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BACTERIAL PATHOGENS INFECTING LAKE WHITEFISH
(*COREGONUS CLUPEAFORMIS*) IN MICHIGAN

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BACTERIAL PATHOGENS INFECTING LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS)* IN MICHIGAN

By

Thomas Peterson Loch

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ABSTRACT

BACTERIAL PATHOGENS INFECTING LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS)* IN MICHIGAN

By

Thomas Peterson Loch

The lake whitefish (*Coregonus clupeaformis*) is a commercially and ecologically invaluable indigenous salmonid within the Great Lakes Basin (GLB). A recent decline in its condition and abundance has alarmed managers, scientists, and the public at large, necessitating research to determine what factors may be involved. Although diseases can significantly impact populations of fishes worldwide, a meager amount of research has focused on pathogens of lake whitefish within the GLB. This study was undertaken to determine what bacterial pathogens are present within four stocks of lake whitefish residing within Lakes Michigan and Huron and whether bacterial infections caused by these microbes vary in prevalence spatially and temporally. *Carnobacterium maltaromaticum*, the etiological agent of pseudokidney disease, *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of furunculosis, and multiple motile, mesophilic *Aeromonas* species, known to cause motile aeromonad septicemia, were among the bacteria isolated from infected lake whitefish within this study. Clinical signs of disease associated with some of these infections were quite severe, and the prevalence of infections caused by a portion of these microbes was at times, quite high. This is considered the first report of *C. maltaromaticum* and *A. salmonicida salmonicida* infecting lake whitefish within the GLB. Implications of these diseases on lake whitefish populations are discussed within.

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GENERAL INTRODUCTION AND OVERVIEW

Historically, lake whitefish (*Coregonus clupeaformis*) have been a staple commercial fishery within the Great Lakes Basin (GLB), beginning in the early 1840s and quickly expanding thereafter (Wells and McLain 1973). Although initial commercial yields of lake whitefish were not formally documented, anecdotal accounts suggest substantially larger yields than those recorded in subsequent years. In the following years, a variety of factors, such as overexploitation and interactions with invasive species, brought about significant declines in lake whitefish abundance. Through the implementation of a number of management strategies, lake whitefish populations rebounded (Ebener 1997) and above average harvests have been experienced (Mohr and Nalepa 2005).

However, recent observations of declines in abundance, condition, and size at age in harvested lake whitefish have caused concern (Hoyle et al. 1999; Pothaven et al. 2001; Madenjian et al. 2002; Mohr and Ebener 2005). Concurrently, there has been significant decline in the abundance of the preferred prey item (*Diporeia* spp.) of lake whitefish (Nalepa et al. 1998), superceded by alterations in phytoplankton composition brought about by the invasion of zebra (*Dresseina polymorpha*) and quagga mussels (*D. bugensis*) into the GLB (Higgins et al. 2005; Bierman et al. 2005).

There is mounting evidence that infectious diseases exert significant pressure on the dynamics of animal populations and communities (Holmes 1996). For example, infections with the fungus *Icthyophonus hoferi* in Atlantic (*Clupea harengus*) and Pacific Herring (*Clupea pallasi*) have caused population declines (Daniel 1933; Sindermann

1958; Mellergaard and Spanggaard 1997; Marty et al. 2003). Additionally, an epizootic of bacterial kidney disease that occurred in Lake Michigan generated mass mortalities in Chinook salmon that reduced the population by 50% or more (Holey et al. 1998).

There are many other examples of diseases affecting fish at the population level, and often, outbreaks of these diseases can be linked to environmental conditions (Lafferty and Holt 2003). Organisms residing in dysfunctional ecosystems do not resist infection as well as their counterparts, which inhabit healthy ecosystems (Folke et al. 2004). Diseases in wild fish populations also result from complex interactions among pathogens, their hosts, and the environment (Snieszko 1973; Hedrick 1998; Reno 1998). To this end, I postulate that significant alterations in the Great Lakes' ecosystem (e.g. food-web shifts, interactions between exotic and native species, etc.) may have placed lake whitefish populations at an increased risk for disease epizootics.

One problem with the determination of a disease's effect on a population of organisms is the paucity of baseline data regarding the incidence and prevalence of diseases in aquatic organisms (Lafferty et al. 2004). The lack of information on diseases affecting coregonines, in general, and the GLB lake whitefish specifically, makes efforts to determine the effects that diseases are having and have had on their populations next to impossible.

Study objectives

This study focused on the isolation and identification of potential bacterial pathogens infecting GLB lake whitefish. In order to elucidate any temporal or spatial associations, lake whitefish were sampled from four stocks (two from Lake Michigan and two from Lake Huron) at four different times per year (October-December; January-March; April-June; July-September). Specific objectives were to: 1) isolate *Carnobacterium maltaromaticum*, *Aeromonas salmonicida* subspecies *salmonicida*, and motile *Aeromonas* spp. infecting lake whitefish using standard bacteriological methodologies and subsequently identify them using morphological, physiological, and biochemical analyses; 2) determine if the detected bacterial infections of lake whitefish varied seasonally or by stock.

This thesis consists of five chapters. The first chapter will summarize the background information and the available body of knowledge on the thesis' topic. The second chapter deals with the first reported isolation of *Carnobacterium maltaromaticum* from lake whitefish. The third chapter reports on the isolation of *A. salmonicida* subspecies *salmonicida* from lake whitefish. The fourth chapter deals with the isolation and identification of four motile *Aeromonas* species, as well as a number of un-classified presumptive *Aeromonas* spp. from lake whitefish. Finally, I shall present my conclusions and potential directions for future research.

CHAPTER ONE

REVIEW OF LITERATURE

I. Lake whitefish background information

Lake whitefish (*Coregonus clupeaformis*), a member of the family Salmonidae and the subfamily Corgeninae, is among a decreasing number of salmonid species considered indigenous to the Great Lakes Basin (GLB). Other endemic coregonines collectively known as deepwater ciscoe, such as *C. nigripinnis*, *C. johannae*, *C. zenithicus*, *C. alpenae*, *C. reighardi,* and *C. kiyi*) were once plentiful within the GLB, but have dramatically declined in numbers or have been extirpated following the invasion of non-native fishes (Wells and McLain 1972). Despite these declines, *C. clupeaformis* is the most plentiful coregonine inhabiting the GLB; the lake herring (*C. artedi*) and the bloater (*C. hoyi*), are still present in some areas, albeit at significantly lower numbers than historically reported (Smith 1970). *Coregonus clupeaformis* is also native to Alaska and most of Canada. Its range extends south into New England and central Minnesota. Additionally, lake whitefish have been introduced into a number of locales, such as areas of the Northwestern United States, including Montana, Idaho, and Washington (Page and Burr 1991).

The lake whitefish is non-anadromous and is characterized by its coarse scales numbering <110 on the lateral line, two dorsal fins, including an adipose fin, and does not have teeth on the maxillary bone (Moyle and Cech 2000). Lake whitefish can weigh

up to 9000 grams, measure up to 800 mm, and live up to 25 years, with sexual maturity occurring by 6-7 years of age (Page and Burr 1991). *Coregonus clupeaformis* is primarily benthivorous, feeding predominantly on the amphipod, *Diporeia* spp. (Hart 1931; Reckahn 1970), as well opossum shrimp (*Mysis relicta),* and various gastropod and pelecypod molluscs (Ihssen et al. 1981; Brandt 1986; Christie et al. 1987). *Diporeia* spp. are endemic amphipods that are extremely nutritious, having high lipid and caloric contents (Johnson 1988, Cavaletto et al. 1996, Pothaven et al. 2001), and were once one of the most abundant benthic invertebrates inhabiting the cold, offshore regions within the Great Lakes (Cook and Johnson 1974).

Historically, lake whitefish were an important fishery within the Great Lakes, until the 1940's, when yields dramatically declined. The lake whitefish fishery continued to struggle, and in the 1960s-1970s, populations reached all time lows (Mohr and Nalepa 2005). The reasons for these drastic declines varied among Lakes, but were largely attributed to overexploitation, interactions with invasive species, such as the sea lamprey (*Petromyzon marinus*), rainbow smelt (*Osmerus mordax*), and alewife (*Alosa pseudoharengus*), and water quality/habitat degradation (Mohr and Nalepa 2005). Through various lake management strategies, such as control of the sea lamprey, improved commercial fishery management, suppression of non-indigenous planktivores via the introduction of exotic salmonids, and phosphorous abatement, the lake whitefish fishery rebounded and produced above average harvest yields from the 1980s until present (Mohr and Nalepa 2005).

Currently, lake whitefish are the most commercially valuable fish species within GLB (Bronte et al. 2003; Hoyle 2005; Schneeberger et al. 2005; Cook et al. 2005; Mohr

and Ebener 2005). A notable decline in lake whitefish condition, growth rate, and size at age has been reported in parts of Lake Michigan (Pothaven et al. 2001; Madenjian et al. 2002) and Lake Ontario (Hoyle et al. 1999). Furthermore, observations of depressed growth rates and poor condition were reported in parts of Lake Huron (Mohr and Nalepa 2005). Concurrently, substantial changes within the Great Lakes' food web were reported. Exotic zebra and quagga mussels (*Dresseina polymorpha* and *D. bugensis*) have driven significant changes in both phytoplanktonic and filamentous algal abundance, as well as alterations in water quality (Higgins et al. 2005; Bierman et al. 2005). Simultaneously, a number of studies have demonstrated declines in numbers of *Diporeia* spp. within the Great Lakes, with the exception of Lake Superior, to the point where certain areas once rich in *Diporeia* are now devoid of these organisms (Dermott and Kerec 1997; Nalepa et al. 1998; Lozano et al. 2001). The high filtering rates of dreissenids have caused marked declines in diatoms within Lake Erie, Saginaw Bay, and Lake Ontario, causing an abundance of cyanobacteria to be present in these areas (as cited in Ebener and Arts 2007). When available, *Diporeia* spp. feed heavily upon diatoms, which are a rich source of essential fatty acids (EFAs). These are subsequently assimilated, thus providing an excellent source of EFA to both the *Diporeia* spp. themselves, and those organisms that prey upon them (e.g. lake whitefish). Essential fatty acids are integral for maintenance of cell membrane integrity, improvement of cold tolerance, mitigation of the stress response, and also contribute to a healthy immune system function in fish and higher vertebrates (Snyder and Hennessey 2003).

Additional factors implicated in the decline in lake whitefish condition include density-dependent mechanisms (Madenjian et al. 2002), climate/temperature change, and

parasitism (Mohr and Nalepa, 2005). Furthermore, movement of lake whitefish to areas outside of their normal range in search of *Diporeia* spp. can change population distributions, reduce weight, growth, and recruitment (State of Great Lakes 2005), which may have broader implications on increased susceptibility to disease.

II. Potential roles of disease in the decline of fisheries

1) General overview

There is mounting evidence that infectious diseases play an important role in the dynamics of animal populations and communities (Holmes 1996). In general, organisms inhabiting healthy, functioning ecosystems may resist infections better than those inhabiting dysfunctional ecosystems (Folke et al. 2004). Malnutrition (Beck and Levander 2000), thermal stress from climate change (Harvell et al. 1999), and toxic chemicals (Khan 1990) are among many stressors that can suppress immune functions, a pertinent risk for animals lacking sufficient energy (Rigby and Moret 2000), thereby increasing susceptibility to infectious disease. Hence, stressed individuals should be, generally, more susceptible to infection (Scott 1988; Holmes 1996).

