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MICROBIAL COMMUNITIES ASSOCIATED WITH THE ZEBRA MUSSEL (DREISSENA POLYMORPHA) IN THE LAURENTIAN GREAT LAKES BASIN (USA)

presented by

ANDREW D. WINTERS

has been accepted towards fulfillment
of the requirements for the

Master of Science degree in Fisheries and Wildlife

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11/25/2008
Date

MICROBIAL COMMUNITIES ASSOCIATED WITH THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) IN THE LAURENTIAN GREAT LAKES BASIN (USA)

By

Andrew D. Winters

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Fisheries and Wildlife

2008

ABSTRACT

MICROBIAL COMMUNITIES ASSOCIATED WITH THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) IN THE LAURENTIAN GREAT LAKES BASIN (USA)

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Bacterial communities within zebra mussel (*Dreissena polymorpha*) samples collected from four waterbodies within the Laurentian Great Lakes basin (the Huron River, Crystal Lake, Lake Vineyard, and Loon Lake) were analyzed. Bacterial community composition and structure were determined by sequencing the 16S rRNA (rDNA) gene from amplified community bacterial DNA extracted from mussel samples. Analysis of sequences revealed the presence of 384 phylogenetically diverse bacterial groups belonging to 15 subdivisions in zebra mussel samples. Our results reveal that bacterial communities associated with zebra mussels not only vary from one waterbody to the other but also from one zebra mussel organ to the other, with the gut having the greastest diversity. Additionally, our results suggest that the zebra mussel harbor potentially pathogenic bacteria for many aquatic and terrestrial animals, such as *Aeromonas* spp., *Clostridium* spp., *Escherichia coli*, *Flavobacterium* spp., *Legionella pneumophila*, *Mycobacterium* sp., *Mycoplasma* sp., *Rickettsia*-like bacteria, *Shewanella putrefaciens*, *Shigella* sp., *Ureaplasma* sp., and *Yersinia ruckeri*. This work constitutes the first account of bacterial community structure in the zebra mussel in its new geographic range in the Laurentian Great Lakes basin.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my colleague and friend, Dr. Mohamed Faisal under whose supervision I chose this topic and began the thesis. With his enthusiasm, his inspiration, and his great efforts to explain things clearly and simply, he helped to make me the person I am today. He has always provided encouragement, sound advice, good teaching, and good company. I will always be indebted to him.

I am grateful to Dr. Terrance Marsh and his entire laboratory. Spending a year in his laboratory was one of the greatest learning experiences I have ever had. I am especially grateful to Dr. Sang-Hoon Kim, Fan Yang, and Natasha Isaacs.

I wish to thank my committee member, Dr. Orlando Sarnelle for meeting with me to discuss my project and assisting in writing this thesis. His expert advice is greatly appreciated.

My sincere thanks are due to my professor, Dr. Scott Winterstein, for his guidance in helping me choose the most appropriate statistical tests for my analyses.

I wish to thank Dr. Faisal's laboratory for providing a stimulating and friendly environment in which to learn and grow. I am especially grateful to Michelle Gunn, Tom Loch, Carolyn Schulz, Kolina Riley, and Wei Xu. Wei Xu was particularly helpful showing patience when explaining difficult concepts to me.

The financial support of National Sea Grant College Program from the National Sea Grant NOAA, U.S. Department of Commerce is gratefully acknowledged for funding this study under the project entitled "Identification of Diseases and Host Defense Mechanisms in the Zebra Mussel (*Dreissena polymorpha*)" (Project #: R/NIS-13).

I owe my loving thanks to my wife, Blair N. Winters. Without her encouragement and understanding it would have been impossible for me to finish this work.

Last and most importantly, I wish to thank my parents, Michelle M. Winters and David L. Winters. They bore me, raised me, taught me, and loved me. To them I dedicate this thesis.

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GENERAL INTRODUCTION AND OVERVIEW

Native to Eastern Europe, the zebra mussel (*Dreissena polymorpha*) is an invasive filter-feeding bivalve mollusk that has become established in many places throughout the world. Since the first report of zebra mussels in Lake Saint Clair, Michigan in the mid 1980's they have spread throughout the United States and into Canada causing an estimated $1,000,000,000 in damages annually (Pimentel *et al.*, 2000). Due to the large impact zebra mussels have on ecosystems and infrastructures much effort has been directed towards research to discover a pathogen that may be used as an effective biological control agent against these nuisance mussels. Still, despite concerted efforts by managers and scientists, an effective strategy to eradicate or control zebra mussel populations has yet to be developed, implemented, and practiced.

Although much research has been conducted regarding the ecology and physiology of zebra mussels since they were first discovered in the Laurentian Great Lakes Basin (LGLB), little is known regarding the unique microbial communities associated with these mussels. Furthermore, there has been little research conducted regarding the microbial flora of the zebra mussel. Most investigations regarding microbial communities in the zebra mussel have used classical bacterial culture methods are limited in their ability to accurately describe the microbial flora of zebra mussels because they only account for species of heterotrophic bacteria that can propagate *in vitro*. These limitations created the need to find an alternate approach to accurately measure diversity of microbial populations within freshwater mussels. The aim of the present study was to use advanced molecular methods to provide a detailed description of the microbial communities associated with the zebra mussel in its new habitat in the

LGLB.

This thesis consists of four chapters. The first chapter will summarize the background information and the available body of knowledge on the thesis, topic. The second chapter deals with identifying which bacteria are associated with the zebra mussel in the LGLB. The third chapter is a report on the differences among bacterial species abundance and bacterial community diversity detected among three zebra mussel sample types. Finally, I shall present my conclusions and potential directions for future work.

CHAPTER ONE

REVIEW OF LITERATURE

I. Zebra mussel background information

Of the more than 140 non-indigenous species of plants, fish, algae, and mollusks that have invaded North American freshwaters since the 1800s, the zebra mussel (*Dreissena polymorpha*) has caused the most dramatic consequences. Due to its rapid range expansion (Roberts, 1990; Ludyanskiy *et al.*, 1993; Nalepa and Schloesser, 1993) this invader have caused negative ecological impacts that ranged from the displacement of native unionid clams to large-scale community and ecosystem changes as energy and nutrient flows are redirected by the mussel's filter-feeding (Mackie, 1991). In addition, this nuisant species has caused severe economic losses. In the USA, zebra mussels have invaded and clogged water intake pipes and water filtration and electric generating plants causing an estimated $100,000,000 in damages annually (Pimentel *et al.*, 2000).

Native to the Caspian Sea and the Ural River (Russia and Kazakhstan), zebra mussels were first described by Pallas in 1769. Since then, populations have been reported in Hungary, Great Britain, the Netherlands, Poland, France, Canada and the United States. Artificial canals that link many European waterways facilitated their early dispersal. Belonging to the largest subclass (Eulamellibranchia) of the molluscan class Bivalvia, the zebra mussel (*Dreissena polymorpha*) is relatively small with a shell length usually smaller than 30 mm (Morton, 1969a). Zebra mussels have a heterodont shell

hinge, leaflike gills, and well-developed siphons (Nalepa and Schloesser, 1993). The mussel attaches itself to hard surfaces by byssal threads which are secreted from a byssal gland just posterior to the foot (Mackie, 1991). This byssal holdfast distinguishes *Dreissena* species from other freshwater bivalves (McMahon, 1990). The zebra mussel is a highly efficient filter-feeder that feeds on phytoplanktons, micro-zooplanktons, and algae (Nalepa and Schloesser, 1993). Like other freshwater bivalves, zebra mussels are able to discriminate among particles they acquire from the water, such as cyanobacteria (Baker *et al.*, 1998) and inorganic substances (Baker *et al.*, 2000). As they pull water into their inhalant siphon, the water passes over and through the gills where particles are trapped and sorted with cilia and mucous (Morton, 1969b). Cilia transport desirable particulate matter to the gut, while non-food particles are combined with a mucus coating called pseudofeces (Reeders and Bij de Vaate, 1990). Pseudofeces is ejected out the incurrent siphon and particle-free water discharged out the exhalent siphon (Baker *et al.*, 2000; Ten Winkel and Davids, 1982). This highly efficient form of filter-feeding by the zebra mussel has enormous impacts on the ecosystem for it decreases phytoplankton biomass (Fahnenstiel *et al.*, 1995), increases the number of toxigenic species of bacteria, like *Microcystis aeruginosa* (Sarnelle *et al.*, 2005; Juhel *et al.*, 2006) and *Clostridium botulinum* (Pérez-Fuentetaja *et al.*, 2006), and alters bacterial community structures in their surrounding environment (Frischer *et al.*, 2000).

II. Negative impact of the zebra mussel on the Great Lakes Ecosystem

1) Harmful algal blooms.

Numerous studies have indicated that the filtering activities of zebra mussels play an important role in the development of blooms of toxic algal species (Raikow *et al.*, 2004; Sarnelle *et al.*, 2005; Juhel *et al.*, 2006). Algal toxins such as microcystins are lethal to many animal species and can cause liver toxicity in humans. In a survey of 61 Michigan lakes of varying nutrient levels that contain or lack zebra mussels, Raikow *et al.* (2004) demonstrated a significant effect of *D. polymorpha* filter-feeding on the dominance of *Microcystis aeruginosa* (P =0.016) but no effects of *D. polymoropha* on total cyanobacterial dominance were observed (P = 0.25).

In this context, Juhel *et al.* (2006) conducted an experiment to determine the preferential selection of zebra mussels for non-toxic strains of phytoplankton. They showed that toxic *M. aeruginosa* were selectively rejected compared to non-toxic diatoms. Microscopic analysis of the pseudofecal ejecta showed that the proportion of *M. aeruginosa* relative to *Asterionella formosa* was high in the pseudofeces and even higher in the 'pseudodiarrhoea' when a mixed diet was given to the mussels. Juhel *et al.* (2006) concluded that the selective rejection of zebra mussels would tend to enhance the presence of toxic *Microcystis aeruginosa* in mixed phytoplankton assemblages.

2) Increased mortalities among fish and aquatic birds due to *Clostridium botulinum* Type E Toxin.

It is believed that the combined affects of increased water clarity and nutrient enrichment by dressenids results in an increase in aquatic macrophytes. When macrophytes decay or undergo respiration at night, they create anoxic conditions in the water. Inland lakes infested with zebra mussels have been shown to have lower dissolved oxygen concentrations (Raikow, 2002). These conditions create an ideal environment for anaerobic bacteria to grow. One of these microbes is *Clostridium botulinum*, the organism that produces botulinum toxin.

It has been suggested that zebra mussels are concentrating the toxin and are passing it up the food-web possibly leading to avian and fish die-offs. (Getchell *et al.*, 2006; Pérez-Fuentetaja *et al.*, 2006) Pérez-Fuentetaja *et al.* (2006) used PCR (polymerase chain reaction) to test for the presence of *Clostridium botulinum* type E spores in lake sediments and in several invertebrates, including mussels, aquatic worms, and even fly larvae from Lake Erie. Pérez-Fuentetaja *et al.* (2006) demonstrated the presence of spores in zebra mussel tissue points to the possibility that they may play a role in the transmission of botulism to upper trophic levels.

In another study, Getchell *et al.* (2006) employed the Quantitative PCR (QPCR) assay to detect *Clostridium botulinum* type E in apparently healthy fish from the lower Great Lakes after a recent fish kill of species, such as the freshwater drum, *Aplodinotus grunniens*, and the round goby, *Neogobius melanostomus*, two fish that are known to feed

on zebra mussels. They concluded that moribund fish may play a large role in transmitting type E toxins in piscivorous birds of the lower Great Lakes.

3) Increased fecal bacterial concentrations in the environment.

Through their filter-feeding nature, dreissenids and unionids mussels have the ability to accumulate bacteria to concentrations several times higher than in surrounding water (Al-Jebouri and Trollope, 1984; Turick *et al.*, 1988). Therefore, both bivalves have been used to monitor water quality and detect fecal contamination in waterbodies (Al-Jebouri and Trollope, 1984; Turick *et al.*, 1988; and Selegean *et al.*, 2001). For example, Turick *et al.* (1988) conducted an experiment to determine if the unionid eastern Elliptio mussel (*Elliptio complanata*) could concentrate *Escherichia coli* from water. They stated that within five hours of exposure the mussels could accumulate concentrations of *E. coli* up to five-times greater than the surrounding water in a variety of freshwater stream environments near the Atlantic slope. After 50 hours, concentrations were up to 15-times greater than the surrounding water. Turick *et al.* (1988) concluded that mussels could serve as a record of recent episodes of fecal contamination because of their ability to concentrate bacteria. In the same context, Selegean *et al.* (2001) conducted field and laboratory experiments to examine the use of zebra mussels as an indicator of elevated bacterial concentrations in highly urbanized areas such as the Clinton River watershed (Michigan, USA). In laboratory experiments, the same authors exposed zebra mussels to a diluted solution of raw sewage. In field experiments, caged mussels were placed in the river for several days. In both field and laboratory studies *E. coli* in mussel homogenate

samples were periodically enumerated using the plate count method. The authors demonstrated that maximum concentrations of *E. coli* in mussel tissue are reached within a few hours after initial exposure and are retained for a few days. Selegean *et al.* (2001) concluded that watershed managers could reduce the sampling frequency normally required to identify critical *E. coli* sources by sampling caged mussels in a river and its tributaries thereby providing a more cost-effective river monitoring strategy for bacterial contamination.

III. Diseases and bacteria associated with bivalves:

There have been several reviews of diseases affecting bivalve mollusks (Lauckner, 1983; Sparks, 1985; Fisher, 1988; Gibbons and Blogoslawski, 1989; Sindermann, 1990; Bower, 1992; Perkins, 1993; Cheng, 1993; Bower *et al.*, 1994; Ford and Tripp, 1996; Elston *et al.*, 1999; McGladdery, 1999; McGladdery *et al.*, 2006; U.S. Fish and Wildlife Service, The American Fisheries Society-Fish Health Section, 2007). These articles mainly discuss diseases of marine bivalves, and most of the isolated pathogenic bacteria have not been reported from freshwater mussels. Unlike marine bivalves, little is known about bacterial diseases of freshwater bivalves. As raising mussels in captivity continues to gain commercial importance worldwide, so may the understanding of bacteria as a cause for disease in mussels.

The largest single sector of the U.S. marine aquaculture industry is molluscan shellfish culture, which accounts for about two-thirds of total U.S. aquaculture production. With current shellfish production taking place mainly on land

(http://aquaculture.noaa.gov), the growing demand for mussels (http://aquaculture.noaa.gov), will force shellfish farmers to increase animal densities in rearing facilities creating "stressful" environments that contribute to facultative bacterial infections in mussels causing economic losses. Similarly, as zebra and quagga mussel populations continue to spread negatively impacting ecosystems and economies the study of bacteria as a cause for disease in dreissenids has also become important. Unfortunately, obtaining evidence for pathogenicity of bacteria in freshwater bivalves has been difficult.

Quite often bivalves sampled from apparently healthy populations harbor diverse groups of bacteria. For example, Starliper and Morrison (2000) and Starliper (2001, 2005) showed that the number of bacterial species present in unionids varies depending on the bacterial species present in the aquatic environment from which they were isolated. Starliper and Morrison (2000) demonstrated that *Aeromonas* spp. and *Pseudomonas* spp. were the predominant genera of bacteria isolated from unionids in the Ohio River. Counts of total bacteria on cytophaga medium ranged between 1.07×10^5 and 4.99×10^5 CFU/g of soft mussel tissue. Additionally, cells with correct morphology for *Renibacterium salmoninarum*, causative agent bacterial kidney disease in fish, were detected from soft mussel tissues from the Ohio River in all six trials using the direct fluorescent antibody test. Sparks *et al.* (1990) isolated bacteria from unionid stomach, hemolymph, midgut, gill, mouth, and mantle samples and determined that *Aeromonas hydrophila* was the most commonly isolated bacteria in both healthy and apparently moribund unionids sampled from the Illinois and Mississippi rivers. Sparks *et al.* (1990) also reported that none of the bacterial species found only in moribund mussels were

isolated from more than two specimens. Similarly, Chittick *et al.* (2001) cultured bacteria from the digestive gland of the unionid *Elliptio complanata* sampled from two waterways in North Carolina and found that *Aeromonas hydrophila* was most commonly cultured.

