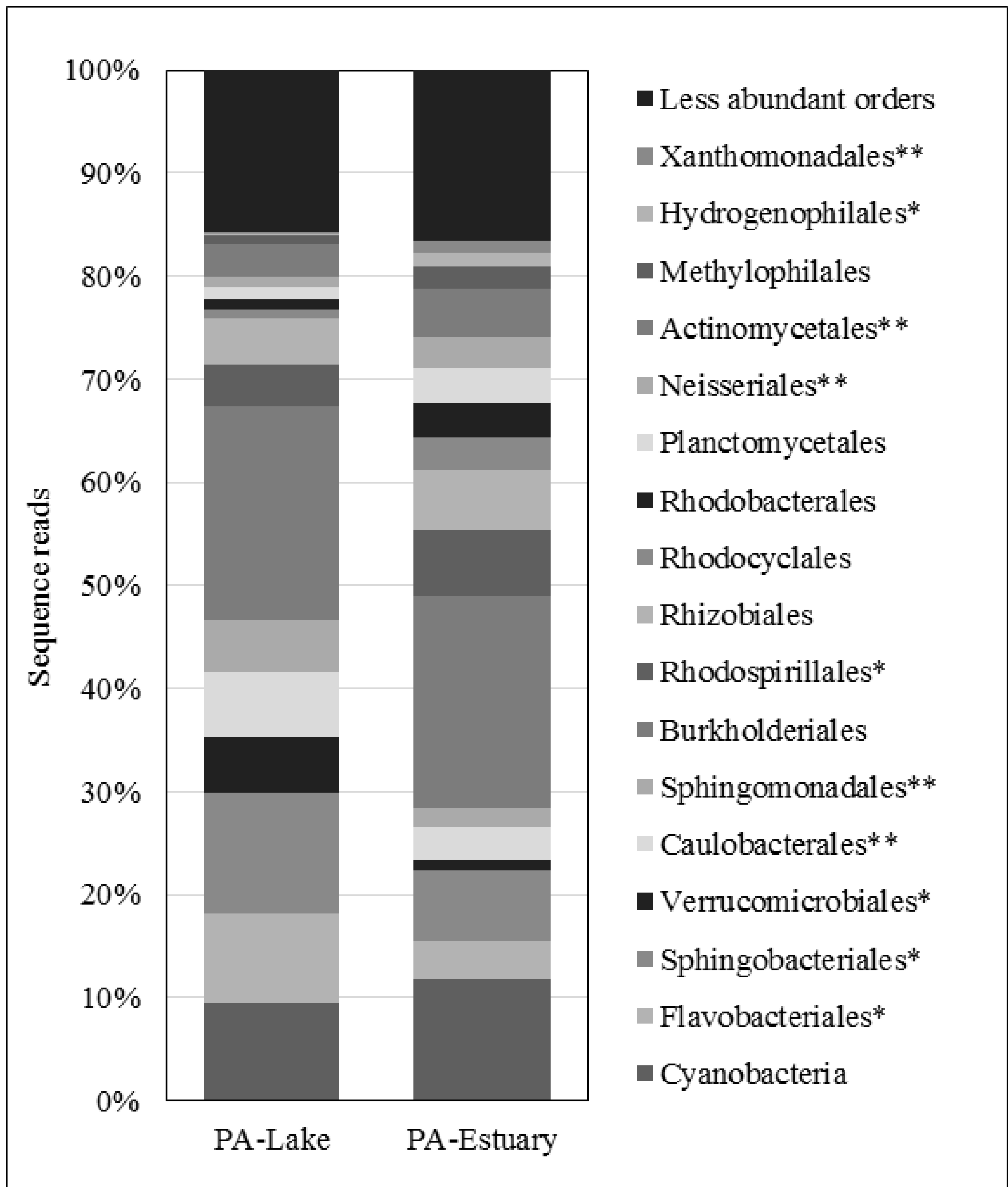
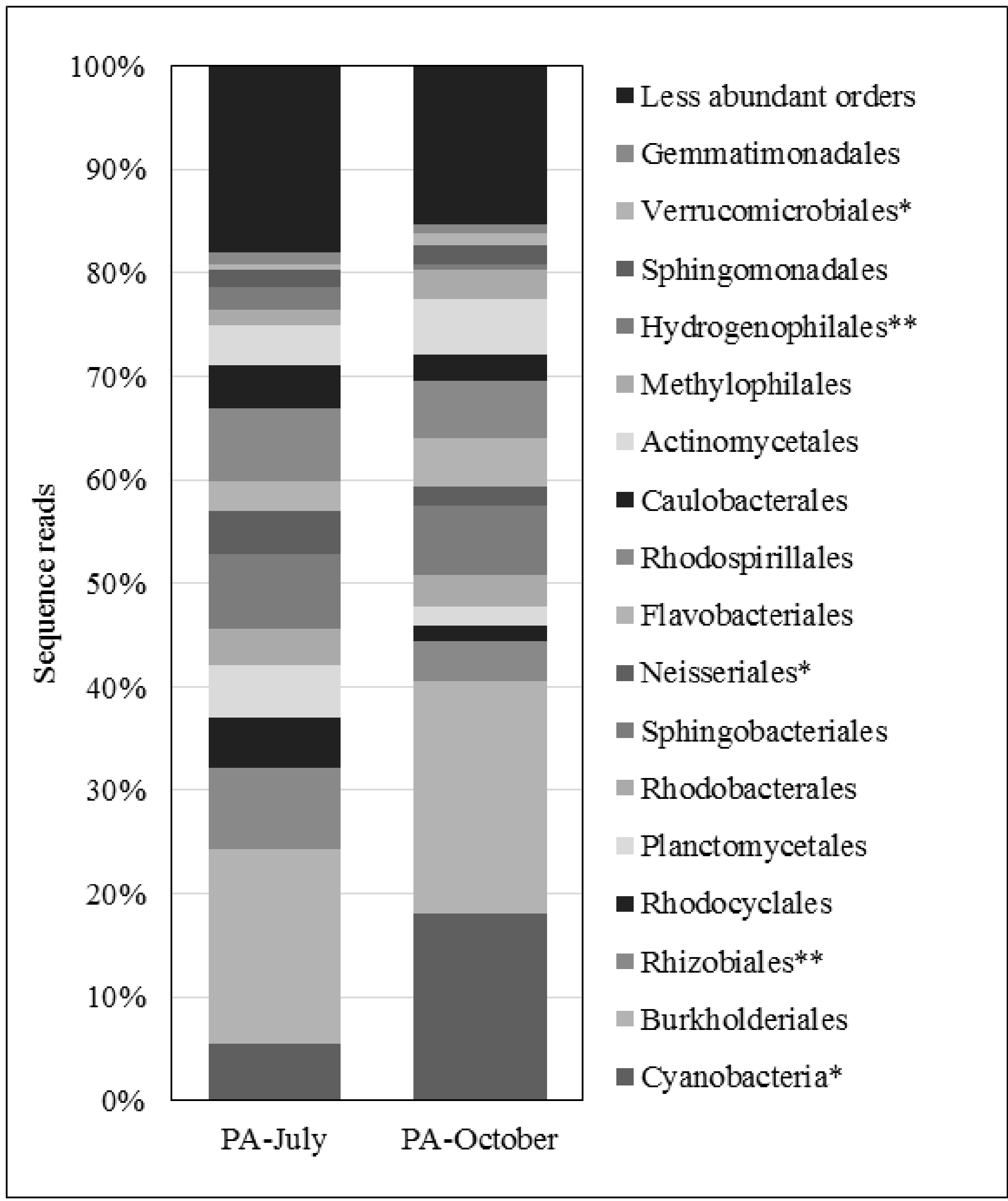

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sequence reads
PA-Lake
PA-Estuary
Less abundant orders
Xanthomonadales**
Hydrogenophilales*
Methylophilales
Actinomycetales**
Neisseriales**
Planctomycetales
Rhodobacterales
Rhodocyclales
Rhizobiales
Rhodospirillales*
Burkholderiales
Sphingomonadales**
Caulobacterales**
Verrucomicrobiales*
Sphingobacteriales*
Flavobacteriales*
Cyanobacteria

Figure 2-7. Distribution of the 17 most abundant bacterial orders found in particle-associated (PA) bacterial communities in the Duluth harbor (estuary) during July and October, 2013, with 19,505 sequence reads per sample. Differences in the abundance of bacterial orders between of particle-associated bacterial communities in July and October were determined by Metastats and are indicated by * ($p < 0.05$) and ** ($p < 0.01$).


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sequence reads
PA-July
PA-October
Less abundant orders
Gemmatimonadales
Verrucomicrobiales*
Sphingomonadales
Hydrogenophilales**
Methylophilales
Actinomycetales
Caulobacterales
Rhodospirillales
Flavobacteriales
Neisseriales*
Sphingobacteriales
Rhodobacterales
Planctomycetales
Rhodocyclales
Rhizobiales**
Burkholderiales
Cyanobacteria*

CHAPTER THREE

Seasonal Dynamics of Water and Aggregate-Associated Bacterial Communities in Nearshore Lake Superior and the Duluth-Superior Harbor

INTRODUCTION

Suspended aggregates are ubiquitous and abundant in marine, estuarine, lacustrine and riverine ecosystems, and consist of a mixture of organic particles, microbes, clay, and sediments (Alldredge and Silver, 1988). The size of these particles range from 1.0 µm to 100 mm, including the macroscopic forms (> 500 µm in length), known as marine or lake snow aggregates, and micro-aggregates, which are less than 500 µm in length (Simon *et al.*, 2002). Sinking aggregates in lakes and oceans transport nutrients, energy, carbon, and other materials from surface waters to deep water and ultimately to benthic habitats (Fowler and Knauer, 1986; Kiørboe *et al.*, 2001). These macro-aggregates are usually highly colonized by various communities of bacteria, phytoplankton, flagellates, and protozoans compared to an equal volume of surrounding seawater (Silver *et al.*, 1978). Aggregate-associated and free-living bacterial communities were considered as two different eco-types due to their different functions in carbon production, degradation of detrital organic matter, and nutrient cycling (Alldredge and Silver, 1988; Azam *et al.*, 1993; Grossart and Simon, 1993). It was observed that particle-attached bacteria accounted for the majority (about 90%) of total bacterial carbon production, and were highly associated with extracellular enzyme activity in the water column of the Columbia River estuary, and these bacteria were 10 to 100 times more active than free-living

bacteria (Crump and Baross, 1996; Crump *et al.*, 1998). In addition, particle-attached bacteria are important for transferring these POM into the detrital food web they account for most of the heterotrophic bacterial degradation of particulate organic matter (Crump *et al.*, 1998).

Studies of bacterioplankton seasonal dynamics in both marine and freshwater environments are very important because these studies can provide fundamental data to comprehensively understand the linkage of microbial diversity to ecosystem functioning. For instance, seasonal studies of bacterial communities could indicate pronounced seasonal patterns in bacterial communities, and may also identify the reoccurrence of certain bacterial phylotypes over multiple seasons.

The seasonal dynamics of free-living or particle-associated bacterial communities in freshwater habitats are still less studied. In one study, Crump *et al.* (2003) found that the bacterioplankton community composition in Toolik Lake on the North Slope of Alaska shifted seasonally according to variations in the source and lability of DOM. They found two shifts in community composition. One occurred in near surface waters in spring with a large influx of labile terrestrial DOM associated with snow melt water. The second shift happened in early summer after the flux of terrestrial DOM when the phytoplankton community developed. In a different study, Allgaier and Grossart (2006) found that free-living bacterial communities were influenced by seasonal changes more than particle-associated bacterial communities in four lakes in northeastern Germany.

Although many studies have examined the temporal distribution of bacterioplankton communities in marine and some freshwater ecosystems, very little is

known about seasonal patterns of lacustrine bacterial communities, especially in large lakes like Lake Superior. Lake Superior is the largest freshwater lake in the world by surface area (82,100 km^2) (Schertzer and Rao, 2009), and is unproductive and highly oligotrophic in terms of nutrient status compared with the other Laurentian Great Lakes (Munawar and Munawar, 2001). One study of annual changes of abundance, cell shapes and biovolumes, and heterotrophic production of bacterioplankton in the western arm of Lake Superior observed that bacterial abundance and cell biovolumes were related to water temperature but there was no consistent seasonal pattern in the rate of bacterial production (Hicks *et al.*, 2004).

Since the 1970s, a few studies of microorganisms in Lake Superior have focused on the bacterioplankton abundance, cell size, and production, but only limited studies have been completed regarding the diversity and structure of Lake Superior microbial communities. Pascoe and Hicks (2004) demonstrated that eplimnetic picoplankton communities in Lake Superior were different than similar communities in the other four Laurentian Great Lakes during August using quantitative whole community DNA:DNA hybridizations. The composition of bacterial communities especially in nearshore areas of Lake Superior and the Duluth-Superior Harbor is virtually unexplored, especially for bacterial communities associated with particles and flocculent aggregates.

The Duluth-Superior Harbor, located at the confluence of the St. Louis River and Lake Superior, is the farthest-inland freshwater port, and also the largest and busiest port on the Laurentian Great Lakes (Welch, 2012). The harbor is hydrologically influenced by Lake Superior seiche oscillations, with amplitudes ranging from 3 cm to 25 cm and a 7.9-

h seiche period in the lower St. Louis River (Stortz and Sydor, 1980). The Duluth-Superior Harbor is a highly dynamic ecological system. It receives large amounts of dissolved organic matter and suspended solids from the St. Louis River, and is also subjected to a high degree of anthropogenic change (Rao and Schwab, 2007). Along with the discharges of ballast water, wastewater, storm water and riverine input, non-indigenous and even possibly harmful bacterial species might be introduced into the Duluth-Superior Harbor, and then flow into Lake Superior.

Two studies have examined spatial changes in bacterial communities within the Duluth-Superior harbor (Welch 2012, LaPara *et al.* 2011). Both studies found three distinct bacterial assemblages in the river, harbor, and lake environments using community fingerprinting techniques; either T-RFLP or ARISA, respectively. However, more comprehensive survey of bacterial community composition using more advanced sequencing techniques is needed to better understand seasonal dynamics of bacterial communities on particles in this harbor and Lake Superior.

The study presented here investigated the seasonal dynamics of attached and free-living bacterial community composition from October 2012 to November 2013 at two sites in nearshore Lake Superior and the Duluth-Superior Harbor. I collected natural sinking particles (SP) using Imhoff settling cones, artificial aggregates (AA) by rolling water samples (modified from Shanks and Edmondson, 1989), as well as aggregate-free water (AFW), which was the water portion above artificial aggregates that formed. Similarities and differences among these three types of bacterial communities were compared based on the taxonomic information gained from bacterial 16S rRNA gene

sequence data. I also looked for correlations between several environmental parameters and changes in these bacterial communities over time. To my knowledge this is the first comprehensive study of seasonal changes found in bacterial communities in nearshore Lake Superior and the Duluth-Superior Harbor.

MATERIALS AND METHODS

Site description and sample collection

Two sites were chosen for comparing bacterial communities in the nearshore Lake Superior and the Duluth-Superior Harbor (Figure 3-1). Site MQ (46°52'51" N, 91°55'07" W) is located in the McQuade small craft harbor (on the lake side of the pier) on the north shore Lake Superior, which is 9 miles north of the Duluth Harbor entry. Site BB (46°45'05" N, 92°06'06" W) is located within the Duluth-Superior Harbor under the Blatnik Bridge. These two sites represent nearshore Lake Superior and the Duluth-Superior Harbor, respectively. Surface water samples (20 liter) were collected by immersing plastic carboys below the water surface approximately every four weeks at the two sites from October 2012 to November 2013 (except during the ice cover period from late December to March). Water samples were refrigerated until returned to the lab. Water samples were preserved or analyzed within 1 to 4 hours of collection.

Bacterioplankton measurements

Bacterial abundance. Triplicate 10 ml water sample from each site were preserved with 37% (w/v) formaldehyde solution, which were stored in the dark at 4°C

for up to 2 weeks. A small portion (0.5-1 ml) of each preserved sample was placed on a black polycarbonate filter (25 mm dia., 0.22 µm pore; GE Water & Process Technologies, Trevose, PA), and 200 µl 4'6-diamidino-2-phenylindole (DAPI; 10 µM final concentration) was added to stain DNA in microbial cell for 5 minutes. The stained prokaryotic cells were filtered onto the membrane filter under low pressure (15 cm Hg), and were counted using a Nikon Eclipse 80i epifluorescence microscope (Porter and Feig, 1980).

Bacterial community composition analysis

Collection of sinking particles. The second portion of water from each site was used to collect natural sinking particles (SP). Duplicate 1 L water samples from each site were gently transferred to Imhoff settling cones held upright on a rack, and SP were allowed to settle for one day. Particles were collected in 15 ml centrifuge tubes attached at the bottom of each settling cone and refrigerated at 4℃ overnight. Afterwards, the upper 13 ml were siphoned from centrifuge tubes, leaving 2 mL at the bottom of each containing collected particles sample (Lyons *et al.*, 2005). All the centrifuge tubes were stored at -80°C until DNA extraction could be extracted.

Formation of artificial aggregates in rolling cylinders. The last portion of water from each site was used to fill triplicate 1 L Pyrex glass cylinder bottles. These bottles were rotated horizontally (1.5 rpm) for 2 d on a roller table at room temperature on the lab bench top to generate visible aggregates (i.e. "lake snow"; modified from Shanks and Edmondson, 1989). After one day, the contents of all bottles were allowed to

settle for 1 h before two portions of the water in each bottle were separated. The upper 900 mL water in each bottle was siphoned out and operationally defined as “aggregate-free water (AFW)”, while the bottom 100 ml containing the settled aggregates was defined as the “artificial aggregates (AA)”. Bacterial cells in AFW were collected by filtering through a Duropore membrane filter (47 mm dia., 0.22 µm pore), while AA and associated bacteria were collected after centrifuging the lower 100 ml water samples at 3,000 rpm for 5 min (Tang *et al.*, 2009). Membrane filters were stored in Whirl-Pak® bags (Nasco, Fort Atkinson, WI) and the aggregate pellets in centrifuge tubes were stored at -80°C until DNA extraction.

DNA extraction. A PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) was used for all DNA extractions because aggregates can contain organic material, clay particles, and humic acids, which all could inhibit PCR amplifications (Kirk *et al.*, 2004). For water-associated bacteria, each filter was cut into small pieces with sterilized scissors, and all filter pieces were used to extract DNA following the manufacturer’s protocol. For sinking aggregates and artificial aggregate-associated bacteria, each centrifuge tube was centrifuged at 3,000rpm for 5 min., the supernatant was slowly removed from the tube by pipetting, and the remaining pellet was used for DNA extraction following the manufacturer’s protocol. Extracted DNA from all the samples were frozen in microcentrifuge tubes at -80°C until further analysis.

Illumina sequencing and analysis. The replicate DNA extracts from each sample type were pooled and sent to the University of Minnesota Genomics Center for paired-end multiplex sequencing on an Illumina MiSeq platform. The V5-V6

hypervariable regions of the 16S rRNA gene were amplified with the BSF784/R1064 primer set. The amplicons were purified and pooled in equal amounts for paired-end sequencing at the read length of 300 bp. Raw sequences were obtained as .fastq files and submitted to the National Center for Biotechnology Information Sequence Read Archive. Sequencing results are available through GenBank BioProject PRJNA357124. Sequence reads were analyzed on the Minnesota Supercomputing Institute server with the mother software program, version 1.27.0 (Schloss *et al.*, 2009). Forward and reverse sequences were paired by using fasta-join, and the uninformative and primer sequences were trimmed off from original sequence reads. All sequences with abundances < 2 were removed before further analysis. The remaining sequences were aligned to the SILVA database (release 119) (Pruesse *et al.*, 2007). Gaps and uninformative sequences were removed, and chimeras were also removed using UCHIME (Edgar *et al.*, 2011). The reasonable sequence reads were randomly subsampled from each sample to normalize the differences in numbers of sequence reads between each sample, while still capturing as much genetic diversity as possible. The processed sequences were then taxonomically classified into operational taxonomic units (OTUs) at the ≥ 97% sequence similarity level by furthest neighbor clustering using the Wang method and the Ribosomal Database Project (RDP) taxonomic database release 9 (Cole *et al.*, 2009).

Environmental parameter measurements

Chlorophyll *a*. Triplicate portions of water from each site were filtered on GF/F glass filter filters, and photosynthetic pigments were extracted with 90% (v/v) acetone at

-20°C for 18-24 h. 90% acetone (v/v) was made by mixing 900 ml of acetone with 100ml of Milli-Q water. Pigments extracts were analyzed using a Turner 10-AU fluorometer (Turner Designs, Sunnyvale, CA) before and after acidification with 6N HCl (v/v). Acidification was used to correct for interference by pheophytin a, which is the common degradation product of chlorophyll *a*. Relative fluorescent units (RFU) were used to calculate chlorophyll a (µg/L) concentrations (Lorenzen, 1967).

Particulate organic carbon (POC), particulate organic nitrogen (PON), and dissolved organic carbon (DOC). One POC sample from each site was collected on a GF/C glass-fiber filter (Whatman, 47mm, 1.2 µm pore size, pre-combusted and pre-weighed) by filtering water until the filter clogged (1 to 2 liter for the lake sites and 200 to 500 ml for the harbor sites). GF/C glass fiber filters are usually used for collection of suspended solids in natural water (Aas and Hicks, 1993). These filters were stored at -20°C until POC could be analyzed using a Costech ESC 4010 elemental analyzer as described in Zigah *et al.* (2011). In addition, a 40 ml portion of each filtrate was collected in acid-washed and combusted brown glass vials, and acidified to pH 2 with 40 µl 6N HCL to remove inorganic carbon and leave the non-purgeable organic carbon in the water sample. All processed samples were stored at 4°C for up to 2 weeks until DOC was measured using a TOC-VCSH/SCN Total Carbon Analyzer (Shimadzu, Columbia, MD) as described by Zigah *et al.* (2011).

Transparent exopolymer particles (TEP). TEP concentrations in water were determined by the Alcian blue staining spectrophotometric method (Passow and Alldredge, 1995). Triplicate fresh water samples from each site were gently filtered

through polycarbonate filters (47 mm dia., 0.4 µm pore; GE Water & Process Technologies, Trevose, PA) at constant 150 mmHg vacuum. Particles on filters were stained for less than two second with 0.5 ml of 0.02% alcian blue solution in 0.06% acetic acid (pH=2.5), and then rinsed with Milli-Q water. The filters were placed into vials containing 6 ml of 80% (v/v) sulfuric acid, and the vials were swirled for at least 2 hrs. Absorption of the solution was measured using a spectrophotometer (GENESYS 20, Thermo Spectronic) at 787 nm. Concentrations of TEP were in units of "µg/L Xeq", which relates the amount of absorbed Alcian blue to the equivalent weight of the xanthan gum (8GX, Sigma-Aldrich).