However, an opposing prediction occurs when looking at things from the view point that epizootics are more likely to occur and will have a greater impact on populations with higher host densities (Anderson and May 1986). Thus, as stress depresses population densities, the chance of an epizootic occurring also decreases because contact rates between infected and uninfected individuals are reduced (Lafferty

and Holt 2003). For instance, a swim bladder nematode, *Cystidicola stigmatura*, which parasitized lake trout (*Salvenius namaycush)* was purportedly extirpated from the Great Lakes after numerous stressors (see previous section) had reduced trout populations below transmission threshold levels (Black 1983). On the other hand, some stressors, such as eutrophication, may increase host parasitism through elevated host densities (Lafferty and Holt 2003). Eutrophication is the most commonly observed stressor found in association with increased parasitism in both invertebrates and fishes (Lafferty 1997). Other possible effects of stressors include adverse consequences on the parasite itself, or increased host susceptibility to toxins if already parasitized (Guth et al. 1977; Stadnichenko et al. 1995). For example, some toxic metals and chemicals have been reported to have negative effects on intestinal helminthes (Lafferty 1997) and free-living stages of parasites (Evans 1982), while having negligible effects on their host species.

Thus, diseases in wild fish populations result from complex interactions among pathogens, the hosts they infect, and the environment within which they all reside. (Snieszko 1973; Hedrick 1998; Reno 1998). The fact that different pathogens respond to stressors or environmental changes in different ways, and that stressors can increase or decrease disease, are both essential in understanding the effects diseases may have at the population-level (Lafferty et al. 2004). For example, stressors tend to have mitigating effects on infectious diseases when transmission is mainly between members of the same population and host specificity is high, but tend to aggravate the effects of infectious diseases when pathogens are generalists or persist in a resistant environmental reservoir (Lafferty and Holt 2003). As such, the ecosystems within the GLB that are undergoing

significant alterations (e.g. food-web shifts, interactions between exotic and native species, etc.) may be at an increased risk for various epizootics caused by some pathogens, but may be less susceptible to others.

Globally, a number of diseases caused by parasites, bacteria, fungi, and viruses have generated large scale epizootics and/or mortalities in fishes. For example, the parasitic monogenean fluke, *Gyrodactylus salaris*, decimated native Norwegian salmon populations after being introduced with imported Atlantic salmon (*Salmo* salar) smolts from Sweden (Johnsen and Jensen 1986; Sattuar 1988). Native populations of Norwegian Atlantic salmon were in great peril, forcing Norwegian authorities to destroy all resident fish in 30 rivers in an attempt to eradicate the parasite (Hindar et al. 1991). Similarly, ichthyophoniasis, caused by the fungus *Ichthyophonus hoferi*, has been associated with population declines in Atlantic herring (*Clupea harengus*; Daniel 1933; Sindermann 1958; Mellergaard and Spanggaard 1997) and Pacific herring (*Clupea pallasi*; Marty et al. 2003). Marty et al. (2003) linked two disease epizootics caused by *Icthyophonus hoferi* and viral hemorrhagic septicemia virus in Pacific Herring inhabiting Prince William Sound, Alaska, to increased natural mortality and poor subsequent recruitment in the years following these outbreaks. Moreover, some aquatic disease epizootics have caused enough devastation to spur mitigating legislation. Such was the case in the issuance of the 1937 Diseases of Fish Act of Great Britain, which was enacted in response to widespread furunculosis, a serious disease caused by *Aeromonas salmonicida*, within wild fish populations in the United Kingdom (Hill 1996).

A decade ago, massive fish kills involving the Atlantic Menhaden (*Brevoortia tyrannus*) occurred over a number of years in the Chesapeake Bay and its tributaries

(Hargis 1985; Blazer et al. 1999). Such acute mortalities were originally believed to be caused by the toxin-producing heterotrophic dinoflagellate *Pfisteria piscicida* (Burkholder et al. 1992, 1995, 2001), yet further investigations provided evidence that the mortalities are caused by the oomycete, *Aphanomyces invadans* (Kiryu et al. 2002).

As is the case globally, many diseases present within the GLB can have significant effects on fishes at the population level. Viral hemorrhagic septicemia virus (VHSV), the etiological agent of viral hemorrhagic septicemia (VHS), is a rhabdovirus that causes significant pathology in infected hosts and is capable of generating large scale mortality events. Viral hemorrhagic septicemia virus has invaded the Great Lakes, spreading from the St. Lawrence River to the straights of Mackinac (Lake Huron), causing massive fish kills in Great Lakes muskellunge (*Esox masquinongy masquinongy*), lake whitefish, walleye (*Stizostedion vitreum*), gizzard shad (*Dorsoma cepadianum*), yellow perch (*Perca flavescens*), and freshwater drum (*Aplodinotus grunniens*) (Elsayed et al. 2006; Faisal et al. in prep; Whelan 2007).

Infectious pancreatic necrosis virus (IPNV, Birnaviridae) is another disease that has caused widespread infection and mortality in freshwater and marine fishes worldwide and has been isolated from many species of fish and invertebrates (Faisal and Hnath 2005). Although the presence of IPNV is necessary for disease to ensue, many other factors are also important, such as viral strain, environment, and age of the host species (Jarp et al. 1994; Hill 1982; Smail et al. 1992). This vertically transmitted virus causes epizootics in a number of cultured fishes, including members of the genera *Oncorhynchus*, *Salmo*, *Salvelinus*, (Reno 1999) and has been isolated from numerous sites within the GLB (Beyerle and Hnath 2002). Among the few GLB recovered isolates

that have been further analyzed, mortalities of up to 88% were generated in brook trout (*Salvelinus fontinalis*) subjected to waterborne exposure (McAllister 2003).

Another viral disease of fishes with serious implications is the large mouth bass virus (LMBV). A member of the Family Iridoviridae, this ranavirus has been identified as the causative agent of mortalities within numerous bodies of water within the southern United States (Faisal and Hnath 2005). In 2000, substantial die-offs involving largemouth bass (*Micropterus salmoides*) in a lake bordering Michigan and Indiana were attributed to the LMBV (Grizzle and Brunner 2003), and subsequent evaluations indicate that this virus was spreading northward, westward, and eastward in southern Michigan (Faisal and Hnath 2005). This disease is primarily manifested within the swim-bladders of infected individuals, where it causes varying degrees of hemorrhage (Faisal and Hnath 2005), with or without the presence of a brown/yellow exudate (Grizzle and Brunner 2003).

The microsporidians *Glugea hertwigi* and *Glugea anomala* have caused large mortality events in rainbow smelt (*Osmerus mordax*) within Lakes Erie and Ontario and in three-spine stickleback (*Gasterostreus aculeatus*) in Michigan's upper-peninsula, respectively (Nepszy et al. 1978; Faisal unpublished data). *Myxobolus cerebralis*, the etiological agent of whirling disease, is a myxosporidian that can cause substantial mortalities in both captive and wild fish populations (Gilbert and Granath 2003). For example, Rognlie and Knapp (1998) implicated *M. cerebralis* to cause a 90% decrease in wild rainbow trout (*Onchorhynchus mykiss*) residing in a stretch of the Madison River, Montana. This parasite has a two stage life cycle, during which it alternates between oligochaete and teleostean hosts (Gilbert and Granath 2003), and is one of the most

pathogenic myxosporidians known to fish (Hedrick et al. 1998). Rainbow trout are most susceptible to the disease, although many other salmonids, such as brook trout, brown trout (*Salmo trutta*), chinook salmon, and Atlantic salmon are also infected (Gilbert and Granath 2003). In 1966, whirling disease was detected in 3 commercial hatcheries within Michigan (Yoder 1972). Despite great efforts to eradicate and contain this disease, *M. cerebralis* was detected in the Manistee and Ausable rivers; however, the clinical form of the disease has never been observed within the GLB (Faisal and Hnath 2005).

Bacterial diseases can also have major impacts on fish populations within the GLB. For example, bacterial kidney disease (BKD), caused by a Gram positive diplobacilli known as *Renibacterium salmoninarum*, has been reported from areas wherever susceptible salmonid populations reside (Fryer and Sanders 1981; Klontz 1983), including the GLB. As early as 1955, BKD was reported in Michigan's brook trout (Allison 1958), and was later isolated from other salmonids inhabiting the GLB, such as coho salmon (*Onchorhynchus kisutch*), as well as chinook (*Oncorhuncus tshawytcha*) and kokanee salmon (*Oncorhuncus nerka*; Maclean and Yoder 1970). In the late 1980's, a substantial epizootic of BKD occurred in Chinook salmon residing within Lake Michigan (Holey et al. 1998). Additionally, Eissa (2005) was able to isolate the bacterium from hatchery raised brook trout. Bacterial kidney disease has also been reported in salmonids of the genus *Coregonus* (Jonas et al. 2002). *Renibacterium salmoninarum* has also been isolated from non-salmonid fish species, including Lake Ontario sea lamprey (*Petromyzon marinus*; Eissa et al. 2004). The epizootic of BKD that occurred in Lake Michigan generated mass mortalities in Chinook salmon that reduced the population by 50% or more (Holey et al 1998). Factors that likely contributed to this

outbreak were heavy parasitic infection rates, elevated densities of Chinook salmon, and depressed levels of prey species, namely alewives (*Alosa pseudoharengus*; Holey et al. 1998; Hansen and Holey 2001).

A recent example of disease effects at the population level is the massive fish kill that involved common carp (*Cyprinus carpio*) in the St. Lawrence River, Quebec, Canada (Monette et al. 2006). Over 25,000 dead or moribund carp were removed from a small portion of the affected areas, while true numbers of affected individuals were most probably higher. These deaths were ultimately attributed to opportunistic bacterial infections by *Aeromonas hydrophila* and *Flavobacterium* spp. that were secondary to immuno-supression brought about by physiologic (i.e., spawning) and environmental (i.e. high temperatures and low water levels) stressors.

Another group of bacterial diseases that generate more mortality than all other pathogens combined within the hatcheries of the state of Michigan are caused by members of the genus *Flavobacterium* (Records of Michigan DNR Fish Health Laboratory; Faisal and Hnath 2005). *Flavobacterium columnare*, *F. branchiophilum*, and *F. psychrophilum,* the etiological agents of columnaris disease, bacterial gill disease, and cold-water disease, respectively, are yellow-pigmented, filamentous, Gram negative bacilli that are capable of generating varying degrees of morbidity and mortality in both captive and wild fish populations (Shotts and Starliper 1999). For example, *F. columnare* reportedly caused 6% mortality in cultured tiger muskellunge (male *Esox lucius* x female *Esox masquinongy*) within a 7 day period (Pecor 1978), severe fin erosion in cultured walleye (Clayton et al. 1998), and was responsible for significant mortalities in a number of wild fishes (Becker and Fujihara 1978; Fijan 1968; Chen et al. 1982). Moreover, *F.*

columnare was isolated from spawning Chinook salmon at multiple weirs in Michigan, where it was associated with varying degrees of pathological effects (Loch and Faisal, unpublished data). *Flavobacterium psychrophilum* is responsible for high annual losses of production coho salmon, brown trout and rainbow trout, particularly in facilities that utilize open water sources reaching cold temperatures (near 0°C) in winter months. Cold water disease has been reported to cause 85-90% mortalities in hatchery lake trout (Schachte 1988). *Flavobacterium branchiophilum* is also capable of generating high mortalities in cool and cold water fish species (Shotts and Starliper 1999), but is more typically associated with high morbidity (up to 100%) via a chronic proliferative response in gill epithelia (Noga 2000).

2) Specific bacterial pathogens

a) *Carnobacterium* spp.