There has only been one study describing the diversity of microbial communities associated with the zebra mussel (Frischer *et al.*, 2000). In the study, zebra mussel tissue homogenate, fecal material and surrounding sediment from the Hudson River, NY were used as a template for PCR amplification and clone library analysis. Frischer *et al.* (2000) stated that a dominance of bacteria belonging to the β- and γ- classes of Proteobacteria was apparent in the zebra mussel homogenate and fecal samples but not in sediment samples from the Hudson River. The authors concluded that zebra mussels may have the ability to enrich certain types of bacteria and that the interactions between zebra mussels and microbial communities remain unclear.

One problem with linking bacteria to disease in bivalves is the normal finding of bacteria in apparently healthy mussels and the rapid change in the bacterial composition in unionids after death (Grizzle and Brunner, 2007). In one study, Jenkinson and Ahlstedt (1987) investigated die-offs of multiple unionid species in the Tennessee River. They observed large numbers of bacteria in the connective tissue and in the digestive gland of apparently diseased mussels. Although no bacteria were observed inside tissue cells, a few bacteria were observed inside phagocytic hemocytes. Several bacterial species including *Acinetobacter* sp., *Aeromonas hydrophila, Bacillus* sp., *Citrobacter fruedii, Hafnia alvei, Klebsiella* spp. *Pseudomonas* sp. and *Vibrio* spp. were isolated from dead or dying mussels but none could be correlated to pathogenicity. The most notable

infection was reported in dying Blue-point mussels (*Amblema plicata)* samples, in which *A. hydrophila* was isolated in higher numbers than any bacteria observed in the study.

Scholla *et al.* (1987) also investigated the same unionid die-off in the Tennessee River. In this study, healthy mussels were compared with apparently unhealthy mussels, which were defined as those whose valves did not close completely or did not remain closed after manual stimulation. Although the number of coliform bacteria did not differ significantly between healthy and sick mussels, a 10-fold increase was observed in the number of total bacteria in apparently sick mussels than in healthy ones (5.15×10^5 versus 5.46×10^4 colony forming units CFU/g). A 10-fold increase was also observed in unidentified gram-negative, yellow-pigmented bacilli present in cultures taken from tissue samples from apparently sick mussels (1.49×10^4 versus 1.48×10^3 CFU/g). Attempts were made to determine if the yellow-pigmented bacteria could cause illness in healthy mussels via cohabitation of healthy and sick mussels. An increase in the total number of bacteria as well in the number of yellow-pigmented bacteria was observed in the previously healthy mussels after cohabitation with the sick mussels. Scholla *et al.* (1987) reported that the number of mussels used in the study was too small to draw any conclusions about pathogenicity.

In the study of Sparks *et al.* (1990), multiple die-offs of 20 different species of unionids that occurred in the Illinois and Mississippi rivers were investigated. They stated that seven taxa of bacteria were isolated from healthy mussels and 10 taxa were isolated from moribund mussels. Fuller (1974) identified fungi-like bacteria as a major bacterial pathogen for freshwater mussels, particularly for parasitic larval mussels (glochidia) under conditions of siltation and nutrient enrichment. In terms of

pathogenesis, there have only been few experimental studies on unionids so most of the information on this topic is for zebra mussels (which do not have a *glochidial* stage of life).

In an experimental study performed on the unionid *A. plicata* (Starliper and Morrison, 2000), mussels were exposed to two fish pathogens, *Aeromonas salmonicida*, the causative agent of furunculosis in teleosts (Cipriano and Bullock, 2001), and *Renibacterium salmoninarum*, the causative agent of Bacterial Kidney Disease in salmonid fish species (Fryer and Sanders, 1981). The two pathogens were introduced into the water and flow was temporarily shut off for 24 hours. None of the mussels died during the three-week challenge suggesting that the selected bacteria are not pathogenic to the Unionids used in the study. Starliper (2005) determined that it is possible for freshwater mussels to harbor *A. salmonicida* several days after initial exposure thereby acting as a vector for furunculosis and recommended a 30-day quarantine of freshwater bivalves destined for relocation.

IV. Use of bacteria as a biological control agent for zebra mussels

Due to the large impact zebra mussels have on ecosystems and infrastructures much effort has been directed towards research to discover a pathogen that may be used as an effective biological control agent against these nuisance mussels. In this context, Toews *et al.* (1993) collected zebra mussels from three locations in the Great Lakes and found *Pseudomonas*, *Aeromonas*, and *Bacillus* to be the predominate genera of bacteria in both living and dead mussels as well as in lake water. Additionally, Toews *et al.*

(1993) employed experimental infection of zebra mussels via inoculation of water containing mussels with bacterial suspensions of both low and high concentrations (10^6 CFU/mL of water and 10^4 CFU/mL of water respectively) of *Serratia liquefaciens* and *E. coli*. Bacterial concentrations were verified by periodically sampling water and carrying out dilution plate counts. Mussels exposed to low and high concentrations of *S. liquefaciens* became byssally detached and lay moribund at the bottom of the aquariums, while similar concentrations of *E. coli* produced no effects. Toews *et al.* (1993) stated that the *S. liquefaciens* strain they tested is lethal to mussels and that the concentrations used in the experiment caused 100% mortality. Toews *et al.* (1993) concluded that their preliminary findings suggest that the use of the potentially pathogenic bacteria in the control of zebra mussels warrants further investigation. Although no one has advocated intentional introduction of pathogenic bacteria into lakes and streams, the use of biological control agents against zebra mussels may be useful in confined areas such as industrial cooling water systems.

Maki *et al.* (1998) conducted a study to determine whether five species of *Aeromonas* were pathogenic to zebra mussels. In their experiment, Maki et al. (1998) used *A. jandaei*, *A. veronii*, and *A. media* originally isolated from dead zebra mussels, and two additional species, *A. salmonicida* subspecies *salmonicida* and *A. hydrophila*. Multiple mussels were then inoculated with either bacterial suspensions or sterile lake water as a control. Inoculations were accomplished via injection in the dorsal section of the mantle cavity. Mussels were then placed in sterile lake water and incubated at 10°C for a maximum of eight days. All mussels injected with *A. jandaei*, *A. veronii*, and *A. media* died after eight days. All mussels injected with *A. salmonicida* subspecies

salmonicida and *A. hydrophila* died after six days. The percentages of mussel mortality from *Aeromonas spp.* were statistically significant as compared to the control group ($P < 0.003$). The authors concluded that a number of *Aeromonas* spp. can be pathogenic to zebra mussels if injected into the mantle cavity and that zebra mussels may act as act as a reservoir for these bacteria in freshwater environments.

Gu and Mitchell (2002) isolated 17 genera of bacteria from fresh zebra mussels that had been collected from Lake Erie and mussels that had been placed in aquaria under varying conditions. They stated that that *Pseudomonas* spp. were dominant under natural conditions, and *Aeromonas* spp. and *Shewanella* spp. became more prevalent under conditions of crowding, temperature elevation, or starvation, suggesting these bacteria were more virulent opportunistic pathogens for zebra mussels. The authors concluded that mussels may be reservoirs of opportunistic pathogenic microorganisms for aquatic organisms and humans, and that further research is required to better understand microbial ecology of zebra mussels. In addition, extra-cellular proteins produced by bacteria have been proven to be toxic to zebra mussels (Genthner *et al.*, 1997; Singer *et al.*, 1997; Molloy, 2001).

Genthner *et al.* (1997) exposed zebra mussels to varying concentrations of *Bacillus alvei* (ATCC 2271), a strain of *Bacillus* that is repeatedly isolated from bee larvae affected with European foul brood. Mussels were exposed to 1.0% suspension for 48 hours. After 48 hours no mussels had died. Although, histopathological examination showed no evidence of bacterial infection in the gut, gills, and gonads, the digestive epithelial cells appeared to be atrophied and were observed to be sloughing off. Genthner

et al. (1997) concluded that a bacterial toxin was the cause because there was no indication of an active infection of the mussel tissues.

Molloy (2001) indicated that live and dead cultures of *Pseudomonas fluorescens*, a bacterium commonly isolated from North American waters, are equally lethal to *Dreissena polymorpha* and *Dreissena bugensis*. One hundred mussels *(D. polymorpha* or *D. bugensis)* were placed in a glass bottle containing aerated water and treated with different concentrations of bacterial cells. Mortalities between 83.5% and 98.4% were achieved when the bottles were treated with bacteria at a target concentration of 200 ppm (bacterial mass/L) and maintained under aerated conditions for 48 hr. Molloy (2001) concluded that the bacterial strain should be equally effective at killing zebra mussels in power plant pipes, regardless of which species is present. Still, despite concerted efforts by managers and scientists, an effective strategy to eradicate or control zebra mussel populations has yet to be developed, implemented, and practiced.

V. Bacterial communities associated with bivalve mollusks

Bacterial communities associated with bivalves traditionally have been compared by analyzing isolates cultured on bacterial media. Once cultured, isolates are clustered into taxonomic groups based on phenotypic and genotypic characteristics. Comparisons of species composition, species richness, and community structure are then calculated for each sample. This method, though valuable, is limited in its ability to accurately evaluate these relative measures of bacterial community diversity in a sample because it only accounts for culturable species of bacteria. The uncultured fraction has been recognized

to be a major component of all microbial communities. For example, it has been well documented for pathogens like *Salmonella enteritidis* (Roszak *et al.*, 1984), *Vibrio cholera* (Colwell, 1996), and *V. vulnificus* (Oliver *et al.*, 1991) that bacteria may quickly enter a nonculturable state upon exposure to environmental factors such as salinity and temperature.

There has only been one study describing the diversity of microbial communities associated with the zebra mussel (Frischer *et al.*, 2000). In the study, zebra mussel tissue homogenate, fecal material and surrounding sediment from the Hudson River were used as a template for PCR amplification and clone library analysis. Frischer *et al.* (2000) stated that a dominance of bacteria belonging to the β- and γ- classes of Proteobacteria was apparent in the zebra mussel homogenate and fecal samples but not in sediment samples from the Hudson River. The authors concluded that zebra mussels may have the ability to enrich certain types of bacteria and that the interactions between zebra mussels and microbial communities remain unclear.

Most microbial studies on bivalves have relied on classical culture methods aimed at identifying only heterotrophic bacteria from whole-mussel homogenate samples and do not accurately describe finer details of the biological components of a given habitat (Al-Jebouri and Trollope, 1984; Jenkinson and Ahlstedt, 1987; Scholla *et al.*, 1987; Turick *et al.*, 1988; Sparks *et al.*, 1990; Toews *et al.*, 1993; Maki *et al.*, 1998; Frischer *et al.*, 2000; Starliper and Morrison, 2000; Selegean *et al.*, 2001; Gu and Mitchell, 2002; Starliper, 2001, 2005). It is estimated that >99% of microorganisms observable in nature typically are not cultivated by using standard bacteriological techniques (Amann *et al.*, 1995). Keeping in mind the diversity of bacteria that cause disease in marine bivalves

(McGladdery, 1999), it seems likely that some major groups of pathogenic bacteria (e.g., Rickettsiales, Chlamydiales, and Mycoplasmatales) have been overlooked in freshwater bivalves (Grizzle and Brunner, 2007).

In conclusion, culture-dependent investigations are limited in their ability to accurately assess microbial diversity in zebra mussels both qualitatively and quantitatively. If a more epidemiological approach is to be taken, it is necessary to first adequately understand the balance of ecological, physiological, and pathological interrelations between indigenous bacteria and zebra mussels before a novel biological control agent or management strategy can be discovered. A better understanding of the phylogenetic diversity within bacterial communities associated with zebra mussels will help to understand the full extent of the zebra mussel's impacts on the Laurentian Great Lakes ecosystem and to identify and track disease in zebra mussel populations. Considering the environment and the filter-feeding activity of the zebra mussel, I chose to employ a molecular approach to identify the structure of bacterial communities associated with zebra mussels in the Laurentian Great Lakes basin (LGLB).

VI. Molecular approach to study microbial diversity

The analysis of the 16S rRNA (rDNA) gene, aided by using PCR to amplify target sequences in environmental samples, has enabled ecologist to provide better estimates of bacterial diversity. Methods that rely on selective amplification of 16S rRNA (rDNA) gene sequences have rapidly replaced cultivation as a way to compare diversity in microbial communities because they objectively relate organisms and allow for a more

accurate description microbial community composition, richness, and structure in environmental samples. One molecular technique used to study microbial communities is 16S rDNA gene cloning (Benloch *et al.*, 1995; DeLong *et al.*, 1993; Fuhrman *et al.*, 1993; Pedersen *et al.*, 1996).

In the context of the present study, the term cloning will be used to describe the process of isolating a defined DNA sequence and obtaining multiple copies of it *in vivo*. By inserting 16S rRNA (rDNA) into competent bacterial cells, like *E. coli*, genetic variation in a bacterial population can be estimated. The process begins with the insertion of amplified community PCR products into a plasmid vector through the process of ligation. After ligation, plasmids are introduced into competent bacterial cells through the process of transformation. Competent bacterial cells are then incubated and screened for positive transformation. Transformants are then prepared and sequenced resulting in a collection of 16S rRNA (rDNA) gene sequences from various genotypes from within the community being investigated. Finally, these sequences are compared to 16S gene sequences contained in various publicly available databases such as BLAST (National Center for Biotechnology Information) and the Ribosomal Database Project (http://rdp.cme.msu.edu) (Maidak *et al.*, 2000). These widely used databases allow researchers to identify bacterial species and to estimate genetic variation in the sample population through the comparison of query sequences with thousands of sequences in the databases.

Numerous studies have investigated the phylogenetic overlap between bacteria identified from environmental samples by culture and by 16S rDNA cloning (Benloch *et al.*, 1995; Chandler *et al.*, 1997; Pedersen *et al.*, 1996; Stackebrandt *et al.*, 1993; Suzuki

et al., 1997; Wilson and Blitchington, 1996; Dunbar *et al.*, 1999). With the exception of Suzuki *et al.* (1997), all of the listed studies reported increases in the number of identifiable bacterial phylotypes when a larger number of clones, rather than cultivated isolates, were sequenced. In two of these studies as much as 59% of the bacteria identified by 16S rDNA gene cloning were not identified in culture collections (Wilson and Blitchington, 1996; Chandler *et al.*, 1997). Stackebrandt *et al.* (1993) reported that bacteria that are customarily isolated from the Subtropical Australian soil were represented in the clone library in small numbers or were not detected at all. Similarly, Suzuki *et al.* (1997) reported that many bacterioplankton species are not readily culturable by standard methods. All of these studies clearly indicate that culture-independent 16S gene cloning is more effective at identifying microbial community composition in environmental samples (Table 1.)

Study	Sample type	# phylotypes / # isolates sequenced	# phylotypes / # clones sequenced
Benloch *et al.*, 1995	seawater	3/14	28/48
Pedersen *et al.*, 1996	soil	5/20	12/155
Wilson and Blitchington, 1996	human colon	21/48	27/59
Suzuki *et al.*, 1997	seawater	36/127	11/58
Chandler *et al.*, 1997	soil	20/100	98/744
Dunbar *et al.*, 1999	soil	33/50	154/200

Table 1. Results of six studies that compare culture and non-culture methods of assessing microbial diversity in environmental samples.

VII. Objectives of this study

There are three objectives for this study: 1) to identify which bacterial species, including pathogens, are associated with the zebra mussels in the Laurentian Great Lakes basin (LGLB) 2) to determine if differences exist in bacterial assemblages in mussel homogenates based on the waterbody sampled 3) to determine if significant differences in microbial community structure exists among three mussel sample types harvested from zebra mussels collected from a single site. The aim of this study is to provide, for the first time, insights into the microbial ecology of the zebra mussel in its new environment in the LGLB.

CHAPTER TWO

MOLECULAR CHARACTERIZATION OF BACTERIAL ASSEMBLAGES ASSOCIATED WITH THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) IN THE LAURENTIAN GREAT LAKES BASIN (USA)

ABSTRACT

Although a number of studies have attempted to describe bacterial communities associated with the zebra mussels (*Dreissena polymorpha*), little is known about their bacterial assemblages in their new range in the Laurentian Great Lakes basin. In the present study, bacteria were identified in mussel samples collected from one river and two inland lakes in Michigan's Lower Peninsula (USA): the Huron River, Crystal Lake, and Lake Vineyard. Bacteria were identified by sequencing the 16S rRNA (rDNA) gene of amplified community bacterial DNA extracted from pooled mussel homogenate samples. Over 170 16S gene sequences were checked for similarity to existing 16S sequences contained in two public databases: the Ribosomal Database Project and BLAST. A total of 35 genera belonging to 12 bacterial subdivisions were detected in mussel homogenates. Bacteria belonging to the phyla Cyanobacteria, Firmicutes, and the α-, β-, γ-, and δ- classes of Proteobacteria were present the three mussel groups. Samples also contained sequences from bacteria that are either currently unclassified or have yet to be added to existing databases. Large abundances of Bacteroidetes (>38%) were detected in the Huron River sample only while large abundances of Actinobacteria

(>30%) were detected in the Lake Vineyard sample only. On the other hand, bacteria belonging to the phyla Planctomycetes, Verrucomicrobia, Chloroflexi, Acidobacteria were only present in the Huron River sample. Potentially pathogenic bacteria for aquatic and terrestrial animals, like *Aeromonas* spp., *Clostridium* spp., *Escherichia coli*, *Flavobacterium* spp., *Shewanella putrefaciens*, *Shigella* sp., and *Yersinia ruckeri*, were detected in mussel homogenates suggesting that zebra mussels may contribute to the spread of these pathogens. In conclusion, bacteria associated with zebra mussels appear to vary from one waterbody to the other, probably reflecting the microbial diversity in the surrounding environment. Data generated in this study constitutes the first account of bacterial assemblages in the zebra mussel in its new geographic range in the Laurentian Great Lakes basin.