Statistical analyses

Ordination analysis. The distance matrices between samples were calculated using the Bray-Curtis measure of dissimilarity (Bray and Curtis, 1957) and imported into PC-ORD (v6.08) software for nonmetric multidimensional scaling (NMS) analysis. Five environmental parameters measured at each sample site were analyzed with distance matrices in PC-ORD to examine relationships between bacterial community structure and environmental parameters. In NMS plots, samples with greater similarity are closer in ordination space, and relationships between environmental parameters and bacterial communities were performed using a joint-plot overlay of a second matrix containing the environmental parameters.

Correlation analysis. To determine the relationships between the 5 environmental parameters and bacterial community structures, the Mantel test was used

to calculate Pearson product-moment correlation coefficients (*r*) and the corresponding *p* values using PC-ORD (v6.08). The Mantel test is usually used to evaluate statistical correlations between the environmental variables and ordination scores. Spearman rank correlations were calculated to examine relationships between environmental parameters and the abundances of specific lineages, which were performed using the online software package PAST (Hammer *et al.*, 2001). All statistical analyses used a $p<0.05$ statistical significance level.

Bacterial community richness, diversity and evenness. Bacterial community richness (total number of unique OTUs), Shannon-Wiener diversity, and Shannon-index based evenness were calculated for all samples based on following equations using mothur version 1.27.0 (Schloss *et al.*, 2009).

$$H' = -\sum_{i=1}^{S} \frac{n_i}{N} \times \ln \frac{n_i}{N}$$

$$J' = \frac{H'}{H'_{max}} = \frac{H'}{\ln S}$$

where *H'* and *J'* are Shannon-Wiener diversity and evenness indices respectively. *S* is the number of observed OTUs, n_i is the number of individuals in OTU *i*, and *N* is the total number of individuals in the community.

Analysis of molecular variance (AMOVA) and similarity percentage (SIMPER). AMOVA is a method to determine whether the centers of two groups are more separated than the variation among samples within the same group by using molecular markers (Excoffier *et al.*, 1992). AMOVA was performed using Mothur,

version 1.27.0 (Schloss *et al.*, 2009). SIMPER analysis was used to evaluate the average dissimilarity in separated groups determined by the AMOVA analysis. The dissimilarity percentage was measured using a Bray-Curtis measure, which was calculated using the online software PAST (Hammer *et al.*, 2001). SIMPER analysis was also used to identify the contribution of each bacterial taxon to the dissimilarity between groups.

Detection of differential abundant features (Metastats). Significant differences between different assemblages in abundance of phyla or orders were determined by the online software program Metastats (White *et al.*, 2009), with 1000 permutations and $p < 0.05$ as the statistical significance level.

RESULTS

Overview of bacterial communities at nearshore Lake Superior and inner Duluth-Superior Harbor sites

Subsamples of 7,043 partial 16S rRNA gene sequences from bacterial communities on sinking particles (SP), artificial aggregates (AA), and in aggregate-free water (AFW) collected from sites in nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB) from October 2012 to November 2013 were further analyzed to avoid the sample-specific differences in sequence reads. The average coverage of Illumina sequences for all samples was estimated to be 97% ± 2%, which indicated sampling of these communities was very thorough and only a few unique sequences probably remained unsampled (Table 3-3). Combining data from sites and all sample dates, an average of 397 ± 165, 742 ± 286, and 490 ± 123 unique OTUs were identified

during the study on SP, AA, and in AFW bacterial communities, respectively. The AA bacterial community had more OTUs (both p's < 0.01) and a higher richness than the AFW and SP communities, considering both sites and all sample dates.

Considering all dates and both sites together, the Shannon-Wiener diversity index for the AA bacterial community (mean = 4.82 ± 0.50) was higher than both the AFW (mean = 4.30 ± 0.22) and SP (mean = 4.09 ± 0.72) bacterial communities (both p's < 0.01) during most of the year, except in October 2012 and August 2013 when a large number of cyanobacterial sequences were present in the AA bacterial community at the BB site (Figure 3-4). Considering all dates and both sites, there was no difference (all p's > 0.05) in Shannon-Wiener based evenness between the SP (0.69 ± 0.10), AA (0.73 ± 0.06), and AFW (0.70 ± 0.03) bacterial communities.

All identified OTUs could be assigned to bacterial phyla and families at the 97% identity level. Considering all samples, more bacterial phyla (20 ± 4) and families (101 ± 21) were represented in the AA compared to the AFW (16 ± 3 and 74 ± 14, respectively) and SP (15 ± 3 and 72 ± 18, respectively) bacterial communities (both p's<0.05). The *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, and Cyanobacteria/Chloroplast were the most abundant phyla represented in all three of these bacterial communities (i.e., SP, AA, and AFW).

Seasonal dynamics of bacterial communities on SP, AA, and in AFW

Nonmetric multidimensional scaling (NMS) ordinations were built based on distance matrices between samples of SP, AA, and AFW bacterial communities collected

in 2012 and 2013 from the lake (MQ) and harbor (BB) sites (Figure 3-2). Based on these ordinations, seasonal changes were observed on SP, AA and in AFW bacterial communities, which showed clear changes between cold and warm seasons (Figure 3-2). These seasonal changes were more pronounced on SP and AA bacterial communities at both lake and harbor sites and least pronounced in the AFW community at the lake site (Figure 3-2). Seasonal dynamics were usually less pronounced in AFW than AA bacterial communities, which could be seen in the ordinations from each sites where variation among AFW bacterial community samples was substantially smaller than in AA community samples (Figure 3-3). More specifically, sequences from *Proteobacteria* were more abundant from June to August than in other months in all bacterial communities at both sites (Figures 3-4 to 3-6). Cyanobacteria/Chloroplast sequences were most abundant in AA communities between November and December at the lake site, and between August to October at the harbor site (Figure 3-5); Sequences from the *Actinobacteria* usually most abundant between October and November in most samples (Figures 3-4 to 3-6). However, some bacterial phyla demonstrated different seasonal patterns depending upon the type of particle they resided on. For example, in bacterial communities on SP, sequences from *Verrucomicrobia* reached peaks toward the end of the year (from October to December), while in communities on AA these sequences often more abundant around July (Figure 3-4 and 3-6).

Spearman's rank correlation coefficient (ρ) was used to determine the correlation between the six most abundant bacterial phyla. In SP communities, the abundance of proteobacterial sequences was negatively correlated with the abundance of cyanobacterial

sequences (ρ = -0.97, $p < 0.05$) at the harbor site, while the abundance of *Verrucomicrobia* sequences were positively correlated with the abundance of *Planctomycetes* sequences at both the lake and harbor sites (ρ = 0.77, $p < 0.05$ and ρ = 0.62, $p < 0.05$ respectively) (Figure 3-4). The abundance of cyanobacterial sequences in AA communities was negatively correlated with *Proteobacteria* and *Bacteroidetes* sequences at both sites (lake site: ρ = -0.88, $p < 0.05$ and ρ = -0.71, $p < 0.05$; harbor site: ρ = -0.73, $p < 0.05$ and ρ = -0.93, $p < 0.05$) (Figure 3-5). The number of sequences from the *Proteobacteria* was negatively correlated with the abundance of *Actinobacteria* and *Verrucomicrobia* sequences in the AFW community at only the lake site (ρ = -0.89, $p < 0.05$ and ρ = -0.96, $p < 0.05$, respectively), while the abundance of sequences from the *Proteobacteria* was negatively correlated with the abundance of *Bacteroidetes* sequences at only the harbor site (ρ = -0.82, $p < 0.05$) (Figure 3-6). The abundance of Cyanobacteria/Chloroplast sequences in AFW were positively correlated with the abundance of *Planctomycetes* sequences at both the lake and harbor sites (ρ = 0.89, $p < 0.05$ and ρ = 0.89, $p < 0.05$ respectively) (Figure 3-6).

Overall, a seasonal reoccurrence pattern was observed for only one major bacterial phylum. In both 2012 and 2013, Cyanobacteria/chloroplast sequences in AA communities were more abundant between November and December than at other times of the year at the lake site, and between August and October at the harbor site (Figure 3-5). However, discernable seasonal reoccurrence patterns of the other major bacterial phyla were not observed in any of the three bacterial community types at either field site (Figures 3-4 to 3-6). Seasonal reoccurrence patterns, however, could not be evaluated for

all months because samples were only taken from October 2012 to December 2013 during two consecutive years.

Comparison of bacterial communities on SP, AA, and in AFW

Analysis of molecular variance (AMOVA) and SIMPER analyses were used to compare the average dissimilarities between the bacterial communities at different sample locations, and detect the families that contributed most to these dissimilarities (Tables 3-1 and 3-2). Bacterial communities on SP and AA, as well as communities on AA and in AFW were significantly different from each other at both the lake and harbor sites (Table 3-1). Richness, Shannon-Wiener diversity and evenness indices were higher for bacterial communities on AA compared to SP or in AFW in most months at the both lake and harbor sites (Table 3-3). To obtain more detailed information regarding the phylogenetic composition of SP, AA, and AFW bacterial communities, all the partial 16S rRNA gene sequences obtained by Illumina sequencing were assigned to bacterial families for all samples. Sequences from several families within the *Proteobacteria* phylum were more abundant on SP compared to AA at both the lake and harbor sites, including the *Comamonadaceae*, *Pseudomonadaceae*, *Oxalobacteraceae*, and *Caulobacteraceae*, while Cyanobacteria/Chloroplast sequences were more abundant on AA than SP at both lake and harbor sites (Table 3-1, Figure 3-4 and 3-5). Some bacterial families in AFW communities compared to AA communities were more abundant at both the lake and harbor sites including *Microbacteriaceae* and *Acidimicrobiaceae* within the *Actinobacteria phylum*; *Comamonadaceae*, *Candidatus_Pelagibacter*, *Methylophilaceae*,

and *Burkholderiaceae* with the *Proteobacteria* phylum; and *Opitutaceae* within the *Verrucomicrobia* phylum, while Cyanobacteria/Chloroplast were more abundant on the AA than AFW at both lake and harbor sites (Table 3-1, Figure 3-5 and 3-6).

Comparison of the bacteria communities at the lake and harbor sites

Each of the three types of bacterial communities (SP, AA, and AFW) were well separated (i.e. different) at the lake (MQ) and harbor (BB) sites (Figure 3-2). AMOVA analyses also demonstrated that each of these bacterial communities was different between the lake and harbor sites (all $p's < 0.05$; Table 3-2). However, the richness, Shannon-Wiener diversity, and evenness indices were not different between the lake and harbor sites for any bacterial community type (all $p's > 0.05$).

To obtain more detailed information about the differences between bacterial community types at the lake and harbor sites, partial 16S rRNA gene sequences obtained by Illumina sequencing were assigned to bacterial families. SIMPER analysis compared the average dissimilarities between the bacterial communities of the two sample locations, and detected which bacterial families contributed most to these dissimilarities (Table 3-2). Cyanobacteria/Chloroplast sequences were more abundant at the harbor site compared to the lake site for SP, and AFW communities (Table 3-2). In both SP and AA communities, sequences from the *Sphingomonadaceae* and *Cytophagaceae* were more abundant at the harbor site than the lake site. More sequences from the *Comamonadaceae*, *Microbacteriaceae*, and *Acidimicrobiaceae* families, but fewer

sequences from the *Burkholderiaceae* were found in AA and AFW communities at the lake site compared to the harbor site (Table 3-2).

The bacterial communities and environmental parameters

Five environmental parameters, chlorophyll *a* (Chl *a*), transparent exopolymer particles (TEP), dissolved organic carbon (DOC), particulate organic carbon (POC), and particulate organic nitrogen (PON), as well as prokaryotic abundance were measured in water samples collected at the lake (MQ) and harbor (BB) sites from 2012 to 2013 (Table 3-4). Overall, higher values for all environmental parameters measured as well as prokaryotic cell abundance were observed in the Duluth-Superior Harbor compared to nearshore Lake Superior. Although the habitats of the two sample sites are very different from each other, environmental parameters at both sample sites changed from 2012 to 2013 but followed similar patterns. Most environmental parameters had higher values during the warm season (from June to October) and lower values during cool seasons (November, December, April and May) (Table 3-4). Many environmental variables, such as chlorophyll a, POC and PON, reached their highest values between June and August, while some other parameters, such as TEP and DOC, had higher values between May and June. The concentrations of most environmental parameters decreased after October during the winter months and increased again before the following June. Thus, most environmental parameters demonstrated seasonal patterns in both nearshore Lake Superior and the Duluth-Superior Harbor.

Ordination analysis was used to determine relationships between these environmental parameters and different bacterial community structures at the two sample sites. The Pearson's correlation coefficient between these environmental variables and each type of bacterial community at each sample site were calculated using a Mantel test (Table 3-5). Only a few environmental parameters were correlated with each bacterial community type, and these correlations were more common for communities at the nearshore Lake Superior site (MQ). For example, DOC, POC and PON were all correlated with the AA bacterial community in nearshore Lake Superior (all *p's* < 0.05), but not correlated with AA communities in the harbor or other community types (SP or AFW communities) in either the harbor or lake. The structure of AFW bacterial communities was correlated with chlorophyll *a* concentration at both lake and harbor sites (both *p's* < 0.05), but bacterial communities residing on particles or aggregates (i.e. SP and AA communities) did not show any correlation with chlorophyll *a* concentration.

Spearman's rank correlation coefficient (ρ) were calculated to explore relationships between environmental parameters and the 6 most abundant bacterial phyla (*Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, and Cyanobacteria/Chloroplast) found in all types of bacterial communities (SP, AA and AFW). In SP bacterial communities from nearshore Lake Superior site, the abundance of *Bacteroidetes* sequences were positive correlated with the TEP concentrations ($\rho = 0.87$, $p < 0.01$), while abundances of *Actinobacteria* sequences at the harbor site were negatively correlated with PON ($\rho = -0.68$, $p = 0.04$). The abundance of cyanobacterial sequences in the AA bacterial communities were positively correlated with chlorophyll *a*

concentrations from the Duluth-Superior Harbor (ρ = 0.63, p = 0.04). *Planctomycetes* and *Verrucomicrobia* sequences were both positively correlated with TEP concentration (ρ = 0.74, $p < 0.01$ and ρ = 0.71, p = 0.01 respectively) at the same site. *Actinobacteria* sequence abundance was negatively correlated with POC and PON concentrations (ρ = -0.70, p = 0.02 and ρ = -0.69, p = 0.02 respectively). In the AFW bacterial community from nearshore Lake Superior, the abundance of *Proteobacteria* sequences was positively correlated with chlorophyll *a* concentrations (ρ = 0.82, p = 0.03), while *Actinobacteria* sequences were negatively correlated with chlorophyll *a* concentrations (ρ = -0.93, p <0.01). *Planctomycetes* sequences in AFW communities at this site were negatively correlated with PON concentration (ρ = -0.83, p = 0.04). Considering AFW communities from the Duluth-Superior Harbor, *Planctomycetes* sequences were positively correlated with chlorophyll *a* concentration (ρ = 0.89, p = 0.01), *Bacteroidetes* sequences were negatively but *Proteobacteria* sequences were positively correlated with TEP concentrations (ρ = 0.780, p = 0.04 and ρ = -0.82, p = 0.03 respectively), and Cyanobacteria/Chloroplast and *Planctomycetes* sequences were both positively correlated with POC and PON concentrations (POC: ρ = 0.96, $p < 0.01$ and ρ = 0.86, $p < 0.01$ respectively; PON: ρ = 0.92, p = 0.01 and ρ = 0.87, p = 0.02 respectively).

DISCUSSION

Environmental parameters and bacterial community composition

Regardless of several methodological limitations of community fingerprint techniques, annually reoccurring or pronounced seasonality of bacterial communities

have been widely observed in coastal ocean, eutrophic lake, and river settings (Crump *et al.*, 2003; Yannarell *et al.*, 2003; Rösel et *al.*, 2012). Similarly, our results also demonstrated some seasonal dynamics of the bacterioplankton communities in both sample sites (Figure 3-2). A number of studies of seasonal changes in microbial communities revealed that the major driver of bacterial dynamics in marine and freshwater systems was the physical or chemical changes over seasons in the aquatic environments. For instance, water temperature, dissolved oxygen, and nitrogen sources were responsible for distinguishing Lake Mendota bacterial communities seasonally (Shade *et al.*, 2007). In this study, it is worth mentioning that one of the most damaging floods in Duluth history occurred on June 19-20, 2012, and one of our sampling periods started after this heavy flood. This extreme weather event resulted in huge amount of stormwater and clay runoff along the shoreline of Lake Superior, which may affect our earlier data. In our study, chlorophyll *a* (Chl *a*) concentration reached maximum value between July to October in both 2012 and 2013 for both lake and harbor sample sites, and even higher Chl *a* concentrations were observed in 2012 summer and fall than 2013. This phenomenon was very reasonable because phytoplankton population is usually positively correlated with higher temperature and nutrient concentration. Similar trends were also found in annual changes of dissolved organic carbon (DOC), particulate organic carbon and nitrogen (POC and PON), except that the values of these parameters started to increase in late spring, which may be associated with snow melting and the transfer to the river and lake of organic compounds that accumulated within the watershed during the winter. The seasonal changes in transparent exopolymer particles (TEP) concentration

also included higher values during spring and summer, in spite of quite different values between 2012 and 2013 at lake and harbor sites. The site located at the nearshore Lake Superior had higher TEP concentrations in 2012 summer, while the sample site within the Duluth-Superior Harbor had pronounced higher values in 2013 summer. TEP is one type of exopolymeric substances (EPS), which is usually the polysaccharides that are generated and released by some species of phytoplankton and bacteria. Therefore, different environmental conditions may favor different types of algal or bacterial species, which would cause different amounts of TEP excreting by these species. In addition, TEP consumption by bacteria could also affect TEP concentrations. Ramaiah *et al.* (2000) found a close relationship between bacterial abundance and concentrations of TEP in Arabian Sea, which indicates TEP is available as a food source for bacterial metabolism.

Although all the environmental parameters we measured demonstrated certain seasonal dynamic patterns from 2012 summer to 2013, we did not see much of them reflected in bacterial community composition (BCC) in both lake and harbor sites based on our statistical analyses between environmental parameters and BCC. In our study, only limited variables (chlorophyll *a*, DOC, POC, PON and TEP concentrations) were measured throughout the whole investigation. Therefore, there may be other environmental parameters that we did not measure in this study that actually influence BCC. For example, it was widely found that water temperature was the strongest driver that could affect seasonal dynamics in BCC (Shade *et al.*, 2007; Rösel *et al.*, 2012), because temperature or seasonal changes would influence bacterial respiration, production, and therefore, community composition (Kent *et al.*, 2004; Crump and

Hobbie, 2005). The other aspect not focused upon in his study is nutrient concentrations. Nitrogen (NO_2^-, NO_3^-, and NH_4^+) and phosphorus are two major nutrients that could limit bacterioplankton growth. Multiple studies have showed seasonal changes of nutrient concentrations can strongly affect BCC in lakes (Newton and McMahon, 2011; Rösel *et al.*, 2012), as different nutrient conditions can favor specific bacterial groups (Cotner and Biddanda, 2002). This phenomenon can become even pronounced when available nutrients become limiting to bacterioplankton (Rösel *et al.*, 2012). Therefore, in future studies more environmental variables should be taking into consideration to investigate the relationships between environmental drivers and BCC.

Diversity of bacterial community could also be influenced by biotic factors, including competitions between species and trophic interactions (Kent *et al.*, 2004; Newton *et al.*, 2006). Multiple previous studies observed dramatic declines in bacterial diversity during macroplankon blooms (Sommer, 1993; Höfle, *et al.*, 1999), suggesting that this decreasing trend was either caused by zooplankton grazing or associated with the dominance of single or multiple algal species in phytoplankton communities (Hansson, *et al.*, 1994; Yannarell, *et al.*, 2003). In our study, bacterial richness and diversity of AA bacteria communities decreased during late spring or early summer at the lake site (June to July 2013), and late summer (August 2013) at the harbor site (Table 3-3), which were synchronous with highest chlorophyll *a* concentration in 2013 (Table 3-4). However, this synchronous response was not observed on SP and in AFW bacterial communities at both sites.

Seasonal dynamics of bacterial communities of SP, AA and AFW at the lake and harbor sites

The NMS ordinations demonstrated very clear seasonal changes of bacterial communities on SP, AA and in AFW at both lake and harbor sites (Figure 3-2). In addition, it was observed that the variations among bacterial communities in AFW were less pronounced than SP and AA bacterial communities, indicating AFW bacterial communities were very consistent throughout the year, while bacterial communities associated with SP and AA had more clear seasonal patterns (Figure 3-3). In AFW, bacterial communities showed more variations over time at the Duluth-Superior Harbor site as compared to the nearshore Lake Superior site (Figure 3-2). However, SP and AA bacterial communities showed almost equal variations over time at the two sample sites (Figure 3-2).

Previous studies indicated that there are four main types of lake aggregates identifiable by microscope observation, which were composed of algal cells or debris, molts or dead body of zooplankton, colony-forming cyanobacterial species, as well as some other unidentified organic and inorganic materials (Grossart and Simon, 1993). Usually, seasonal changes resulted in variation in aggregates composition by influencing the zooplankton, phytoplankton and cyanobacteria compositions in aquatic environments. Aggregates with various composition may harbor different bacterial species, therefore showing distinctive seasonal changes. This assumption is very consistent with our observations here: bacterial community communities on SP or AA at the lake and harbor sites were different from each other, and both showed clear seasonal patterns.

Overall, the seasonal differences of richness and biodiversity of three different bacterial communities among the sites were very clear. At the lake site, both the SP and AA bacterial communities had the highest bacterial richness in late summer or early fall (August to October), while at the harbor site, these bacterial communities had highest richness in early summer (June) (Figure 3-4 and 3-5). However, the bacterial community in AFW presented highest richness in late spring or early summer (May to June) at both lake and harbor site (Figure 3-6).

In this study, we observed some major bacterial phyla on SP, AA and in AFW communities that all showed seasonal variations. It is worth mentioning that at the harbor site, the bacterial richness of the AA community decreased strongly in late summer or early fall (August or October) (Figure 3-5). This seasonal pattern was mainly reflected as the changes of cyanobacteria/chloroplast OTUs from 2012 and 2013, which was also highly correlated with the annual changes of concentrations of chlorophyll *a*. The bloom of cyanobacteria or other small phytoplankton often occur in eutrophic condition during warmer months, which would explain two dramatic peaks of cyanobacteria/chloroplast sequences in October 2012 and August 2013. AA associated cyanobacteria/chloroplast at the lake site also increased at the end of each sample year. Because the water temperature of Lake Superior is relatively low in early winter, these peaks of cyanobacteria/chloroplast sequences could be related to either available nutrient concentration or other factors that we did not investigate.