Lactobacilli and other closely related species are Gram positive, non-spore-forming rods that constitute a normal component of the gastrointestinal and urogenital flora of vertebrates, including fish (Ringo et al. 1995; JoÈborn 1998; Ringo and Gatesoupe 1998; Gonzalez et al., 2000). In fish, lactobacilli have also been associated with mortalities, septicemias, chronic inflammation, and necrotic changes within internal organs (Rucker et al. 1953; Ross & Toth 1974; Cone, 1982). Based on extensive genetic studies, biochemical fermentation patterns, and association with fish diseases, Hiu et al. (1984) proposed a new *Lactobacillus* species; *L. piscicio*la. Studies demonstrated that *L.*

pisciciola was associated with post spawning morbidity and mortality, kidney granulomas, massive chronic inflammation, and pseudomembrane formation (Herman et al. 1985; Michel et al. 1986; Humphrey et al. 1987). Mature salmonids seem to be the most susceptible to infection, though infections of fry and fingerlings have also been reported (Hiu et al. 1984). Additionally, Michel et al. (1986) isolated the bacterium from the common carp.

Lactobacillus spp. went through a number of taxonomic changes when Collins et al. (1987) grouped *Lactobacillus divergens, L. piscicola,* as well as some catalase-negative, non-spore forming bacilli from poultry into a new genus; *Carnobacterium.* The main characteristics separating *Carnobacterium* spp. from typical lactobacilli are their inability to grow on acetate media and their production of oleic acid rather than *cis*-vaccenic acid (Hiu et al. 1984). *Carnobacterium pisciola* has been isolated from salmonid and non-salmonid species suffering from a range of infections, some of which were associated with high mortalities (Baya et al. 1991; Starliper et al. 1992; Toranzo et al. 1993). Due to kidney granulomas and pseudomembranes that are often associated with *C. pisciola* infections, the disease in salmonids is often refered to as "pseudokidney disease" (Austin and Austin 1987; Noga 2000). Based on recent phenotypic and genotypic analyses by Mora et al. (2003), it was concluded that *Lactobacillus maltaromicus*, originally isolated from milk, and *Carnobacterium piscicola,* were synonyms. Therefore, Mora et al. (2003) reclassified these species into a combination *novum, Carnobacterium maltaromaticum.*

An interesting property of *C. maltaromaticum* is the ability of some strains to produce a 126 kilo dalton protein, piscicolin, which has antimicrobial properties (Gibbs et

al 2004). The effects of piscicolin *in vivo* in fish are currently unknown; however, in a study by Ingham et al (2003), the antibacterial activity of systemically injected piscicolin 126 was studied in mice experimentally infected with *Listeria monocytogenes*. This study showed a statistically significant reduction in clinical signs associated with listeriosis, as well as a decrease in the number of recovered colony-forming-units in individuals that were injected with P126 15 minutes before and 30 minutes after being experimentally infected with *L. monocytogenes*.

b) *Aeromonas* spp.

Most species belonging to the genus *Aeromonas* are oxidase-positive, facultatively anaerobic, glucose-fermenting, Gram-negative bacilli that are ubiquitous to the aquatic environment, including fresh, brackish, and marine waters (Hazen et al 1978; Alonso et al 1994). They are associated with disease in both poikilothermic and homeothermic organisms, including an increasing number of human case reports (Austin and Adams 1996; Janda and Abbott 1996). A number of the motile *Aeromonas* spp. can cause infections in fish, typically termed motile aeromonad septicemia (Cipriano 2001; Aoki 1999). Manifestation of disease is frequently associated with various environmental or physiological stressors, such as warm water temperatures, poor water quality, parasitic infections, or poor over-wintering conditions (Austin and Adams 1996; Aoki 1999; Noga 2000).

Disease manifests as a generalized septicemia typical of other gram-negative rods, with acute, chronic, and sub-clinical forms (Cipriano 2001). Pathological effects induced

by infections with members of this complex include fin/tail rot, dermal ulceration, and hemorrhagic septicemias. These septicemias are characterized by small surface lesions, which may lead to sloughing of scales, hemorrhaging in the gills and anus, ulcers, abscesses, exophthalmia, and abdominal swelling (Austin and Adams 1996). The presence of ascites within the peritoneum, anemia, and swelling of the kidney and liver may also occur (Austin and Adams 1996). Motile aeromonads are also part of the natural intestinal microflora present within healthy fish (Trust et al. 1974); thus, their presence is not necessarily indicative of disease.

The heterogeneous complex of *Aeromonas* species currently resides within the family *Aeromonadaceae* (Colwell et al. 1986) and has undergone many reclassifications within the last three decades (Carnahan 1993). In the mid to late 1970s, the majority of aeromonads belonged to one of two groups; those that grew at 35-37°C, were motile, and were primarily associated with human infections, known as the mesophiles, and those that grew better at lower temperatures and were responsible for infections in fish, termed the pscychrophiles (Janda and Abbot 1998). Since then, the genus *Aeromonas* has undergone much taxonomic flux and the expansion of species belonging to this group has been quite astounding (Janda and Abbott 1998).

Currently, the genus contains the following species according to the latest edition of *Bergey's Manual of Systematic Bacteriology* (Holt et al. 2000): *Aeromonas hydrophila, Aeromonas bestiarum, Aeromonas salmonicida, Aeromonas caviae, Aeromonas media, Aeromonas eucrenophila, Aeromonas sobria, Aeromonas veronii (biovars veronii and sobria), Aeromonas jandaei, Aeromonas schubertii, Aeromonas trota, Aeromonas allosaccharophila, Aeromonas encheleia, Aeromonas popoffii,* as well

as two homology groups without species names, *Aeromonas* sp. HG11 and *Aeromonas* sp. HG13. Additionally, three novel species have recently been described; *Aeromonas culicicola* (Pidiyar et al. 2002), *Aeromonas simiae* (Harf-Monteil et al. 2004), and *Aeromonas molluscorum* (Minana-Galbis et al. 2004).

In addition to the significant taxonomic changes and expansion in the number of species within the genus *Aeromonas*, the heterogeneity in biochemistry, genetics, and serology among and between species are substantial (Cipriano 2001). For this reason, as well as the lack of clear-cut phenotypic tables useful for the dichotomization of the numerous groups of aeromonads, both newly and previously described (Abbott et al. 2003), identification is never a simple process.

A number of *Aeromonas* spp. are associated with fish disease. For example, *A. sobria*, and *A. hydrophila* have been recovered from fish exhibiting signs of Epizootic Ulcerative Disease (EUI; McGarey et al. 1991), and *A. veronii* biovar *sobria* has recently been implicated as the possible etiological agent of EUI (Rahman et al. 2002). *Aeromonas sobria* was recovered by Toranzo et al. (1989) from a large mortality event involving adult gizzard shad (*Dorosoma cepedianum*). *Aeromonas hydrophila* has also been recovered from a wide range of freshwater fish and marine fish (Austin and Adams 1996; Aoki 1999). Additionally, *A. bestiarum*, *A. salmonicida*, and *A. veronii* were retrieved from common carp, albeit at varying degrees of virulence (Kozinska et al. 2002). The isolation of *A. allosacchaophila* from diseased elvers was reported by Martinez-Murcia et al. (1992). Moreover, *Aeromonas* spp. cause disease in many warm water fishes, including minnows, baitfishes, channel catfish (*Ictalurus punctatus*), striped

bass (*Morone saxatalis*), large mouth bass (*Micropterus salmoides*; Cipriano 2001), and tilapia (*Oreochromis niloticus*; Faisal et al. 1984; 1989).

Although infections by motile aeromonads predominate in warmer temperatures, infections can occur in cool and cold water species. For example, *Aeromonas sobria* was reported to cause significant mortalities in farmed perch (*Perca fluviatilis*) by Wahli et al. (2005) and Michel (1981) isolated a motile aeromonad from diseased perch. Paniagua et al. (1990) demonstrated that some motile aeromonads recovered from river water were virulent to rainbow trout (*Oncorhynchus mykiss*) upon experimental infection.

A number of virulence factors associated with motile aeromonads have been described. The presence of adhesins in strains of *A. hydrophila, A. sobria,* and *A. caviae* were demonstrated to facilitate adherence to animal cell lines with mucous-cell receptors, and the proportion of *A. hydrophila* strains able to bind was significantly greater than that of the *A. sobria* and *A. caviae* strains (Ascencio et al. 1998). The same authors were also able to correlate specific binding of collagen, fibronectin, and laminin to isolates from diseased fish. Moreover, *A. hydrophila* can attach to selected fish cells, such as erythrocytes, and tissue proteins via adhesins that are very specific for D-mannose and L-fucose side chains on surface polymers (Trust et al. 1980; Ascensio et al. 1991). Furthermore, a surface array protein (S-layer) was described from virulent isolates of *A. hydrophila* (Dooley and Trust 1988) and from motile aeromonads recovered from clinically diseased catfish (Ford and Thune 1991). These S-layers are known to confer a degree of protection from lytic action by complement and bacteriophages, and reduce phagocytosis by leukocytes (Dooley et al 1988).

Extracellular products (ECPs) that play varying roles in virulence are also produced by some motile aeromonads. For instance, ECPs with hemolytic and proteolytic activity are capable of eliciting pathology when injected into fish (Allan and Stevenson 1981). Extracellular metallo-proteases and serine-proteases have also been identified (Nieto and Ellis 1986) and have been suggested to increase invasiveness, protect the pathogen against the bacteriocidal effects of serum, and provide nutrients for growth following host tissue destruction (Leung and Stevenson 1988). Many other ECPs have also been identified, such as enterotoxins, hemolysins, hemagglutinins, and endotoxins (Cahill 1990).

Aeromonas salmonicida is a non-motile, psychrophilic, obligate pathogen of fish that causes furunculosis and other diseases that devastate populations of numerous fishes, including including salmon, trout, grayling, char (Cipriano and Bullock 2001), and whitefish (*Coregonus* spp.) from Finland (Rintamäki & Koski 1987). Additionally, infections caused by *Aeromonas salmonicida* have been reported in an increasing number of non-salmonid species (Fijan 1972; Bootsma et al. 1977; Shotts et al. 1980; Wiklund 1995; Bernoth and Korting 1992; Wilson and Holliman 1994). *Aeromonas salmonicida* also causes carp erythrodermatitis (Fijan 1972), cutaneous ulcerative disease in goldfish (Shotts et al. 1980), and "head ulcer disease" in Japanese eels (Kitao et al. 1984).

In fish, diseases caused by *Aeromonas salmonicida* have been studied for quite some time, beginning in 1890, when Emmerich and Weibel described a bacterium associated with diseased trout within a hatchery. Initially known as *Bacillus salmonicida* (syn. *Bacterium salmonicida* and *Bacterium trutta*) (Emmerich and Weibel 1890), *Aeromonas salmonicida* has become one of the most studied bacterial piscine pathogens.

Furunculosis, so-named for the presence of boil-like lesions that are known as furuncles, which may be present in the musculature (Austin and Adams 1996). Additional clinical signs include hemorrhages within the fins, musculature, orbits of the eye, and liver, exopthalmia, lethargy, bloody discharge from the nares and anus, swelling of the spleen, and renal necrosis. Rates of mortality associated with these chronic infections are typically low (McCarthy and Roberts 1980). Another form of disease caused by *A. salmonicida*, termed acute furunculosis, manifests as a septicemia accompanied by melanosis, inappetance, lethargy, petechiae in the fins, and varying degrees of splenomegaly. Mortalities typically occur within 2-3 days (McCarthy 1975). Furthermore, a peracute form has also been found in association with high mortalities in salmonid fingerlings (McCarthy and Roberts 1980; Austin and Austin 1993). An intestinal manifestation of furunculosis has also been described, in which intestinal inflammation and anal inversion occurs (Amlacher 1961). Latent forms of *A. salmonicida salmonicida* infection, recently termed covert infections (Hiney et al. 1997), have also been identified. These individuals develop clinical disease only under certain conditions of environmental and physical stress. Covertly infected individuals act as reservoirs for the disease, capable of transmitting the disease to uninfected hosts (Hiney et al 1997).