2) INTRODUCTION

Since the zebra mussel (*Dreissena polymorpha*) was first introduced into Lake St. Clair, Michigan (USA) in the late 1980's, it has spread to a large number of watersheds in North America, including the Mississippi, Hudson, St. Lawrence, Ohio, Cumberland, Missouri, Tennessee, Colorado, and Arkansas Rivers. This spread is believed to be facilitated by ships and recreational vessels unknowingly transferring this intruding bivalve mollusk to new locations. Due to rapid range expansion (Roberts, 1990; Ludyanskiy *et al.*, 1993) this invasive bivalve mollusk has caused devastating ecological impacts that range from habitat destruction to large-scale community and ecosystem changes as energy and nutrient flows are redirected by the mussel's filter-feeding nature (Mackie, 1991).

The highly efficient form of filter-feeding performed by the zebra mussel is believed to cause significant decline in phytoplankton biomass thereby adversely affecting the food-web (Nalepa and Schloesser, 1993). Like other freshwater bivalves, zebra mussels are also able to differentiate the particles they filter; rejecting some microbes and passing others to the digestive tract. As a result, shifts in microbial populations such as increases in the number of toxigenic bacteria, like *Microcystis aeruginosa* have occurred in waters heavily infested with zebra mussels (Raikow *et al.*, 2004; Sarnelle *et al.*, 2005; Juhel *et al.*, 2006). The influence of zebra mussels on microbial communities in the Laurentian Great Lakes basin (LGLB) has not been fully elucidated, neither have the bacterial assemblages of zebra mussels in their new home range been identified. To this end, the first objective of the present study is to use 16S rRNA (rDNA) gene sequencing to identify and compare bacterial assemblages associated

with zebra mussels collected from three waterbodies in the LGLB.

Considering the large number of bacterial species that can be associated with a bivalve mollusk and those that cannot be cultured *in vitro* (Amann *et al.*, 1995), we opted to employ the 16S rRNA (rDNA) gene sequence analysis to identify phylogenetically diverse bacteria in zebra mussel homogenate samples. This approach has been widely used in studying bacterial community structure in samples where bacterial diversity is expected to be rich such as activated sludge, human intestine, and soils (reviewed in DeSantis *et al.*, 2006).

Efforts have been directed towards identifying the role of zebra mussels in the epidemiology of bacteria of potential pathogenicity to the zebra mussel as well as other aquatic and terrestrial organisms. In this context, Gu and Mitchell (2002), who used conventional culture techniques, reported the presence of *Aeromonas* spp., *Pseudomonas* spp., and *Vibrio anguillarum* among 17 genera of bacteria in homogenates collected from zebra mussels residing in Lake Erie, New York (USA). Unfortunately, the limitations inherited in conventional bacteriological methods did not allow a wide-scale identification of the bacterial flora of the zebra mussel in its new range in the LGLB. Therefore, the second objective of this study is to identify the presence of bacteria of potential pathogenicity associated with zebra mussels in the three waterbodies in Michigan, USA.

3) MATERIALS AND METHODS

a) Zebra mussel and sampling sites and sample processing

Between May 6-13, 2006, five mussels were collected from each of the Huron River, Crystal Lake, and Lake Vineyard (Figure 1). The mussels were collected manually from a depth of 1.5 meters. Mussels were placed in separate five-gallon buckets containing fresh aerated lake water from the respective water-body and brought to the laboratory for processing. Each mussel was taxonomically identified according to the morphological features described by Morton (1969a) and immediately processed.

For this study, three waterbodies were selected for sampling based on the fact that zebra mussels had been settled in them for a relatively long time (over eight years, www.miseagrant.umich.edu/ais/lakes, Figure 1). The time elapsed between settlement and sampling should allow for the stability of microbial communities associated with zebra mussel populations that had adapted to the chosen sites. The Huron River (43.322° N, -83.797° W, Washtenaw County) is a large river system (233,099 ha) that flows through the southeast Michigan counties of Oakland, Ingham, Livingston, Washtenaw, Monroe, and Wayne into Lake Erie. Approximately 162 lakes ranging in size from one to 261 hectares drain into the Huron River (Michigan Department of Natural Resources Fisheries Division, 2002). Huron River mussel samples were collected in a narrow section of the river below the city of Ann Arbor where the principal sources of pollution include discharge from wastewater treatment plants and industrial and manufacturing companies (Michigan Department of Natural Resources Fisheries Division, 2002). High

nutrient concentrations, primarily phosphorus, and localized low dissolved oxygen have been reported near the location sampled (Michigan Department of Natural Resources Fisheries Division, 2002). The watershed that feeds Crystal Lake (44.659N, -86.156W, Benzie County) is small (11,390 ha). Fertilizers and sewage outflows are minimal, and the lake's water is exceptionally clear (http://www.clwa.us/about_watershed.htm). The water quality of Crystal Lake is extremely oligotrophic compared to other inland lakes in Michigan (Cooperative Lakes Monitoring Program, 2005). Crystal Lake mussel samples were collected from a small boulder on the sandy bottom. Lake Vineyard (42.093N, -84.442W, Jackson County) is within the River Raisin watershed (278,052 ha). The River Raisin watershed, where agriculture and urbanized areas accounted for roughly 76% of land uses in 2000, is home to more than 140,000 people (http://www.city-data.com/township/Columbia-Jackson-MI.html). These watershed pressures have created sediment, nutrient, and pathogen loads and flow instability not only in the River Raisin but also in lakes within the watershed (http://riverraisin.org/about/management_plan.html). Whole mussel tissues were aseptically excised from the shells and dissections were performed with the aid of a dissecting scope. Mussel samples were homogenized with a sterile glass rod, pelleted (15,000 g centrifugation), and stored in 80% ethanol (ETOH) at -20°C prior to extraction of genetic material.

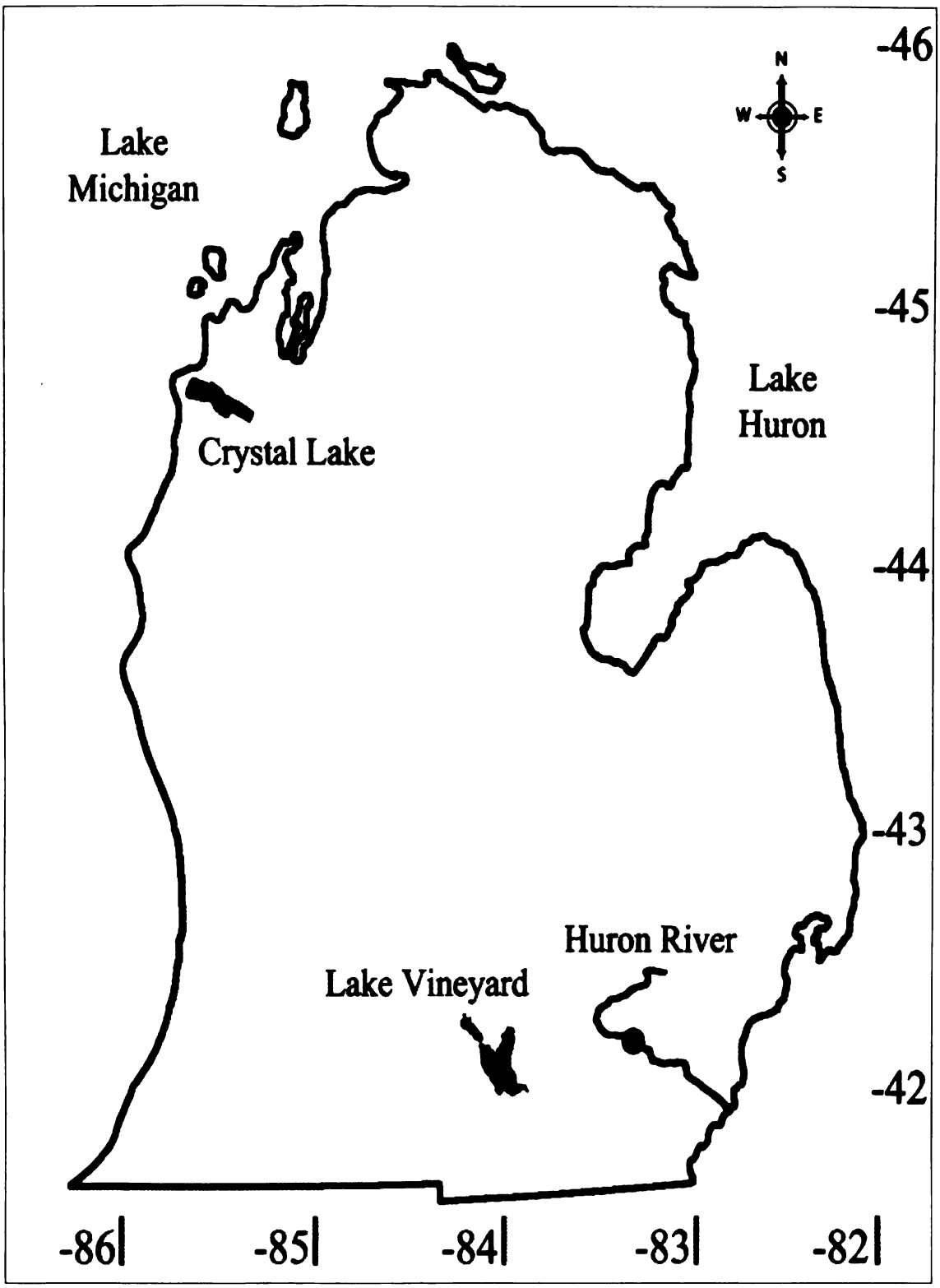

Figure 1. Map of Michigan's Lower Peninsula showing sampling sites where zebra mussels were collected (numbers on x and y axes represent latitude and longitude).

b) Sequence analysis of the 16S rRNA (rDNA) gene

Genomic bacterial community DNA was harvested from mussel homogenates using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) following the manufacturer's protocol. PCR amplification of bacterial 16S genes was performed using the universal eubacterial primer set 27f-1387r (27f: 5'-AGAGTTTGATC(AC)TGGCTCAG-3' and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.*, 1998). PCR mixtures (25µl/reaction) contained 20 pmol 27F and 1387R primers, 22mM Tris-HCL (pH 8.4), 55mM KCL, 1.65 mM $MgCl_2$, 220 µM dNTP's, 0.55 units recombinant *Taq* DNA Polymerase, and 20-50 ng template DNA (all reagents from Invitrogen Life Technologies, Carlsbad, CA, USA unless otherwise stated). PCR amplification was carried out for 30 cycles of 94°C for 4 min., 56°C for 30 sec. and 72°C for 1.5 min. and final 7 min. incubation at 72°C (modified after Sambrook and Russell, 2001).

The expected size of PCR products was 1.36 kb. PCR products were used to construct 16S gene clone libraries using a TOPO TA Cloning Kit® (with pCR®2.1-TOPO® vector and One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) following the manufacturer's protocol. All clones were cultured on Luria-Bertani agar plates (Fisher Scientific Inc., Pittsburgh, PA) containing 50 µg/ml Kanamycin as directed by the protocol supplied in the TOPO TA Cloning Kit®. Clones were screened for positive transformation with PCR using the primer set M13 forward (5'-GTT TTC CCA GTC ACG AC-3') and M13 reverse (5'-CAG GAA ACA GCT ATG ACC-3'). A total of 192 screened clones were submitted to the Michigan State University Genomic

Technology Support Facility (GTSF) for cell preparation and sequencing on a 3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequences were aligned and classified using the Ribosomal Database Project II (RDP II) Release 9.47 produced by Wang *et al.* (2007). RDP-II provides aligned and annotated rRNA sequences, derived phylogenetic trees and taxonomic hierarchies, and analysis services through its server available at http://rdp.cme.msu.edu. The RDP Classifier uses a naive Bayesian classifier to assign sequences to the RDP Taxonomy. It provides taxonomic assignments from domain to genus, with confidence estimates for each assignment. Eighty-seven clones for the Huron River, 52 clones for Crystal Lake, and 32 clones for Lake Vineyard were successfully aligned. Multiple sequences were rechecked with BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information of the National Institute of Health (http://www.ncbi.nlm.nih.gov/BLAST).

4) RESULTS

Sequence analysis of 16S rDNA sequences (about 800 bp) revealed the presence of 12 bacterial groups (the α-, β-, γ-, and δ- classes of Proteobacteria and the Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, Verrucomicrobia phyla of bacteria) in mussel homogenate samples harvested from zebra mussels collected from the Huron River, Crystal Lake, and Lake Vineyard (tables 2-5). Clones belonging to the Phylum Proteobacteria accounted for 24.2% of all bacteria detected with the greatest proportions of Proteobacteria in the β- and γ- classes.

Clone libraries from different water-bodies revealed unique microbial assemblages associated with the water-bodies sampled (Figure 2). The Crystal Lake library had the highest abundance of β- and γ- Proteobacteria (9.3% and 9.3% respectively). Potential zebra mussel pathogens such as *Aeromonas hydrophila* subspecies *hydrophila* and *Aeromonas salmonicida* subspecies *salmonicida* (Maki *et al.*, 1998) were only detected in the Huron River library. The Huron River library had the highest abundance (38.3%) of Bacteroidetes of all the sites sampled. The Lake Vineyard library had the highest abundance (31.0%) of Actinobacteria. Ubiquitous environmental bacteria belonging to the phylum Bacteroidetes were most abundant (38.3%) in the Huron River library while none of these bacteria were detected in the Lake Vineyard library. Bacteria belonging to the class γ-Proteobacteria were detected in all mussel samples were most abundant (9.3%) in the Huron River library. Cyanobacteria appeared in all three libraries and were most abundant (10.0%) in the Crystal Lake library.

As shown in table 2, α-Proteobacteria detected included the families Anaplasmataceae, Bradyrhizobiaceae, Hyphomicrobiaceae, and Rickettsiaceae. Genera detected included *Bradyrhizobium* sp., *Hyphomicrobium* sp., and unclassified members of the Anaplasmataceae, Rhizobiales, Rickettsiales subdivisions of bacteria. β-Proteobacteria detected included the families Commamonadaceae, Burkholderiaceae, Neisseriaceae, Rhodocyclaceae, Methylophilaceae, and Incarte sedis 5 families of bacteria (Table 2). Genera detected included *Curvibacter*, *Chitinibacter*, *Dechloromonas*, *Polynucleobacter*, *Rhodofera*, and two species of *Methylophilus*. γ-Proteobacteria detected included members of the families Aeromonadaceae, Enterobacteriaceae, Moraxellaceae, Oceanospirillaeae, and Shewanellaceae (Table 3). Genera detected included *Acinetobacter*, *Aeromonas*, *Escherichia, Shewanella*, *Shigella,* and *Yersinia* sp. As displayed in table 3, δ-Proteobacteria detected included members of the families Bdellovibrionaceae and Desulfobulbaceae. The only classifiable genus of δ-Proteobacteria detected was *Bdellovibrio*.

As shown in Table 4, Acidobacteriaceae detected included the genera *Gp3*, *Gp4*, *Gp8* (Acidobacteriaceae). Actinobacteria detected included members of the families Microbacteriaceae and Propionibacteriaceae (Table 4). Genera detected included *Cryobacterium* and *Propioniobacterium*. Many sequences (>40) were determined to be an unclassified member of the family Microbacteriaceae. Among Bacteroidetes detected, are members of the families Bacteroidaceae and Flavobacteriaceae (Table 4). Genera detected included *Bacteroides*, *Cloacibacterium*, *Flavobacterium*, and *Mariniflexile*. Genera detected included Clostridium, and *Lactobacillus*. As shown in table 5, Chloroflexi detected included the family Caldilineacea. The only genus detected was

Caldilinea sp. As shown in table 5, the only genus of Cyanobacteria detected was *Bacillariophyta* sp. (Chloroplast). Firmicutes detected included members of the Clostridiaceae and Lactobacillaceae families. Genera detected included *Lactobacillus* and *Clostridium* (Table 5). The only member of the phylum Planctomycetes detected was an unclassified member of the family Planctomycetaceae, while the only member of the phylum Verrucomicrobia detected was the genus Subdivision 3 genera incertae sedis (Subdivision 3) (Table 5).