Comparison of bacterial communities on SP, AA and in AFW

Compared to several other interannual or seasonal analyses of bacterial community composition in aquatic ecosystems, which were mainly based on the relative abundance of individual populations that was from different techniques of community fingerprints (Schauer *et al.*, 2003; Yannarell *et al.*, 2003; Henriques *et al.*, 2006), we applied Illumina next-generation sequencing of the highly varied region of the v5-v6 16S rRNA gene, which on average provided 95% coverage of total population. In addition, unlike some previous studies, which only focused on total bacterial community (van der Gucht, *et al.*, 2001; Yannarell, *et al.*, 2003), we studied three different eco-types of bacterial community: settling particle (SP), artificial aggregates (AA), and aggregate free water (AFW). Overall, this study could provide higher resolution and more comprehensive results regrading phylogenetic composition of bacterial communities at both nearshore Lake Superior and the Duluth-Superior Harbor sites and their temporal variability.

Because marine or lake snow is fragile, traditional sample techniques or subsequent handling methods could destroy them easily (Nishizawa *et al.*, 1954; Riley, 1963). Since we are not capable of collecting aggregates via scuba divers, and Lake Superior is very oligotrophic with very small amounts of suspended organic matter to form aggregates naturally, the best way to study lake snow for us is to generate artificial ones in the lab as a biological model of the real thing (Shanks and Edmondson, 1989). Besides artificial aggregates, using Imhoff Cones provides a reliable method to collect more rapidly sinking and larger aggregates. One of the most important roles that sinking

aggregates play is cycling and transferring of suspended organic matter from the ocean's surface to its benthic layers (Fowler and Knauer, 1986; Alldredge and Silver, 1988). Therefore, the characteristics of sinking particles and organic aggregates that are suspended in water column could be very distinctive, which may suggest the bacterial communities associated these two type of aggregates maybe also different.

So far, very few studies have been done to investigate bacterial communities associated with naturally sinking particles, as well as artificial organic aggregates in freshwater environments. The artificial aggregates made in rolling cylinders have similar morphology and composition, as well as bacterial colonization patterns compared to natural lake snow aggregates by the microscopic examination, which indicates artificial aggregates are very good models for simulating natural aggregates (Weiss *et al.*, 2002). Although both natural sinking particles and artificial aggregates are considered as "hotspots" for diverse microorganisms, our results revealed that bacterial communities on SP and AA were significantly different from each other at both the nearshore Lake Superior and the Duluth-Superior harbor sites. More specifically, we found that in both sample sites, AA bacterial community had significantly higher total unique OTUs (bacterial richness) than SP bacterial communities. The Shannon-Wiener diversity and evenness indices were also found to be higher in AA than SP bacterial communities. Although SP and AA were both types of particles, the main difference between these two types of particles is that sinking particles are naturally settled out in aquatic ecosystem, while artificial aggregates were artificially formed with the help of turbulence during the

rotation. This difference could cause distinctive bacterial communities to be attached to each type of particles.

Cyanobacteria/chloroplast dominated AA communities and were more abundant in AA than SP communities. Cyanobacteria/chloroplast have been found, in previous studies, to contribute to the particle-attached fraction due to their larger cell size (>5.0 μm in diameter) and their tendency to form colonies (Rösel *et al.*, 2012; also see Chapter 2 results and discussion). In this study, most cyanobacteria/chloroplast sequences were found in AA, which also emphasizes their tendency to form colonies and aggregates with the help of turbulence as well as the effect of their cell size.

Interestingly, our results demonstrated that the abundance of the families *Comamonadaceae* (*Betaproteobacteria*), *Oxalobacteraceae* (*Betaproteobacteria*), *Caulobacteraceae* (*Alphaproteobacteria*), and *Pseudomonadaceae* (*Gammaproteobacteria*) in sinking particles was substantially higher than that in artificial aggregates. Similar results have been observed in a study showing that the relative fraction of *Betaproteobacteria* in aggregates collected from sediment traps were significantly higher than that on natural lake snow (Weiss *et al.*, 1996). Weiss *et al.* (1996) concluded the possible reasons for these results were because the lake snow aggregates were collected 3 days earlier and 30 m above the aggregates collected from sediment trap, the aggregates in the trap were older and colonized by growing *Betaproteobacteria*. Several reasons may explain why bacterial communities on SP and AA were different from each other. In this study, artificial aggregates were formed in rolling cylinders, where these aggregates could experience simulated shear force and

turbulence. Therefore, we expected the process of bacterial colonization on these aggregates to be highly dynamic, and only bacterial species that could tolerant turbulence would colonize on these aggregates. Sinking particles were collected by a natural sinking process, which is more gentle and might favor an attached bacterial community continuing to grow and propagate. In addition, during the process of sedimentation, particles are more prone to be influenced by grazing of zooplankton. Multiple studies have shown that the relative abundance of *Alphaproteobacteria* increased because of high zooplankton grazing (Šimek *et al.*, 1999; Jürgens and Jeppesen, 2000; Langenheder and Jürgens, 2001; Salcher *et al.*, 2005; Comté *et al.*, 2006). This trend was suggested to be associated with the tendency of members within the class *Alphaproteobacteria* to form filaments, aggregates, or "*Caulobacter*-like stalked cells" (Šimek *et al.*, 1999; Salcher *et al.*, 2005; Hahn, *et al.*, 2010). In this study, sequences from the *Caulobacteraceae* (*Alphaproteobacteria*) were detected more commonly on SP than AA communities, which was consistent with the conclusions of these previous studies. Overall, although both SP and AA are particles from in aquatic systems, bacterial communities attached to AA were planktonic and suspended in the water column, while bacterial communities attached to SP entered sediments. These differences may make bacterial communities on AA have some distinctive features compared to SP communities.

The differences of bacterial community compositions of free-living and particle-associated bacteria have been observed in multiple studies in a variety of aquatic environments including marine, estuarine, riverine, and lacustrine (Riemann and Winding, 2001; Bouvier and del Giorgio, 2002; Henriques *et al.*, 2006; Rösel *et al.*, 2012; Rösel

and Grossart, 2012; Mou *et al.*, 2013). Multiple previous studies found that the taxonomy of the bacterial community attached to particles was more diverse than free-living bacterial communities. These findings were very consistent with the results of our spatial investigation of bacterial communities compositions along the transect from the St. Louis River to nearshore Lake Superior (Chapter 2). In this study, it was not very surprised to find that the AA bacterial communities were more diverse than the AFW communities at both lake and harbor sites. There were significant differences between bacterial communities in AA and AFW at both lake and harbor environments. For example, sequence reads of the families *Comamonadaceae* (*Betaproteobacteria*), *Microbacteriaceae* (*Actinobacteria*), *Candidatus Pelagibacter* (*Alphaproteobacteria*), *Burkholderiaceae* (*Betaproteobacteria*), *Methylophilaceae* (*Betaproteobacteria*), *Acidimicrobiaceae* (*Actinobacteria*), *Opitutaceae* (*Verrucomicrobia*) and *Cryptosporan-giaceae* (*Actinobacteria*) were all more abundant in AFW bacterial communities at both lake and harbor sites. Some of these families are considered to have free-living life history strategies in water based on the results of several previous studies. For example, *Microbacteriaceae*, *Cryptosporangiaceae* and *Acidimicrobiaceae* are affiliated with the orders of *Acidimicrobiales* (*acIV*) and *Actinomycetales* (*acI*). These two major lineages are very dominant in lake *Actinobacteria* (Warnecke *et al.*, 2005; Allgaier and Grossart, 2006), and members within *acI* clusters accounted for large fractions of free-living communities in four lakes of the Mecklenburg Lake District (Northeastern Germany) (Allgaier *et al.*, 2007). *Candidatus_Pelagibacter*, also known as *SAR11* clade within the class of *Alphaproteobacteria*, constituted free-living fraction bacteria (Thrash *et al.*,

2011), and counted for very common members of *Alphaproteobacteria* in freshwater lake 16S rRNA gene libraries (Zwart *et al.*, 2002), which agreed with the results in our study. *Burkholderiaceae* (Pnec) is the best-studied freshwater lake clade within the *Betaproteobacteria*, and recently some members of the genus *Polynucleobacter* with the *Burkholderiaceae* family were reported as free-living subspecies (Hahn *et al.*, 2009). However, less is known about the ecology of the family *Methylophilaceae* (betIV), and only few studies have revealed members within this family that were free-living cells (Giovannoni *et al.*, 2008). Members of the family *Methylophilaceae* are obligate aerobic and restricted facultative methylotrophs that can only utilize methanol or methylamine instead of methane as their solo carbon and energy sources (Anthony, 1982). Few studies have been done regarding the distribution in aquatic environments of family *Opitutaceae* (*Verrucomicrobia*), which may due to their lower abundance in freshwater lakes.

Comparison of bacterial communities between the lake and harbor sites

Although the richness, diversity and evenness of three types of bacterial communities were very similar at both lake and harbor sites, the compositions of these bacterial communities were very different. We observed that cyanobacteria/chloroplast were more abundant at the harbor than the lake site in all bacterial communities on SP, AA and in AFW. This is not surprising due to the higher chlorophyll *a* and TEP concentrations we measured at the harbor site relative to the lake site.

The family *Cytophagaceae* (within *Bacteroidetes*) and *Sphingomonadaceae* (within *Proteobacteria*) had more sequence reads at the lake than the harbor site in both

SP and AA bacterial communities. Sphingomonads are considered as typical members occurring in freshwater habitats (Newton *et al.*, 2011). Some species within this group have ability to utilize small organic matter formed by the degradation or photolysis by sunlight exposure of humic substances, which are highly abundant in the lakes. Members of the family *Cytophagaceae* are heterotrophic and widely distributed in marine, freshwater, and terrestrial habitats (McBride, *et al.*, 2014). Many members of this family have the ability to digest or degrade complex biopolymers, such as proteins and polysaccharides, and several previous studies observed the appearance of many sequenced DGGE bands related to the groups of *Cytophaga-Flavobacterium* cluster after a massive cyanobacterial bloom (Van Hannen, *et al.*, 1999), and an intensive dinoflagellate bloom along the southern California coast (Fandino, *et al.*, 2001). However, all these studies only focused on total bacterial communities instead of separation between particle-associated or water-associated communities. In our study, we did not observe similar patterns between the family *Cytophagaceae* or the phylum *Bacteroidetes* and cyanobacteria/chloroplast in bacterial communities on SP and AA, suggesting they may perform differently than the total bacterial community structure.

Although overall the total abundance of *Proteobacteria* sequences on AA and in AFW communities was not significantly different between two sites, some families within this taxon presented variations. For example, a higher number of sequences from the *Comamonadaceae* family but fewer *Burkholderiaceae* were found at the lake site compared to the harbor site. The family of *Comamonadaceae* and *Burkholderiaceae* are both very large and diverse groups, and some members of them have been found in many

lakes ranging from an oligotrophic lake in Alaska to shallow eutrophic lakes in Belgium, by either using a 16S rRNA targeted FISH probe or 16S rRNA gene sequencing techniques (Newton *et al.*, 2011). There was one notable peak of the *Comamonadaceae* family in AA bacterial communities that occurred in early spring (April) at both lake and harbor sites (data not shown); this may be associated with the runoff of snow melting along with other accumulative nutrients and organic matters. The phylum of *Actinobacteria* (such as the family of *Microbacteriaceae* and *Acidimicrobiaceae*) was more abundant in the nearshore communities. The abundance of *Actinobacteria* were observed to be associated with high UV transparency (Warnecke *et al.*,2005), as well as low nutrient concentrations (Haukka, *et al.*, 2006). Consistent with these previously observed trends, more *Actinobacteria* sequences were found at the lake site with higher UV penetration and lower nutrient concentrations compared to the harbor site, which is highly loaded with suspended organic matters and exhibits lower transparency.

Conclusions

Overall, bacterial communities on SP, AA, and in AFW all demonstrated some seasonal changes from 2012 to 2013. One of the most striking findings was the difference between bacterial communities in naturally sinking particles (i.e., SP) and artificial aggregates (AA) generated by rotating bottles of water. This finding indicates that turbulence that causes particles to form aggregates controls the composition of bacterial communities that reside on these particles. In addition, we also found bacterial communities on artificial aggregates (AA) were very different from those in aggregate-

free water (AFW). These differences may be caused by the tendency of certain bacterial communities to form aggregates with the help of turbulence. Based on all the environmental parameters we measured, we found nearshore Lake Superior and the Duluth-Superior harbor had dissimilar trophic states, therefore, the bacterial communities at two sites also had substantially distinctive compositions and structures as we expected. In the future, to obtain more comprehensive results about the seasonal patterns it will be better for us to collect samples in a long run of 2-3 years to observe more clear seasonality and the reoccurrence of some bacterial groups. It's also better to compare bacterial communities in aggregate-free water and sinking particle-free water fractions to detect any differences or similarities of the composition or diversity of bacterial communities. In addition, by comparing bacteria communities attached to particles by serial filtration and those associated with artificial aggregates using rolling cylinders, their similarities or difference could enhance our knowledge regarding characteristics of methodology to studying suspended aggregates.

TABLES

Table 3-1. Comparisons between three different bacterial communities types associated with sinking particles (SP), artificial aggregates (AA) and aggregate-free water (AFW) from nearshore Lake Superior and the Duluth-Superior Harbor. AMOVA analysis was used to compare two communities, which were considered significantly different if $p < 0.05$. Total dissimilarity (D) between bacterial communities is shown. Bacterial taxa that contributed most to the dissimilarities between different bacterial communities types at lake (MQ) and harbor (BB) sites were identified using Metastats (all *p's* < 0.05).

Sample site	Group comparison	*p* value	*D* (%)	Abundant bacterial taxa that contribute to dissimilarities between compared groups
MQ	SP vs. AA	<0.001	58.22	*Comamonadaceae* ^{S*}, Cyanobacteria/Chloroplast ^{A*}, *Oxalobacteraceae* ^{S*}, *Caulobacteraceae* ^{S*}, *Pseudomonadaceae* ^{S*}, *Sphingomonadaceae* ^{S*}, *Microbacteriaceae* ^{S*}, *Methylophilaceae* ^{S*}, *Rhodobacteraceae* ^{S*}, *Candidatus_Pelagibacter* ^{S*}, *Burkholderiaceae* ^{S*}, *Acidimicrobiaceae* ^{A*}
	AA vs. AFW	<0.001	55.36	*Comamonadaceae* ^{W*}, Cyanobacteria/Chloroplast ^{A*}, *Microbacteriaceae* ^{W*}, *Candidatus_Pelagibacter* ^{W*}, *Acidimicrobiaceae* ^{W*}, *Methylophilaceae* ^{W*}, *Burkholderiaceae* ^{W*}, *Sphingomonadaceae* ^{A*}, *Opitutaceae* ^{W*}, *Caulobacteraceae* ^{A*}, *Cyclobacteriaceae* ^{W*}, *Oxalobacteraceae* ^{A*}, *Cryptosporangiaceae* ^{W*}, *Rhodobacteraceae* ^{A*},
BB	SP vs. AA	<0.001	56.11	Cyanobacteria/Chloroplast ^{A*}, *Comamonadaceae* ^{S*}, *Pseudomonadaceae* ^{S*}, *Oxalobacteraceae* ^{S*}, *Caulobacteraceae* ^{S*}, *Phycisphaeraceae* ^{A*}
	AA vs. AFW	<0.001	63.79	Cyanobacteria/Chloroplast ^{A*}, *Comamonadaceae* ^{W*}, *Burkholderiaceae* ^{W*}, *Candidatus_Pelagibacter* ^{W*}, *Methylophilaceae* ^{W*}, *Microbacteriaceae* ^{W*}, *Cryptosporangiaceae* ^{W*}, *Opitutaceae* ^{W*}, *Acidimicrobiaceae* ^{W*}, *Anaerolineaceae* ^{W*}

Abbreviations: MQ-McQuade Harbor (nearshore Lake Superior); BB-underneath the Blatnik Bridge (the Duluth-Superior Harbor); AFW-aggregate-free water associated bacterial community; SP-sinking particle associated bacterial community; AA-artificial aggregate associated bacterial community;

^{A*} indicates this family was statistically significant more abundant in AA bacterial community

^{W*} indicates this family was statistically significant more abundant in AFW bacterial community

^{S*} indicates this family was statistically significant more abundant in SP bacterial community

Table 3-2. Comparison between nearshore Lake Superior and the Duluth-Superior Harbor for three different bacterial communities associated with sinking particles (SP), artificial aggregates (AA), and aggregate-free water (AFW). AMOVA analysis was used to compare samples at different sites, which were considered different if $p < 0.05$. Total dissimilarity (D) between bacterial communities at the two sample sites is shown. Bacterial taxa that contributed most to the dissimilarity between each bacterial community type at the Lake Superior and Duluth-Superior Harbor sites were identified using Metastats (all *p's* < 0.05).

Bacterial Communities	p value	D (%)	Abundant bacterial taxa that contribute to dissimilarities between nearshore Lake Superior and the Duluth-Superior Harbor
SP	<0.001	57.21	Cyanobacteria/Chloroplast ^{H*}, *Sphingomonadaceae* ^{L*}, *Cytophagaceae* ^{L*}, *Candidatus_Pelagibacter* ^{L*}, *Rhodospirillaceae* ^{L*}
AA	<0.001	42.38	Cyanobacteria/Chloroplast ^{H*}, *Comamonadaceae* ^{L*}, *Sphingomonadaceae* ^{L*}, *Cytophagaceae* ^{L*}, *Microbacteriaceae* ^{L*}, *Oxalobacteraceae* ^{L*}, *Acidimicrobiaceae* ^{L*}, *Burkholderiaceae* ^{H*},
AFW	<0.001	43.96	*Comamonadaceae* ^{L*}, Cyanobacteria/Chloroplast ^{H*}, *Burkholderiaceae* ^{H*}, *Microbacteriaceae* ^{L*}, *Cryptosporangiaceae* ^{H*}, *Verrucomicrobiaceae* ^{L*}, *Sphingobacteriaceae* ^{H*}, *Acidimicrobiaceae* ^{L*}, *Chitinophagaceae* ^{L*}, *Cyclobacteriaceae* ^{L*}

Abbreviations: SP-sinking particle associated bacterial community; AA-artificial aggregate associated bacterial community; AFW-aggregate-free water bacterial community

^{L*} indicates this family was significant more abundant at the nearshore Lake Superior than at the Duluth-Superior Harbor site

^{H*} indicates this family was significant more abundant at the Duluth-Superior Harbor than at the nearshore Lake Superior site

Table 3-3. Sequencing coverage, richness (*sobs*), Shannon-Wiener diversity (*H'*) and evenness (*J'*) indices were compared among SP, AA and AFW bacterial communities from April to November 2013 at nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB) sites.

Month	Type	Coverage		*sobs*		H'		J'	
		MQ	BB	MQ	BB	MQ	BB	MQ	BB
Apr-13	SP	96%	95%	354	503	4.57	4.20	0.78	0.68
	AA	98%	97%	595	593	4.37	4.16	0.68	0.65
	AFW	99%	97%	350	439	4.11	4.34	0.70	0.71
May-13	SP	95%	91%	300	519	4.48	4.64	0.79	0.74
	AA	95%	96%	708	1108	4.79	5.28	0.73	0.75
	AFW	99%	97%	671	565	4.56	4.37	0.70	0.69
Jun-13	SP	96%	90%	403	702	4.10	4.66	0.68	0.71
	AA	95%	96%	678	1292	4.62	5.42	0.71	0.76
	AFW	98%	96%	610	682	4.28	4.65	0.67	0.71
Jul-13	SP	99%	93%	238	310	3.95	3.55	0.72	0.62
	AA	98%	97%	424	1113	4.65	5.41	0.77	0.77
	AFW	99%	99%	355	578	4.23	4.21	0.72	0.66
Aug-13	SP	93%	98%	545	411	4.76	4.17	0.75	0.69
	AA	98%	97%	1003	227	5.05	1.25	0.73	0.23
	AFW	98%	98%	387	510	4.12	4.44	0.69	0.71
Oct-13	SP	94%	95%	817	380	5.42	3.64	0.81	0.61
	AA	98%	96%	1002	778	5.23	4.26	0.76	0.64
	AFW	96%	99%	383	582	4.18	4.52	0.70	0.71
Dec-13	SP	96%	97%	272	372	3.68	4.48	0.66	0.76
	AA	98%	98%	562	486	4.42	3.76	0.70	0.61
	AFW	99%	99%	370	378	4.35	3.80	0.74	0.64

Table 3-4. Seasonal environmental and biotic parameters in nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB). Standard deviations are shown in parenthesis if applicable.

Date	Prokaryotic cell abundance (×10^6/ml)		Chlorophyll *a* (µg/L)		TEP (µg/L Xeq)		DOC (mg/L)		POC (µg/L)		PON (µg/L)	
	MQ	BB	MQ	BB	MQ	BB	MQ	BB	MQ	BB	MQ	BB
7/11/2012	1.88 (0.35)	2.04 (0.06)	1.31 (0.04)	5.86 (0.55)	132.8 (31.3)	264.7 (73.3)	18.9	27.6	204	770	33	107
10/18/2012	0.39 (0.06)	1.55 (0.18)	1.32 (0.02)	8.50 (0.05)	152.2 (19.0)	539.2 (48.2)	1.3	10.7	138	492	17	67
11/20/2012	0.31 (0.02)	1.83 (0.32)	0.80 (0.01)	5.04 (0.17)	61.1 (9.0)	189.7 (23.4)	1.0	9.7	63	389	11	65
12/20/2012	0.48 (0.05)	1.63 (0.16)	0.89 (0.08)	3.82 (0.10)	71.8 (14.3)	209.2 (13.7)	1.0	9.1	65	320	10	52
4/22/2013	0.22 (0.03)	1.74 (0.31)	0.25 (0.02)	0.52 (0.04)	79.2 (4.3)	202.2 (31.8)	1.1	18.1	61	480	12	61
5/24/2013	0.40 (0.02)	1.77 (0.08)	0.43 (0.03)	1.77 (0.05)	121.9 (11.4)	655.6 (1.5)	3.1	24.6	61	695	18	90
6/25/2013	0.70 (0.06)	2.72 (0.47)	1.32 (0.11)	1.70 (0.04)	79.6 (4.4)	504.3 (21.4)	5.1	23.1	282	938	39	111
7/23/2013	0.38 (0.02)	6.04 (0.76)	0.55 (0.02)	2.50 (0.08)	54.4 (3.1)	585.5 (61.6)	3.0	18.0	135	639	16	89
8/27/2013	1.95 (0.31)	4.73 (0.27)	0.34 (0.01)	2.47 (0.06)	116.2 (15.5)	216.3 (16.4)	2.1	8.8	205	1068	27	157
10/22/2013	0.76 (0.04)	4.65 (0.34)	0.20 (0.01)	0.94 (0.06)	66.3 (5.2)	344.4 (20.1)	5.0	31.0	249	514	27	59
11/30/2013	0.57 (0.02)	4.84 (0.69)	0.19 (0.03)	0.46 (0.04)	54.2 (2.0)	110.7 (58.4)	2.5	42.7	73	233	11	35

Table 3-5. Mantel test analysis showing the relationship between 6 environmental parameters and SP, AA and AFW bacterial communities. The *r* value is the Pearson's correlation coefficient. *p* values printed in bold italic typeface indicate significant correlations ($p < 0.05$).