A number of virulence factors play major roles in the pathogencity of *Aeromonas salmonicida*. Virulence mechanisms fit into two categories; cell-surface structures and extracellular products (ECPs; Hiney and Oliver 1999). *Aeromonas salmonicida* can produce an additional surface protein microcapsule (Kay and Trust 1997), traditionally termed S-Layer, but known as A-layer in *A. salmonicida* for historical reasons (Udey and

Fryer 1978). This 50 kDa protein has been shown to be immunologically conserved among many *A. salmonicida* strains (Kay et al. 1984) and strong evidence in favor of its role as a primary virulence factor exists. The ability of the A-layer to bind immunoglobulins and other extracellular proteins has been purported to "mask" the bacterium's own immunogenic receptors, thus allowing evasion of the host immune system (Kay and Trust 1997). Other reported functions of the A-layer include promotion of bacterial penetration and adhesion, inhibition of complement-mediated lysis, and protection from dessication in low nutrient environments (Hiney and Oliver 1999). Another major component of the cell surface of *A. salmonicida* is lipopolysaccharide (LPS), a major constituent of all Gram-negative bacteria. The exact role that LPS plays in the virulence of *A. salmonicida* is still not fully realized; however, Cipriano and Blanch (1989) reported that only strains with an intact A-layer and LPS were virulent for brook trout.

Extracellular products of *A. salmonicida* play a large role in the pathological effects associated with infection, as evidenced by the killing of susceptible fish upon injection with crude extracellular material (Munro et al. 1980; Ellis et al. 1981). Three main types of ECPs have been identified by Ellis (1997), which are proteases, membrane damaging toxins, and other toxins, such as H-lysin. Among these, glycerophospholipid-cholesterol acyltransferase complexed with LPS has been suggested as the most important factor in lethal toxicity and pathology associated with ECPs (Ellis 1997).

Currently, at least 5 subspecies of *Aeromonas salmonicida* are recognized; -*salmonicida, achromogenes, masoucida, smithia* (Holt et al. 2000), and *pectinolytica* (Pavan et al. 2000). *Aeromonas salmonicida salmonicida* is the etiological agent of

furunculosis, while subspp. *achromogenes, masoucida, smithia* are associated with atypical forms of the disease that often involve external pathology (i.e. dermal ulcerations) with or without septicemia (Cipriano and Bullock 2001). *A. salmonicida pectinolytica* is a relatively new subspecies that was recently isolated from a heavily polluted river near Buenos Aires city but has yet to be reported in association with disease (Pavan et al. 2000).

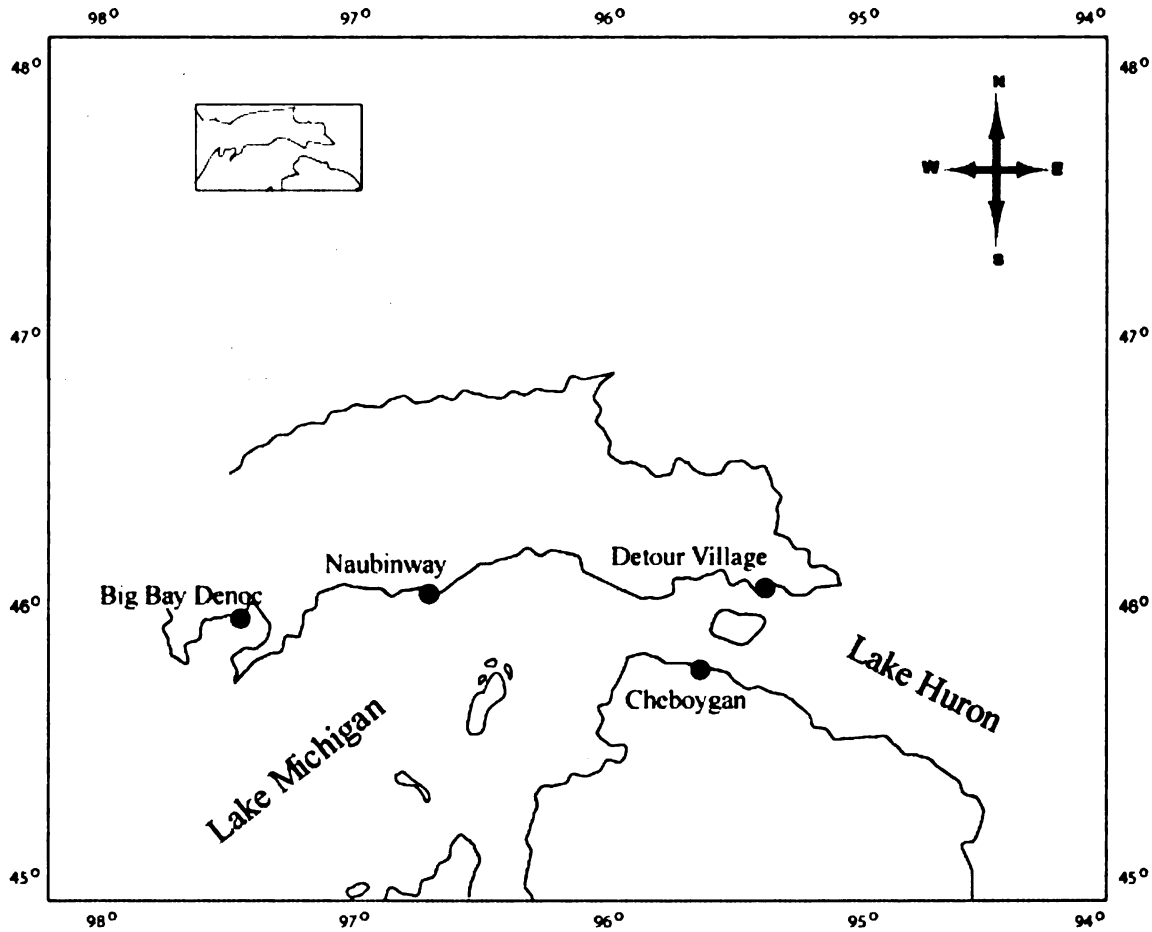

Figure 1. Four sites from which Lake whitefish were collected for this study. The Lake Michigan stocks were from management units WFM-01 (Big Bay de Noc) and WFM-03 (Naubinway); the Lake Huron stocks were from WFH-01 (Cheboygan) and WFH-02 (Detour). These sites were selected due to the large spawning aggregations of whitefish that inhabit these areas, as well as their relevance in commercial fisheries.

CHAPTER TWO

ISOLATION OF *CARNOBACTERIUM MALTAROMATICUM* FROM LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*) IN LAKES MICHIGAN AND HURON

ABSTRACT

Carnobacterium maltaromaticum, the causative agent of Pseudokidney Disease, has been isolated from lake whitefish (*Coregonus clupeaformis*) caught from Lakes Michigan and Huron, USA, during a three-year study. Twenty-three *C. maltaromaticum* isolates were recovered from kidneys and swimbladders of infected fish. The isolates were Gram-positve, nonmotile, facultatively anaerobic, asporogenous rods arranged in palisades. They did not produce catalase, cytochrome oxidase, or H_2S, and did not reduce nitrate. Isolates grew on Cresol Red Thallium Acetate Sucrose Inulin Agar, a selective and differential medium for *Carnobacterium* spp. Variability in mannitol and innulin fermentation, as well as arginine dihydrolase production, considered a key in *Carnobacterium* spp. dichotomization, was observed among our isolates. Amplification of selected 16S and 23S rRNA regions specific to *C. maltaromaticum* and subsequent sequencing of generated amplicons, yielded a 97% nucleotide match with *C. maltaromaticum* sequences deposited in GenBank. Additionally, sequencing of the piscicolin 126 precursor gene yielded a 98% nucleotide match with GenBank sequences. Phylogenetic analyses showed a high degree of relatedness of the lake whitefish isolates

with *C. maltaromaticum*. Fish from which the bacteria was isolated showed splenomegaly, pallor and mottling in the liver, congestion in the spleen and kidney, and opacity/thickening of the swim bladder wall with accumulation of a mucoid exudate within the lumen. Histopathological examination showed congestion within kidneys and spleens, vacuolation and bile stasis within the liver, and varying degrees of hyperplasia within the epithelium of the swim bladder. The prevalence of infection varied significantly between sites and seasons, with the highest infections occurring in winter sampling periods.

INTRODUCTION

Lactobacilli and other closely related species are a normal component of the gastrointestinal and urogenital flora of vertebrates, including fish (Ringo et al. 1995; JoÈborn 1998; Ringo and Gatesoupe 1998; Gonzalez et al. 2000). However, in fish, lactobacilli have also been associated with mortalities, septicemias, chronic inflammation, and necrotic changes within internal organs (Rucker et al. 1953; Ross & Toth 1974; Cone 1982). Based on extensive genetic studies, biochemical fermentation patterns, and association with fish diseases, Hiu et al. (1984) proposed a new *Lactobacillus* species; *L. pisciciol*a. Studies demonstrated that *L. pisciciola* was associated with post spawning morbidity and mortality, kidney granulomas, massive chronic inflammation, and pseudomembrane formation (Herman et al. 1985; Michel et al. 1986; Humphrey et al. 1987). Salmonids seem most susceptible when they are sexually mature, though infections in fry and fingerlings have also been reported (Hiu et al. 1984). Additionally, Michel and colleagues (1986) isolated the bacterium from the zebra danio (*Brachydanio rerio*) and the common carp (*Cyprinus carpio*).

Lactobacillus spp. have gone through a number of taxonomic changes. Collins et al. (1987) grouped *Lactobacillus divergens, L. piscicola,* as well as some catalase-negative, non-spore forming bacilli from poultry into a new genus; *Carnobacterium. Carnobacterium pisciola* was isolated from salmonid and non-salmonid species suffering from a range of infections, some of which were associated with high mortalities (Baya et al. 1991; Starliper et al. 1992; Toranzo et al. 1993). Due to kidney granulomas and pseudomembranes that are often associated with *C. pisciola* infections, the disease in

salmonids is often refered to as "Pseudokidney Disease" (Austin and Austin 1987; Noga 2000). Based on recent phenotypic and genotypic analyses, Mora et al. (2003), concluded that *Lactobacillus maltaromicus*, originally isolated from milk, and *Carnobacterium piscicola,* were synonyms. Therefore, Mora et al. (2003) reclassified these species into a combination *novum, Carnobacterium maltaromaticum.*

Herein, I report on the retrieval of 23 bacterial isolates from kidneys and swim bladders of lake whitefish inhabiting Lakes Huron and Michigan, USA, whose morphological, cultural, and biochemical characteristics resemble those of *C. maltaromaticum* (Mora et al. 2003). This is considered a new host species and geographic location record for *C. maltaromaticum.*

MATERIALS AND METHODS

Fish and sampling. Between the fall of 2003 and the summer of 2006, lake whitefish were collected for bacteriological analyses from 4 representative stocks on four sampling occasions per year (~30 fish/site/season, Table 1). The two Lake Michigan sites, Big Bay de Noc (latitude 4527-4548.20; longitude 8535-8722) and Naubinway (latitude 4553-4603; longitude 8513-8535), and the two Lake Huron sites, Detour Village (latitude 4555-4556.97; longitude 8350.5-8419.12) and Cheboygan (latitude 4540.24-4548; longitude 8424-8437.87) were selected for this study due to their association with large spawning aggregations and accessibility via commercial fishing (Figure 1). A total of 1286 lake whitefish from the four sites were sampled throughout the course of this study.