Bacterial sequences greater than 600 base pairs that were RDP-unclassified were subjected to BLAST searches. As shown in Table 6, RDP-unclassified bacterial sequences from Crystal Lake were similar to *Mycoplasma gallisepticum* R (Firmicutesand *Ureaplasma urealyticum* (Firmicutes). One RDP-unclassified bacterial sequence from Lake Vineyard was also similar to *Ureaplasma urealyticum*. RDP-unclassified bacterial sequences from the Huron River were similar to *Acidovarax* sp. (β-Proteobacteria), *Chlorobium phaeobacteroides* (Bacteroidetes), *Prosthecochloris vibrioformi* (Bacteroidetes), *Cyanothece* sp. (Cyanobacteria), and *Faecalibacterium prausnitzii* (Firmicutes) (Table 6).

5) DISCUSSION

Microbial assemblages of the three mussel groups share many similarities. For example, bacteria belonging to the α-, β-, γ-, and δ- classes of Proteobacteria and the phyla Firmicutes and Cyanobacteria were present in three zebra mussel samples analyzed and appear to be commonly associated with the zebra mussel in the Laurentian Great Lakes basin. This is in agreement with (Frischer *et al.*, 2000), however the author did not report on Cyanobacteria. On the other hand, a few variations were noticed among the three groups. For example, large abundances of Bacteroidetes (>38%) were detected in the Huron River library only while large abundances of Actinobacteria (>30%) were detected in the Lake Vineyard library only. Similarly, bacteria belonging to the phyla Planctomycetes, Verrucomicrobia, Chloroflexi, Acidobacteria were present in the Huron River library only. Furthermore, bacteria belonging to the phylum Bacteroidetes were present in the Huron River and Crystal Lake libraries but not in the Lake Vineyard library.

There are potential explainations for the differences in bacterial assemblage noticed among the three mussel groups. The microbial communities present in the sediment and water column may have played a role in causing such variation. Other environmental factors at the sampling sites such as nutrient loading (reviewed in Reichenbach, 2006), water precipitation, light intensity, (Kennedy and Smith, 1995; Bossio *et al.*, 1998), prevailing temperature (White *et al.*, 1991), pH, oxygen levels, and the presence humic substances (Curtis *et al.*, 1992) could also influence the microbial communities present in zebra mussel samples.

This study is different from other studies that have investigated microbial communities associated with zebra mussels. Most studies have relied on classical culturing methods aimed at identifying only heterotrophic bacteria and do not accurately describe finer details of the biological components of a given habitat (Maki *et al.*, 1998; Gu and Mitchell, 2002). In comparison to these studies, we took a gene sequencing approach and were able to identify a greater number of bacterial species significantly adding to the list of bacteria reported to be associated with zebra mussels. This difference can be attributed to the wide range of bacteria that 16S rRNA (rDNA) gene sequences can identify versus using classical culturing methods.

There has only been one gene sequencing study describing the diversity of microbial communities associated with the zebra mussel (Frischer *et al.*, 2000). In the study, the 16S rRNA (rDNA) gene was sequenced from bacteria present in homogenate samples harvested from zebra mussels collected from a single site in the Hudson River, New York (USA). Detected bacteria were taxonomically reported to the class level only. In the present, study we analyzed zebra mussel samples from three waterbodies and were able to taxonomically report many detected bacteria to the species level. In contrast to Frischer *et al.* (2000), which stated that the β-, and γ- classes of Proteobacteria was apparent in zebra mussel homogenate samples, we detected high abundance Actinobacteria and Bacteroidetes in zebra mussel homogenates.

Based on the results of the present study, it is obvious that the amount of bacterial species detected reveals that microbial communities associated with zebra mussel are rich and reflect the environment sampled. For example, the high abundance of Bacteroidetes observed in the Huron River sample is likely due to the high levels of phosphorus in the

section of the sampled (Michigan Department of Natural Resources Fisheries Division, 2002). Similarly, the high abundance of Actinobacteria in the Crystal Lake sample likely reflects the microbial communities present in that waterbody. Additionally, the higher species richness observed in the Huron River sample may be due to the number of different bacteria transferred as a result of runoff into the large Huron River Watershed.

Also of interest is the number of bacterial pathogens found in the zebra mussel in our study that are known to cause diseases in other organisms such as plants, fish, birds, and humans. Most Actinobacteria detected were genetically similar (92% BLAST score) to the plant pathogen, *Leifsonia xyli* subsp. *xyli* str. CTCB07 (Accession number: NC-006087.1), which causes ratoon stunting disease and affects sugarcane crops worldwide (Brumbley *et al.*, 2002). This is the first report of this bacterium being associated with the zebra mussel.

Bacterial pathogens detected in the zebra mussel in our study that are those known to cause diseases in teleosts were *Aeromonas hydrophila,* which causes motile aeromonas septicemia (Cipriano, 2001), *Aeromonas salmonicida,* which causes Furunculosis (Cipriano and Bullock, 2001), *Flavobacterium johnsoniae,* which causes Flavobacteriosis (Flemming *et al.*, 2007), *Flavobacterium psychrophilum*, which causes Rainbow Trout Fry Syndrome (Lorenzen *et al.*, 1991; Rangdale, 1999) and Bacterial Cold Water Disease (Cipriano and Holt, 2005), and *Yersinia ruckeri*, which causes Enteric Redmouth Disease (Del Cerro *et al.*, 2002). Detecting these disease-associated bacteria in zebra mussels is important because fish stocks play a major role in the economics of the Great Lakes region and the increased spread of fish diseases due to zebra mussels could be economically devasting.

The only pathogen associated with disease in birds was *Mycoplasma gallisepticum*, which causes chronic respiratory disease in poultry (Papazisi *et al.*, 2003). This bacterium was detected in considerable abundance. This is the first report of this pathogen being associated with the zebra mussel, which furthers our knowledge on the ecology of *Mycoplasma* spp.

Bacterial pathogens associated with diseases in humans that were detected are *Bacteroides fragilis*, which is responsible for the majority of all clinical cases of anaerobic sepsis and intraabdominal abscesses (Gorbach and Bartlett, 1974; Polk and Kasper, 1977). *Clostridium botulinum*, which produces the most potent biological toxins known to affect humans, *Escherichia coli*, which causes food poisoning and is occasionally responsible for costly product recalls, *Shewanella putrefaciens*, which causes bacteremia in soft tissues (Pagani *et al.*, 2003), *Shigella* sp., which causes diarrhea, fever, and stomach cramps, and *Ureaplasma urealyticum*, one well-known cause of nongonoccal urethritis (Glass *et al.*, 2000). We also detected a member of the Rickettsiales order of bacteria, a group of bacteria that causes a variety of diseases in humans and many other organisms.

Indeed this study has shed light not only on the dominant pathogens associated with zebra mussels but also on the potential role zebra mussels play in disease ecology. We recommend the use of zebra mussels in monitoring pathogens in the aquatic environment. Limitations of this study were that only a single group of mussels was collected from each site, there was only one sampling event, and the study took place during a single season. Despite these limitations, this study was beneficial. In order to gain a better understanding of the microbial ecology of zebra mussels and its impact to

the Laurentian Great Lakes ecosystem, this study will be followed by a study in which we analyze microbial communities present in multiple zebra mussel tissues.

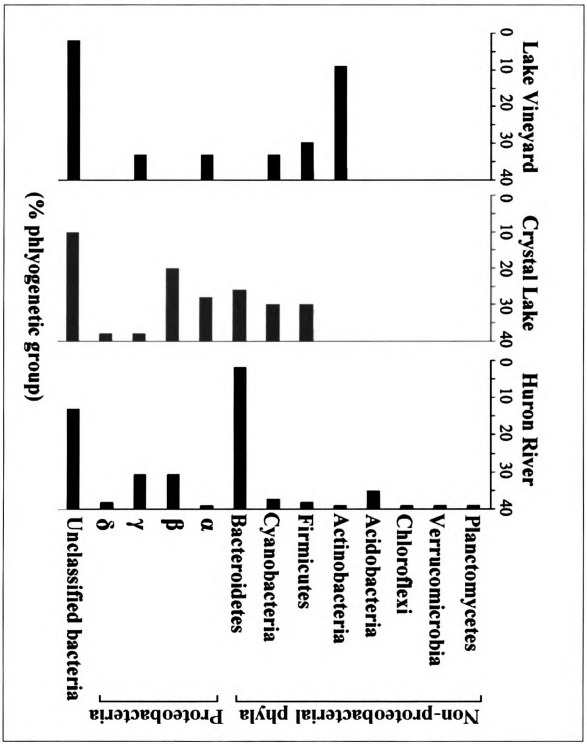

Figure 2. Frequency distribution of 16S rDNA clone libraries constructed from mussel homogenate samples collected from the Huron River, Crystal Lake, and Lake Vineyard using the Ribosomal Database Project.

Class	Family	Subdivision	Huron River	Crystal Lake	Lake Vineyard
α-Proteo-bacteria	Anaplasmataceae	Unclassified Anaplasmataceae			+
	Bradyrhizobiaceae	*Bradyrhizobium* sp.		+	
	Hyphomicrobrobiacae	*Hyphomicrobium* sp.	+		
		Uclassified Rhizobiales		+	
	Rickesttsiaceae	Unclassified Rickettsiales	+		
	Family unknown	Unclassified α-Proteobacteria #1	+		
	Family unknown	Unclassified α-Proteobacteria #2			+
	Family unknown	Unclassified α-Proteobacteria #3		+	
β-Proteo-bacteria	Commamonadaceae	*Curvibacter* sp.		+	
		Rhodoferax ferrireducens	+	+	
	Burkholderiaceae	*Polynucleobacter* sp.	+		
	Neisseriaceae	*Chitinibacter* sp.	+		
	Rhodocyclaceae	*Dechloromonas aromatica*	+		
	Methylophilaceae	*Methylophilus* sp. #1	+		
		Methylophilus sp. #2		+	
	Incarte sedis 5	Unclassified Incarte sedis 5	+		
	Family unknown	Unclassified β-Proteobacteria #1		+	
	Family unknown	Unclassified β-Proteobacteria #2		+	

Table 2. α- and β- Proteobacteria detected in 16S rRNA (rDNA) gene clone libraries constructed from tissue homogenate samples harvested from zebra mussels collected from the Huron River, Crystal Lake, and Lake Vineyard.

Class	Family	Subdivision	Huron River	Crystal Lake	Lake Vineyard
γ- Proteo-bacteria	Aeromonadaceae	*Aeromonas hydrophila* subsp. *hydrophila*	+		
		Aeromonas salmonicida subsp. *salmonicida*	+		
	Enterobacteriaceae	*Escherichia coli*	+		
		Shigella sp.	+		
		Yersinia ruckeri		+	
	Moraxellaceae	*Acinetobacter baumannii*	+		+
	Oceanospirillaceae	Unclassified Oceanospirillaceae	+		
	Shewanellaceae	*Shewanella putrifaciens*	+		+
δ- Proteo-bacteria	Bdellovibrionaceae	*Bdellovibrio* sp.	+		
	Desulfobulbaceae	Unclassified Desulfobulbaceae	+		
	Family unknown	Unclassified δ-Proteobacteria		+	

Table 3. δ- and γ- Proteobacteria detected in 16S rRNA (rDNA) gene clone libraries constructed from tissue homogenate samples harvested from zebra mussels collected from the Huron River, Crystal Lake, and Lake Vineyard.

Phylum	Family	Subdivision	Huron River	Crystal Lake	Lake Vineyard
Acidobacteria	Acidobacteriaceae	*Gp3*	+		
		Gp4	+		
		Gp8	+		
Actinobacteria	Microbacteriaceae	*Cryobacterium* sp.			+
		Unclassified Microbacteriaceae #1			+
	Propionibacteriaceae	*Propionibacterium acnes*	+		
Bacteroidetes	Bacteroidaceae	*Bacteroides fragilis*	+		
	Flavobacteriaceae	*Cloacibacterium* sp.		+	
		Flavobacterium johnsoniae	+		
		Flavobacterium psychrophilum	+		
		Mariniflexile sp.	+		
	Family unknown	Unclassified Flavobacteria #1	+		
	Family unknown	Unclassified Flavobacteria #2	+		
	Family unknown	Unclassified Flavobacteria #3		+	

Table 4. Acidobacteria, Actinobacteria, and Bacteroidetes detected in 16S rRNA (rDNA) gene clone libraries constructed from tissue homogenate samples harvested from zebra mussels collected from the Huron River, Crystal Lake, and Lake Vineyard.

Phylum	Family	Subdivision	Huron River	Crystal Lake	Lake Vineyard
Chloroflexi	Caldilineacea	*Caldilinea* sp.	+		
Cyanobacteria	Chloroplast	*Bacillariophyta* sp.	+	+	+
Firmicutes	Clostridiaceae	*Clostridium butyricum*		+	
	Lactobacillaceae	*Lactobacillus acidophilus*	+		
		Lactobacillus sp. #2	+		
	Family unknown	Unclassified Firmicutes #1			+
	Family unknown	Unclassified Firmicutes #2		+	
Planctomycetes	Planctomycetaceae	Unclassified Plantomycetaceae	+		
Verrucomicrobia	Subdivision 3	Subdivision 3	+		

Table 5. Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, and Verrucomicrobia detected in 16S rRNA (rDNA) gene clone libraries constructed from tissue homogenate samples harvested from zebra mussels collected from the Huron River, Crystal Lake, and Lake Vineyard.

Location	Significant alignments	Phylum	# of sequences
Huron River	*Acidovorax* sp.	β-Proteobacteria	1
	Cyanothece sp.	Cyanobacteria	1
	Chlorobium phaeobacteroides	Bacteroidetes	2
	Faecalibacterium prausnitzii	Firmicutes	2
	Prosthecochloris vibrioformi	Bacteroidetes	1
Crystal Lake	*Mycoplasma gallisepticum*	Firmicutes	11
	Ureaplasma urealyticum	Firmicutes	4
Vineyard Lake	*Prochlorococcus marinus*	Cyanobacteria	2
	Ureaplasma urealyticum	Firmicutes	2

Table 6. Unclassified bacteria in zebra mussel samples from the Huron River, Crystal Lake, and Lake Vineyard using the ribosomal Database Project rechecked using BLAST. (E values are less than 9E-121).

CHAPTER THREE

HETEROGENEITY OF MICROBIAL COMMUNITIES WITHIN THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) IN THE LAURENTIAN GREAT LAKES BASIN (USA)

1) ABSTRACT

Little is known concerning the structure of microbial communities associated with the zebra mussel (*Dreissena polymorpha*) in the Great Lakes basin. In the present study, the structure of microbial communities within zebra mussel gill, gut, and mantle cavity fluid samples collected from Lake Loon, an inland lake in Michigan's Lower Peninsula (U.S.A.). Bacterial community structure was determined by sequencing the 16S rRNA (rDNA) gene from amplified community bacterial DNA extracted from mussel gill, gut, and mantle cavity fluid samples. A total of 735 16S rRNA gene sequences were checked for similarity to existing 16S sequences in two public databases: the Ribosomal Database Project and BLAST. A total of 294 phylotypes belonging to 15 bacterial subdivisions were identified in zebra mussel samples. The phylotype richness and frequency distribution (evenness) of clone libraries were investigated by using a variety of diversity indices. Shannon-Wiener diversity index values (H') for mantle cavity fluid, gill, and gut samples were 2.96, 2.80, and 3.54, respectively, indicating greatest phylogenetic diversity in gut samples. A dominance of bacteria belonging to the class γ-Proteobacteria as

observed in the mantle cavity fluid clone libraries. With the exception of gut compared to mantle cavity fluid, the abundance of Cyanobacteria significantly differed ($P < 0.05$) between all clone libraries. Our results suggest that the zebra mussel may serve as a reservoir for potentially pathogenic bacteria for many aquatic and terrestrial animals, such as *Clostridium* spp., *Escherichia coli*, *Flavobacterium* spp., *Rickettsia*-like bacteria, and *Mycobacterium* sp. This work constitutes the first account of bacterial community structure in the zebra mussel in its new geographic range in the Laurentian Great Lakes basin.