	SP				AA				AFW			
	MQ		BB		MQ		BB		MQ		BB	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Chl a (µg/L)	-0.017	0.514	0.071	0.453	0.075	0.279	0.111	0.300	***0.700***	***0.018***	***0.517***	***0.039***
TEP (µg/L)	-0.013	0.523	-0.114	0.729	0.041	0.374	0.182	0.124	-0.051	0.540	0.230	0.087
DOC (mg/L)	-0.004	0.493	0.096	0.349	***0.504***	***0.026***	0.112	0.295	-0.097	0.569	0.192	0.269
POC (mg/L)	0.126	0.245	-0.138	0.706	***0.348***	***0.024***	0.074	0.345	0.244	0.149	0.165	0.277
PON (mg/L)	0.215	0.169	0.000	0.525	***0.367***	***0.016***	0.182	0.226	0.508	0.036	0.060	0.387
C:N	-0.287	0.914	-0.425	0.978	-0.245	0.901	0.138	0.303	0.085	0.334	-0.102	0.645

FIGURES

Figure 3-1. Map of the western end of Lake Superior and the Duluth-Superior Harbor. Site MQ indicates the McQuade small craft harbor site in the near shore area of Lake Superior. Site BB indicates the sampling site underneath the Blatnik Bridge within the Duluth-Superior Harbor.

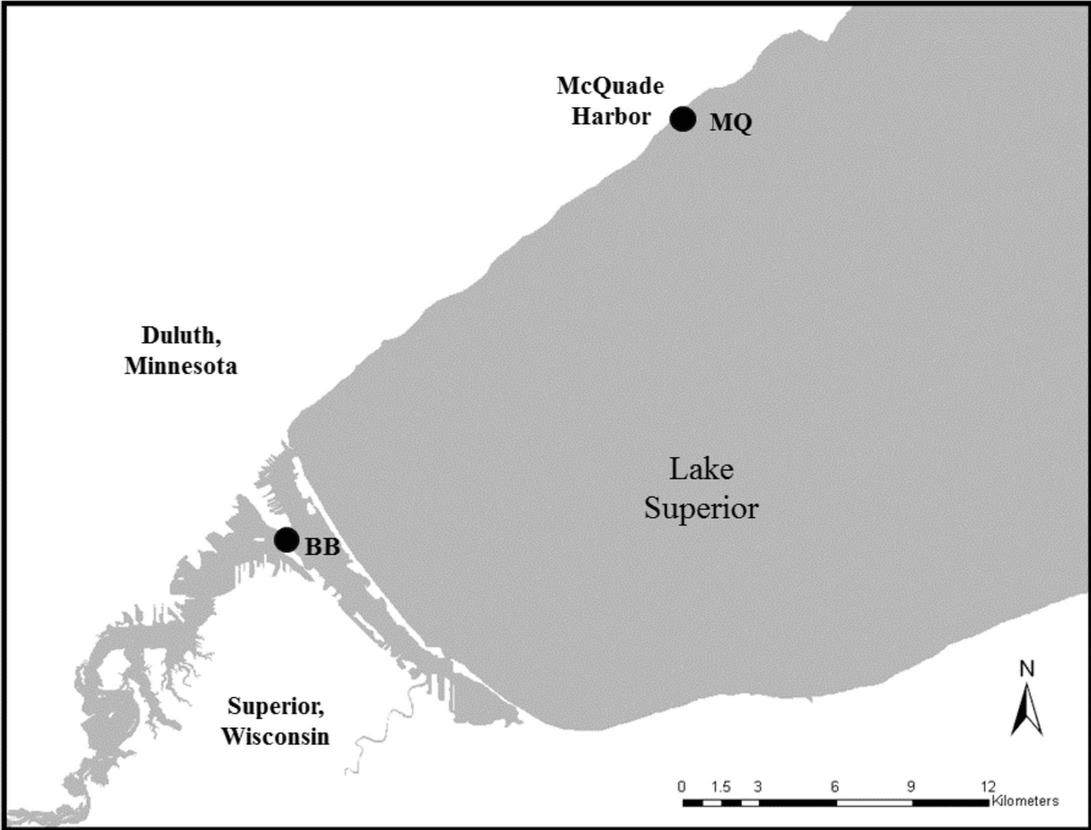
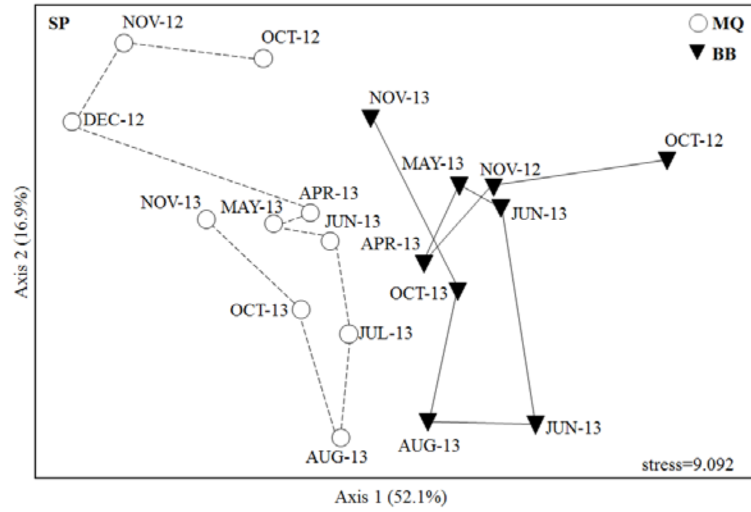
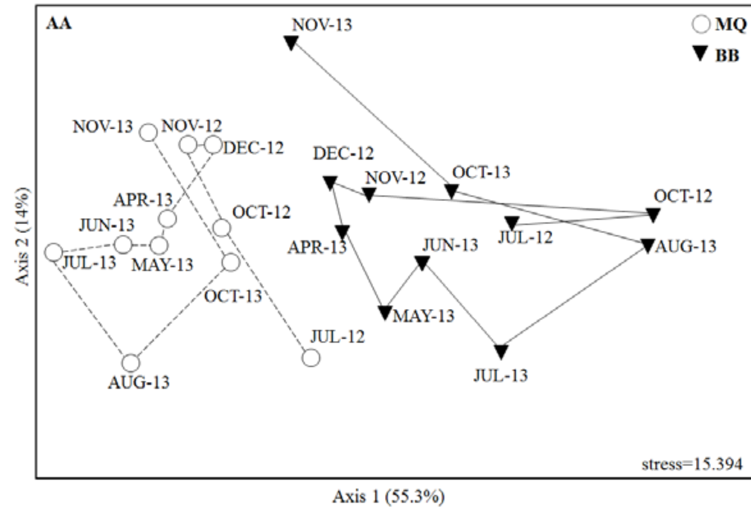

McQuade
Harbor
MQ
Duluth,
Minnesota
Lake
Superior
BB
Superior,
Wisconsin
N
0
1.5
3
6
9
12
Kilometers

Figure 3-2. NMS ordinations among bacterial communities associated with sinking particles (SP), artificial aggregates (AA), and aggregate-free water (AFW) from nearshore Lake Superior (MQ, open circle on the left) and the Duluth-Superior Harbor (BB, solid triangle on the right) during 2012 and 2013. Samples collected in different months are sequentially connected by a dashed (for MQ samples) and solid (for BB samples) line for clarity.


SP
MQ
BB
NOV-12
OCT-12
DEC-12
NOV-13
MAY-13
APR-13
JUN-13
OCT-13
JUL-13
AUG-13
NOV-13
MAY-13
NOV-12
OCT-12
JUN-13
APR-13
OCT-13
AUG-13
JUN-13
stress=9.092
Axis 2 (16.9%)
Axis 1 (52.1%)


AA
MQ
BB
NOV-13
NOV-12
DEC-12
APR-13
OCT-12
JUN-13
JUL-13
MAY-13
OCT-13
JUL-12
AUG-13
NOV-13
DEC-12
NOV-12
OCT-13
OCT-12
JUL-12
APR-13
JUN-13
AUG-13
MAY-13
JUL-13
stress=15.394
Axis 2 (14%)
Axis 1 (55.3%)

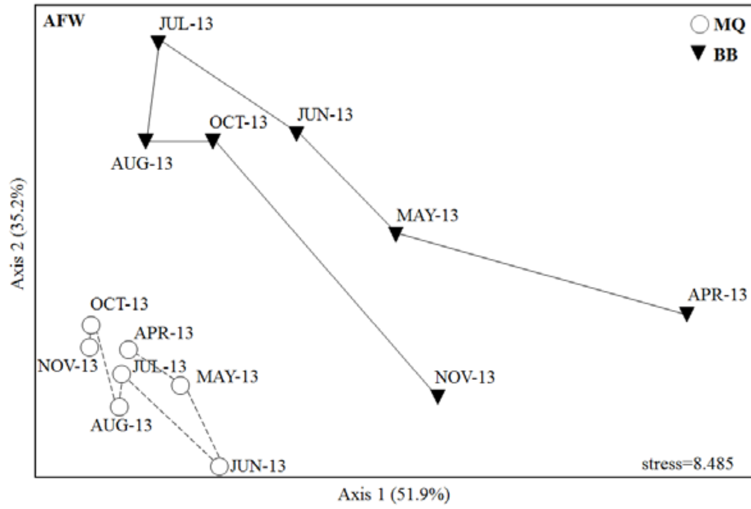
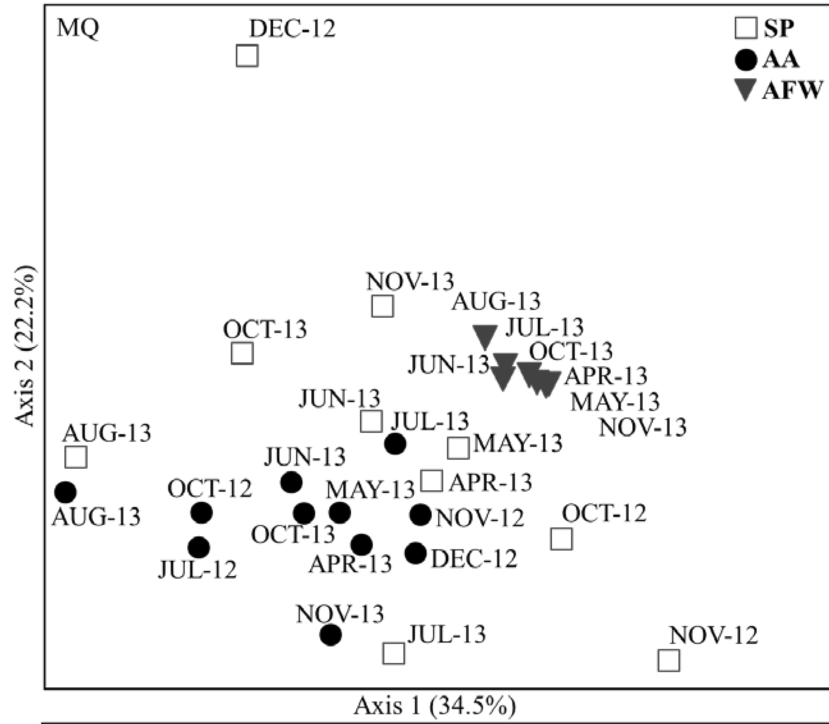

AFW
MQ
BB
JUL-13
JUN-13
OCT-13
AUG-13
MAY-13
APR-13
NOV-13
OCT-13
APR-13
NOV-13
JUL-13
MAY-13
AUG-13
JUN-13
stress=8.485
Axis 2 (35.2%)
Axis 1 (51.9%)

Figure 3-3. NMS ordinations showing differences between bacterial communities on sinking particles (SP), artificial aggregates (AA), and in aggregate-free water (AFW) sampled from nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB) during 2012 and 2013.


MQ
SP
AA
AFW
DEC-12
NOV-13
AUG-13
OCT-13
JUL-13
JUN-13
OCT-13
APR-13
MAY-13
NOV-13
JUN-13
JUL-13
AUG-13
MAY-13
JUN-13
APR-13
OCT-12
MAY-13
AUG-13
NOV-12
OCT-12
OCT-13
APR-13
DEC-12
JUL-12
NOV-13
JUL-13
NOV-12
Axis 2 (22.2%)
Axis 1 (34.5%)

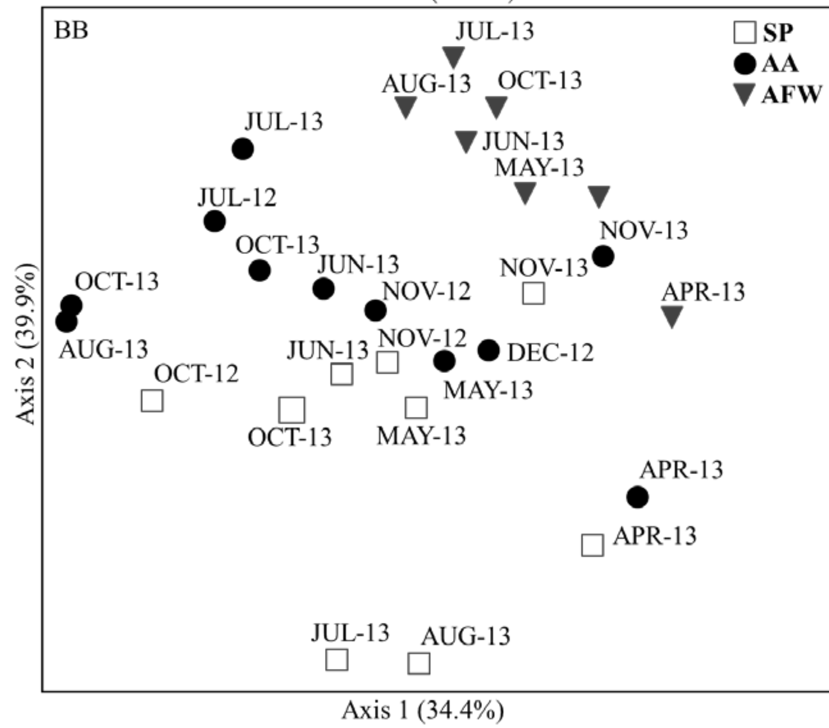
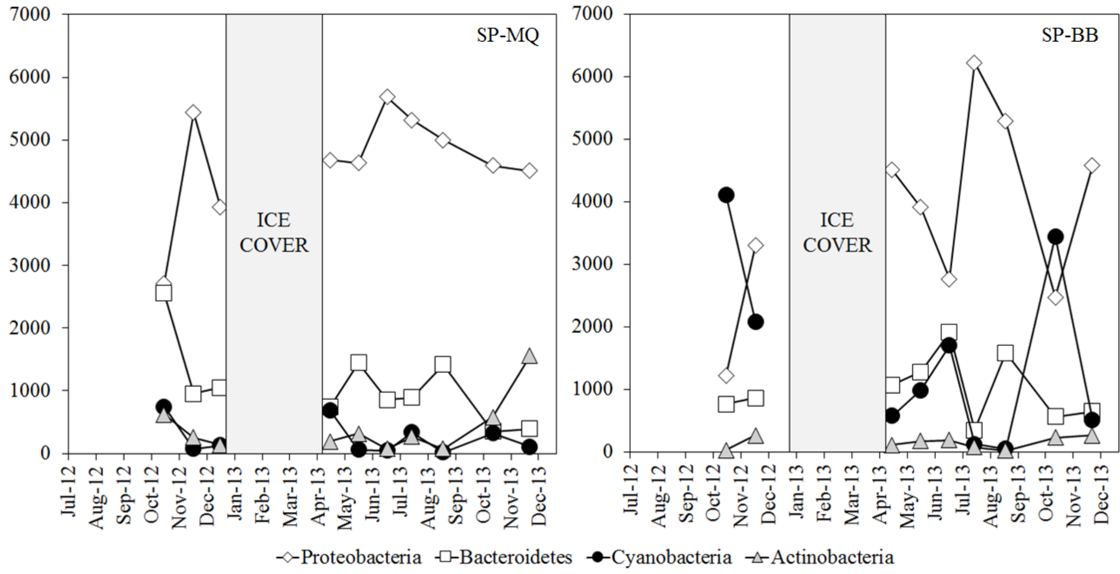

BB
SP
AA
AFW
JUL-13
AUG-13
OCT-13
JUL-13
JUN-13
MAY-13
JUL-12
NOV-13
OCT-13
JUN-13
NOV-13
OCT-13
NOV-12
APR-13
AUG-13
JUN-13
NOV-12
DEC-12
OCT-12
MAY-13
OCT-13
MAY-13
APR-13
APR-13
JUL-13
AUG-13
Axis 2 (39.9%)
Axis 1 (34.4%)

Figure 3-4. Seasonal dynamics of the six most abundant bacterial phyla found in sinking particle (SP) bacterial communities from nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB) in 2012 and 2013.


SP-MQ
SP-BB
ICE COVER
ICE COVER
Proteobacteria
Bacteroidetes
Cyanobacteria
Actinobacteria

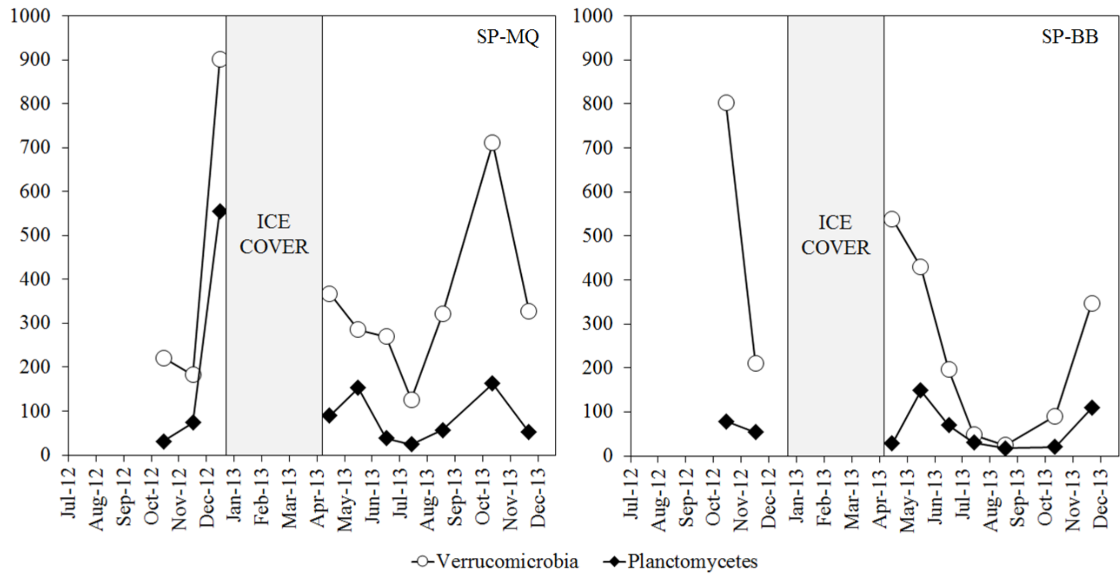
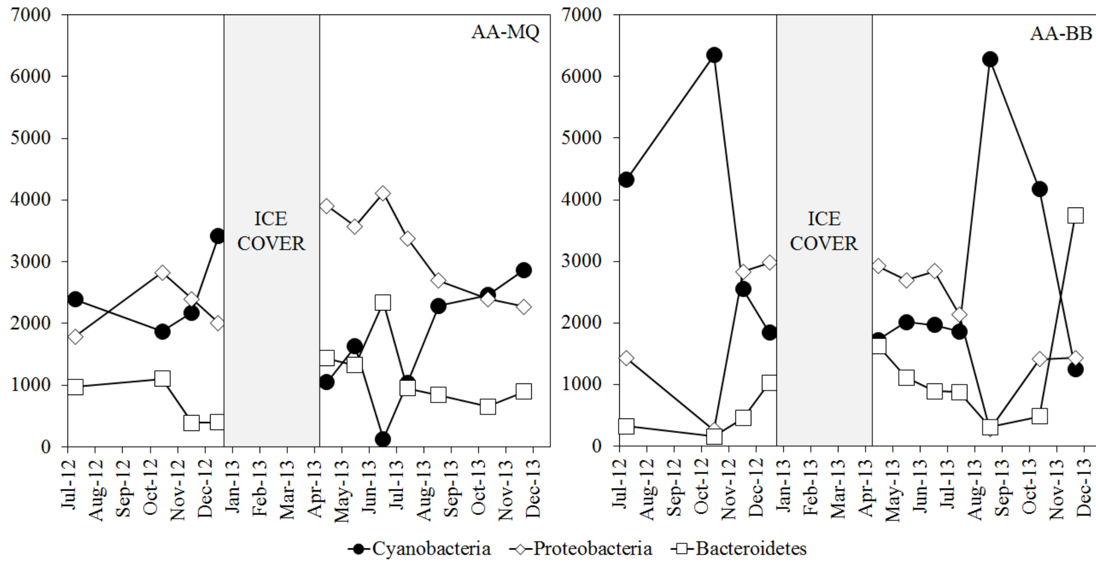

SP-MQ
SP-BB
ICE COVER
ICE COVER
Verrucomicrobia
Planctomycetes

Figure 3-5. Seasonal dynamics of the six most abundant bacterial phyla found in artificial aggregate (AA) bacterial communities generated in the lab with water from nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB) in 2012 and 2013.