Fish were captured using commercial trap nets and, when necessary, commercial gill nets, with only live fish being used for the study. The collected fish were transported alive to the Chippewa Ottawa Research Authority (CORA) Fishery Enhancement Facility near Hessel, Michigan. The fish were then chilled on ice and transported to the Aquatic Animal Health Laboratory at Michigan State University, East Lansing, MI, where they were immediately subjected to thorough internal and external examinations.

Bacterial isolation. Bacterial samples retrieved from lake whitefish were collected from kidneys and visible lesions. Tissue samples were streaked onto Trypticase Soy Agar (TSA; Remel, Lenexa, KS) and Cresol Red Thallium Acetate Sucrose Inulin (CTSI) agar (all ingredients are from Sigma Chemical Co., St. Louis, MO), a selective

and differential medium for *Carnobacterium* spp. (Wasney et al. 2001), and incubated at 22 °C for up to 72 hr. Periodic examination of bacterial growth was recorded, and individual colonies were sub-cultured onto TSA and then incubated for 24 hours at 22°C. A total of 23 isolates were recovered and examined (Table 2).

Biochemical characterization. Isolated bacteria were initially identified using a battery of morphological and biochemical tests, including Gram reaction, cytochrome oxidase, catalase reaction (3% H_2O_2), motility, indole production, hydrogen sulfide production, oxidation/fermentation reaction (BD Scientific, Sparks, MD), methyl red, acetoin production from glucose (Voges-Proskauer), nitrate reduction, citrate utilization, TSI reaction, ONPG (*o*-nitrophenyl-*β*-D-galactopyranoside), lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, esculin hydrolysis, phenylalanine deaminase (BD Scientific), growth on acetate agar (pH 5.4), and growth on Cresol Red Thallium Acetate Sucrose Inulin medium at pH 9.1, as described by Wasney et al. (2001). Production of acid from the following carbohydrates was examined in phenol red broth base at a final concentration of 1%: adonitol, arabinose, cellobiose, dextrose, galactose, glycerol, inositol, innulin, lactose, malonate, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Results were recorded up to 7 days post-inoculation with the following exceptions: methyl red, Voges-Proskauer, indole production, Simmons citrate, and TSI reactions were read at 2 days. All materials and reagents were purchased from Remel Inc. (Lenexa, KS) unless specified otherwise.

Confirmation of isolate identification with polymerase chain reaction and gene sequencing. Representative colonies with the typical morphological and biochemical characteristics of *C. maltaromiticum*, as listed in Bergey's Manual of Determinative Bacteriology (Holt et al. 2000) were selected for this study. Single colonies were resuspended in Trypticase Soy Broth (Remel), and incubated overnight at 22 °C. Their DNA was extracted using a Qiagen DNeasy Tissue Kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's protocol for Gram-positive bacteria. PCR amplification was performed using the following primers: 1) 16S-4 (5′-GCT GGA TCA CCT CCT TTC T-3′) and 23S-7 (5′-GGT ACT TAG ATG TTT CAG TTC C-3′), which anneals to positions 1526-1542 of the 16S rRNA gene and positions 207-189 of the 23S rRNA gene in *E. coli* (Kabadjova et al. 2002; Pelle et al. 2005), and 2) PisA forward (5′-GTC ACA GCA TTG ATG CGT ATC-3′) and PisA reverse (5′-GAT GTG ATA CAG TCA GCA TGT-3′), which anneal to positions 1756-1777 and 2036-2057 on each side of the *pisA* precursor gene for the piscicolin 126 protein produced by *C. maltaromaticum* (*piscicola*)-JG126 strain (Pelle et al. 2005).

DNA template (4 µl) was combined with 2.5 µl of 10X PCR buffer containing 1.5 mMol $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.5 µl dNTP (Invitrogen), 1 µl of each primer, 17.5 µl distilled water, and 0.5 µl *Taq* Polymerase with loading buffer (Denville Scientific, Metuchen, NJ). The PCR amplification program was that of Pelle et al. (2005) with slight modifications, which included an initial denaturation at 94°C for 10 min, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final step of 10 min at 72°C. The amplified products were subsequently run on 1.5% agarose gel for 30 min at

50 volts and then stained with ethidium bromide for viewing via UV exposure. A 1 Kb plus DNA Ladder (Invitrogen) was used as a molecular marker.

Amplicons were purified using QIAquick PCR Purification kit (Qiagen). Upon purification, a portion of the product was used for ligation with pGEM-T vectors (Invitrogen) and incubated at 4°C overnight. The recombinants were then transferred into DH5-α competent cells (Invitrogen). The transformed cells were then heat shocked for 45 seconds in a 42°C water bath and subsequently placed on ice for 2 minutes. S.O.C. medium (Invitrogen) was then added to the cells (0.9 ml at room temperature) and the solution was incubated at 225 rpms at 37°C for 1 hour in an Incubating Shaker (Labnet, Edison, NJ). Thirty µl of isopropyl-beta-D-thiogalactopyranoside (IPTG, Denville Scientific) were then added and 500 µl of the bacterial suspension were aliquoted to SOB + ampicillin agar plates, on which X-gal (Denville) had been previously applied (BD Scientific). Plates were incubated at 37 °C overnight. Single, white, colonies were then picked up, inoculated into 500 µl S.O.B. broth, and incubated at 220 rpms at 37 °C for 4 hours. Clones were then submitted to Michigan State University Research Technology Support Facility for analysis.

Analysis of the sequence data. Generated sequences were analyzed using the BLASTn software from the National Center for Biotechnology Information, (NCBI, Bethesda, MD) to detect homologous sequences. Homology of the generated sequences with that of the database was assessed using the nucleotide database from NCBI. Using the CLUSTAL W program from Molecular Evolutionary Genetics Analysis (MEGA;

version 3.1), a phylogenetic tree was constructed using the Neighbor-Joining method as the bootstrap test of phylogeny.

Histopathological Analyses. Samples of tissue with gross clinical abnormalities were fixed in 10% buffered formalin. The fixed samples were then embedded within paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (Prophet et al. 1992).

Statistical Analyses. Statistical analyses were performed using the Chi-Square analysis of contingency tables through the Sigma Stat software (Jandel Corporation, Carlsbad, CA).

RESULTS

Throughout the course of this study, a number of bacterial species were isolated and characterized based on morphological, biochemical, and molecular attributes. Among these, 23 isolates were presumptively identified as *Carnobacterium maltaromaticum*. These isolates were subcultured onto both TSA and CTSI media. Following 24-48 hours incubation on TSA, at 22°C, the isolates produced colonies that were semi-translucent, whitish, entire, convex, and measured approximately 1-2 mm in diameter. It is noteworthy that upon primary isolation, colonies were semi-translucent, raised, striated, and with irregular margins. Growth on CTSI agar yielded complete, convex colonies surrounded by a yellow halo with a reddish-purple center (Figure 2). In 21 isolates, the number of colonies present upon primary isolation varied from 1-50/10 µl of kidney inoculum. This corresponds to a presence of approximately 1×10^2-5×10^3 colony forming units/g kidney tissue in infected individuals. In the two cases that involved the swim bladder, *C. maltaromaticum* growth was interconnected, profuse, and covered the inoculated area.

Presumptive *C. maltaromaticum* isolates were then subjected to morphological, cultural, and biochemical reactions. All 23 isolates were non-spore-forming, non-motile, short, straight bacilli (1.0-1.5 µm by 0.5 µm) arranged in palisades that did not produce catalase or cytochrome oxidase (Table 3). They were facultative anaerobes that did not produce H_2S, indole, phenylalanine deaminase, lysine decarboxylase, ornithine decarboxylase, did not reduce nitrates, and gave a negative Simmons citrate reaction. They all gave an acid slant over an acid butt Triple Sugar Iron reaction, with no H_2S or

gas being produced, were able to hydrolyze esculin, and were positive for mixed acid fermentation (MR+). Furthermore, all isolates produced acid from cellibiose, dextrose, galactose, maltose, mannose, salicin, and sucrose. In addition, no acid was produced from adonitol, arabinose, rhamnose, or xylose. No growth was achieved upon inoculation of acetate or MacConkey agars.

There were, however, discrepancies among some isolates regarding a few of the biochemical reactions. For example, the DV-1 and DV-8 isolates did not produce arginine dihydrolase, DV-1 did not produce *o*-nitrophenyl-β-D-galactopyranoside, and NB-6, 7, &13, DV-1, 2, 4, &7, and BD-1 did not produce acetoin (VP negative). Additionally, DV-8 produced acid from inositol, DV-3, DV-7, and CH-1 produced acid from raffinose, and DV-7 and DV-8 produced acid from sorbitol. Furthermore, NB-10, DV-1, and DV-5 did not produce acid from melibiose and DV-1 did not produce acid from trehalose or lactose. Other detected variabilities occurred in glycerol, innulin, and mannitol fermentation (Table 3).

Electrophoresis of the amplified rRNA from the 23 isolates generated 600 bp amplicons, as well as the three typical intergenic spacer region bands associated with *C. maltaromaticum* (Figure 3). Moreover, all isolates generated 300 bp bands when the *Pis*A primers were used for amplification (Figure 3).

Sequencing of the 1526-1542 16S rRNA gene stretch, the 207-189 of the 23S rRNA gene stretch, and the *pis*A precursor gene for the piscicolin 126 protein of our whitefish isolates showed high homology with the sequences contained within GenBank (Figure 4 & Figure 5). For example, the 16S rRNA sequence of the *C. maltaromaticum* isolates retrieved from lake whitefish had highly significant homology with the deposited

sequences in GenBank, as evidenced by the expectation value of 0.0 and the 97% nucleotide match of our sequence with it's homologue. The sequence corresponding to *pis*A precursor gene from the lake whitefish isolates had 98% nucleotide similarity with GenBank sequences and an expectation value of e -139.

The sequences generated by representative *C. maltaromiticum* isolates retrieved from lake whitefish using portions of the 16S and 23S rRNA clustered most closely with *C. maltaromaticum* isolates, followed by a cluster of *C. gallinarum* isolates (Figure 6).

The overall prevalence of *C. maltaromiticum* in the four lake whitefish stocks varied by season and by site (Table 4). The overall prevalence of whitefish infected with *C. maltaromiticum* varied among sites in a statistically significant fashion (χ^2=18.587; DF=3; P= < 0.001), with Naubinway having the highest prevalence, followed by Detour Village. There were also statistically significant differences in overall *C. maltaromaticum* infections between seasons (χ^2=27.740; DF=3; P=<0.001), with winter prevalences being the highest, followed by spring. However, no statistically significant differences in the overall prevalence of *C. maltaromaticum* infections between Lakes Michigan and Huron were observed (χ^2=0.611; df=1; P=0.435).

Lake whitefish from Detour Village exhibited waves of low-level infections, with peaks of infection occurring in the spring of 2005 and the winter of 2006, while high prevalence was detected in the Naubinway winter 2005 samples, accounting for nearly 80% of the infections detected within this site throughout the three year study. The only *Carnobacterium* infections that were detected in the other two sites came in the spring of 2006 and were found in only one individual from each sample.

In this study, we report on clinical signs and histopathology noticed in fish where *C. maltaromaticum* was the only pathogen detected. Among the most common clinical signs were mild to severe splenomegaly, varying degrees of hyperemia and friability within the kidney, mottling and/or pallor in the liver, and varying degrees of congestion within the testes. Additionally, of the two fish from which *C. maltaromaticum* was isolated from the swim bladders, copius amounts of an opaque, mucoid, exudate was present (Figure 7), along with hemorrhage and a general thickening/opacity of the swim bladder wall.