2) INTRODUCTION

The zebra mussel (*Dreissena polymorpha*) invaded the Great Lakes basin in 1986 (Hebert *et al.*, 1989) and since then has caused ecological devastation (Nalepa and Schloesser, 1993). Once such impact is the shifts zebra mussels have caused in bacterial community structure in their surrounding environment (Frischer *et al.*, 2000; Lohner *et al.*, 2007). Frischer *et al.* (2000) who used the 16S rDNA (rRNA) sequence to investigate microbial community structure in zebra mussel homogenate, pseudofeces, sediment, and water samples determined that the presence of zebra mussels may increase the abundance of certain bacteria in surrounding sediments. In the same context, Lohner *et al.* (2007) monitored benthic bacterial community structure in the presence of zebra mussels and quagga mussels, (*Dreissena bugensis*) and determined that the presence of dreissenids allowed certain bacterial populations to become dominant in the surrounding benthic environment.

In order to understand the alterations of bacterial communities caused by the presence of zebra mussels, it is necessary to gain a detailed understanding of the microbial ecology within the zebra mussel. Microbial communities associated with the zebra mussel have rarely been investigated and when studied, analysis involved whole mussel homogenate only (Al-Jebouri and Trollope, 1984; Frisher *et al.*, 2000; Selegean *et al.*, 2001; Gu and Mitchell, 2002; Chapter 2). Despite the limitations associated with the use of whole mussel homogenate, important information was retrieved. For example, our previous study (detailed in Chapter 2.) has demonstrated that the microbial communities associated with the zebra mussel are relatively rich and contain phylogenetically distinct

groups of bacteria. Using 16S rRNA (rDNA) sequence analysis, 12 bacterial subdivisions including 35 genera were detected in homogenates of mussels residing in three waterbodies in Michigan's Lower Peninsula (USA).

Despite the wealth of information retrieved from analyzing zebra mussel homogenates, little is known about the differences in bacterial communities within the mussel's body. Experience from human and veterinary medicine has suggested that microbial communities vary from one organ to the other within an organism (reviewed by Savage, 1977). Additionally, it has been demonstrated that microbial communities can even vary in different sections within the same organs (Eckburg *et al.*, 2005). Similar findings were reported in bivalve mollusks. For example, Hernandez-Zarate and Olmos-Soto (2005) demonstrated that bacterial diversity varied among the organ of the Pacific oyster *Crassostrea giga*. Unfortunately, none of the studies performed on zebra mussels have addressed the possible difference in bacterial community structure among multiple mussel organs. Therefore, in this study, we chose to compare the microbial communities present in different zebra mussel organs harvested from mussels collected from a single site to gain a better understanding of variations of microbial communities within the zebra mussel. This type of analysis will provide a better understanding of the filter-feeding activity of the zebra mussel and its impact to the surrounding environment.

As zebra mussels pull water into their inhalant siphon, water passes into the mantle cavity over and through the gills where particles are trapped and sorted with cilia and mucous (Morton, 1969b). Like other freshwater bivalves, zebra mussels are able to discriminate among particles they acquire from the water (Baker *et al.*, 1998; 2000). Cilia then transport desirable particulate matter to the esophagus and into the gut to be

digested (Morton, 1969b). The mantle cavity of the zebra mussel is open to the environment. Therefore, the microbial community present in the mantle cavity fluid reflects the microbial community present in the surrounding environment, while the microbial community present in the gills and gut reflect the mussel's selective filtration processes. The objectives for this study are to determine the differences in microbial community structure of the mussel's three major organ compartments; mantle cavity water, gills, and gut.

3) MATERIALS AND METHODS

a) Zebra mussel sampling sites and sample processing

On October 1, 2006, mussels were collected from Loon Lake, Oakland County, Michigan (42.680N, -83.357W). The mussels were collected manually from a depth of 1.5 meters at random. Loon Lake mussel samples were collected from a small boulder on the sand silt bottom. Loon Lake was selected as a representative of the many glacial lakes in Michigan's Lower Peninsula (Cooperative Lakes Monitoring Program, 2005). Mussels were placed in a five-gallon bucket containing fresh aerated water from Loon Lake. Each mussel was taxonomically identified according to the features described by Morton (1969a), and immediately processed.

Mussels were separated into pools of five for each sample type. Guided by the anatomical features detailed in Morton (1969a), the desired organs from each mussel were dissected aseptically. Mantle cavity fluid was drawn through a 32-gauge needle into the closed mantle cavity of live mussels. Gill and gut samples were harvested aseptically under a dissecting scope and rinsed several times with filter-sterilized (0.2 μm) and autoclaved distilled water prior to processing. Mantle cavity fluid was stored at -20°C until processed. Gill and gut samples were homogenized with a sterile glass rod, pelleted (15,000 X g centrifugation), and stored in 80% ethanol (ETOH) at -20°C prior to extraction of genetic material. Genomic bacterial community DNA was harvested from 30 μl of both homogenized mussel tissue and vortexed mantle cavity fluid using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) following the manufacturer's protocol.

b) Sequence analysis of the 16S rRNA (rDNA) gene

The isolated bacterial DNA was then used as a template for PCR amplification. PCR amplification of the 16S gene was performed using the universal eubacterial primer set 27f-1387r (27f: 5'-AGAGTTTGATC(AC)TGGCTCAG-3' and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi, 1998). PCR mixtures (25µl/reaction) contained 20 pmol 27F and 1387R primers, 22mM Tris-HCL (pH 8.4), 55mM KCL, 1.65 mM $MgCl_2$, 220 µM dNTP's, 0.55 units recombinant *Taq* DNA Polymerase, and 20-50 ng template DNA (all reagents from Invitrogen Life Technologies, Carlsbad, CA, USA unless otherwise stated). PCR amplification was carried out for 30 cycles of 94°C for 4 min., 56°C for 30 sec. and 72°C for 1.5 min. and final 7 min. incubation at 72°C (modified after Sambrook and Russell, 2001). The expected size of PCR products was 1.36 kb. PCR products were used to construct 16S gene clone libraries using a TOPO TA Cloning Kit® (with pCR®2.1-TOPO® vector and One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) following the manufacturer's protocol. All clones were cultured on Luria-Bertani agar plates (Fisher Scientific Inc., Pittsburgh, PA) containing 50 µg/ml Kanamycin as directed by the protocol supplied in the TOPO TA Cloning Kit®. Clones were screened for positive transformation with PCR using the primer set M13 forward (5'-GTT TTC CCA GTC ACG AC-3') and M13 reverse (5'-CAG GAA ACA GCT ATG ACC-3').

A total of 863 clones were sub-cultured on Luria-Bertani agar plates (Fisher Scientific Inc., Pittsburgh, PA) containing 50 µg/ml Kanamycin as directed by the protocol supplied in the TOPO TA Cloning Kit® and submitted to Macrogen© (Seoul,

South Korea) for cell preparation and sequencing. 792 sequences were successfully aligned and classified using the Ribosomal Database Project II (RDP II) Release 9.47 produced by Wang *et al.* (2007). RDP-II provides aligned and annotated rRNA sequences, derived phylogenetic trees and taxonomic hierarchies, and analysis services through its server available at http://rdp.cme.msu.edu. The RDP Classifier uses a Bayesian classifier to assign sequences to the RDP Taxonomy. It provides taxonomic assignments from domain to genus, with confidence estimates for each assignment. Multiple sequences were rechecked with BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information of the National Institute of Health (http://www.ncbi.nlm.nih.gov/BLAST).

c) Statistical analyses

Data normality and equivalency of variance were examined using the algorithms provided in the SAS software package (SAS Institute Inc, Cary, NC). Data normality was also examined using the algorithms provided in the FastGroupII© (http://biome.sdsu.edu/fastgroup/fg_tools.htm). After sequences were aligned, grouped, and identified with Database Project II (RDP II) Release 9.47 (Wang *et al.*, 2007), comparison between the three zebra mussel sample types for differences in bacterial abundances were carried by ANOVA using the the repeated measures linear mixed model algorithms provided in SAS software package (SAS Institute Inc, Cary, NC).

Bacterial community structure among clone libraries was determined using the bioinformatics program FastGroupII© (http://biome.sdsu.edu/fastgroup/fg_tools.htm). FastGroupII© is a web-based tool that allows for dereplication of large 16S rDNA

libraries using several different algorithms, including Percentage Sequence Identity (PSI), PSI with Gaps (Yu *et al.*, 2006), Tree-parsing (or ClustalW global alignment) (Thompson *et al.*, 1994), and Seq-Match (Sequence Match in Ribosomal Database Project) (Cole and Chai, 2003). FastGroupII© also automatically calculates standard diversity and richness indices, including the Shannon-Wiener Index (Shannon and Weaver, 1963), Chao1 (Chao, 1984) and rarefaction (Heck and Belle, 1975).

Bacterial diversity within samples was compared using the Shannon-Wiener Index. The advantage of using the Shannon-Wiener index of diversity is that it takes into account both the number of bacterial groups and the evenness, or relative distribution, of the bacterial groups within the community. The index is increased either by having additional unique bacterial groups (richness), or by having a more equal relative abundance of bacterial groups (evenness) in the community being investigated. Since it is believed that a 3% difference in a 1.36kb 16S rRNA sequence will only affect bacterial identification to the species level, Shannon-Wiener values were based on 97% sequence identity.

Rarefaction analysis, which averages randomizations of the species-accumulation curve, was used to estimate relative diversity among the microbial communities present in zebra mussel gill, gut, and mantle cavity fluid samples. Rarefaction is a technique used for ecological data analysis that compares diversity among samples in two regions when the sampling effort differs (Hurlbert, 1971; Simberloff, 1978) and has shown to be particularly useful for investigations of microbial ecology (Hughes and Hellmann, 2005). The constructed rarefaction curves were used to determine if a reasonable number of clones were sampled and if more intensive sampling will yield additional bacterial groups

(coverage). Additionally, because the number of clones sequenced varied slightly from sample to sample, rarefaction was used to standardize and compare species richness among clone libraries.

The Chao1 richness estimator was used to estimate taxonomic richness among clone libraries. Chao1 is a non-parametric estimator that predicts the point at which an accumulation curve will reach an asymptote allowing for estimations of coverage. Chao1 estimates total richness as

$$S_{Chao1} = S_{obs} + n_1^2 / 2n_2$$

where S_{obs} is the number of observed taxa, n_1 is the number of singletons (species observed once), and n_2 is the number of doubletons (species observed twice) (Chao, 1984). Chao (1984) noted that this index is particularly useful for data sets skewed toward the low-abundance classes, as is likely to be the case with microbes.

Non-parametric estimators, such as the Kruskal-Wallis ranked sum test, compared to rarefaction, provide more meaningful projections of the actual diversity within the sampled environment (Hughes *et al.*, 2001) and were therefore included in this study. When evidence of non-normality was observed, the nonparametric Kruskal-Wallis ranked sum test was used to test the difference in Shannon Weiner diversity index values among clone libraries.

4) RESULTS

a) Sequence analysis of the 16S rRNA (rDNA) gene

Sequence analysis of 735 partial 16S rDNA sequences (about 800 bp) revealed the presence of 15 bacterial groups (the α-, β-, γ-, and δ- classes of Proteobacteria and the Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, Spirochaetes, Genera Incarte sedis OP10, Nitrospira, and Verrucomicrobia phyla of bacteria) in zebra mussel gill, gut, and mantle cavity fluid samples of zebra mussels collected from Loon Lake. Based on a 97% sequence identity, 294 phylotypes belonging to 15 bacterial subdivisions were identified. As displayed in tables 5-8, 66 bacterial genera belonging to 40 families were identified. Additionally, a number of sequences were determined to be unclassified bacteria belonging to a range of taxonomic subdivisions.

A number of bacterial genera were detected in one mussel sample type only. For example, 12 bacterial genera were found in gill samples only; these are *Agrobacterium*, *Acidisphaera Hyphomicrobium*, *Rhodobacter Sphingomonas, Sphingopyxis* (α-Proteobacteria), *Methylobacillus* (β-Proteobacteria), *Anaeromyxobacter*, *Byssovorax* (δ-Proteobacteria), *Fulvibacter*, *Lutibacter* (Bacteroidetes), and *OP10* genera *incarte sedis* (Genera Incarte sedis (tables 7-10). Similarily, bacterial genera found only in gut samples included *Roseomonas*, *Sphingobium* (α-Proteobacteria), *Rhodoferax* sp., (β-Proteobacteria), *Aquicella* sp., *Thermomonas* sp. (γ-Proteobacteria), *Desulphomonile* sp., *Peredibacter sp.* (δ-Proteobacteria), *Actinomyces*, *Conexibacter* (Actinobacteria), *Chlorophyta* (Cyanobacteria), *Magnetobacterium* sp. (Nitrospira) (tables 7-10). Bacterial

genera found only in mantle cavity fluid samples included *Pedomicrobium*, *Roseicyclus* (α-Proteobacteria), *Commamonas*, *Methylovorous* (β-Proteobacteria), *Alkanidiges*, *Enterobacter*, *Rheinheimera*, *Stenotrophomonas*, (γ-Proteobacteria), *Mycobacterium*, *Rhodococcus* (Actinobacteria), *Chryseobacterium*, *Fulviicola* (Bacteroidetes), *GPI*, *GPXIII*, (Cyanobacteria), *Leptonema* sp. (Spirochaetes) (tables 7-10).

All three sample types contained 16S rRNA (rDNA) gene sequences of unknown phylogenetic affiliations beyond the Domain of Bacteria. RDP-unclassified bacterial sequences greater than 600 base pairs that produced significant BLAST matches are shown in tables 11-13. RDP-unclassified bacterial sequences were similar to the β- γ-, and δ- classes of Proteobacteria and the phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Planctomycetes, and Spirochaetes (BLAST). The majority of unclassified bacterial sequences (62%) produced significant match scores for the δ-Proteobacteria and Firmicutes groups of bacteria.

b) Statement of variability

Although we sequenced a total of 735 16S rRNA (rDNA) sequences from three zebra mussel sample types (~250 sequences for each sample type), the Shannon-Wiener diversity index and Chao1 richness estimator (FastGroupII©) show a high level of variability is present among replicate samples (Table 14) suggesting an inadequate sample size. Sequencing a greater number of clones per sample and more samples would define the communities in greater detail and smooth out variability from limited sampling. This being said, we still found it worthwhile to statistically compare bacterial

abundance and microbial community diversity in gill, gut, and mantle cavity fluid samples.

b) Comparison of bacterial abundances and statistical analyses

We compared bacterial abundance (figures 3-6) and diversity of bacterial communities associated with zebra mussel sample gill, gut, and mantle cavity fluid to gain a better understanding of the microbial ecology of zebra mussels. Keeping in mind that all sample sizes and treatments for each sample type were the same, any significant differences in microbial community structure present would reflect a specific bacterial group's association with a specific sample type. All mussel samples were aseptically dissected and equal volumes (200 µl) for all sample types were used for extraction of genetic material. Further more, all PCR and cloning reactions were performed using the same reaction conditions. Therefore, direct comparison of the microbial community structure present in gill, gut, and mantle cavity fluid clone libraries should reveal a detailed description of the microbial communities associated with mussels.

Each sample type displayed unique bacterial assemblages (figures 7-9). The dominant bacterial group in gill clone libraries was unclassified bacteria ($P = 0.0067$) followed by Actinobacteria ($P = 0.0298$). These RDP-unclassified bacterial sequences were determined to belong to the γ-, and δ- classes of Proteobacteria and the phyla Actinobacteria, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, and Spirochaetes, (Table 12). 94% of Actinobacteria in the gill clone libraries were unclassified members of the family Microbacteriaceae. The dominant bacterial group in

the gut clone libraries was unclassified bacteria ($P < 0.0001$) followed by Cyanobacteria ($P < 0.0001$), γ-Proteobacteria ($P < 0.0001$), and Planctomycetes ($P = 0.0433$). RDP-unclassified bacterial sequences in the gut clone libraries were determined to belong to the α-, γ-, and δ- classes of Proteobacteria and the phyla Actinobacteria, Cyanobacteria, Firmicutes, Planctomycetes, and Spirochaetes (Table 13). Most Cyanobacteria detected in the gut clone libraries belong to the genera *Bacillariophyta* (48%) and *GpIIa* (30%). The dominant bacterial group in the mantle cavity fluid clone libraries was γ-Proteobacteria ($P < 0.0001$). 80% of γ-Proteobacteria detected in the mantle cavity fluid clone libraries was members of the order Psuedomonadales. 77% Psuedomonads detected in the mantle cavity fluid was *Acinetobacter baumanii.*

Comparison of bacterial abundance between the three zebra mussel sample types showed significant differences in abundances of Cyanobacteria (Figure 10) and γ-Proteobacteria (Figure 11). The abundance of Cyanobacteria was significantly different between the gill and mantle cavity fluid clone libraries ($P = 0.0347$) while the abundance of Cyanobacteria clones did not significantly differ between the gut and the mantle cavity fluid ($P = 0.9072$) clone libraries. Additionally, the abundance of γ-Proteobacteria was significantly different between all clone libraries ($P < 0.001$).