AA-MQ
AA-BB
ICE COVER
Cyanobacteria
Proteobacteria
Bacteroidetes

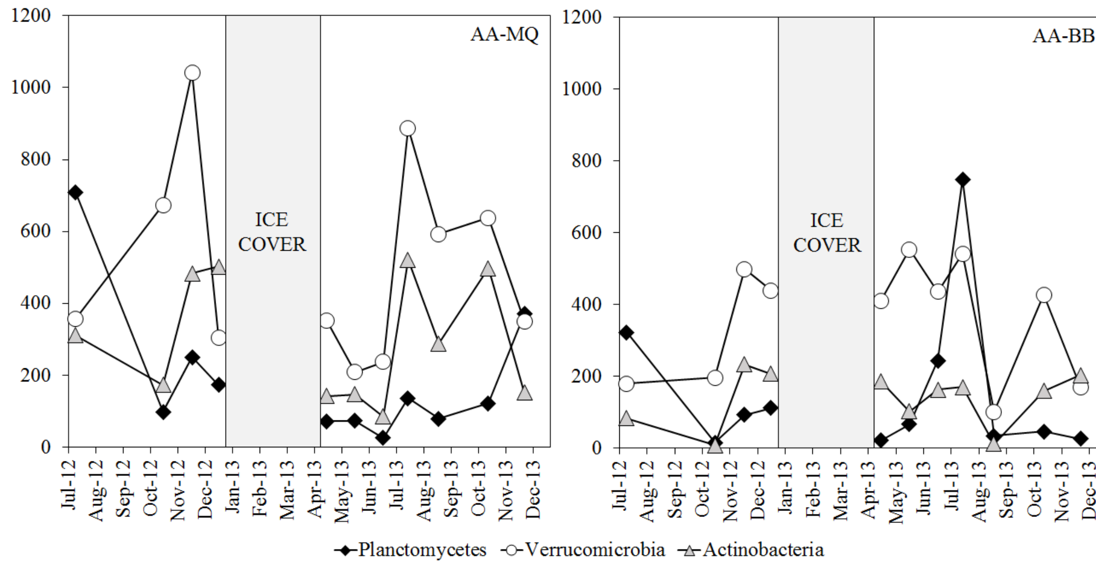
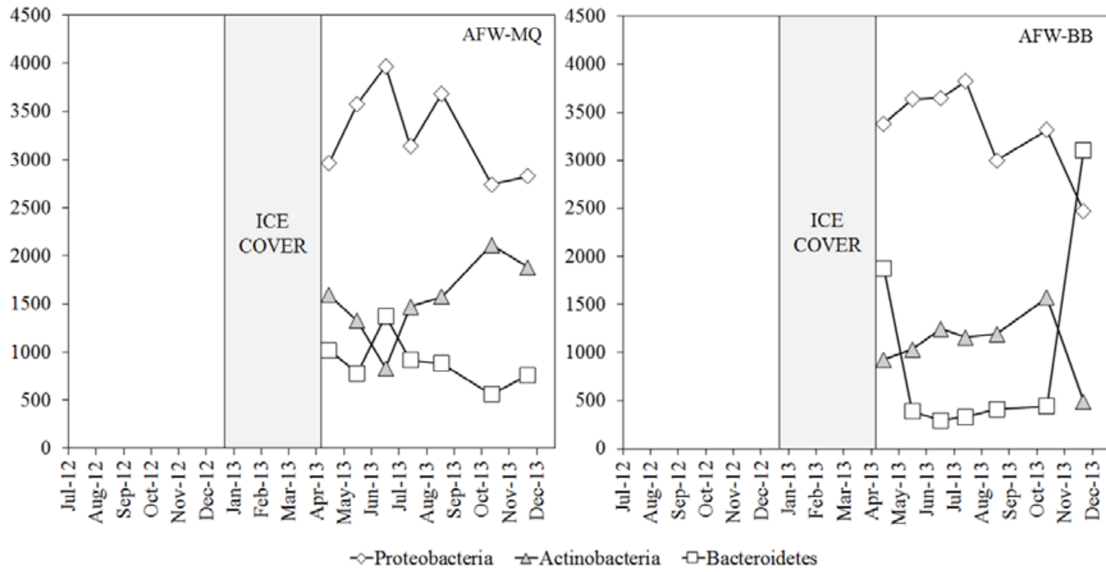
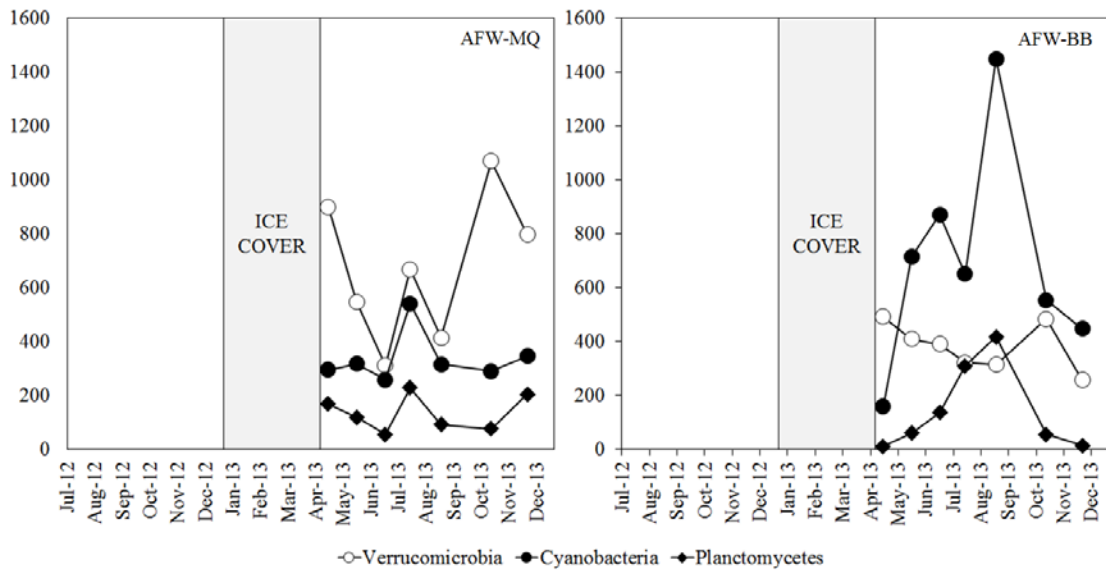

AA-MQ
AA-BB
ICE COVER
Planctomycetes
Verrucomicrobia
Actinobacteria

Figure 3-6. Seasonal dynamics of the six most abundant bacterial phyla found in aggregate-free water (AFW) bacterial communities from nearshore Lake Superior (MQ) and the Duluth-Superior Harbor (BB) in 2012 and 2013.


AFW-MQ
AFW-BB
ICE COVER
Proteobacteria
Actinobacteria
Bacteroidetes


AFW-MQ
AFW-BB
ICE COVER
Verrucomicrobia
Cyanobacteria
Planctomycetes

CHAPTER FOUR

Examining Succession of Bacterial Communities and Testing the Theory of Island Biogeography on Freshwater "Microscopic Islands"

INTRODUCTION

Macroscopic flocculent aggregates are ubiquitous in aquatic ecosystems and often referred to as 'marine snow' in oceans or "lake snow" in freshwater environments. Generally, they are composed of dead or living zooplankton or phytoplankton, detritus, other organic and inorganic matter. Flocculent aggregates are always enriched with organic materials and other nutrients (Trent *et al.*, 1978), and highly colonized compared to the surrounding water with diverse microorganisms, such as bacteria, algae, flagellates, and ciliates (Shanks and Trent, 1979; Caron *et al.*, 1982; Alldredge and Silver, 1988; Alldredge and Gotschalk, 1990; Grossart and Simon, 1993). Heterotrophic bacteria are capable of decomposing organic matter within the aggregate, which could indicate that aggregates are important microhabitats for the decomposition of particulate organic matter and nutrient cycling in water columns (Shanks and Trent, 1979; Gotschalk and Alldredge, 1989; Herndl, 1992). Marine snow also transfers carbon and nutrients from the surface water column to benthic layers in oceans (Fowler and Knauer, 1986; Alldredge and Silver, 1988). More recent studies have found that macroscopic aggregates in lakes have very similar trophic functions to those found in oceans (Grossart and Simon, 1993; Grossart, 1995; Weiss *et al.*, 1996).

Flocculent aggregates could be considered “microscopic islands” in water columns that provide favorable microenvironments and nutritional conditions for a variety of aquatic bacteria. MacArthur and Wilson proposed a theory to predict the species richness and dynamics on islands that is based on rates of migration and arrival and eventually extinction that affect species establishment (MacArthur and Wilson, 1967). The MacArthur-Wilson Theory of Island Biogeography predicts that the community richness (the total number of species) on islands is the dynamic equilibrium between successful colonization of new species and extinction of resident species. The species richness is relatively constant on islands but depends upon their size and distances from sources of colonizing species.

Many previous studies have tested the Theory of Island Biogeography using animal communities found on either traditional islands, such as islands in oceans, or untraditional islands - any ecosystem with suitable habitat surrounded by unsuitable habitat, such as the peaks of mountains, isolated springs in the desert, or natural parks surrounded by urban environments (Simberloff and Wilson, 1970; Diamond, 1974; Lomolino *et al.*, 1989; Thornton, 1997; Fore and Guttman, 1999; Whittaker, 2000; Ohmura *et al.*, 2006; Kebede *et al.*, 2007). However, very few studies have been applied this theory to microbial communities (but see Bell *et al.*, 2005; Reche *et al.*, 2005), especially bacterial communities on microscopic islands (e.g., flocculent aggregates; but see Lyons *et al.*, 2010; Kramer *et al.*, 2013).

Unlike geographically fixed, “traditional” and “untraditional” large-scale islands, microscopic islands like flocculent aggregates may be transported by vertical and

horizontal currents in aquatic environments. As a result, colonized aggregates will be exposed to different microbial communities at the confluence of streams, rivers, lakes, and oceans, and may be colonized by new species as they are transported, and thus approach a new species equilibrium. This situation is analogous to a geographically fixed island become closer to a new source of potential colonizers. Schweitzer *et al.* (2001) revealed that *Beta*- and *Alpha-Proteobacteria* were the most dominant phylotypes found on lake snow in Lake Constance by using fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes. During the colonization of this lake snow both of these groups showed a distinct succession. Today, by using more advanced DNA sequencing technique, dynamics of bacterial community succession on aggregates during colonization could be more fully studied.

In this study, several lab experiments were designed to (1) determine whether the theory of island biogeography applies at the microscopic scale and (2) examine the dynamic succession of bacterial communities on different flocculent aggregates during colonization and transport. Artificial aggregates and aggregate-free water were generated in the lab using water samples from nearshore Lake Superior and the Duluth-Superior Harbor (DSH). Lake Superior is very oligotrophic, with lower concentrations of dissolved and particulate organic matter and nutrients than most lake systems. By contrast, DSH is a highly dynamic aquatic ecosystem with large amounts of suspended matter and a high degree of anthropogenic influence. I have previously found that both environmental conditions and bacterial community structure on particles from the nearshore of Lake Superior were significantly different than those from the DSH (see

Chapters 2 and 3). Aggregates from the DSH are transported out to Lake Superior by outflow from the St. Louis River and conversely aggregates from Lake Superior are transported into the DSH by seiches. One of my objectives was to simulate the situation of a previously colonized island being exposed to a new source of colonizers by mixing aggregates formed and colonized in one water source with aggregate-free water containing potential colonists from the opposite reciprocal source. Changes in bacterial community composition were followed during these experiments using 16S rRNA gene sequence analysis to identify bacterial community richness, diversity and composition on the aggregates.

MATERIALS AND METHODS

Study site and sampling

Water samples used for generating aggregates were collected from nearshore Lake Superior and the Duluth-Superior Harbor (Figure 4-1). The lake site was the McQuade small craft harbor (46°52'51" N, 91°55'07" W) on the north shore of Lake Superior, 9 miles north of Duluth, MN (USA). The harbor site was located within the Duluth-Superior Harbor under the Blatnik Bridge (46°45'05" N, 92°06'06" W). Water samples (20 L) were collected just below the water surface using plastic carboys. Both sites were sampled in September and October 2012, as well as April and May 2013. All water samples were stored at 4°C before aggregates were generated and the experiments started.

Formation of aggregates and aggregate-free water

Artificial aggregates were generated in the lab with the water from nearshore Lake Superior (lake aggregates) and the Duluth-Superior Harbor (harbor aggregates). A one-liter sterile glass media bottle with 1 L of natural water from either the lake or harbor was incubated at 13ºC and rolled horizontally (1.5 rpm) for two days to form visible aggregates (Shanks and Edmondson, 1989). After two days, flocculent aggregates that formed in the bottles were allowed to settle for 1 hr to create an operationally defined aggregate fraction (bottom 100 ml) and water fraction (upper 900 ml). The water fraction was carefully siphoned and disposed.

To create aggregate-free water with free-living bacteria, water from either the lake or harbor was filtered through 2 μm-pore Nuclepore polycarbonate membrane filters (47 mm dia., Whatman, England) under low pressure (15 cm Hg) to remove large particles and the bacteria bound to them, leaving behind free-living bacteria. These filtered water samples were designated "aggregate-free" water.

Experimental design

Two sets of experiment were completed to examine bacterial colonization of aggregates over time. In the first experiment, by using organic aggregates, we were testing the influence of colonizer distance on island bacterial richness. During the process of colonizing of an island, different concentrations of potential colonizers could have different probabilities to colonize to this island. The higher the number of potential colonizers, the easier it will be for them to colonize to the island, which could be

analogous to closer distance for them to colonize. Therefore, we made different free-living background bacterial concentrations to mimic different distances of islands from a colonization source analogous to a mainland. Two questions were asked in this experiment: 1) Is there a relationship between bacterial richness and the distance between microscopic islands and bacterial colonizers? 2) Do aggregates from harbor and lake environments show similar patterns? In this experiment, approximately half of lake and harbor aggregate-free water was autoclaved to kill active microbial cells. This sterile water was used to dilute aggregate-free water with free-living active microbes to form different bacterial concentrations, which would be proxies for different distances of islands (particles) from the source of potential colonizing species (bacterial cells). For both the lake and harbor samples, mixtures of 10%, 50%, 70%, and 100% aggregate-free water with active microbes were made (Table 1). If the bottle is labelled with 100% natural bacterial cells, it was filled with 1 L of nonsterile aggregate free water with no dilution; if the bottle is labelled with 10%, it was filled with 100 ml of nonsterile aggregate-free water, and 900 ml sterile aggregate-free water to dilute the original natural bacterial cells to 10%. The same number of aggregates (ca. number = 25) which formed with water from the opposite source were carefully transferred into different bacterial concentration treatments by using pipette. All treatment bottles were incubated and rolled using the same conditions that were used for generating the aggregate particles. The aggregates from one bottle from each treatment were harvested at the beginning of the experiment (day 0), and again after 5, 10, 20, and 30 days from each treatment bottle using a 1-ml plastic pipette whose dispensing tip was removed to avoid destroying

aggregates. These aggregate samples from each treatment were stored in 15 ml centrifuge tubes at -80ºC before further processing.

The second set of experiments was designed to examine the succession of bacterial communities on aggregates during colonization independent of distance between islands as considered by the Theory of Island Biogeography. Two questions were asked in this experiment: (1) Are there changes in bacterial community richness and diversity during recolonization of aggregates? (2) Do bacterial communities on lake or harbor aggregates show similar successional trends? In this experiment, generated lake aggregates were carefully transferred into aggregate-free harbor water, as well as aggregate-free lake water as control. As a comparison, harbor aggregates were carefully transferred into aggregate-free lake water, as well as aggregate-free harbor water as control (Figure 4-2). All treatment bottles were incubated using same conditions that were used for generating aggregate particles (see above). The aggregates in each treatment bottle were harvested at the beginning of the experiment (day 0), and after 8, 16, 24, and 32 days from each treatment bottle using a 1-ml plastic pipette whose dispensing tip was removed to avoid destroying aggregates. All aggregate samples were stored in 15 ml centrifuge tubes at -80ºC before further processing.

16S rRNA gene Illumina sequencing

Each 15 ml tube with collected aggregates was centrifuged at 3,000 rpm for 5 min. The supernatant was carefully removed from each tube by pipetting, and then the pellet was used for DNA extraction. A PowerSoil DNA Isolation Kit (MO BIO

Laboratories Inc., Carlsbad, CA) was used for all DNA extractions because aggregates collect or are made with organic material, clay particles, or humic acids, which all could be inhibit PCR amplification (Kirk *et al.*, 2004). Extracted DNA from all the samples were frozen in microcentrifuge tubes at -80°C until further analysis.

The V6 hyper-variable region of the 16S rRNA was amplified in triplicate with tagged PCR primers (967F and 1046R). 967F was in form of a cocktail of five modified primers (5'-CNACGCGAAGAACCTTANC, 5'- CAACGCGAAAAACCTTA, 5'-CAACGCGCAGAACCTTACC, 5'-ATACGCGAR¬GAACCTTACC, and 5'-CTAACCGANGAACCTYACC), which was described to increase the number of taxa that matched primer sequences (Teske and Sørensen, 2008). The 1046R reverse primer (5'-ID-CGACRRCCATGCANCACCT) had a barcoded Illumina adapter sequence (ID) including a six-base multiplexing identification barcode that is unique to each sample but identical for replicates of each sample (Bartram *et al.*, 2011). The PCR amplification mixture (50 µl/reaction) contained: 1 µL 200 µM dNTPs (Promega, Madison, WI); 5 µl 10× PCR buffer (Denville Scientific, South Plainfield, NJ); 1 µl 5.0 units of Choice Taq DNA polymerase (Denville Scientific, South Plainfield, NJ); 2 µl 0.2 µM concentration of each PCR primer; and 11 µl nuclease-free water. Fifty ng of sample DNA were added to amplification mixtures at the last step. PCR amplification was conducted in a BioRad DNA Engine Thermal Cycler using the calculated control method. After an initial denaturation at 95°C for 5 min, 25 cycles of PCR of 95°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 30 sec were conducted. Finally, a 2 min extension at 72°C ended the PCR cycle. PCR products were cleaned using an Ultraclean

PCR Clean-up DNA Purification Kit (MoBio Laboratories Inc., Carlsbad, CA). The concentration and quality of the PCR products were assessed using a Nanodrop 2000c spectrophotometer (Wilmington, DE).

The triplicate PCR products with unique tags were pooled to create the Illumina library for each bacterial community sample, and then were sent to University of Minnesota Biomedical Genomics Center for running paired-end multiplex sequencing on an Illumina MiSeq platform. Sequences were obtained as .fastq files and submitted to the National Center for Biotechnology Information Sequence Read Archive. Sequencing results are available through GenBank BioProject PRJNA357264. Sequence reads were analyzed on the Minnesota Supercomputing Institute server with mothur version 1.27.0 (Schloss *et al.*, 2009). The uninformative and primer sequences were trimmed off from original sequence reads, and different samples were sorted out based on barcodes specific to each sample. Sequences were aligned to the SILVA database (Pruesse *et al.*, 2007), and then chimeras were also removed from sequences by using UCHIME (Edgar *et al.*, 2011). The same number of sequences were randomly subsampled from each sample sequence library to normalize differences in numbers of sequence reads obtained for each sample while still capturing as much genetic diversity as possible. The processed sequences were then taxonomically classified into operational taxonomic units (OTUs) at the $\geq$ 97% sequence similarity level using furthest neighbor clustering by using the Wang method and the Ribosomal Database Project (RDP) taxonomic database release 9 (Cole *et al.*, 2009). All sequences with abundances $<$ 2 were removed before further analysis.

Data Analysis

Ordination analysis. The community difference matrices between samples over time and across dilutions were calculated using the Bray-Curtis measure of dissimilarity (Bray and Curtis, 1957) and imported into PC-ORD version 6.08 software for nonmetric multidimensional scaling (NMS) analysis. In NMS plots, samples with greater similarity are closer in ordination space.

Bacterial community richness, diversity and evenness. Bacterial community richness (total number of unique OTUs) and Shannon-Wiener diversity index were calculated for all samples based on following equations using mothur version 1.27.0 (Schloss *et al.*, 2009).

$$H' = -\sum_{i=1}^{S} \frac{n_i}{N} \times \ln \frac{n_i}{N}$$

where *H'* is Shannon-Wiener diversity index. *S* is the number of observed OTUs, n_i is the number of individuals in OTU i, and *N* is the total number of individuals in the community.

Analysis of molecular variance (AMOVA). AMOVA is a method to detect whether the centers of two groups are more separated than the variation among samples in the same group by utilizing molecular markers (Excoffier *et al.*, 1992). AMOVA analyses were performed using mothur version 1.27.0 (Schloss *et al.*, 2009).

Detection of differential abundant features (Metastats). Significant differences between different samples in abundance of phyla or orders were determined by the online

software program Metastats (White *et al.*, 2009), with 1000 permutations and $p < 0.05$ as the statistical significance level.

RESULTS

Influence of colonizer distance on the bacterial richness of lake aggregates

By making 4 treatments with lake aggregates and harbor water with different bacterial concentrations (100%, 70%, 50%, and 10%), we were testing how different distances between a microscopic island and its potential colonizing sources would affect bacterial community richness on the aggregates. We used 16S rRNA gene sequencing techniques to examine the bacterial community composition on aggregates, which could be used for obtaining bacterial richness (total number of unique OTUs) and the Bray-Curtis index describing the pairwise dissimilarity between communities. Overall, a total of 64,976 16S rRNA gene V6 hypervariable region sequence reads were obtained by Illumina next-generation sequencing, and all these sequences were taxonomically assigned to a average of total 3930 ± 418 unique OTUs for each sample. The coverage of each sample was estimated at 96.9 ± 0.3%, which indicated in this study our sampling was very thorough and only few OTUs remained unsampled.

Although the total number of unique bacterial OTUs in each bacterial concentration changed over time, bacterial richness on lake aggregates was always greater in the treatment with 100% bacterial concentration, followed by 70%, 50% and 10% bacterial concentration treatments respectively (Figure 4-3). Therefore, it seems that bacterial richness was greater with closer colonizer distances for the lake aggregates.

Two dimensions of NMS (Nonmetric Multidimensional Scaling) ordinations were generated by the distance matrix of dissimilarity between samples, which showed there were clear differences in the dynamics of the bacterial communities over time (Figure 4-4). The variations between samples on the same sample date became more pronounced as the experiment proceeded, with the greatest variation on day 20 and some decrease in variation on day 30. For example, in day 0 and 5, the variation between different treatments were very small. After 10 days, these variations became much greater (Figure 4-4). There was a clear pattern of rapid and large increases of Bray-Curtis dissimilarity of each community with respect to the initial community from day 5 to day 10, and a slow increase from day 10 to day 30 (Figure 4-5). Although the community dissimilarity became more and more stable toward the end of experiments, the dissimilarity between the initial and final communities within each concentration and between communities of different concentrations became very large, which was similar to the pattern found in NMS ordinations. It was also found that the samples within the treatment with 100% bacterial concentration had an overall highest dissimilarity with respect to the initial community composition. The samples within 10% treatment had the lowest dissimilarity, while the 70% and 50% treatments had intermediate dissimilarities with respect to the initial community composition (Figure 4-5).

All the sequences were taxonomically assigned to bacterial phyla (39 ± 3). Across all the sample times, the number of sequence reads from the *Betaproteobacteria* and *Deltaproteobacteria* were highest in the treatment with 100% bacterial concentration, and these species became less abundant in lower bacterial concentration treatments. The taxa

of *Alphaproteobacteria* and *Gammaproteobacteria* were less abundant in higher bacterial concentration treatments, and these taxa became more abundant in lower bacterial concentration treatments (Figure A-1).

Influence of colonizer distance on the bacterial richness of harbor aggregates

A similar experiment was carried out by mixing harbor aggregates with aggregate-free lake water of different bacterial concentrations (100%, 70%, 50% and 10%) to examine whether bacterial community richness on harbor aggregates would have similar dynamic patterns just as lake aggregates had. Overall, a total of 53,873 16S rRNA gene V6 hypervariable region sequence reads were subsampled, and all these sequences reads were taxonomically assigned to on average of total 4184 ± 269 unique OTUs for each sample. The coverage of each sample was estimated at 95.8 ± 0.2%, which indicated in this study our sampling was very thorough and only few OTUs remained unsampled.