In stained tissue sections of infected fish, there was mild to moderate hepatocyte degeneration due to cytoplasmic vacuolation, as well as mild bile stasis, within the livers (Figure 8 & Figure 9). Both spleen and kidney tissues had varying degrees of congestion (Figure 10 & Figure 11). In the cases where *C. maltaromiticum* was isolated from fluid within the swim bladder, thickening of the swim bladder wall with fibrous connective tissue, some degree of neovascularization, as well as fibrin and cellular debris within the lumen, were evident. Additionally, the epithelial lining of the swim bladder exhibited varying degrees of hyperplasia (Figure 12).

DISCUSSION

The morphological, biochemical, and molecular assays performed in this study confirm that the 23 isolates discussed were indeed *C. maltaromiticum*. This report is considered the first in which *C. maltaromiticum* was obtained from lake whitefish within the Great Lakes. *C. maltaromiticum* could potentially be the cause of the moderate pathological effects observed in the infected lake whitefish tissues. This is not totally surprising, as *C. maltaromiticum* has been reported to cause diseased conditions and mortalities (Cone 1982; Hiu et al. 1984; Herman et al. 1985; Michel et al. 1986; Baya et al. 1991; Starliper et al. 1992; Toranzo et al. 1993). The isolation of *C. maltaromiticum* from kidneys and swim bladders of lake whitefish indicated that the infection may have been systemic in nature.

When comparing the prevalence of *C. maltaromiticum* infections in the four stocks of lake whitefish, there was a statistically significant difference among the four sites. The highest prevalence was found within the Naubinway populations, but it is interesting to note that most of the infections were detected in the winter of 2005 samples. We were unable to link other environmental factors to the high presence of *C. maltaromiticum* at this particular site and time. However, in a parallel study conducted by Sutton and Koops (ongoing study), lake whitefish sampled from four stocks within Lake Michigan, 2 of which were Naubinway and Big Bay de Noc, fish from the Naubinway site had the lowest levels of docosahexaenoic acid (DHA) within dorsal muscle tissues, which is known to play an important role in both neural development and retinal function (Dr. Michael Arts, National Water Research Institute, Enivornment

Canada, pers. comm.). Additionally, levels of eicosapentaenoic acid (EPA) were significantly lower in Naubinway females, while EPA levels in males were significantly different from individuals collected from two of the four sites. EPA is a fatty acid precursor of the anti-inflammatory prostaglandin G3, thus having pertinent effects on the functionality of portions of the immune system (Dr. Michael Arts, pers. comm.).

A high prevalence of *C. maltaromaticum* infections was observed in the winter of 2005 in Naubinway whitefish samples. It is of interest to note that no infections were detected during the fall sampling periods, when spawning is occurring, as previous research has proposed a link between post spawning stress and manifestation of disease caused by *C. maltaromaticum* (Cone 1982; Herman et al. 1985; Starliper et al. 1992). However, the fall sampling times corresponded to a period during which the fish were still gravid/ready to spawn, which would mean that our winter sampling periods would correlate more closely with a "post-spawning" time period. Thus, while a multitude of factors likely play a role in the ability of *C. maltaromaticum* to generate disease, spawning stress could potentially be a key predisposing and/or initiating factor.

Among the isolates that were retrieved in this study, some variations were observed in their respective phenotypic characteristics, specifically, a number of the carbohydrate fermentation reactions. Variability in biochemical profiles has also been reported in previous studies that involved fermentation reactions of *Carnobacterium* spp. (Hiu et al. 1984; Michel et al. 1986; Starliper et al. 1992; Toranzo et al. 1993). Although some of the phenotypes varied only by a single fermentation reaction, the substantial variability that I observed may indicate that a number of strains are present in the Great Lakes basin. Furthermore, two biochemical reactions that have been reported to be key

in the dichotomization of *C. divergens* and *C. maltaromiticum,* inulin and mannitol fermentation (Montel et al. 1991), were variable among our isolates, despite the strong homology between our sequences and that of GenBank. Some of the biochemical variations seen in *Carnobacterium* spp. have been attributed to the use of different basal mediums (Baya et al. 1991; Toranzo et al. 1993); however, it is possible that these newly isolated strains of *C. maltaromiticum* may truly have a multitude of biochemical phenotypes that were not detectable genotypically due to the relatively small stretch of DNA that was sequenced in this study. Thus, while current biochemical identification schemes are useful for presumptive identification of *Carnobacterium* spp., molecular techniques may, in some cases, be necessary for definitive identification.

Concurrent with the biochemical variation discussed above was the variability in pathological effects potentially associated with the *C. maltaromiticum* infections in lake whitefish. While a typical pseudokidney disease was not seen, the isolation of this bacterium from the kidneys, along with the histopathological changes seen in the tissues of infected individuals, suggests a systemic infection. For example, the vacuolation of hepatocytes can be associated with the inhibition of protein synthesis, energy depletion, disaggregation of microtubules, or shifts in substrate utilization (Hinton and Lauren, 1990). Furthermore, bile stasis can be an indication of inappetance (Andicoberry et al. 1999) that may have resulted from an ongoing *C. maltaromitiucm* infection. Additionally, the apparent congestion observed within some of the kidneys and spleens is common in systemic bacterial infections due to an increase in angiogenic factors and other substances that increase recruitment of cells that are integral in mounting an immune response (Kumar et al. 2005) In the swim bladders infected with this bacterium,

signs of chronic irritation, such as hyperplasia, fibrin deposition, and neovascularization, and additional pathological effects, such as fibrin and cellular debris within the lumen, again illustrate the potential for disease generation.

The sequence generated from our isolates for the *pisA* precursor gene were nearly identical to the sequences presently contained within GenBank, with the occurrence of only a four base pair difference throughout the entire ~300 bp sequence. Additionally, the 97% nucleotide match and extremely low E-value between the rRNA stretch sequenced from our isolates and that of GenBank's indicate that our isolates are closely related to previously retrieved *C. maltaromiticum* isolates for which sequences were deposited into GenBank. However, there were portions of our sequence that deviated from the deposited sequence. These differences in genotype, as well as the variability in biochemical phenotypes, may suggest that lake whitefish isolates are distinct from previously deposited *C. maltaromiticum* isolates, some of which were also retrieved from fish.

In conclusion, *C. maltaromaticum* was isolated from lake whitefish inhabiting Lakes Michigan and Huron for the first time, a new occurrence in this host species and geographical location. Infected lake whitefish exhibited a number of clinical and histopathological alterations that could potentially have been caused by these infections, although further investigation fulfilling Koch's postulates is required. Although the detected prevalence throughout the course of the study was not extremely high (1.8%), infection prevalence did reach 20.8% in one seasonal sample. This finding may illustrate that *C. maltaromaticum* can potentially infect large portions of endemic lake whitefish

populations if conditions favorable to an epizootic occur. Implications of these outbreaks of disease are currently unknown and warrant further investigation.

	Collection Site			
Sampling Period	**Big Bay de Noc**	**Naubinway**	**Cheboygan**	**Detour Village**
Fall 2003	35	30	30	34
Winter 2004	29	0	32	10
Spring 2004	30	30	20	30
Summer 2004	22	20	30	20
Fall 2004	26	30	26	30
Winter 2005	16	30	15	30
Spring 2005	30	30	30	30
Summer 2005	30	30	28	30
Fall 2005	30	30	0	30
Winter 2006	30	23	30	30
Spring 2006	30	30	30	30
Summer 2006	30	30	30	30

Table 1. Representative seasons during which lake whitefish were collected and the number of individuals sampled from each of the four sites throughout the course of this study.

Sampling Date	Collection Site	Organ	Isolate ID #
2/12/2005	Naubinway	Kidney	NB-1
"	Naubinway	Kidney	NB-2
"	Naubinway	Kidney	NB-3
"	Naubinway	Kidney	NB-4
"	Naubinway	Kidney	NB-5
"	Naubinway	Kidney	NB-6
"	Naubinway	Kidney	NB-7
"	Naubinway	Kidney	NB-8
"	Naubinway	Kidney	NB-9
"	Naubinway	Kidney	NB-10
6/1/2005	Naubinway	Kidney	NB-11
"	Naubinway	Kidney	NB-12
6/7/2005	Detour	Kidney	DV-1
"	Detour	Kidney	DV-2
8/23/2005	Detour	Swim Bladder	DV-3
1/12/2006	Detour	Kidney	DV-4
"	Detour	Kidney	DV-5
"	Detour	Kidney	DV-6
3/28/2006	Naubinway	Kidney	NB-13
5/25/2006	Bay de Noc	Swim Bladder	BD-1
5/31/2006	Detour	Kidney	DV-7
"	Detour	Kidney	DV-8
6/15/2006	Cheboyghan	Kidney	CH-1

Table 2. Dates, sites, organs of isolation, and identification numbers of *Carnobacterium maltaromaticum* isolates retrieved from lake whitefish throughout the course of this study.

Catalase	0%	H_2S	0%
Cytochrome Oxidase	0%	Indole	0%
Lysine Decarboxylase	0%	Motility	0%
Ornithine Decarboxylase	0%	Methyl Red	100%
Arginine Dihydrolase	90%	Voges Proskauer	63%
ONPG	95%	Bile Esculin	100%
Phenyalanine Deaminase	0%	Gas Production from Glucose	0%
Nitrate Reduction	0%	Acid over Acid TSI Reaction	100%
Growth On:			
TSA	100%	MacConkey Agar	0%
CTSI	100%	Acetate Agar	0%
Acid Production from:			
Adonitol	0%	Mannitol	50%
Arabinose	0%	Mannose	100%
Cellobiose	100%	Melibiose	79%
Glucose	100%	Raffinose	10%
Galactose	100%	Rhamnose	0%
Glycerol	45%	Salicin	100%
Inositol	5%	Sorbitol	10%
Innulin	55%	Sucrose	100%
Lactose	95%	Trehalose	95%
Maltose	100%	Xylose	0%

Table 3. Percentage of *Carnobacterium maltaromaticum* isolates recovered from infected lake whitefish that were positive for characteristics of interest. All tests were incubated at 22°C, with results being recorded at up to 7 days post-inoculation with the following exceptions: methyl red, Voges-Proskauer, indole production, Simmons citrate, and TSI reactions were read at 2 days. Production of acid from carbohydrates was examined in phenol red broth base at a final concentration of 1%. All materials and reagents were purchased from Remel Inc. (Lenexa, KS) unless specified otherwise. TSA=trypticase soy agar; CTSI=cresol red thallium acetate sucrose innulin medium; TSI=triple sugar iron medium; ONPG= *o*-nitrophenyl-*β*-D-galactopyranoside.

Collection Site	Season				Site Total
	Fall	Winter	Spring	Summer	
Big Bay de Noc	0% (0/91)	0% (0/75)	1.1% (1/90)	0% (0/82)	**0.30% (1/338)**
Naubinway	0% (0/90)	20.8% (11/53)	2.2% (2/90)	0% (0/80)	**4.2% (13/313)**
Cheboygan	0% (0/56)	0% (0/77)	1.3% (1/80)	0% (0/88)	**0.33% (1/301)**
Detour Village	0% (0/94)	4.3% (3/70)	4.4% (4/90)	1.3% (1/80)	**2.4% (8/334)**
SeasonTotal	**0% (0/331)**	**5.1% (14/275)**	**2.3% (8/350)**	**0.3% (1/330)**	**1.8% (23/1286)**

Table 4. Percent prevalence of *Carnobacterium maltaromiticum* infections in lake whitefish by site and season, as well as the total prevalence, throughout the course of the study. (Nov. 2003-Aug. 2006).

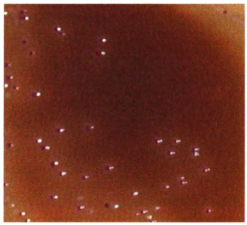

Figure 2. Appearance of *Carnobacterium maltaromaticum* colonies recovered from lake whitefish on Cresol Red Thallium Acetate Sucrose Inulin Medium (CTSI) (Wasney et al. 2001). All isolates produced convex, entire colonies with a reddish-purple center that were surrounded by a yellow halo.