Although comparison of Shannon-Weiner diversity index values using the nonparametric Kruskal-Wallis rank sum test revealed no significant difference in diversity based on sample type ($H = 2.75$ > critical chi square table value), analysis of phylotype richness using both Chao1 and rarefaction revealed differences among composite clone libraries constructed for each sample type (a total of three composite clone libraries, Table 15). Chao1 richness estimates show that the gut library had a

relatively high level of phylotype richness whereas the gill and mantle cavity fluid libraries had lower levels of phylotype richness. Chao1 estimates of richness values for the composite mantle cavity fluid, gill, and gut libraries were 387.682, 856.500, and 305.980 respectively and showing greater evenness in the gut library and revealing the greatest coverage (sequencing a greater number of clones will not produce a greater number of phylotypes) was obtained for the gut library. In agreement with Chao1 estimates of richness, rarefaction analysis also revealed higher phylogenetic richness in gut samples and lower phylogenetic richness in gill and mantle cavity fluid libraries. Based on a 97% sequence similarity, rarefaction revealed that, for a sample size of 265 mantle cavity fluid, 254 gill, and 273 gut sequences, each library respectively contained 104, 96, and 133 phylotypes, showing the greatest phylogenetic richness is in the gut library.

5) DISCUSSION

Bacteria belonging to the Cyanobacteria, α-Proteobacteria, γ-Proteobacteria, and δ-Proteobacteria taxa were present in all clone libraries and appear to be commonly associated with zebra mussels in the Laurentian Great Lakes basin (LGLB). Similarly, all clone libraries contained a large proportion of sequences (11-28%) with close affiliations to 16S rRNA gene sequences of unknown phylogenetic affiliations. While the majority of RDP-unclassified bacterial sequences (62%) were similar to the δ-Proteobacteria and Firmicutes groups of bacteria, many unclassified bacterial sequences were similar to the β-Proteobacteria, Bacteroidetes, Cyanobacteria, and Firmicutes groups of bacteria. It is possible that these sequences represent strains of bacteria that have never been cultured and described before.

Frischer *et al.* (2000), who sequenced the 16S rDNA gene to study microbial diversity in zebra mussel homogenates collected from the Hudson River (New York), stated that a dominance of β- and γ-Proteobacteria was apparent in the zebra mussel homogenate and fecal samples. In comparison to Frischer *et al.* (2000) who taxonomically reported bacteria in zebra mussel samples to the class level only, a more advanced primer set enabled us to detect 66 genera of bacteria, including a number of bacteria that have never before been reported in zebra mussel samples. As in Frischer *et al.* (2000), abundances of γ-Proteobacteria were observed in many samples confirming these bacteria are commonly associated with zebra mussels. In terms of a dominance of γ-Proetobacteria, the microbial community observed in the mantle cavity fluid samples was most similar to the communities Frischer *et al.* (2000) observed in zebra mussel

homogenates.

Lower abundances of Cyanobacteria and γ-Proetobacteria were detected in the gill clone libraries which can be attributed to our repeated rinsing of gill tissues to limit our study to bacteria that are intimately associated with gill tissues and to exlude those that are present as a part of the mixture to be filtered by the gills. On the other hand, the high abundances of Actinobacteria detected in the gill clone libraries likely reflect an intimate symbiotic association between an unclassified member of the Microbacteriaceae family (Actinobacteria) and the gills of zebra mussels. These findings further confirm the heterogeneity of bacterial community structure among different compartments of mussels.

The low variance associated with replicate gut clone libraries provided a high level of statistical confidence proving a good description of the microbial communities present in the gut of zebra mussels was attained. This low variance suggests a high level of homogeneity exists in the microbial communities associated with the gut of zebra mussels in Loon Lake. Futhermore, the high abundance of Cyanobacteria present in the gut clone libraries suggests zebra mussels select these bacteria over the other bacteria detected, probably for feeding reasons. This finding provides insight into how zebra mussels can alter their surrounding environment.

Comparison of bacterial community diversity associated zebra mussel gill, gut, and mantle cavity fluid samples collected from Loon Lake also revealed a number of interesting findings. Our results show that microbial communities associated with zebra mussel gill, gut, and mantle cavity fluid samples are quite different in terms of phylogenetic richness and eveness. The gut library had a relatively high level of

phylotype richness whereas the gut and mantle cavity fluid libraries had lower richness values. For sample sizes of 254, 273, and 265 clones, the gill, gut, and mantle cavity fluid libraries had estimated diversities of 96,133, and 104 phylotypes respectively. This difference in richness could possibly be attributed to the number of different bacterial species that accumulate in zebra mussel gut tissues as a result of mussel feeding activity.

A strong predominance of sequences (such as Actinobacteria and γ-Proteobacteria for gill and mantle cavity fluid samples respectively) indicated a lower evenness (or relative abundance of each phylogenetic group in a community) of phylotype distributions and affected the Shannon-Weiner diversity index, in particular. Still, the Shannon-Weiner (H') values for the composite clone libraries also indicated that the gut clone library had greater bacterial diversity than the gill and mantle cavity fluid clone libraries. Because estimates of both phylogenetic richness and evenness for individual clone libraries showed greater richness and evenness for the gut library, the greater estimated diversity for the composite gut library is believable

Results of the present study further confirm that zebra mussels harbor phylogenetically diverse groups of microorganisms and suggest that it is possible for zebra mussels to act as a reservoir for potentially pathogenic bacteria for many aquatic and terrestrial animals, such as *Clostridium* spp., *Escherichia coli*, *Flavobacterium psychrophilum*, *Legionella pneumophila*, *Mycobacterium* sp., *Mycoplasma* sp., and *Ureaplasma* sp. In addition to these bacteria, we also detected a member of the Rickettsiales order of bacteria, a group of bacteria that are believed to be associated with mortalities in *Diporeia* (Amphipoda) populations in the Great Lakes region (Messick *et al.*, 2004). Whether this constitutes a reservoir of infection for *Diporeia* or not remains to

be elucidated.

In conclusion, we were able to determine the structure of microbial communities associated with zebra mussel tissues and compartments collected from a single site. We were also able to determine significant differences in microbial community structure exist among the three sample types. This work constitutes the first account about bacterial community structure in multiple zebra mussel body parts. Because little is known regarding microbial communities associated with the zebra mussel, the information gained in this study serves as baseline information on the structure of the microbal communities associated with zebra mussels in the LGLB. Information gained in this study will help in understanding the potential role of zebra mussels in disease ecology and food-web shifts in ecosystems. Furthermore, the findings of this study provide insight into the microbial ecology of the zebra mussel and its possible impacts to the Great Lakes ecosystem.

Class	Family	Sudivision	MCF	Gill	Gut
α – Proteobacteria	Acetobacteraceae	*Acidisphaera* sp.		+	
		Roseomonas sp.			+
		Unclassified Acetobacteraceae		+	
		Unclassified Acetobacteraceae	+		
	Hyphomicrobiaceae	*Hyphomicrobium* sp.		+	
		Pedomicrobium sp.	+		
		Unclassified Hyphomicrobiaceae			+
	Rickettsiaceae	*Orienta* sp.		+	+
		Unclassified Rickettsiales	+		
	Rhizobiaceae	*Agrobacterium* sp.		+	
		Rhizobium sp.	+	+	
		Uclassified Rhizobiales	+	+	+
	Rhodobacteraceae	*Rhodobacter sphaeroides*	+		+
		Rhodobacter sp. #2		+	
		Roseicyclus sp.	+		
		Unclassified Rhodobacteraceae	+	+	
	Sphingomonadaceae	*Sphingobium* sp.	+	+	+
		Sphingomonas sp.		+	
		Sphingopyxis sp.		+	
		Unclassified Sphingomonadaceae	+	+	
	Family unknown	Unclassified α-Proteobacteria #1			+
	Family unknown	Unclassified α-Proteobacteria #2			+
	Family unknown	Unclassified α-Proteobacteria #3			+
	Family unknown	Unclassified α-Proteobacteria #4			+
	Family unknown	Unclassified α-Proteobacteria #5	+		
	Family unknown	Unclassified α-Proteobacteria #6	+		
β – Proteobacteria	Comamonadaceae	*Commamonas* sp.	+		
		Rhodoferax ferrireducens			+
		Rhodoferax sp. #2			+
	Methylophilaceae	*Methylophilus* sp.	+		+
		Methylovorous sp.	+		
		Methylobacillus flagellatus		+	
	Oxalobacteraceae	Unclassified Oxalobacteraceae		+	
	Family unknown	Unclassified Burkholderiales		+	
	Family unknown	Unclassified β-Proteobacteria	+		+

Table 7. α– and β– Proteobacteria detected by sequencing the 16S rRNA gene isolated from zebra mussel gill, gut, and mantle cavity fluid (MCF) samples collected from Loon Lake.

Class	Family	Sudivision	MCF	Gill	Gut
γ – Proteobacteria	Chromatiaceae	*Rheinheimera* sp.	+		
	Coxiellaceae	*Aquicella* sp.			+
		Rickettsiella	+		+
	Enterobacteriaceae	*Enterobacter cloacae* subsp. *cloacae*	+		
	Legionellaceae	*Legionella pneumophila*	+	+	+
	Moraxellaceae	*Acinetobacter baumannii*	+		+
		Alkanidiges sp.	+		
	Oceanospirillaceae	Unclassified Oceanospirillaceae			+
	Pseudomonadaceae	*Psuedomonas entomophila*	+		
		Pseudomonas syringae pv. *phaseolicola*			+
	Vibrionaceae	Unclassified Vibrionaceae	+	+	+
	Xanthomonadaceae	*Dokdonella* sp.	+	+	
		Stenotrophomonas sp.	+		
		Thermomonas sp.			+
		Unclassified Xanthomonadaceae		+	+
	Family unknown	*Unclassified* γ-Proteobacteria #1	+	+	+
	Family unknown	*Unclassified* γ-Proteobacteria #2			+
	Family unknown	*Unclassified* γ-Proteobacteria #3			+
	Family unknown	*Unclassified* γ-Proteobacteria #4			+
	Family unknown	*Unclassified* γ-Proteobacteria #5			+
	Family unknown	*Unclassified* γ-Proteobacteria #6			+
	Family unknown	*Unclassified* γ-Proteobacteria #7			+
	Family unknown	*Unclassified* γ-Proteobacteria #8			+
	Family unknown	*Unclassified* γ-Proteobacteria #9	+	+	
δ – Proteobacteria	Bacteriovoracaceae	*Peredibacter* sp.			+
	Myxococcaceae	*Anaeromyxobacter dehalogenans*		+	
	Polyangiaceae	*Byssovorax* sp.		+	
	Syntrophaceae	*Desulphomonile* sp.			+
	Family unknown	Unclassified Myxococcales	+		
	Family unknown	Unclassified δ-Proteobacteria #1	+	+	+
	Family unknown	Unclassified δ-Proteobacteria #2		+	
	Family unknown	Unclassified δ-Proteobacteria #3		+	
	Family unknown	Unclassified δ-Proteobacteria #4			+

Table 8. γ– and δ– Proteobacteria detected by sequencing the 16S rRNA gene isolated from zebra mussel gill, gut, and mantle cavity fluid (MCF) samples collected from Loon Lake.

Phylum		Classification	MCF	Gill	Gut
Acidobacteria	Acidobacteriaceae	Gp3	+		+
		Gp6	+		+
		Gp10	+	+	+
Actinobacteria	Actinomycetaceae	*Actinomyces* sp.			+
	Microbacteriaceae	Unclassified Microbacteriaceae		+	+
	Mycobacteriaceae	*Mycobacterium* sp.	+		
	Nocardiaceae	*Rhodococcus* sp.	+		
	Rubrobacteraceae	*Conexibacter* sp.			+
	Family unknown	Unclassified Actinomycetales	+	+	+
	Family unknown	Unclassified Micrococcineae		+	
	Family unknown	Unclassified Actinobacteria #1	+	+	+
	Family unknown	Unclassified Actinobacteria #2			+
Bacteroidetes	Crenotrichaceae	*Crenothrix* sp.	+	+	+
	Cryomorphaceae	*Fulviicola* sp.	+		
		Fulvibacter sp.# 2		+	
	Flavobacteriaceae	*Chryseobacterium* sp.	+		
		Cloacibacterium sp.		+	+
		Flavobacterium johnsoniae	+		+
		Flavobacterium psychrophilum	+		+
		Lutibacter sp.		+	
	Flexibacteraceae	Unclassified Flexibacteraceae	+		
	Family unknown	Unclassified Bacteroidetes #1	+	+	
	Family unknown	Unclassified Bacteroidetes #2	+		+
Chloroflexi	Caldilineacea	*Caldilinea* sp.	+	+	

Table 9. Acidobacteria, Actinobacteria, Bacteroidetes, and Chloroflexi detected by sequencing the 16S rRNA gene isolated from zebra mussel gill, gut, and mantle cavity fluid (MCF) samples collected from Loon Lake.

Phylum		Classification	MCF	Gill	Gut
Cyanobacteria	Chloroplast	*Bacillariophyta* sp.	+	+	+
		Chlorophyta sp.			+
	Family I	*GPI*	+		
	Family II	*GPIIa*	+		+
	Family XI	*GPXI*	+		+
	Family XIII	*GPXIII*	+		
	Family unknown	Unclassified Cyanobacteria #1	+		+
	Family unknown	Unclassified Cyanobacteria #2	+	+	+
Firmicutes	Clostridiaceae	*Clostridium beijerinckii*		+	
		Unclassified Clostridiaceae		+	
	Incertae Sedis XII	Unclassified Incertae Sedis XII		+	
	Family unknown	Unclassified Bacillales	+		
Gen. inc. sed. OP10	OP10	*OP10* gen. inc. sed.		+	
Nitrospira	Nitrospiraceae	*Magnetobacterium* sp.			+
		Nitrospira sp.	+	+	+
Planctomycetes	Planctomycetaceae	*Blastopirellula* sp.	+	+	+
		Gemmata sp.	+	+	+
		Isophaera sp.		+	
		Pirellula sp.	+	+	+
		Planctomyces sp.	+	+	+
		Unclassified Plantomycetaceae	+	+	+
Spirochaetes	Leptospiraceae	*Leptonema* sp.	+		
Verrucomicrobia	Subdivision 3	Subdivision 3 gen. inc. sed.	+	+	+
	Opitutaceae	*Opitutus* sp.		+	

Table 10. Cyanobacteria, Firmicutes, Gen. incarte sedis OP10, Nitrospira, Planctomycetes, Spirochaetes, Verrucomicrobia detected by sequencing the 16S rRNA gene isolated from zebra mussel gill, gut, and mantle cavity fluid (MCF) samples collected from Loon Lake.

Accession #	Significant alignments	Phylogenetic group	# of sequences
NC_008578.1	*Acidothermus sp.*	Actinobacteria	1
NZ_ABDY01000013.1	*Clostridium perfringens*	Firmicutes	1
NZ_AAYW02000018.1	*Clostridium* sp.	Firmicutes	15
NC_008255.1	*Cytophaga hutchinsonii*	Bacteroidetes	1
NZ_AAVG01000086.1	*Geobacter lovleyi*	δ-Proteobacteria	1
NC_005125.1	*Gloebacter violaceus*	δ-Proteobacteria	1
NZ_AAZV01000073.1	*Leptolyngbya valderiana*	Cyanobacteria	1
NZ_AAYK01000142.1	*Mycobacterium tuberculosis*	Actinobacteria	1
NC_007498.2	*Pelobacter carbinolicus*	δ-Proteobacteria	1
NC_005027.1	*Rhodopirellula baltica*	Planctomycetes	1

Table 11. RDP-unclassified bacterial 16S rRNA (rDNA) gene sequences rechecked using BLAST for zebra mussel mantle cavity fluid samples collected from Lake Loon (E values are less than 1E-129).