On lake aggregates, bacterial richness remained higher with closer colonizer distances on the aggregates. However, this trend was not found in the treatments with different dilution of bacterial concentration (100%, 70%, 50% and 10%) on harbor aggregates (Figure 4-6). Bacterial richness of different treatments fluctuated across the entire experiment, and the treatment with no dilution (100%) did not always have higher bacterial richness (Figure 4-6).

The NMS ordinations indicated the differences between the 4 different dilutions were insignificant all the time (Figure 4-7). Therefore, it seems that the differences between different sample times were greater than the differences between different

percentage treatments on both lake and harbor aggregates (Figure 4-5 and 4-7). The community dissimilarities of all treatments showed pronounced increasing trends over times, and overall the treatment with 10% bacterial concentration had higher community dissimilarity compared to other treatments (Figure 4-8).

All 16S rRNA gene sequencing reads were assigned to bacterial phyla (36 ± 2) among all samples. Combining all the sample times, overall, we found the 100% bacterial concentration treatment had higher number of sequences of *Betaproteobacteria* and *Gammaproteobacteria* but lower abundant *Alphaproteobacteria* and *Deltaproteobacteria* compared to the treatments with lower bacterial concentration (Figure A-1).

Examine succession of bacterial communities during recolonization on lake and harbor aggregates

In this experiment, by mixing lake aggregates with aggregate-free harbor and lake water as well as mixing harbor aggregates with aggregate-free lake and harbor water, we were examining succession of bacterial communities during recolonization on lake and harbor aggregates. Overall, a total of 65,165 16S rRNA gene V6 hypervariable region sequence reads were subsampled from each sample. The mean ± standard deviation of total unique OTUs on lake aggregates mixed with lake water and harbor water were 4,424 ± 217 and 3,578 ± 684 compared to 2,897 ± 123 and 2,753 ± 374 for harbor aggregates mixed with harbor water and lake water. The coverage of each sample was estimated at 97.4 ± 0.5%, which indicated our sampling was very thorough and only a few OTUs remained unsampled.

The NMS ordinations indicated clear and distinct differences between bacterial communities on the lake and harbor aggregates (Figure 4-9), which was also statistically significant by applying AMOVA analysis ($p's < 0.05$). In addition, the dynamics of bacterial communities on lake aggregates were much more pronounced than that on harbor aggregates, and bacterial community succession was clearly happening on lake aggregates. However, there was no apparent succession in bacterial communities on harbor aggregates in either harbor water or lake water. The bacterial communities on lake aggregates mixing with lake water were significantly different from those on aggregates maxing with harbor water ($p's < 0.05$), while the bacterial communities on harbor aggregates did not differ from each other or through time ($p's = 0.178$). Bacterial community richness and Shannon-Wiener Diversity index on lake aggregates were higher than those on harbor aggregates in general (Figure 4-10; Figure A-1). It seems that in most cases the richness and diversity of on harbor aggregates did not change very much during the process of recolonization, but only the diversity indices became greater towards the end of experiment (Figure 4-10; Figure A-2). Bacterial richness and diversity on lake aggregates in lake water remained very stable throughout the experiment, while those on lake aggregates in harbor water decreased over times (Figure 4-10; Figure A-2).

Although in most cases the richness on aggregates did not change very much during the recolonization, based on the taxonomic data at phylum or class level, we could see clear succession of some dominant bacterial groups. The average value of assigned bacterial phyla on lake aggregates was 39 ± 2, which was significantly higher than the average value of those on harbor aggregates (34 ± 2). The 3 most abundant bacterial taxa

(the proportions were >10%) examined on lake aggregates in both treatments were *Betaproteobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria*, while Cyanobacteria/Chloroplast, *Betaproteobacteria*, and *Alphaproteobacteria* were the most abundant phyla found on harbor aggregates. Among the top 10 most abundant bacterial taxa, the phyla of *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Firmicutes* generally had higher abundance on lake aggregates than on harbor aggregates (Figure 4-11). However, harbor aggregates were heavily colonized by cyanobacteria/chloroplast sequences (48.2% ± 12.5%) which were much prevalent on harbor aggregates than those on lake aggregates (2.1% ± 0.6%) (Figure 4-11). The sequence abundance of *Bacteroidetes* and *Acidobacteria* on the two types of aggregates were not significantly different (both *p's* > 0.05). In most cases, some phyla, such as the *Alphaproteobacteria* and *Actinobacteria*, showed generally decreasing abundances over time on lake aggregates regardless of the type of water they were mixed with, while the sequence reads of *Gammaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes* generally increased over times (Figure 4-11). On harbor aggregates, sequences from the *Betaproteobacteria*, *Deltaproteobacteria*, *Firmicutes*, and *Acidobacteria* generally became more abundant over time, while sequences from the *Gammaproteobacteria* followed the opposite trends, regardless of the type of water being mixed with the aggregates (Figure 4-11). The number of sequences number from some other phyla, such as the *Alphaproteobacteria* and *Actinobacteria*, fluctuated throughout the experiment without a consistent pattern (Figure 4-11).

Sequences from the Cyanobacteria/chloroplast group increased and reached plateau before day 16 or 24, and then decreased until the end of the experiment (Figure 4-11)

There were three bacterial taxa on lake aggregates which showed significant differences between treatments of mixing with lake water and harbor water regardless of the temporal dynamics. The abundance of the *Betaproteobacteria* and *Actinobacteria* were higher in the treatment in which lake aggregates were mixed with lake water compared to those on lake aggregates mixed with harbor water. The phylum of *Gammaproteobacteria* followed the opposite trend: their abundance was higher on lake aggregates mixing with harbor water. There was no significant difference of bacterial abundance on harbor aggregates in two treatments.

DISCUSSION

Test the theory of island biogeography using organic aggregates

The Theory of Island Biogeography that was developed by MacArthur and Wilson in 1967 described the equilibrium of island population between immigration (colonization) and emigration (extinction), and explained the influence of island area distance on both immigration and emigration. We applied this theory to flocculent aggregates, which could be considered as microscopic islands in aquatic environments, and studied how island distance relative to colonizers (background bacterial density) affects bacterial richness on flocculent aggregates, which are already colonized by various bacteria species.

In our experiments, we adjusted the background bacterial density into different percentages to simulate different distances between island and bacterial sources, and we found that different percentages of background bacterial concentration affected bacterial richness on aggregates that generated from lake but not from harbor water. When lake aggregates mixed with harbor water, bacterial richness was greater with nearer colonizer distance. However, this pattern was not found on harbor aggregates in lake water.

According to the Theory of Island Biogeography, the number of species that coexist on an island is determined by the balance between immigration and extinction. Usually, for an island of a given size, the immigration/colonization rate of new species is influenced by the distance of the island from a colonizing source, and the extinction rate depends on the number of species already present on the island. Therefore, nearer islands will be colonized by more species than more distant islands, and the chances of extinction will be greater on smaller than on larger islands. In the experiment with lake aggregates and harbor water, we tried to use similar sized lake aggregates for all treatments to control for the effect of island size. Since the bacterial richness and diversity decreased across the experiment (Figure 4-3), the immigration rates of new species appear smaller than the extinction rates, indicating the bacterial species in harbor water cannot easily migrate to the lake aggregates in this experiment. It's possible that the bacterial OTUs in harbor water are very similar to those already colonized on lake aggregates so we can not detect any new bacterial OTUs over times. The other possible reason maybe that during the course of the experiment the islands (lake aggregates) were always less favorable for harbor bacteria to live on, which may become even worse towards the end of the

experiment so even less bacterial species would like to attach to the island. But overall, the bacterial richness was higher on lake aggregates with higher background bacterial concentration, which is consistent with the prediction of island biogeography theory with respect to distance between islands.

In the experiment with lake aggregates and harbor water, mixing harbor aggregates with lake water, we found the richness of bacterial communities fluctuated during the course of the experiment, and did not show very clear dynamic patterns. Therefore, during the course of this experiment, there may be some immigration or extinction process happened on harbor aggregates, but the overall bacterial richness did not change that much during the course of this experiment. In addition, unlike lake aggregates in harbor water, we found the bacterial richness was very similar among all treatments with different background concentrations, which did not follow the theory of island biogeography.

Overall, aggregates from the lake and harbor performed differently in this experiment, and only lake aggregates in harbor water supported the predictions of the island biogeography theory. There may be several reasons for these differences. The physical and chemical characteristics of aquatic environments from Lake Superior and the Duluth-Superior Harbor differed in many aspects. For example, temperature, turbidity, as well as the concentration of chlorophyll a, DOC, POC, and TEP are all higher within the harbor than in lake. In addition, aggregate-associated and free-living bacterial communities are very different between these two sites. Several previous studies have worked on lake aggregates in Lake Constance and found that on different types of

aggregates (such as zooplankton-derived or cyanobacterial aggregates), bacterial abundance and bacterial protein production varied greatly, and different ages of aggregates could harbor different sizes or shapes of bacteria (Grossart and Simon, 1993; Grossart and Simon, 1998a). They also found four major types of aggregates based on their component particle composition, and these aggregates could associate with algal cells or debris, zooplankton molts or carcasses, cyanobacterial colonies, and other unidentifiable particles (Grossart *et al.*, 1997). In our experiment the chemical composition of aggregates that formed with lake or harbor water was unknown, but because the environmental conditions in lake and harbor displayed significant variations, we could assume the aggregates formed in these two environments have different chemical composition and size. Therefore, they could be colonized or favored by different types of bacterial species. These differences may have resulted in fewer bacterial species in harbor water that could successfully colonize lake aggregates. Another possible reason might be due to higher nutrient and organic matter concentrations in harbor water compared to lake water. It may be possible that harbor water was rich enough that the bacteria do not need to attach to aggregates as a favorable microhabitat. In contrast, harbor aggregates may not only be suitable for their native bacterial species but also favor some new bacterial species from lake water, resulting in net extinction on lake aggregates but clear immigration onto harbor aggregates across the course of experiment.

The size of aggregates could be another reason for the differences between different sets of experiments. In the experiments of testing the island biogeography

theory, harbor aggregates were relatively larger than lake aggregates (no measurement, only observations by naked eyes), which may be due to higher concentration of organic or inorganic suspended particles in the harbor than the lake. One study has shown a clear positive relationship between the size of aggregates and the microbial species functional diversity on the aggregates (Lyons *et al.*, 2010). Therefore, compared to lake aggregates, the extinction rates on harbor aggregates are expected to be smaller and thus could harbor more bacterial species.

The other difference we found between lake or harbor aggregates lies in the dynamics of dissimilarities in the bacterial communities during the course of the experiment. The Bray-Curtis dissimilarities of bacterial communities (compared to day 0) with different background bacterial concentrations along times also varied a lot during the course of the experiment. Overall, the dissimilarities of bacterial communities on lake aggregates increased rapidly and greatly during the first half of the experiment, but remained very stable after the 10th day; however, during the whole experiment, the dissimilarities of bacterial communities on harbor aggregates showed a consistently increasing trend. During the process of colonization on aggregates, the bacterial communities with different background concentrations became more different when compared to the original communities, and these dissimilarities of bacterial communities on lake aggregates became stable earlier than those on harbor aggregates.

16S rRNA gene sequencing results of bacterial communities on aggregates showed that the abundance of some bacterial taxa increased with background bacterial concentrations, while some others did not, and even showed an opposite pattern. For

example, in both lake and harbor aggregates, sequences from the *Betaproteobacteria* were more abundant in 100% bacterial concentrations, while more sequences from the *Alphaproteobacteria* were found in the 10% concentrations (Figure A-1). Kramer *et al.* (2013) also found the number of target pathogenic bacterial cells on a majority of aggregates increased with initial higher bacterial densities indicating rapid bacterial accumulation and reproduction on aggregates. The abundance of bacterial cells on aggregates are relevant to species mortality and detachment caused by interactions among species such as lytic phage production or inhibitions from bacterial cells (Grossart *et al.*, 2004; Riemann and Grossart, 2008). In our study, we found *alphaproteobacterial* sequences were more abundant on aggregates with lower bacterial concentrations. The most plausible reason why sequences from this bacterial class were more abundant on aggregates with lower bacterial concentrations may be related to their lower competitive ability. Therefore, when the background concentrations of other bacterial species are higher, the species from this class may be less competitive with other bacterial taxa, so less of them colonized on aggregates. When the background bacterial cell numbers of competitors are lower, the competition between species may become less intense so members within the *Alphaproteobacteria* started to heavily colonize aggregates. In addition, members of the *Alphaproteobacteria* are suggested to be resistant to zooplankton grazing, competitive under conditions with low nutrient/substrate availability, and also capable to degrade complex organic materials (Newton *et al.*, 2011). All these characteristics may explain why *Alphaproteobacteria* can survive well in a free-living life style but are still able to attach to aggregates.

Examine succession of bacterial communities during recolonization

Aggregates have two main roles in aquatic ecosystems. First, they serve as microenvironments and harbor diverse microbes. Second, they are transport agents due to their significant roles in transferring particulate matter derived from surface to deeper water layers in lake ecosystems. Several previous studies have compared the dynamics of microbial community structure on lab-made aggregates with lake snow collected in sediment traps, which usually represented older aggregate material (Weiss *et al.*, 1996; Schweitzer *et al.*, 2001). They all found bacterial community structure on lab-made aggregates presumably reflects ones on lake aggregates during sinking and aging through the water column (Weiss *et al.*, 1996; Schweitzer *et al.*, 2001). For example, bacterial community on aggregates during the early incubation presumably reflected the structure on lake snow in the epilimnion and upper hypolimnion (Weiss *et al.*, 1996). It was also found at the end of incubation bacterial community structure on lab-made aggregates was very similar with that on lake snow collected in sediment trap (Weiss *et al.*, 1996). Therefore, investigating changes of bacterial community structure on aggregates during the incubation could provide some useful information about changes of bacterial community during their sinking or aging processing, as well as their association with dynamics of biochemical composition and dissolved organic matter release of aggregates. Bacteria made up more than 80% of all microbial cells, as well as some other microorganism, such as flagellates, ciliates, and protists, on lake snow. Therefore, during

the process of aging of lake snow, grazing pressure from bacterivorous protozoans also strongly affect bacterial abundance or community structure on aggregates.

Previous studies have examined bacterial community structure on lab-made aggregates in time series experiments by using *in situ* hybridization with rRNA-targeted fluorescent oligonucleotide probes, which were only specific for *Alpha*-, *Beta*- and *Gammaproteobacteria* (e.g., Weiss *et al.*, 1996; Schweitzer *et al.*, 2001). By applying 16S rRNA gene Illumina next-generation sequencing to our colonization experiments, we could provide more comprehensive results regarding dynamics of taxonomic composition of bacterial communities during the colonization process. In this study, by mixing lab-made aggregates with the original and different water sources, we could not only test the re-colonization process on aggregates, but also examine whether different water source would affect the re-colonization process on different aggregates.

The distinct succession of the bacterial community on aggregates could be associated with bacterial ability to decompose or digest organic matter on aggregates. Schweitzer *et al.* (2001) found bacterial members on lake snow started to consume DAA (dissolved amino acid) from the surrounding water on the day the aggregates formed. With increasing abundance of *Betaproteobacteria*, and decreased *Alphaproteobacteria*, DAA started to be released from aggregates into the surrounding water. This process is assumed to reflect decomposition of POM from aggregates. Some members of *Betaproteobacteria* are able to hydrolyze aggregate-associated combined amino acids, and release them into surrounding water. These combined amino acids can then be utilized by other heterotrophic bacterial species. *Acidovorax facilis*, *Hydrogenophaga*

palleroni, and *Duganella zoogloeoides*, which were all within the *Burkholderiales* order, were identified as the closest members who have hydrolytic enzymes and are capable of utilizing a variety of carbon sources and labile DOM, such as amino acids, protein and carbohydrates (Schweitzer *et al.*, 2001). This can explain why *Betaproteobacteria*, especially *Burkholderiales* are the most dominant phylotypes on aggregates (with the exception of the cyanobacteria/chloroplast group on harbor aggregates), and the abundance of this group either remained higher in abundance or increased on aggregates throughout the colonization process. Some other bacterial species, such as *Myxobacteria* within the *Deltaproteobacteria*, are able to release hydrolytic enzymes to decompose different and complex biopolymers (Shimkets *et al.*, 2006). This is consistent with our observation that at the later stage of our experiment, these bacterial species became more abundant; most likely, they are starting to degrade organic compounds which are rather refractory to decompose by other bacteria. Although some members of *Bacteroidetes* are also considered to have high potential for degrading complex organic matters (Reichenbach, 2006), in our study, we only identified 2.3% to 8.6% of total sequence reads belonging to this phylum, and they remained relatively stable throughout the experiment. Some orders within the class of *Alphaproteobacteria*, such as *Sphingomonadales* and *Rhodobacterales*, are considered to be able to utilize more refractory DOC (Allers *et al.*, 2007; Basta *et al.*, 2005; Weinbauer *et al.*, 2006), thus have an oligotrophic lifestyle (Lauro *et al.*, 2009). In our study, the relative abundance of *Alphaproteobacteria* on lake aggregates were found to decrease over time. The microenvironments on aggregates are very nutritional with high amounts of organic

materials compared to surrounding waters, and are more attractive to bacterial species that heavily depend on labile DOM but not members of the *Alphaproteobacteria* with an oligotrophic lifestyle. Therefore, after detachment of *Alphaproteobacteria* from aggregates, *Gammaproteobacteria* in surrounding water, such as the opportunistic *Pseudomonadales,* whose abundance continually increased throughout the experiment, may have started to colonize the aggregates. The overall richness of bacterial communities on lake aggregates generally decreased over time, which could be presumable due to the growth of opportunistic groups that were selected by conditions on aggregates during the incubations. However, this pattern of decreasing bacterial richness over time was not found on harbor aggregates. Although bacterial richness fluctuated, it remained relatively stable until the end of experiments, which may indicate that aged harbor aggregates are more favorable for free-living bacteria to colonize compared to aged lake aggregates.

Differences between lake and harbor aggregates during the process of colonization

Based on the results we showed in the previous chapters, the environmental conditions and bacterial community structure at the site of nearshore Lake Superior and within the Duluth-Superior Harbor are very different. In the second experiment, bacterial community on lake and harbor aggregates showed very different colonization patterns over times. Regardless of water sources that were used for mixing aggregates, there were pronounced variations of the bacterial communities on lake aggregates during the colonization, while the bacterial communities on harbor aggregates did not show this

clear pattern. There were significant differences of bacterial communities on lake aggregates when mixed with lake and harbor water, while no such difference was found in harbor aggregates. Overall, bacterial communities on lake aggregates had a higher number of unique OTUs (bacterial richness) as well as a larger Shannon-Wiener diversity index (Figure 4-10; Figure A-2) compared to these on harbor aggregates. We found harbor aggregates were heavily colonized by on average 48.2% ± 12.5% by cyanobacteria/chloroplast sequences throughout the colonization process no matter which type of water source the aggregates were mixed with. *Beta*- and *Alphaproteobacteria* were also numerically abundant during the process but only accounted for on average 22.1% ± 11.7% and 12.0% ± 3.4% of total bacterial sequence reads, and the rest of bacteria taxa only accounted for about 18% of total. Therefore, even though there were some changes of abundance of some bacterial taxa during the colonization process, due to consistent higher abundance of cyanobacteria/chloroplast, the overall bacterial community structure on harbor aggregates did not show large changes during the experiment.

In contrast, although *Betaproteobacteria* were a very dominant taxon on lake aggregates and accounted for 36.8% ± 6.5% of total bacterial sequences, members of the *Alpha*- and *Gammaproteobacteria* also accounted for substantial portions of the bacterial community (22.2% ± 4.3% and 16.9% ± 10% of bacterial sequences, respectively). Based on ordination graphs and statistical analysis, we observed significant differences in bacterial community structure on lake aggregates when mixed with lake water or harbor water, but such significant differences were not found on harbor aggregates.

Cyanobacteria/chloroplast was substantially more abundant on harbor aggregates than lake aggregates and in harbor water compared to lake water. However, when harbor aggregates were mixed with lake water, the abundance of cyanobacteria/chloroplast were very similar with these on aggregates mixed with harbor water. In contrast with this, when lake aggregates were mixed with harbor water, the abundance of cyanobacteria/chloroplast did not increase as we expected, but remained lower throughout the experiment. This is presumably due to members of cyanobacteria/chloroplast having their own preferences for specific aggregates to colonize, and natural chemical composition of harbor aggregates is more favorable for these members to colonize.

Most dominant bacteria taxa on aggregates during the process of colonization

Weiss *et al.* (1996) found that *Betaproteobacteria* usually dominated the bacterial community on aggregates, and were numerically higher abundant in aged aggregates. We also found *Betaproteobacteria* were very dominant in both types of aggregates, especially on lake aggregates, on which this group constituted largest proportion of all bacterial sequences. The abundance of *Betaproteobacteria* on lake aggregates in general decreased during the course of each experiment, while those on harbor aggregates increased. It was assumed from previous studies that the older organic matters, which usually had polymer-rich substrates on them, could harbor specialized aggregates-associated bacterial members and allow them propagated even more. This presumably could explain the increasing trends showing on harbor aggregates. We assume that decreasing trend of

Betaproteobacteria on lake aggregates may reflect less nutrient/organic matters available to support this group of bacteria to grow.

In our study, *Alphaproteobacteria* on both lake and harbor aggregates were found to be more abundant on younger aggregates, which was very consistent with results from some previous studies (Grossart and Simon, 1998; Weiss *et al.*, 1996). In our study, *Rhodobacterales*, *Sphingomonadales*, *Rhizobiales*, *Caulobacterales*, and *Rhodospirillales* are the major members of *Alphaproteobacterial* community. All members except *Rhodobacterales* greatly decreased over time (data not shown).

The class of *Deltaproteobacteria* increased on both lake and harbor aggregates, and were more abundant on lake aggregates than on harbor ones. The order of *Myxococcales* accounted for large proportion of the *Deltaproteobacterial* community, and it displayed very similar dynamic patterns during colonization process as the rest of the *Deltaproteobacteria*. The members within this order digest insoluble organic subtracts by releasing extracellular enzymes, and aggregation of these bacterial cells allow accumulation of enzymes resulting in increasing feeding efficiency, especially when nutrients became limited. This presumably explains higher abundance of this order in older aggregates and less nutrient-rich lake environments.

The abundance of *Gammaproteobacteria* on lake aggregates increased throughout the experiment while those on harbor aggregates decreased. *Pseudomonadales* and *Xanthomonadales* are two orders that constituted the majority of *Gammaproteobacterial* community. *Pseudomonadales* increased rapidly on lake aggregates when mixed with harbor water at the beginning of experiment, while those on aggregates mixed with lake

water only increased towards the end of experiments. Similarly, the abundance of *Xanthomonadales* on all lake aggregates increased, but they only increased rapidly on aggregates mixed with harbor water. Overall, members within *Pseudomonadales* and *Xanthomonadales* were highly colonized on older aggregates, and we assume harbor water is the potential source of these members.