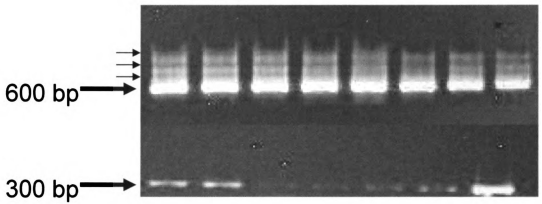

Figure 3. PCR patterns of *Carnobacterium maltaromaticum* isolates retrieved from lake whitefish. The approximate corresponding band size is denoted on the left with the large arrows. The three small arrows in the upper left corner correspond to the typical intergenic spacer region band pattern associated with *C. maltaromaticum* (Pelle et al. 2005).

Figure 4. Portion of 16S and 23S rRNA sequence of *Carnobacterium maltaromiticum* isolates recovered from lake whitefish (denoted by WF) aligned with a *C. maltaromaticum* sequence within Genbank (denoted by 2). The sequences were aligned using the BLASTn software from the National Center for Biotechnology Information, (NCBI) and analyzed using the nucleotide database from NCBI. Homology of the generated sequences with that of the database was assessed using the nucleotide database from NCBI. E-value=0.0; Nucleotide homology= 97%. Black=identical nucleotides; grey=nucleotide with similar characteristic (i.e. both are purines, etc); white=nucleotides differ.

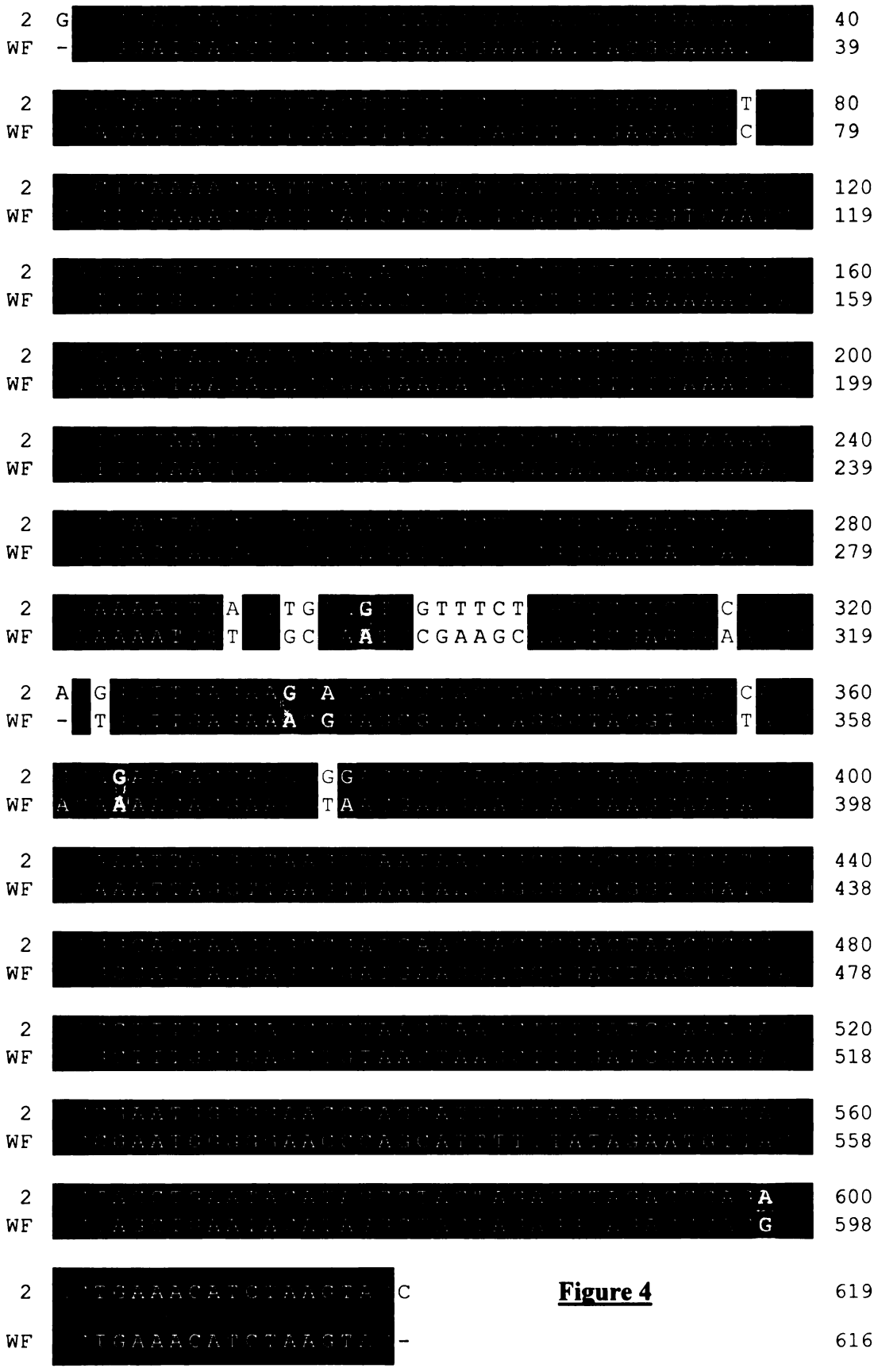
2 G 40
WF - 39
2 T 80
WF C 79
2 120
WF 119
2 160
WF 159
2 200
WF 199
2 240
WF 239
2 280
WF 279
2 A TG G GTTTCT C 320
WF T GC A CGAAGC A 319
2 A G G A C 360
WF - T A G T 358
2 G GG 400
WF A A TA 398
2 440
WF 438
2 480
WF 478
2 520
WF 518
2 560
WF 558
2 A 600
WF G 598
2 C 619
WF - 616

Figure 4

Figure 5. Putative piscicolin 126 precursor gene sequence of *Carnobacterium maltaromiticum* isolates recovered from lake whitefish (denoted by WF) aligned with the sequence within Genbank (denoted by 1). The sequences were aligned using the BLASTn software from the National Center for Biotechnology Information, (NCBI) and analyzed using the nucleotide database from NCBI. Homology of the generated sequences with that of the database was assessed using the nucleotide database from NCBI. E-value=1x10-138; Nucleotide homology=98%. Black=identical nucleotides; grey=nucleotide with similar characteristic (i.e. both are purines, etc); white=nucleotides differ.

Figure 6. A phylogenetic tree of *C. maltaromaticum* isolates retrieved from lake whitefish based upon portions of the 16S rRNA and 23S rRNA genes. Generated sequences were analyzed using the BLASTn software from the National Center for Biotechnology Information, (NCBI, Bethesda, MD) to detect homologous sequences. Homology of the generated sequences with that of the database was assessed using the nucleotide database from NCBI. Using the CLUSTAL W program from Molecular Evolutionary Genetics Analysis (MEGA) 3.1., a phylogenetic tree was constructed using the Neighbor-Joining method as the bootstrap test of phylogeny. Accession numbers from NCBI precede scientific names.

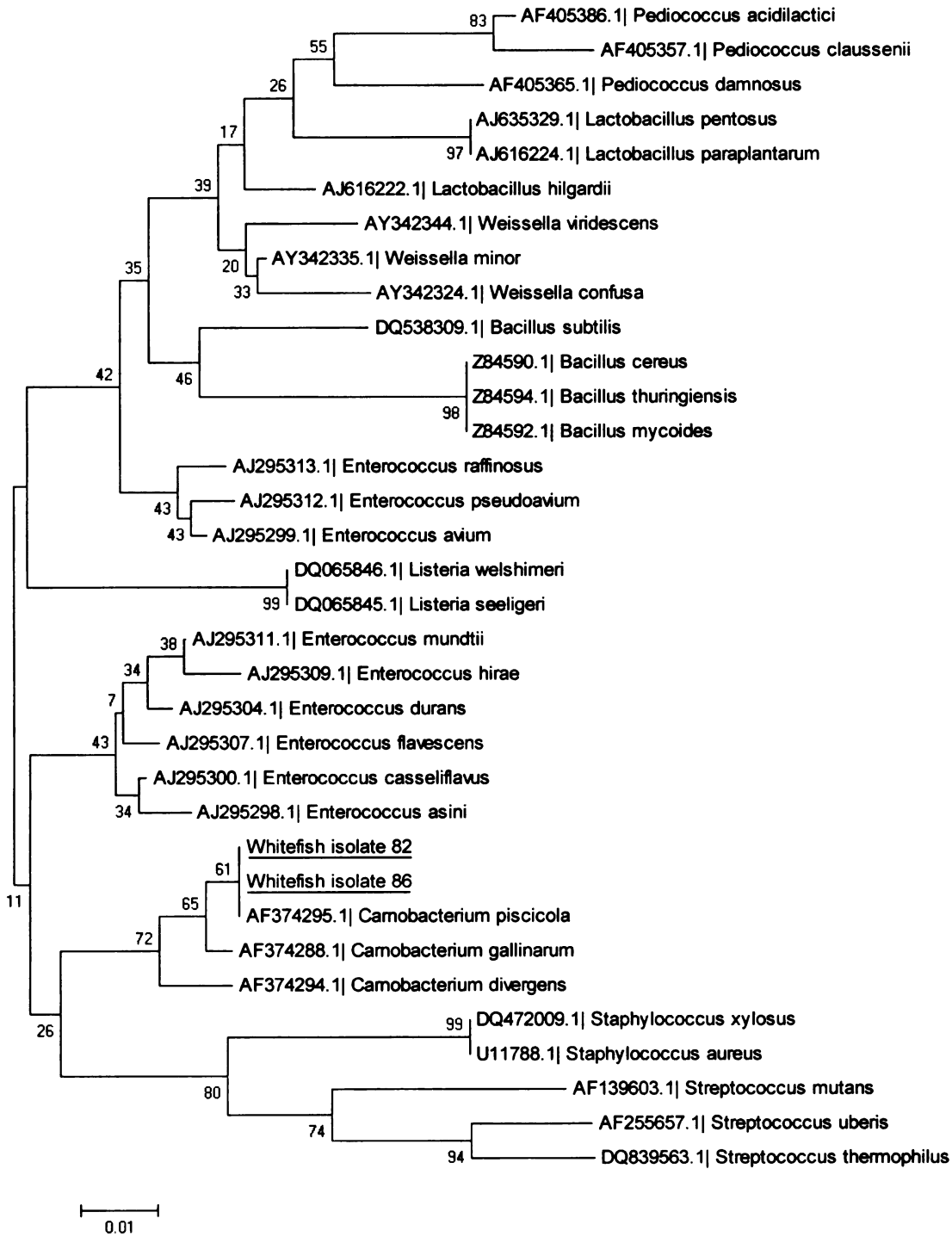
AF405386.1| Pediococcus acidilactici
AF405357.1| Pediococcus claussenii
AF405365.1| Pediococcus damnosus
AJ635329.1| Lactobacillus pentosus
AJ616224.1| Lactobacillus paraplantarum
AJ616222.1| Lactobacillus hilgardii
AY342344.1| Weissella viridescens
AY342335.1| Weissella minor
AY342324.1| Weissella confusa
DQ538309.1| Bacillus subtilis
Z84590.1| Bacillus cereus
Z84594.1| Bacillus thuringiensis
Z84592.1| Bacillus mycoides
AJ295313.1| Enterococcus raffinosus
AJ295312.1| Enterococcus pseudoavium
AJ295299.1| Enterococcus avium
DQ065846.1| Listeria welshimeri
DQ065845.1| Listeria seeligeri
AJ295311.1| Enterococcus mundtii
AJ295309.1| Enterococcus hirae
AJ295304.1| Enterococcus durans
AJ295307.1| Enterococcus flavescens
AJ295300.1| Enterococcus casseliflavus
AJ295298.1| Enterococcus asini
Whitefish isolate 82
Whitefish isolate 86
AF374295.1| Carnobacterium piscicola
AF374288.1| Carnobacterium gallinarum
AF374294.1| Carnobacterium divergens
DQ472009.1| Staphylococcus xylosus
U11788.1| Staphylococcus aureus
AF139603.1| Streptococcus mutans
AF255657.1| Streptococcus uberis
DQ839563.1| Streptococcus thermophilus
0.01

Figure 6.