Accession #	Significant alignments	Phylogenetic group	# of sequences
NC_008578.1	*Acidothermus* sp.	Actinobacteria	4
NC_009633.1	*Alkaliphilus metalliredigens*	Firmicutes	1
NC_008340.1	*Akalilimnicola ehrlichei*	γ-Proteobacteria	1
NZ_AANZ01000021.1	*Blastopirellula marina*	Planctomycetes	2
NC_006156.1	*Borrelia garinii*	Spirocheaetes	1
NZ_AAYW02000018.1	*Clostridium sp.*	Firmicutes	27
NC_009012.1	*Clostridium thermocellum*	Firmicutes	1
NC_009455.1	*Dehalococcoides sp.*	Chloroflexi	1
NC_007519.1	*Desulfovibrio desulfuricans*	δ-Proteobacteria	1
NZ_AAVG01000086.1	*Geobacter lovleyi*	δ-Proteobacteria	1
NC_002939.4	*Geobacter sulfurreducens*	δ-Proteobacteria	1
NZ_AAZV01000073.1	*Leptolyngbya valderiana*	Cyanobacteria	4
NZ_AAVV01000015.1	Marine γ-Proteobacteria	γ-Proteobacteria	1
NC_007498.2	*Pelobacter carbinolicus*	δ-Proteobacteria	1
NC_003888.3	*Streptomyces coelicolor*	Actinobacteria	7

Table 12. RDP-unclassified bacterial 16S rRNA (rDNA) gene sequences rechecked using BLAST for zebra mussel gill samples collected from Lake Loon (E values are less than 2E-107).

Accession #	Significant alignments	Phylogenetic group	# of sequences
NC_008340.1	*Alkalilimnicola ehrlichei*	γ-Proteobacteria	1
NC_009633.1	*Alkaliphilus metalliredigens*	Firmicutes	2
NC_007797.1	*Anaplasma phagocytophilum*	α-Proteobacteria	7
NZ_AANZ01000021.1	*Blastopirellula marina*	Planctomycetes	1
NZ_ABCU01000001.1	*Borrelia afzelii*	Spirocheaetes	5
NZ_ABCY01000002.1	*Borrelia valaisiana*	Spirochaetes	2
NZ_ABCU01000001.1	*Borrelia sp.*	Spirochaetes	1
NC_009943.1	*Candidatus Desulfococcus*	δ-Proteobacteria	1
NC_007503.1	*Carboxydothermus sp.*	Firmicutes	3
NZ_AAYW02000018.1	*Clostridium* sp.	Firmicutes	9
NC_008751.1	*Desulphovibrio vulgaris*	δ-Proteobacteria	1
NZ_AAVG01000086.1	*Geobacter lovleyi*	δ-Proteobacteria	2
NC_002939.4	*Geobacter sulfurreducens*	δ-Proteobacteria	2
NZ_AAZV01000073.1	*Leptolyngbya valderiana*	Cyanobacteria	1
NZ_AAYK01000142.1	*Mycobacterium tuberculosis*	Actinobacteria	2
NC_004829.1	*Mycoplasma gallisepticum*	Firmicutes	7
NC_007498.2	*Pelobacter carbinolicus*	δ-Proteobacteria	1
NC_003888.3	*Streptomyces coelicolor*	Actinobacteria	1
NC_006526.1	*Zymomonas mobilis*	α-Proteobacteria	1

Table 13. RDP-unclassified bacterial 16S rRNA (rDNA) gene sequences rechecked using BLAST for zebra mussel gut samples collected from Lake Loon (E values are less than 2E-148).

Sample	# of sequences	Shannon-Wiener	Chao1
MCF - A	75	2.767	88.167
MCF - B	95	4.141	438
MCF - C	95	1.963	63.1
Gill - A	83	3.229	313.667
Gill - B	79	2.641	136.166
Gill - C	92	2.475	165.667
Gut - A	89	3.265	174.75
Gut - B	89	3.914	256.143
Gut - C	95	3.430	170.5

Table 14. Analysis of diversity and richness for replicate 16S rDNA clone libraries constructed from zebra mussel mantle cavity fluid (MCF), gill, and gut samples collected from a single site in Loon Lake determined using FastGroupII©.

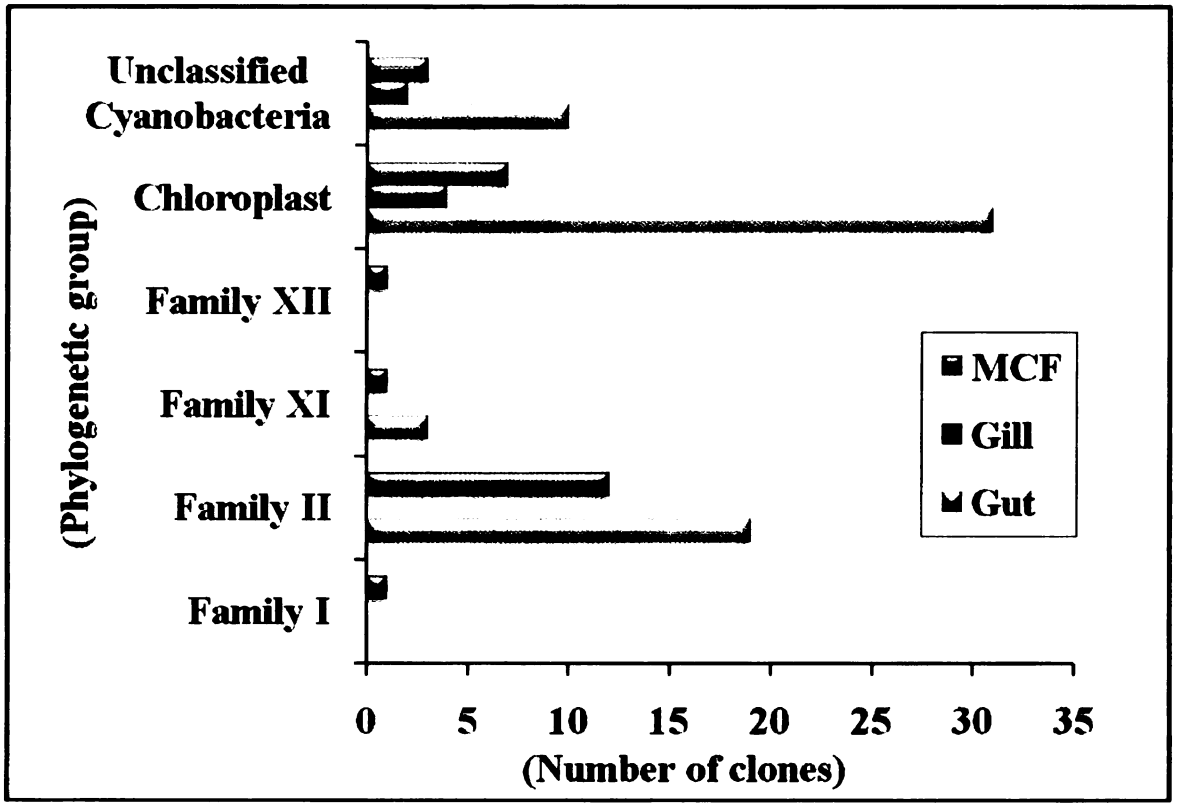

Figure 3. Abundances of Cyanobacteria detected in 16S rDNA (rRNA) clone libraries derived from zebra mussel mantle cavity fluid (MCF), gill, and gut samples collected from a single site in Loon Lake, Oakland County (Michigan, USA).

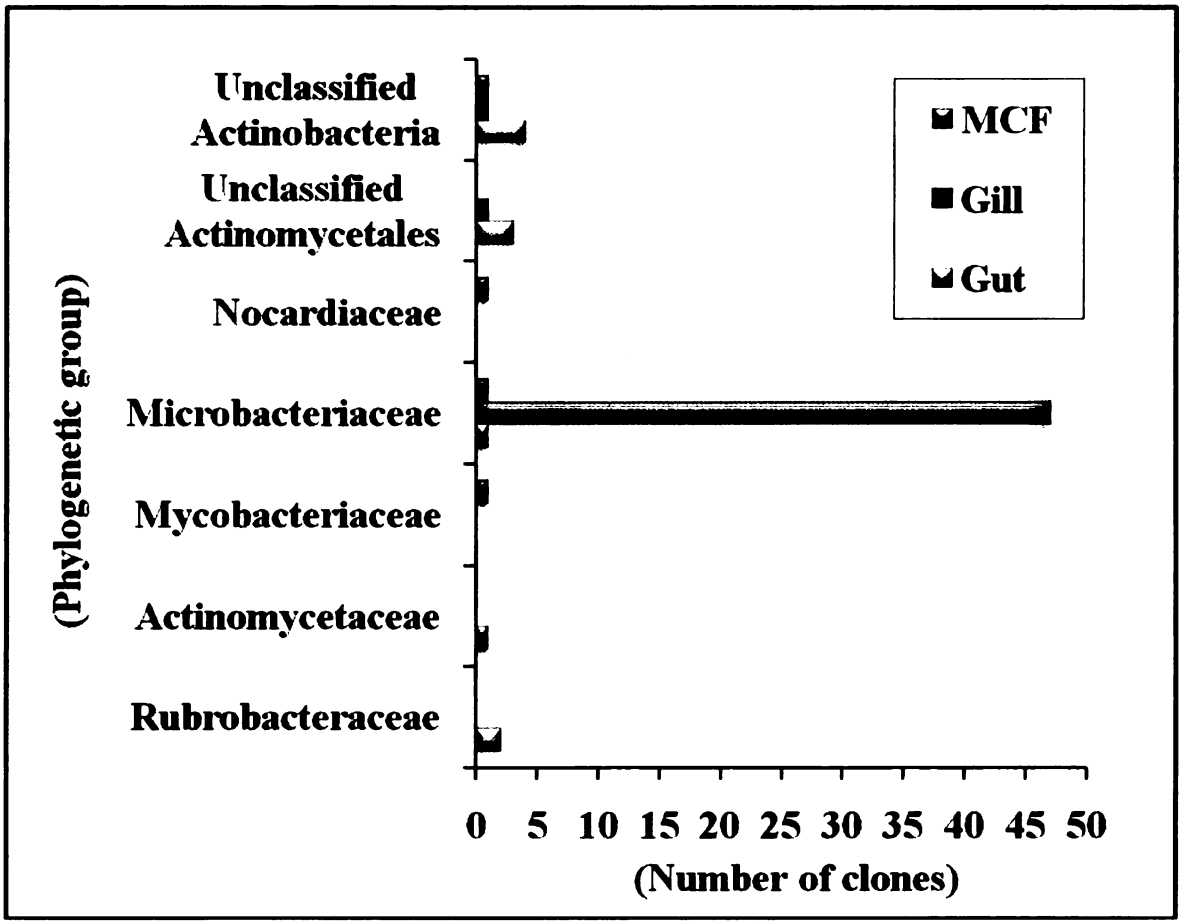

Figure 4. Abundances of Actinobacteria detected in 16S rDNA (rRNA) clone libraries derived from zebra mussel mantle cavity fluid (MCF), gill, and gut samples collected from a single site in Loon Lake, Oakland County (Michigan, USA).

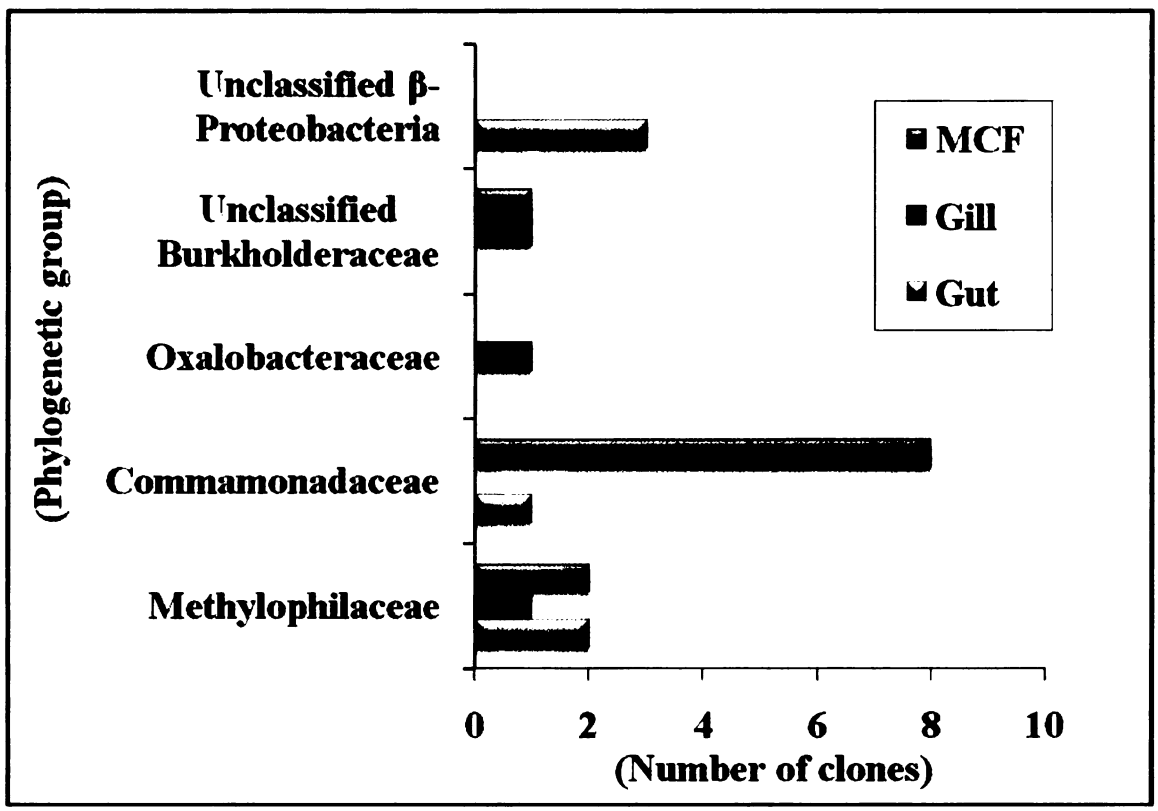

Figure 5. Abundances of the β- class of Proteobacteria detected in 16S rDNA (rRNA) clone libraries derived from zebra mussel mantle cavity fluid (MCF), gill, and gut samples collected from a single site in Loon Lake, Oakland County (Michigan, USA).

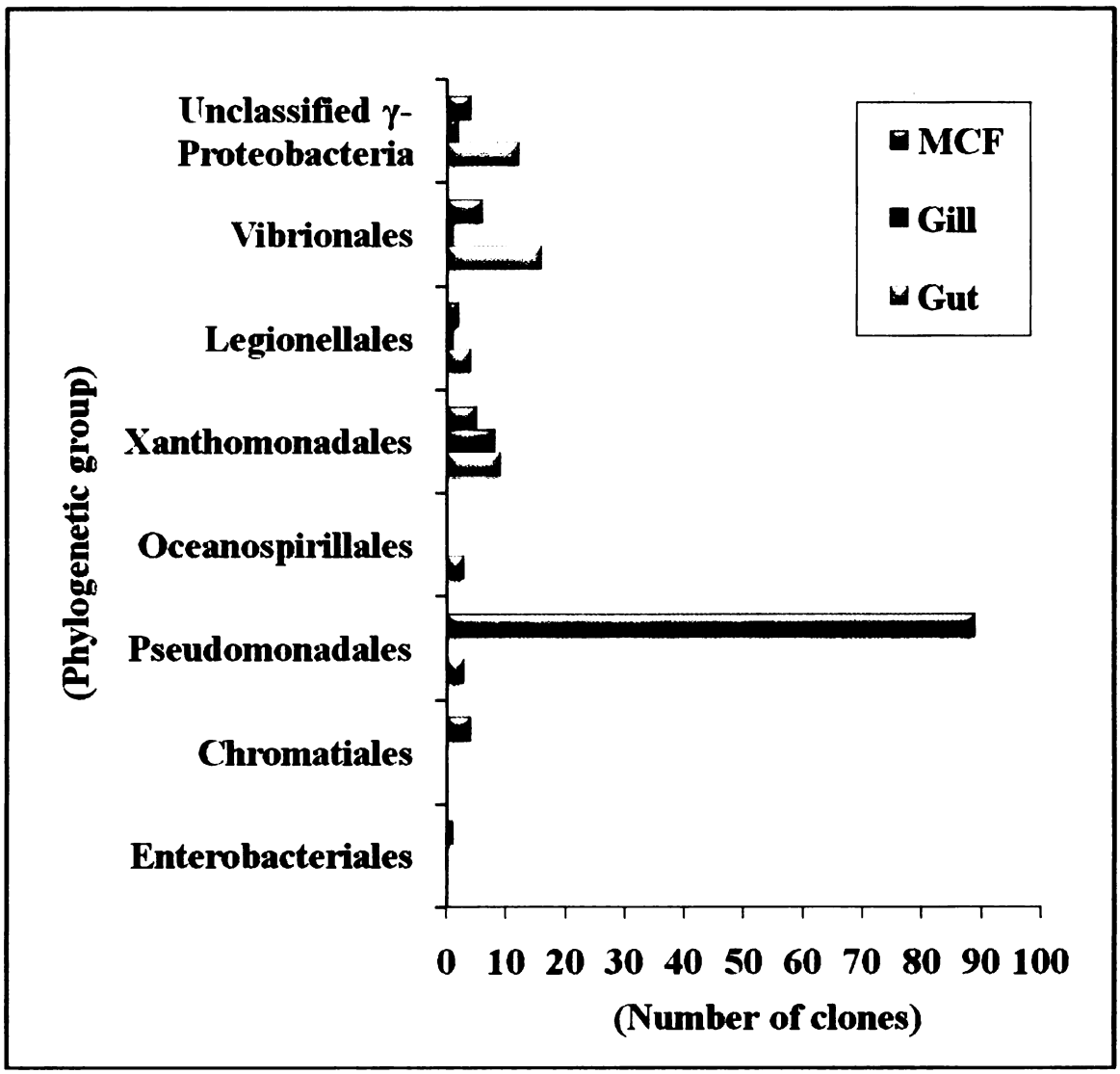

Figure 6. Abundances of the γ- class of Proteobacteria detected in 16S rDNA (rRNA) clone libraries derived from zebra mussel mantle cavity fluid (MCF), gill, and gut samples collected from a single site in Loon Lake, Oakland County (Michigan, USA).