Although several other bacterial taxa, such as *Firmicutes* and *Acidobacteria*, were not as dominant as groups of *Proteobacteria*, they both increased during the course of the experiments, more so on lake aggregates than harbor ones. *Bacteroidetes* and *Verrucomicrobia* show clear trends with lake or harbor aggregates. On lake aggregates, the abundance of *Actinobacteria* in general decreased over time, while on harbor aggregates, the abundance did not change very much.

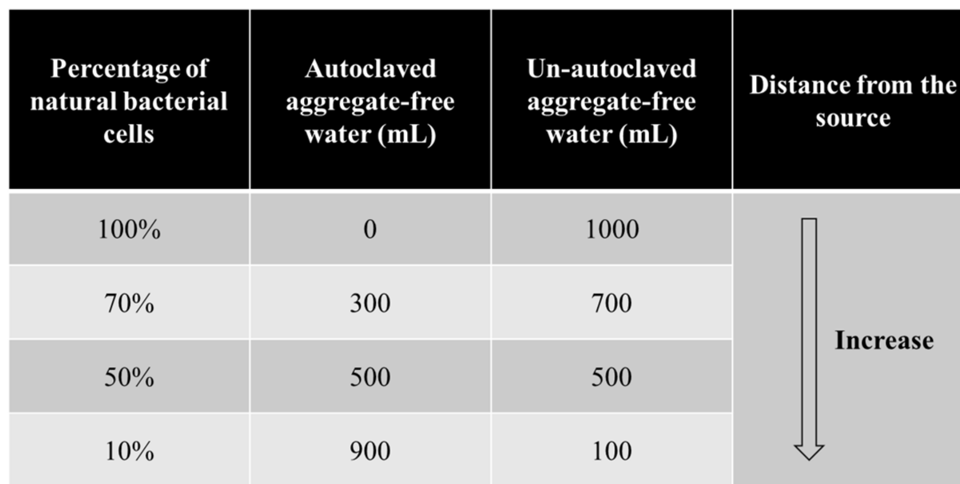
Conclusions

In summary, our studies regarding bacterial community structure on lab-made aggregates could be used to test the theory of island biogeography on these microscopic islands. In the first experiment, which mimicked changes in colonizer distance, the richness of the bacterial community on lake aggregates were higher. However, this pattern was not found in harbor aggregates in lake water. NMS ordinations showed some clear dynamics in bacterial communities on aggregates over time. Overall, changes in bacterial communities over time were greater than differences between communities in treatments mimicking different distances from potential colonizers. In the second experiment examining succession, bacterial richness and diversity on lake aggregates

were different than on harbor aggregates. In addition, succession only appeared to occur in bacterial communities on lake aggregates, and aggregate communities in lake water were different from those in harbor water. This trend was not observed for bacterial communities on harbor aggregates. More experiments with aggregates generated from different water sources collected at different times of the year and with greater replication should be carried out to confirm our results.

TABLES

Table 4-1. Experiment design for testing the influence of different distances from island to bacterial community on aggregates by makeing different percentages of backgroud bacterial concentrations. The higher percentage of bacterial cells, the closer distance of potential colonizer to island.



Percentage of natural bacterial cells	Autoclaved aggregate-free water (mL)	Un-autoclaved aggregate-free water (mL)	Distance from the source
100%	0	1000	Increase ↓
70%	300	700	
50%	500	500	
10%	900	100	

FIGURES

Figure 4-1. Map of sampling station locations on nearshore Lake Superior (lake), and within the Duluth-Superior Harbor (harbor).

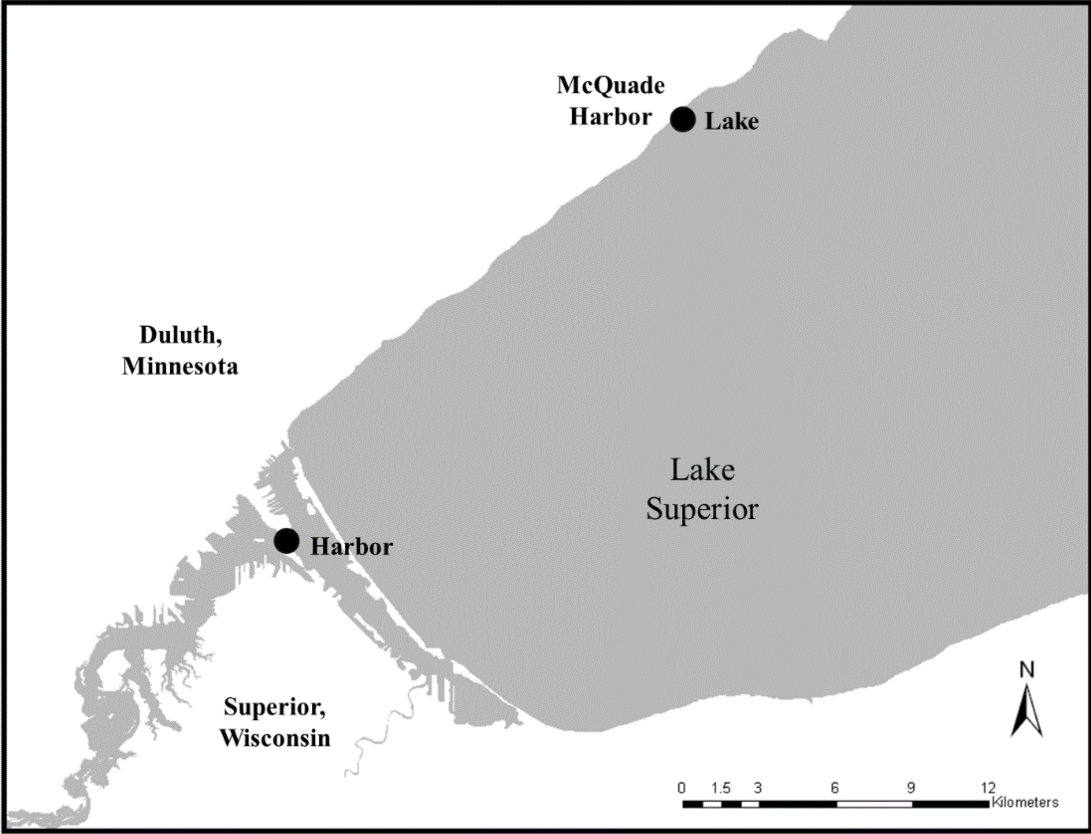

McQuade
Harbor
Lake
Duluth,
Minnesota
Lake
Superior
Harbor
Superior,
Wisconsin
N
0 1.5 3 6 9 12
Kilometers

Figure 4-2. Experiment design to test bacterial community succession during aggregate recolonization.

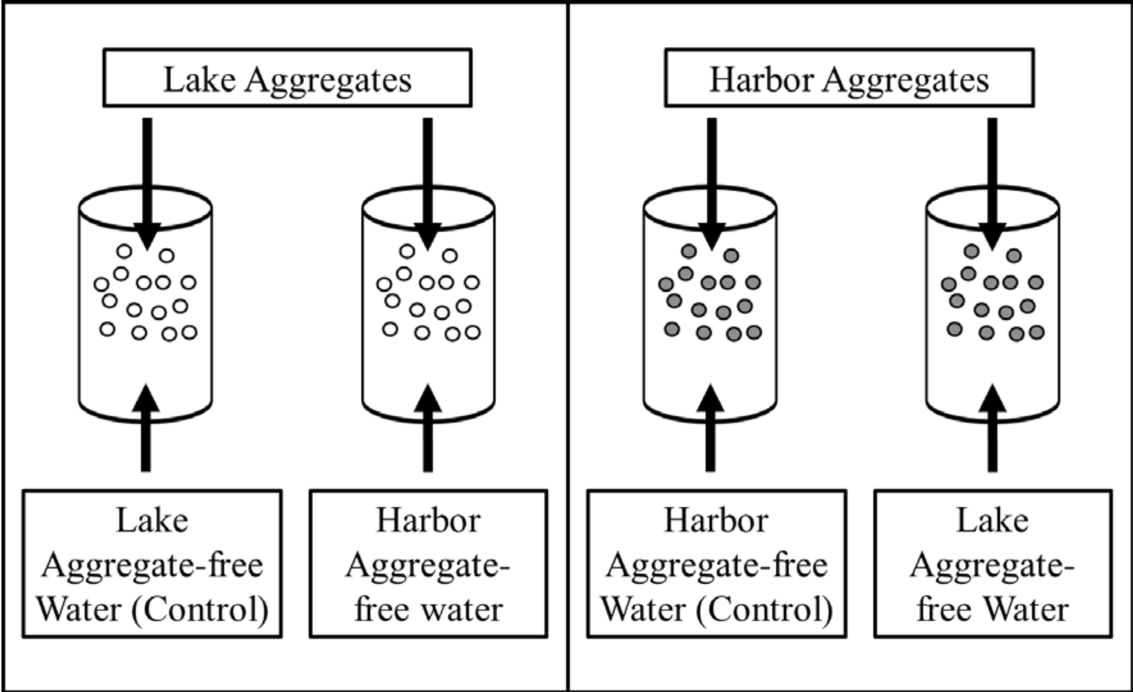

Lake Aggregates
Harbor Aggregates
Lake
Aggregate-free
Water (Control)
Harbor
Aggregate-
free water
Harbor
Aggregate-free
Water (Control)
Lake
Aggregate-
free Water

Figure 4-3. Richness dynamics of bacterial community on lake aggregates when mixed with aggregate-free harbor water over time. Different symbol represented different background bacterial concentration percentages.

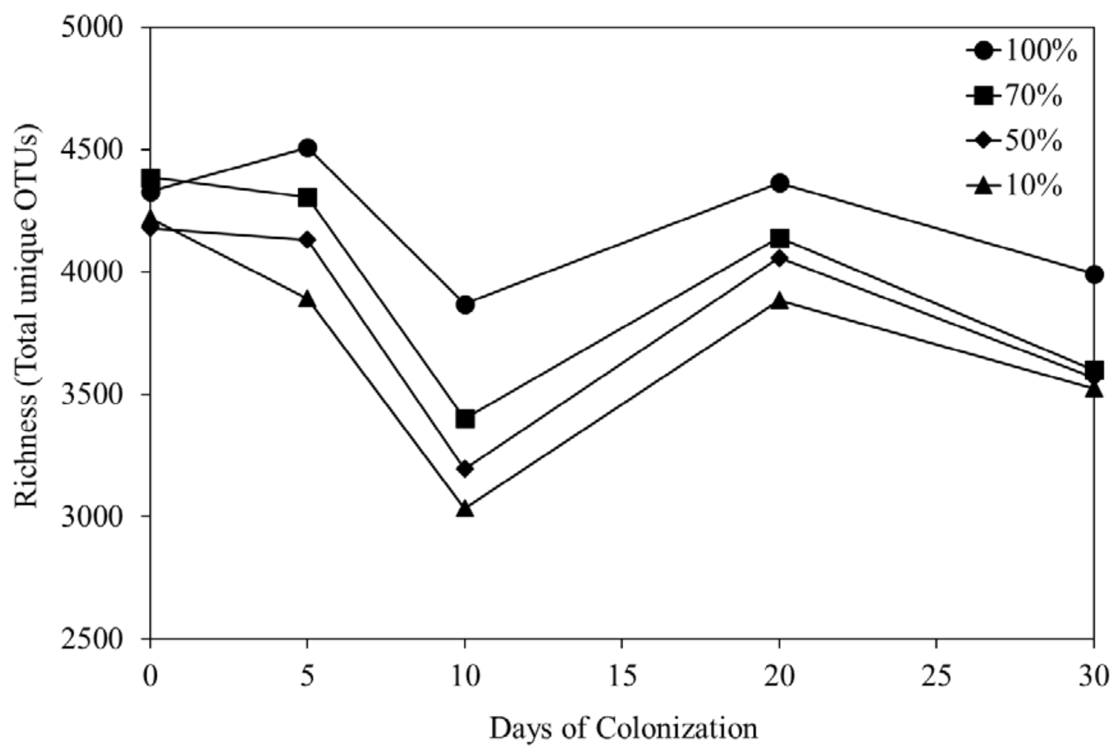

5000
4500
4000
3500
3000
2500
Richness (Total unique OTUs)
0
5
10
15
20
25
30
Days of Colonization
100%
70%
50%
10%

Figure 4-4. Nonmetric multidimensional scaling (NMS) analysis of bacterial community structure dynamics on lake aggregates when mixed with harbor water. The distance matrix used to build up NMS was derived from comparison of OTUs of each sample.

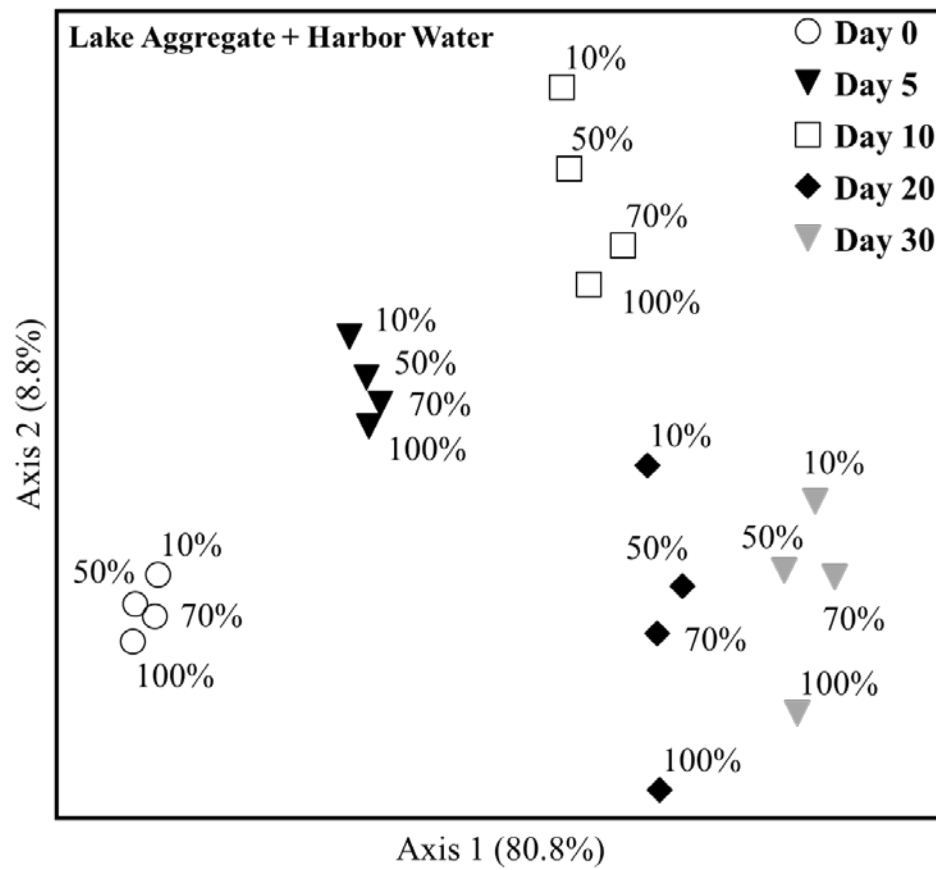
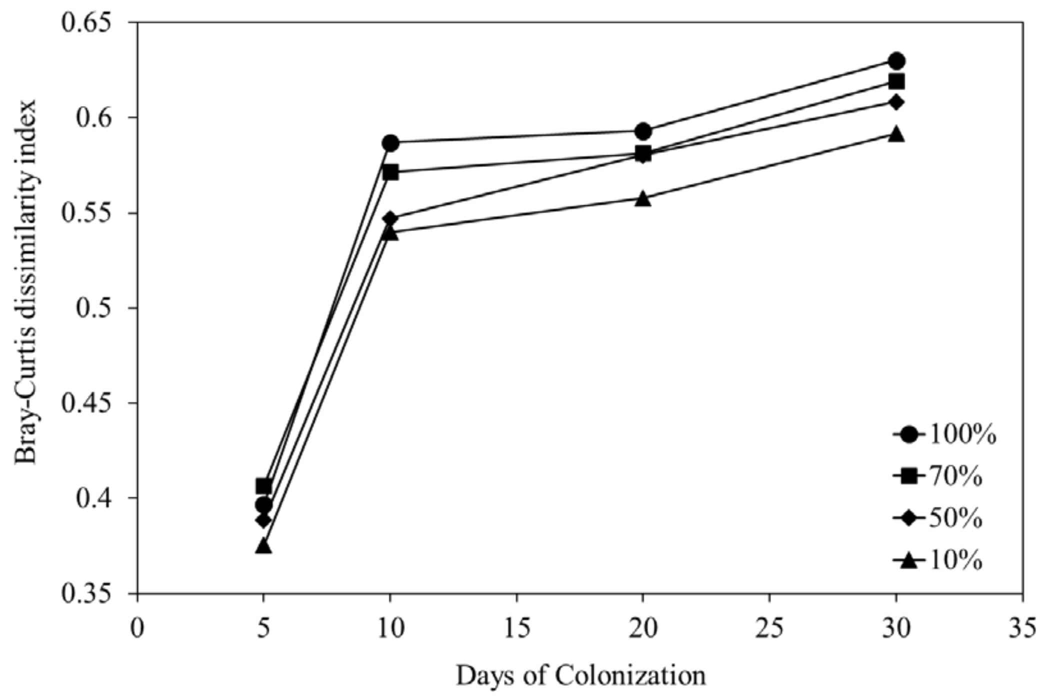

Lake Aggregate + Harbor Water
Day 0
Day 5
Day 10
Day 20
Day 30
10%
50%
70%
100%
Axis 2 (8.8%)
Axis 1 (80.8%)

Figure 4-5. Bray-Curtis dissimilarities are plotted as function of time to show the differences of bacterial community on lake aggregates between each sample point and the beginning (t_0). The dissimilarities of each treatment with different background bacterial concentrations were also compared.



0.65
0.6
0.55
0.5
0.45
0.4
0.35
Bray-Curtis dissimilarity index
0
5
10
15
20
25
30
35
Days of Colonization
100%
70%
50%
10%

Figure 4-6. Richness dynamics of bacterial community on harbor aggregates mixed with aggregate-free lake water over time. Different symbol represented different background bacterial concentration percentages.

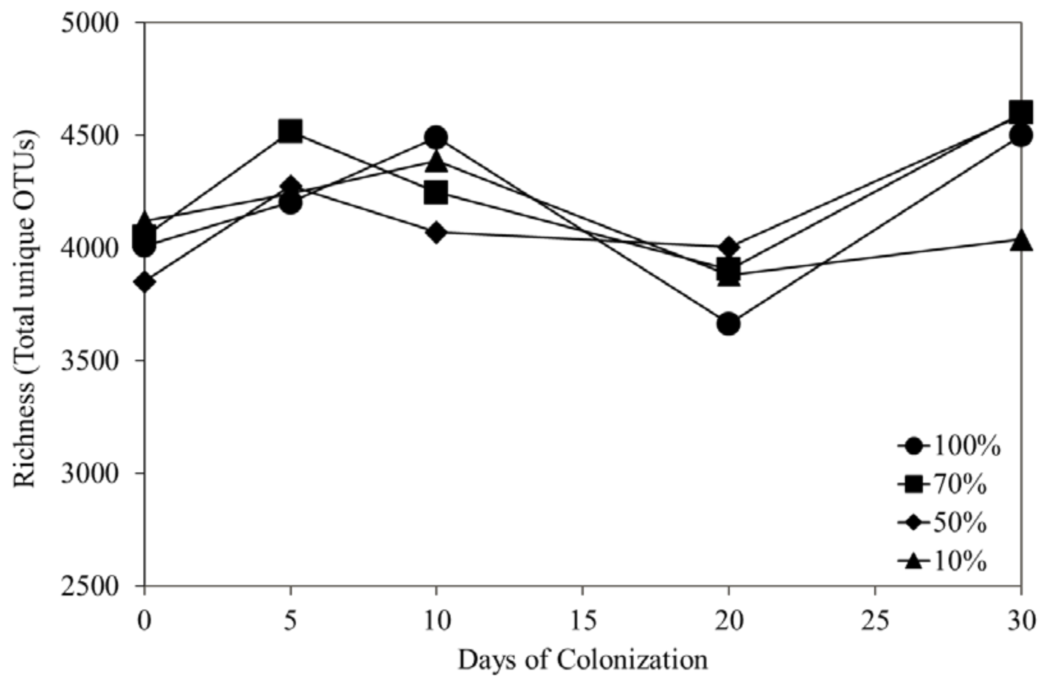

5000
4500
4000
3500
3000
2500
Richness (Total unique OTUs)
0
5
10
15
20
25
30
Days of Colonization
100%
70%
50%
10%

Figure 4-7. Nonmetric multidimensional scaling (NMS) analysis of bacterial community structure dynamics on harbor aggregates mixed with lake water. The distance matrix used to build up NMS was derived from comparison of OTUs of each sample.

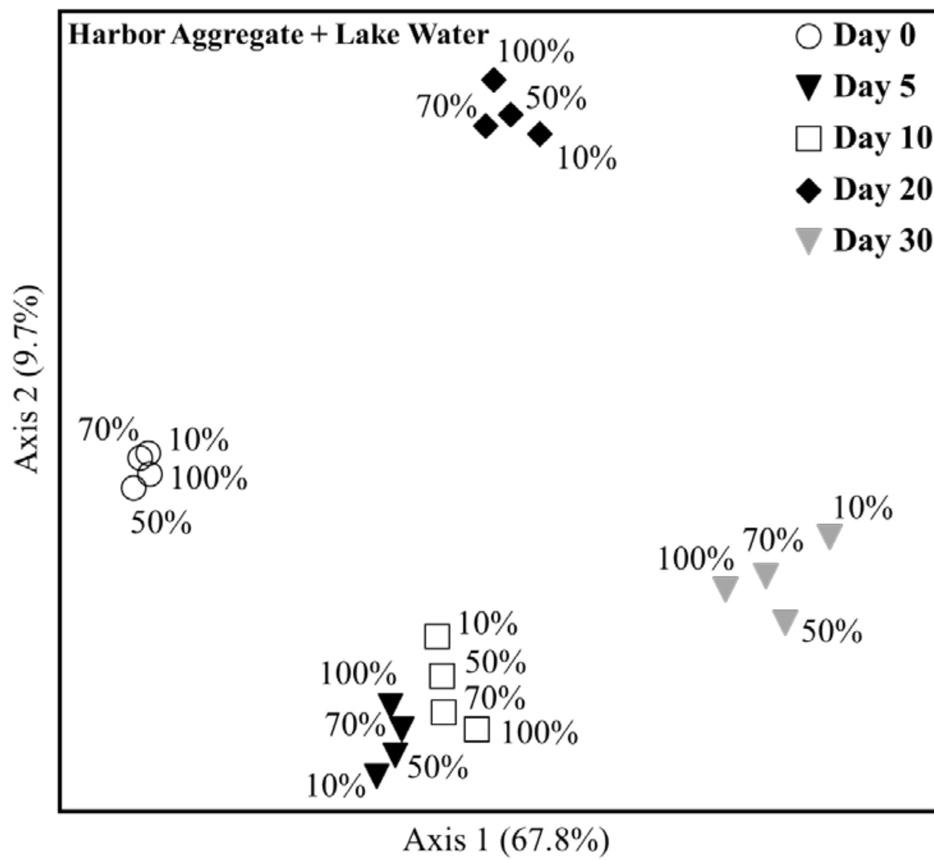

Harbor Aggregate + Lake Water
Day 0
Day 5
Day 10
Day 20
Day 30
100%
70%
50%
10%
70%
10%
100%
50%
10%
70%
100%
50%
10%
50%
100%
70%
70%
100%
50%
10%
Axis 2 (9.7%)
Axis 1 (67.8%)

Figure 4-8. Bray-Curtis dissimilarities are plotted as function of time to show the differences of bacterial community on harbor aggregates between each sample point and the beginning (t0). The dissimilarities of each treatment with different background bacterial concentrations were also compared.