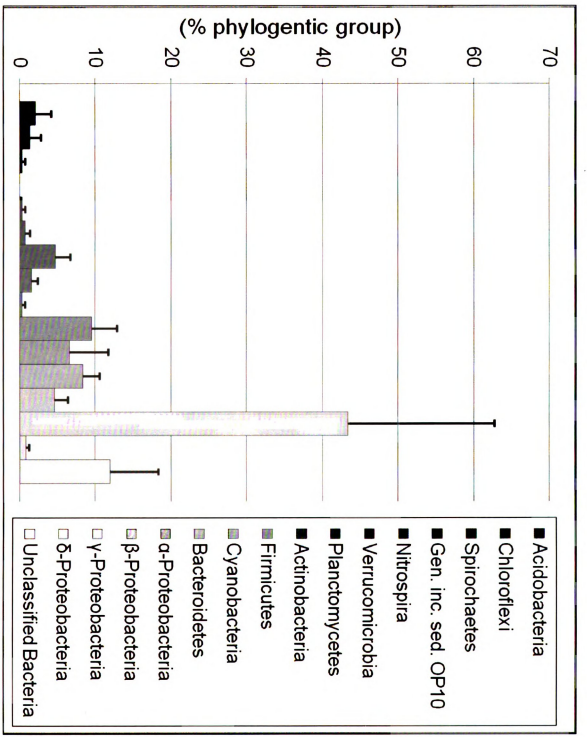

Figure 7. Frequency distribution of bacteria detected in zebra mussel mantle cavity fluid samples collected from Loon Lake based on 16S rDNA clone library analysis (Mean plus standard error).

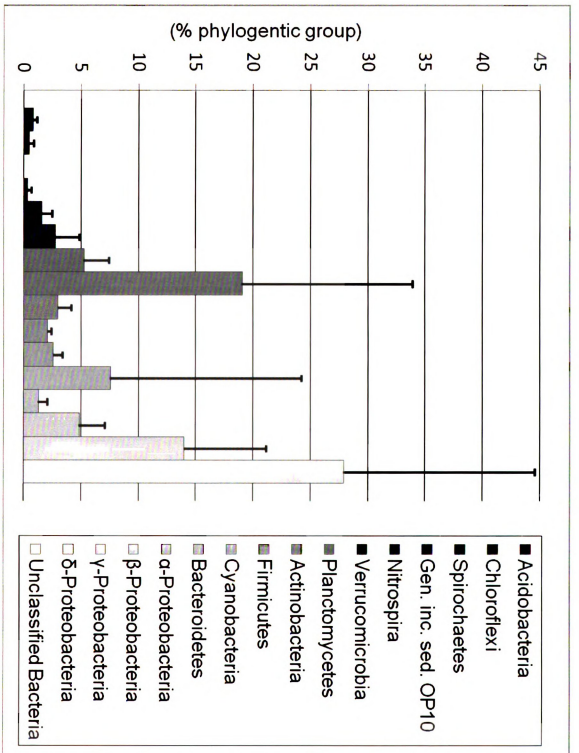

Figure 8. Frequency distribution of bacteria detected in zebra mussel gill samples collected from Loon Lake based on 16S rDNA clone library analysis (Mean plus standard error).

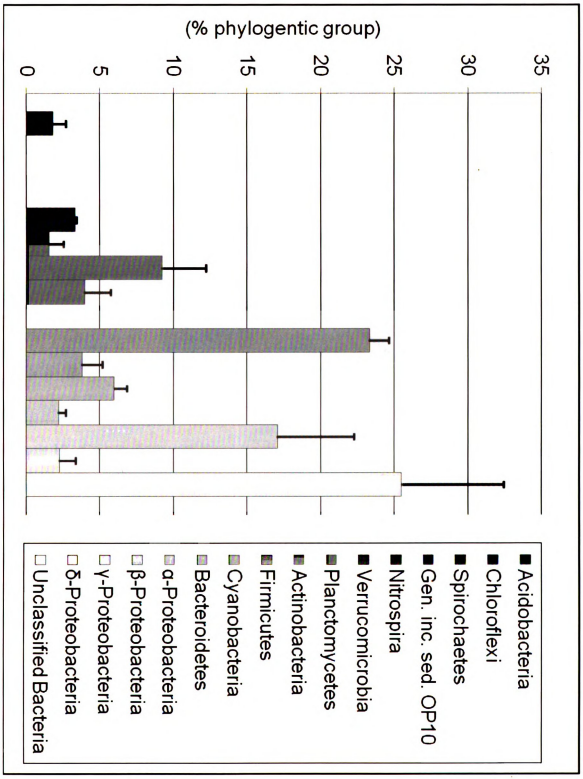

Figure 9. Frequency distribution of bacteria detected in zebra mussel gut samples collected from Loon Lake based on 16S rDNA clone library analysis (Mean plus standard error).

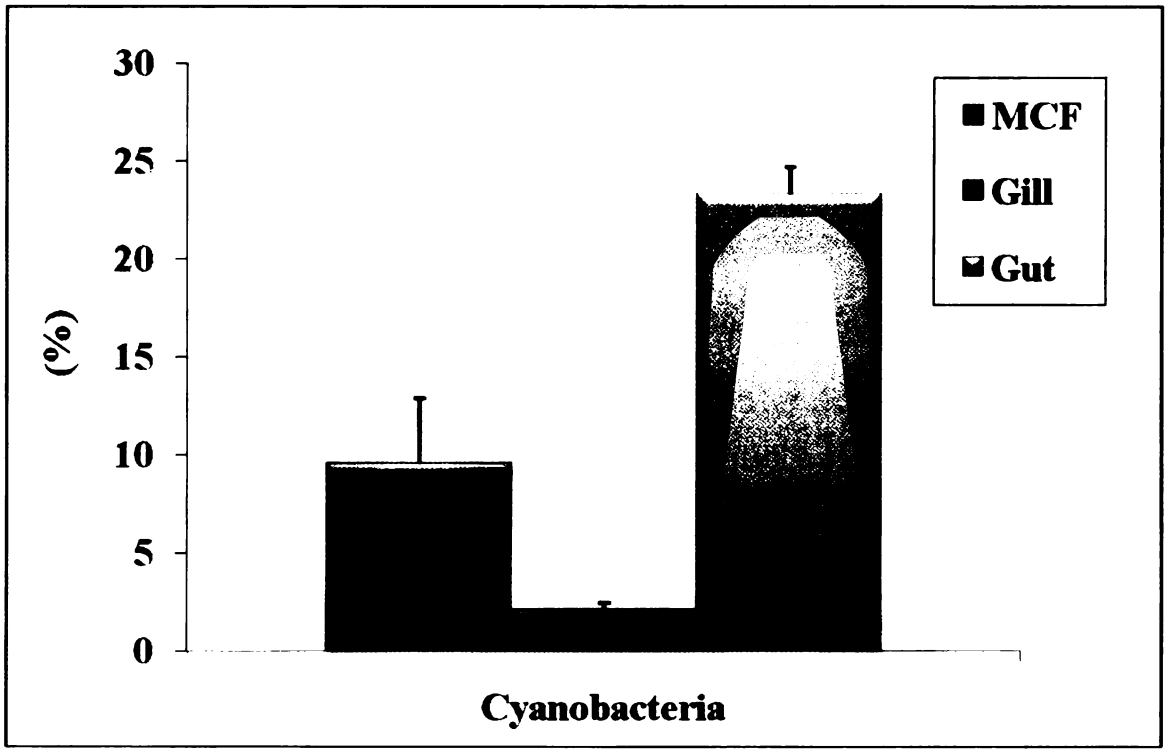

Figure 10. Comparison of mean abundance of Cyanobacteria present in 16S rDNA clone libraries derived from zebra mussel mantle cavity fluid (MCF), gill and gut and samples collected from a single site in Loon Lake, Oakland County (Michigan, USA).

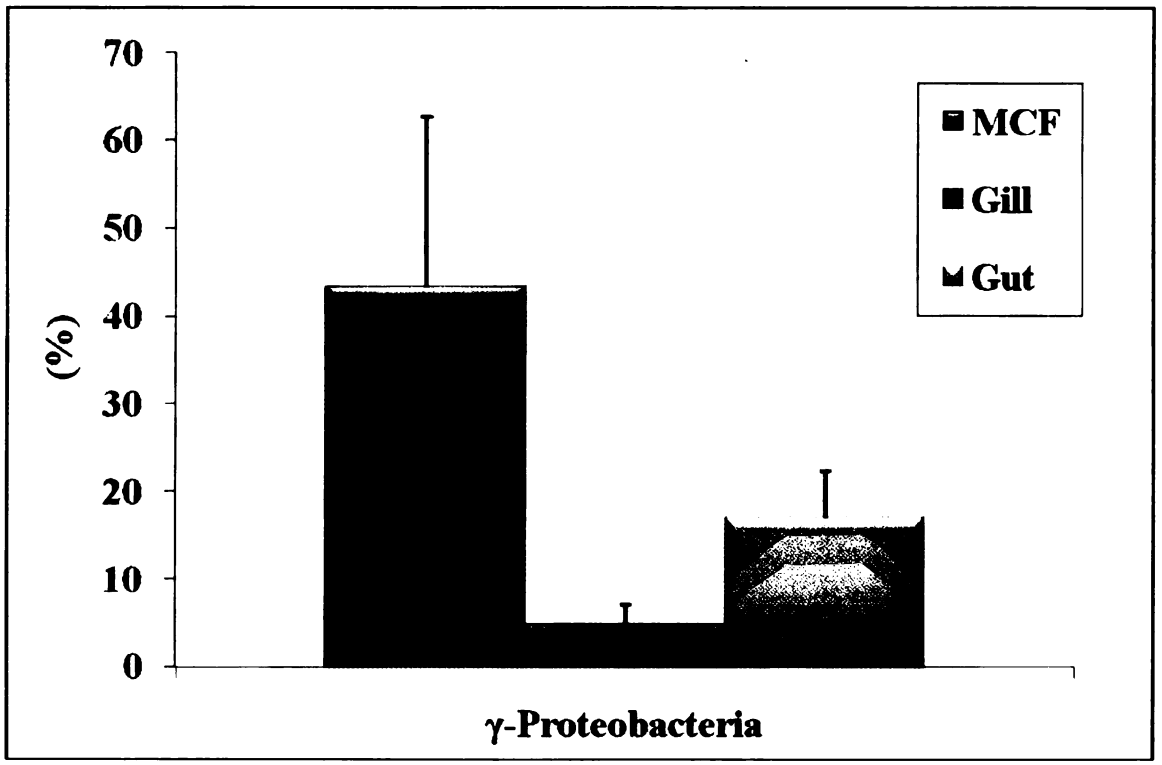

Figure 11. Comparison of mean abundances of the γ- class of Proteobacteria present in 16S rDNA clone libraries derived from zebra mussel mantle cavity fluid (MCF), gill, and gut samples collected from a single site in Loon Lake, Oakland County (Michigan, USA).

Sample	# of sequences	# of Groups	Shannon-Wiener	Chao1
MCF	265	104	2.957	387.682
Gill	254	96	2.782	856.500
Gut	273	133	3.536	305.980

Table 15. Analysis of diversity and richness for composite 16S rDNA clone libraries constructed from zebra mussel mantle cavity fluid (MCF), gill, and gut and samples collected from a single site in Loon Lake determined using FastGroupII©.

CHAPTER FOUR

CONCLUSIONS AND FUTURE RESEARCH

Through this study, it was possible to detect phylogenetically diverse bacteria in multiple zebra mussel samples, and thus gain some understanding on the microbial ecology of the zebra mussel in its new range in the Laurentian Great Lakes basin (LGLB). Bacterial assemblages present in tissue homogenates harvested from zebra mussels collected from one river and two inland lakes in Michigan's Lower Peninsula (USA): the Huron River, Crystal Lake, and Lake Vineyard were described for the first time. A total of 35 genera belonging to 12 bacterial subdivisions are present in the Laurentian Great Lakes mussel population. Potentially pathogenic bacteria for aquatic and terrestrial animals, like *Aeromonas* spp., *Clostridium* spp., *Escherichia coli*, *Flavobacterium* spp., *Shewanella putrefaciens*, *Shigella* sp., and *Yersinia ruckeri*, were also detected suggesting that zebra mussels may contribute to the ecology of these pathogens. While, many bacterial groups were detected in all three waterbodies appearing to be commonly associated with zebra mussels in the LGLB, some bacterial groups were detected in a single waterbody (Huron River) only. This finding suggests the microbial communities associated with zebra mussels vary from one waterbody to the other and likely reflect the microbial diversity in the surrounding environment. Additional research is needed to determine major differences between bacterial assemblages in zebra mussels and those found in the surrounding sediment and water column.

The structure of microbial communities present in three zebra mussel sample types (gill, gut, mantle cavity fluid) was identified. While clone libraries for each zebra mussel sample type had similarities in terms of composition, they clearly differed in terms of community structure. Cyanobacteria were detected in higher abundances in gut samples likely reflecting the diet of the zebra mussel. An unclassified member of the family Microbacteriaceae (Actinobacteria) was detected in considerable abundances in gill samples compared to the other two sample types suggesting this bacterium may be intimately associated with the gills of zebra mussels in Loon Lake. Whether or not this member of the Microbacteriaceae family of bacteria is pathogenic to zebra mussels remains to be elucidated. The sum of this study's results reveals the richness of microbial communities associated with the zebra mussel being 384 phylotypes belonging to 15 bacterial subdivisions.

Further research is required in order to better understand the microbial ecology of zebra mussels in the Great Lakes region. The employment of additional molecular techniques based on direct 16S rDNA amplification that are amenable to replication should provide powerful tools for rapidly investigating alterations in microbial communities due to zebra mussels. For this reason, future work will employ 16S rDNA gene sequencing in combination with terminal restriction fragment length polymorphism (T-RFLP) analysis, a cost-effective and powerful culture independent method of genotyping, to profile microbial communities present in bivalves and amphipods collected from multiple waterbodies in the Great Lakes (Michigan) and Finger Lakes (New York) regions to better understand the role invertebrates play in the transmission of disease and food-web shifts. By comparing trends in microbial community structure

relative to geological, limnological, and temporal data, cause and effect linkages between alterations in ecosystem functioning and changes in microbial community structure induced by zebra mussels will be revealed.

It is possible that particular bacteria are only pathogenic to zebra mussels under certain environmental conditions. By understanding which pathogens are relatively abundant in mussel samples from a particular collected at a particular time of year, more effective bacterial biological control agents that will have limited affects on non-target organism can be used, allowing for these agents to be safely used on a scale larger than water intake lines and pumps. For this reason, researcher should take into account temporal influences on microbial species composition and abundance when constructing 16S rRNA (rDNA) gene clone libraries.

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