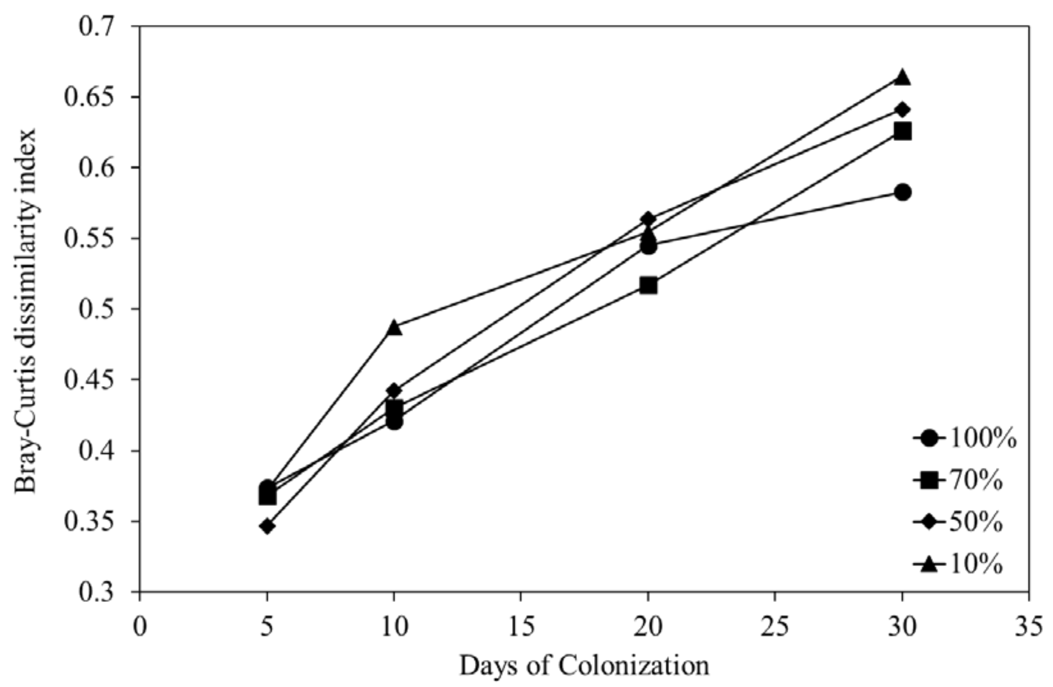
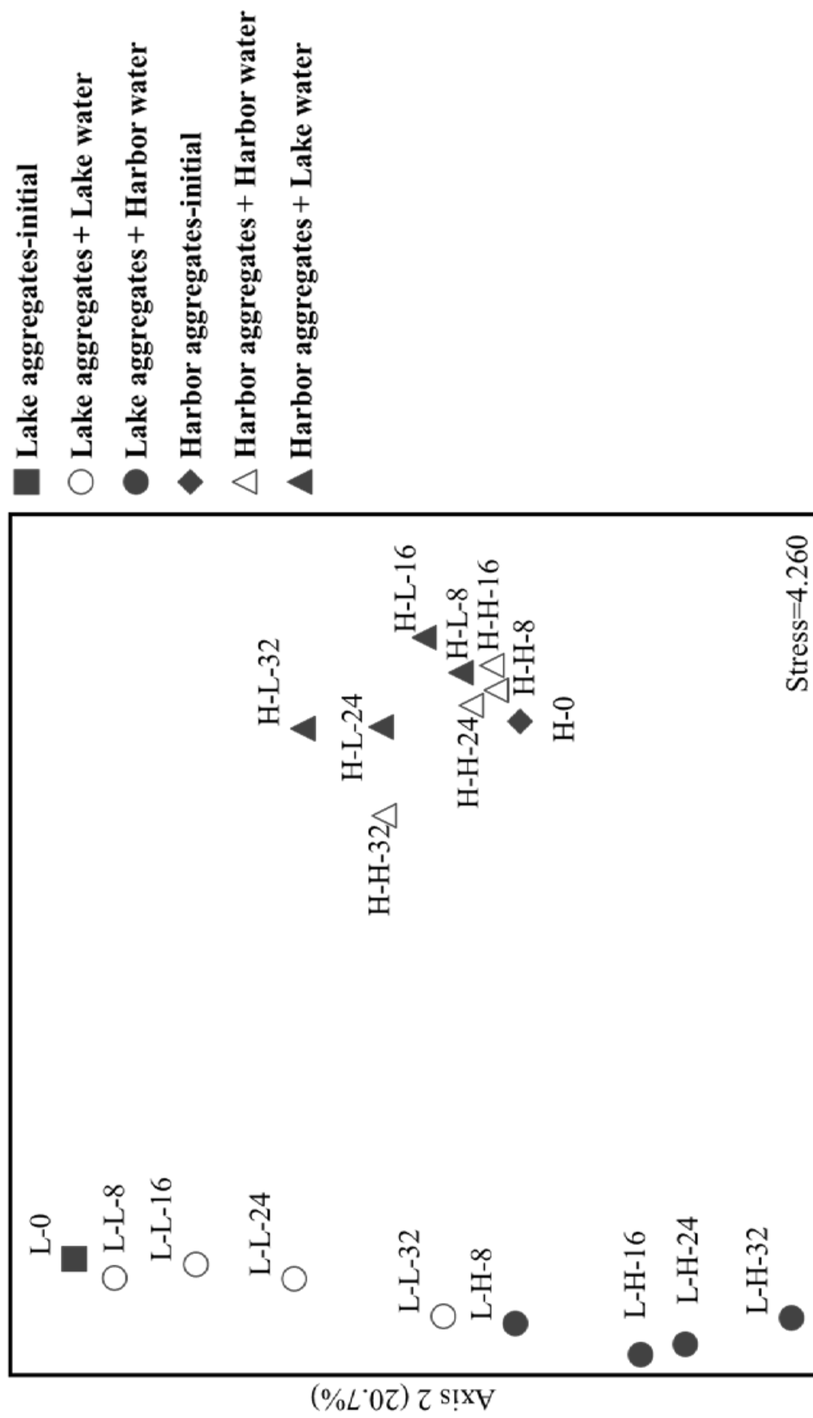
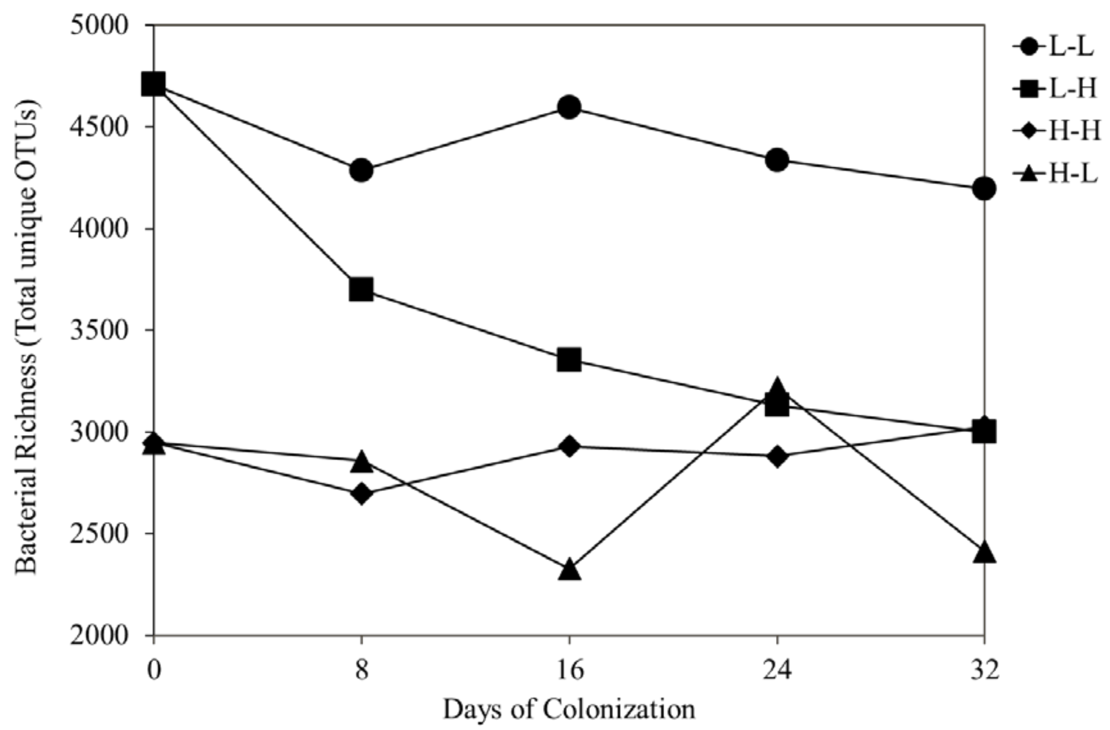

0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
Bray-Curtis dissimilarity index
0
5
10
15
20
25
30
35
Days of Colonization
100%
70%
50%
10%

Figure 4-9. Nonmetric multidimensional scaling (NMS) analysis of bacterial community structure dynamics on lake or harbor aggregates mixed with their original or opposite aggregate-free water. The distance matrix used to build up NMS was derived from comparison of OTUs of each sample.



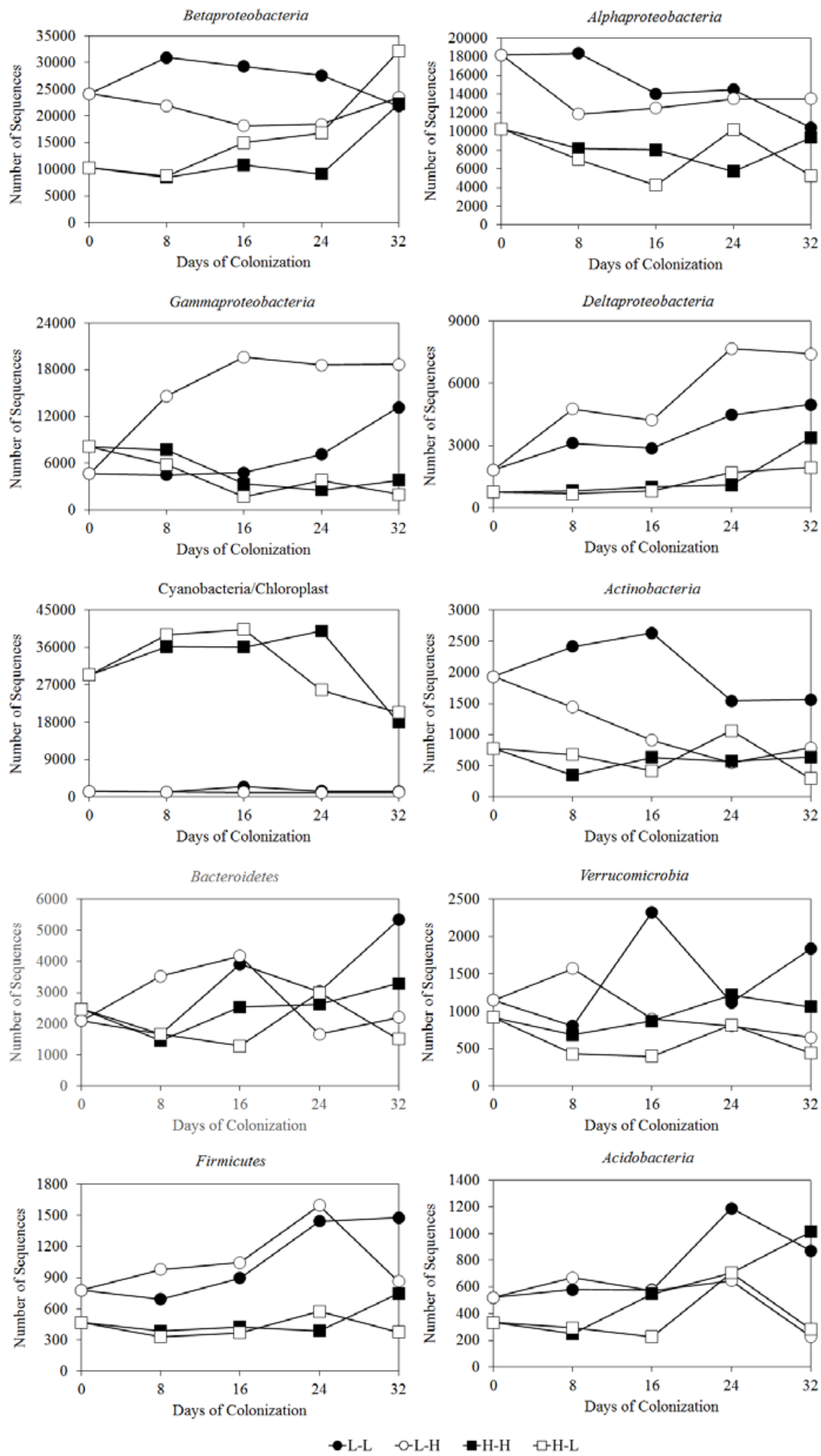
Lake aggregates-initial
Lake aggregates + Lake water
Lake aggregates + Harbor water
Harbor aggregates-initial
Harbor aggregates + Harbor water
Harbor aggregates + Lake water
L-0
L-L-8
L-L-16
L-L-24
L-L-32
L-H-8
L-H-16
L-H-24
L-H-32
H-0
H-H-8
H-H-16
H-H-24
H-H-32
H-L-8
H-L-16
H-L-24
H-L-32
Stress=4.260
Axis 1 (73.7%)
Axis 2 (20.7%)

Figure 4-10. Temporal dynamics of bacterial richness on lake or harbor aggregates mixed with their original or opposite aggregate-free water.



Bacterial Richness (Total unique OTUs)
5000
4500
4000
3500
3000
2500
2000
0
8
16
24
32
Days of Colonization
L-L
L-H
H-H
H-L

Figure 4-11. Dynamics of the 10 most abundant bacterial taxa on lake or harbor aggregates during the process of colonization.


Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Cyanobacteria/Chloroplast
Actinobacteria
Bacteroidetes
Verrucomicrobia
Firmicutes
Acidobacteria
Number of Sequences
Days of Colonization
L-L
L-H
H-H
H-L

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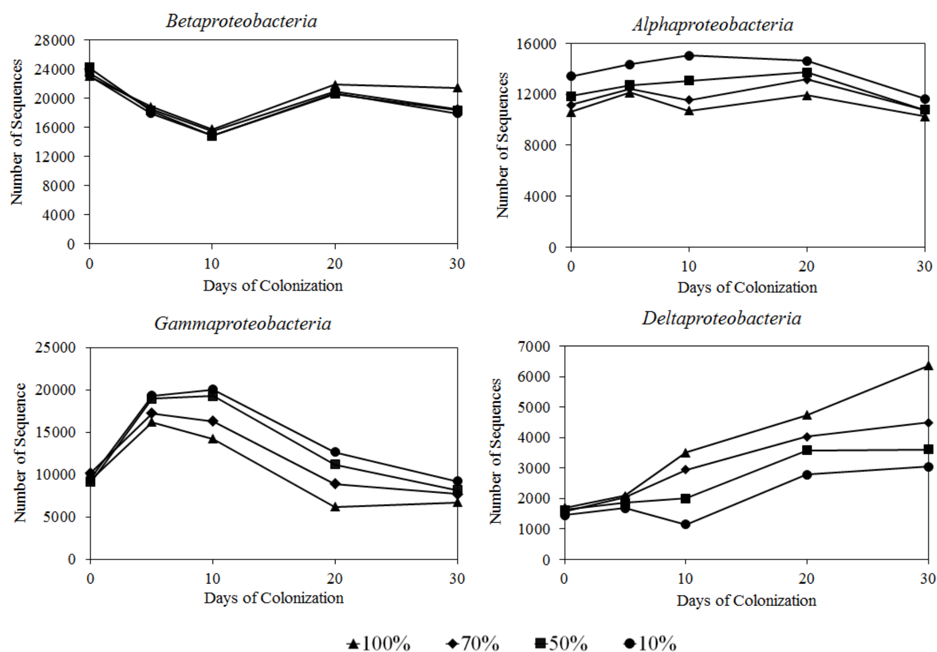
APPENDIX

Figure A-1. Dynamics of several most abundant bacterial taxa on lake aggregates in harbor water (I) or harbor aggregates in lake water (II).


I. Lake aggregates in Harbor water
Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Number of Sequences
Number of Sequence
Days of Colonization
▲100% ◆70% ■50% ●10%

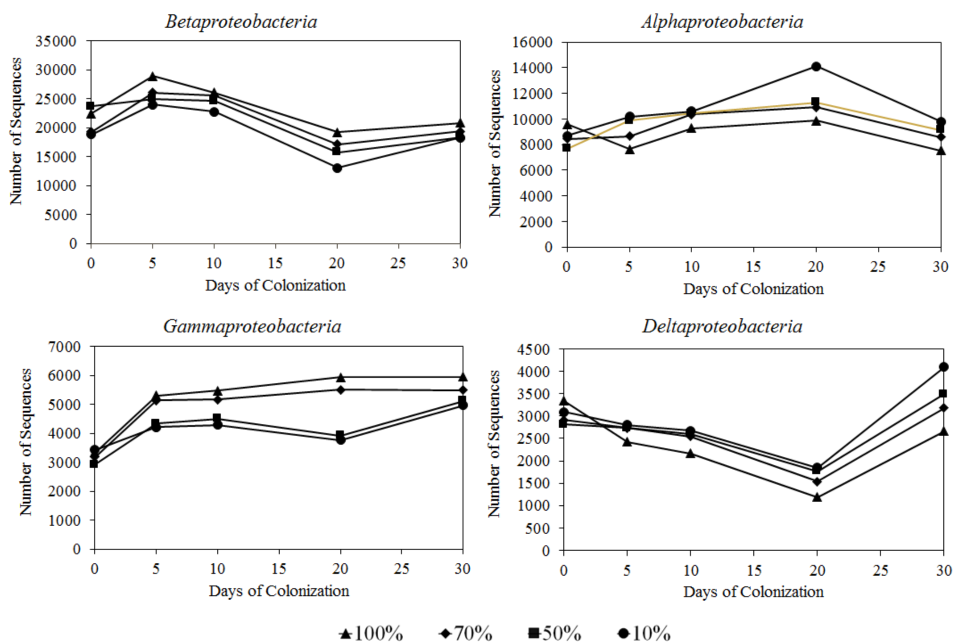
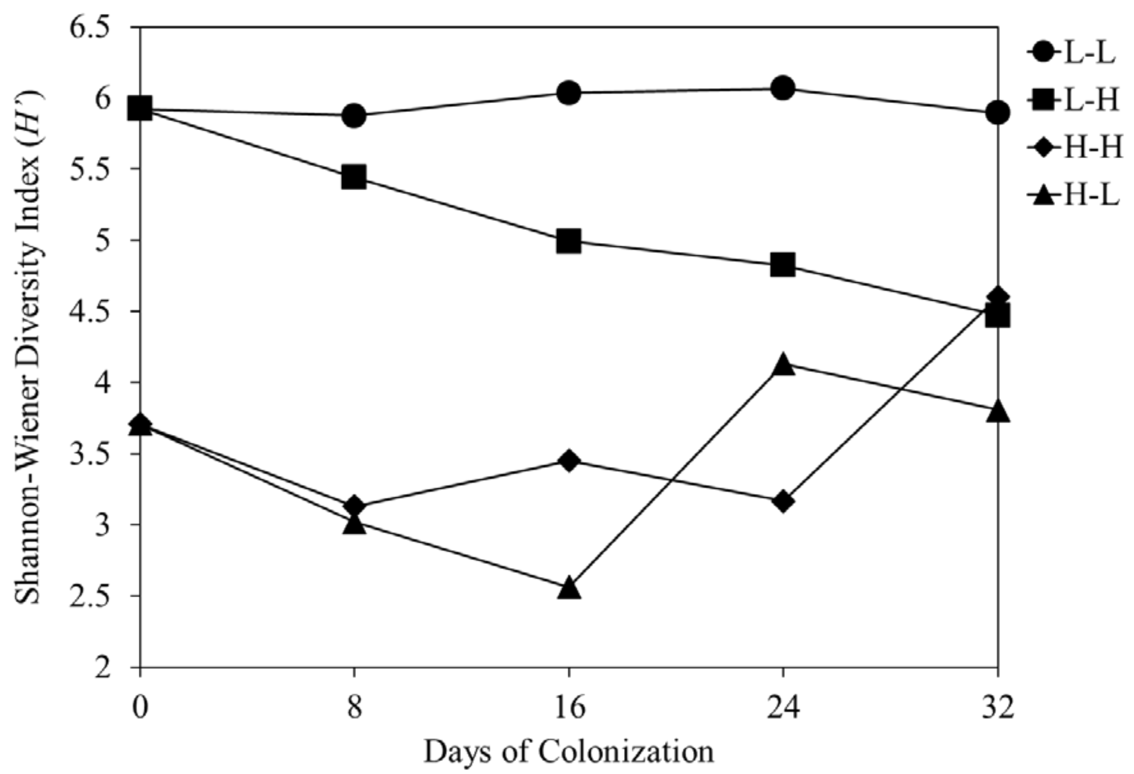

II. Harbor aggregates in Lake water
Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Number of Sequences
Days of Colonization
▲100% ◆70% ■50% ●10%

Figure A-2. Temporal dynamics of Shannon-Wiener Diversity Indices of bacterial communities on lake or harbor aggregates mixed with their original or opposite aggregate-free water.



6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
0
8
16
24
32
Shannon-Wiener Diversity Index (H')
Days of Colonization
L-L
L-H
H-H
H-L