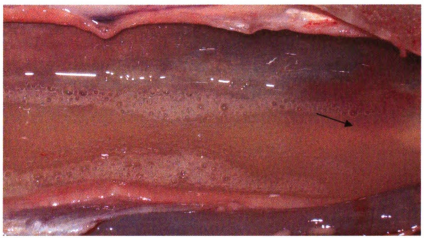

Figure 7. Lumen of a lake whitefish swim bladder filled with a turbid, mucoid exudate from which *C. maltaromaticum* was recovered in pure culture. The walls of the swim-bladder are severely thickened and opaque, with some areas of extensive hemorrhaging also evident (denoted by arrow).

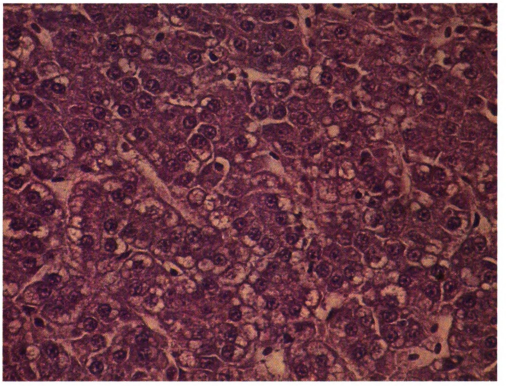

Figure 8. Stained section of a liver from a *C. maltaromaticum* infected lake whitefish exhibiting mild hepatocyte degeneration due to cytoplasmic vacuolation (H&E stain, 20X magnification.

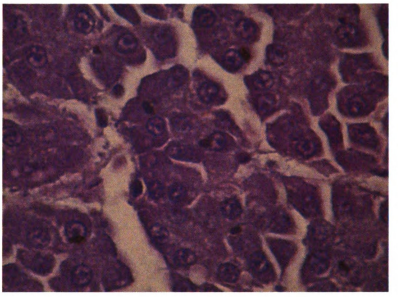

Figure 9. Stained section of a liver from a *C. maltaromaticum* infected lake whitefish exhibiting mild bile stasis (H&E stain, 40X magnification).

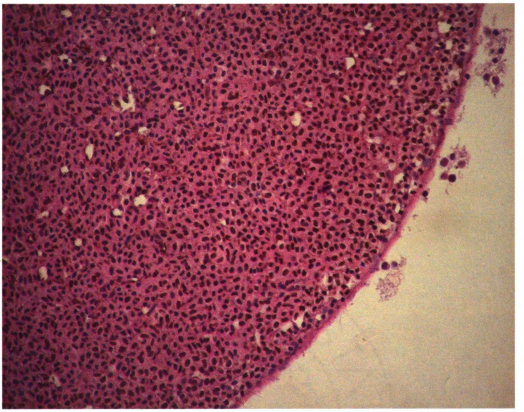

Figure 10. Stained section of a spleen from a *C. maltaromaticum* infected lake whitefish exhibiting congestion and swelling (H&E stain, 20X magnification).

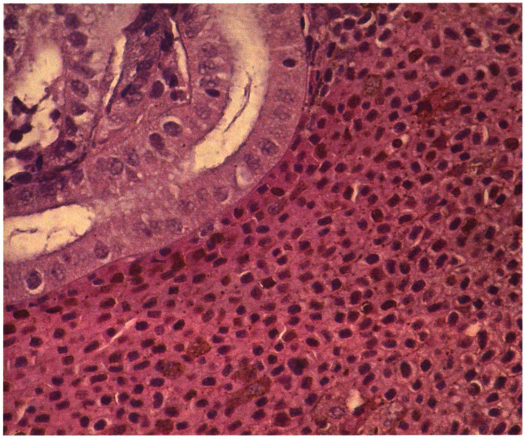

Figure 11. Stained section of the posterior kidney from a *C. maltaromaticum* infected lake whitefish exhibiting mild congestion (H&E stain, 20X magnification).

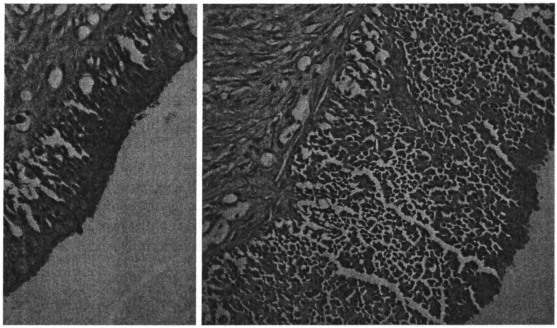

Figure 12. Stained section of a lake whitefish swim-bladder from which *C. maltaromaticum* was isolated exhibiting severe epithelial hyperplasia (right) compared with that of a normal swim-bladder (left; H&E stain, 20X magnification).

CHAPTER THREE

ISOLATION OF *AEROMONAS SALMONICIDA* SUBSPECIES *SALMONICIDA* FROM LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*) IN LAKES MICHIGAN AND HURON

ABSTRACT

Aeromonas salmonicida subspecies *salmonicida,* the etiological agent of furunculosis, has been isolated from lake whitefish (*Coregonus clupeaformis*) collected from Lakes Michigan and Huron, USA, during a three-year study. Four *A. salmonicida* subspecies *salmonicida* isolates were recovered from the kidneys of infected fish. The isolates were Gram-negative, non-motile, coccobacilli that produced a brown diffusible pigment on Trypticase Soy Agar. All isolates produced deep blue colonies on Coomassie Brilliant Blue Agar. Amplification of selected 16S rRNA regions specific to *A. salmonicida* subspecies *salmonicida* via polymerase chain reactions and subsequent gel electrophoresis analyses of the retrieved isolates yielded amplicons of the expected size (512 bp). Kidney/spleen tissues from culture positive lake whitefish, as well as culture negative individuals with furuncle-like lesions, were assayed using the same primers. Three of the four culture positive individuals were also PCR positive, while the remaining culture positive lake whitefish and the culture-negative, furuncle-like lesion individuals were PCR negative. Clinical signs associated with infection included extensive external hemorrhaging, exopthalmia, varying degrees of splenomegaly, hepatic

and renal pallor, spenic and renal congestion, friability of the kidney, fibrinous adhesions on the spleen and liver, as well as severe hemorrhagic enteritis. Histopathological examination of culture positive lake whitefish revealed multi-focal hemorrhage and mild to moderate infiltration of lymphocytes and histiocytes in the fat under the skin and in the muscultature, and massive diffuse congestion within the spleen. Histopathological examination of furuncle-like lesions showed ulceration, necrotizing dermatitis and myositis, along with an infiltration of mixed lymphocytes, macrophages, and infrequent heterophils wihin the skin and underlying musculature, as well as hemorrhage and fibrin deposition; however, no bacterial colonies were observed. Detected *A. salmonicida* subspecies *salmonicida* infections were limited to the Naubinway, Lake Michigan, and Detour Village, Lake Huron sites.

INTRODUCTION

Bacteria belonging to the genus *Aeromonas* are oxidase-positive, facultatively anaerobic, glucose-fermenting, Gram-negative bacilli that are ubiquitous to the aquatic environment, including fresh, brackish, and marine waters (Hazen et al. 1978; Alonso et al. 1994). They have been found in association with disease in both poikilothermic and homeothermic organisms, including humans. In fish, diseases caused by these organisms have been studied for quite sometime, beginning in 1890, when Emmerich and Weibel described a bacterium associated with diseased trout within a hatchery. Initially known as *Bacillus salmonicida* (syn. *Bacterium salmonicida* and *Bacterium trutta*) (Emmerich and Weibel. 1890), *Aeromonas salmonicida* has become one of the most studied bacterial piscine pathogens. This obligate pathogen is the etiological agent of furunculosis and is known to infect a multitude of salmonids, including salmon, trout, grayling, char (Cipriano and Bullock 2001) and whitefish (*Coregonus* spp.) in Finland (Rintamäki & Koski 1987). Additionally, infections caused by *Aeromonas salmonicida* have been reported in an increasing number of non-salmonid species (Fijan 1972; Bootsma et al. 1977; Shotts et al. 1980; Wiklund 1995; Bernoth and Korting 1992; Wilson and Holliman 1994). *Aeromonas salmonicida* has also been reported to cause carp erythrodermatitis (Fijan 1972), cutaneous ulcerative disease in goldfish (Shotts et al. 1980), and "head ulcer disease" in Japanese eels (Kitao et al. 1984).

Currently, at least 5 subspecies of *Aeromonas salmonicida* are recognized; - *salmonicida, achromogenes, masoucida, smithia* (Holt et al. 2000), and *pectinolytica* (Pavan et al. 2000). *A. salmonicida salmonicida* is the etiological agent of furunculosis,

while subspp. *achromogenes, masoucida,* and *smithia* are associated with atypical forms of the disease that often involve external pathology (i.e. dermal ulcerations) with or without septicemia (Cipriano & Bullock 2001). *A. salmonicida pectinolytica* is a relatively new subspecies that was recently isolated from a heavily polluted river near Buenos Aires city but has yet to be reported in association with disease (Pavan et al. 2000).

Furunculosis, so-named for the subacute or chronic form of the disease, is sometimes characterized by the presence of boil-like lesions, known as furuncles, which are present in the musculature (Austin and Adams, 1996). Additional clinical signs associated with these forms of the disease include hemorrhages within the fins, musculature, and liver exopthalmia, lethargy, bloody discharge from the nares and anus, swelling of the spleen, and renal necrosis. Rates of mortality associated with these chronic infections are typically low (McCarthy and Roberts, 1980). Another form of disease caused by *A. salmonicida*, termed acute furunculosis, manifests as a septicemia accompanied by melanosis, inappetance, lethargy, petechiae in the fins, and varying degrees of splenomegaly. Mortalities typically occur within 2-3 days (McCarthy 1975). Furthermore, a peracute form has also been found in association with high mortalities in salmonid fingerlings (McCarthy and Roberts 1980; Austin and Austin 1993). An intestinal manifestation of furunculosis has also been described, in which intestinal inflammation and anal inversion occurs (Amlacher 1961). Latent forms of *A. salmonicida salmonicida* infection, recently termed covert infections (Hiney et al. 1997), have also been identified. These individuals develop clinical disease only under certain conditions of environmental and physical stress. Covertly infected individuals act as

reservoirs for the disease, capable of transmitting the disease to uninfected hosts, yet no clinical signs are present (Hiney et al. 1997).

Herein, I report on the isolation of *A. salmonicida salmonicida* from the kidneys of systemically infected lake whitefish inhabiting Lakes Michigan and Huron, U.S.A. I also report on the attempted detection of *A. salmonicida salmonicida* in culture negative fish, with furuncle-like lesions, using a molecular assay.

MATERIALS AND METHODS

Fish and sampling. Between the fall of 2003 and the summer of 2006, lake whitefish were collected for bacteriological analyses from 4 representative stocks on four sampling occasions per year (~30 fish/site/season, Table 1). The two Lake Michigan sites, Big Bay de Noc (latitude 4527-4548.20; longitude 8535-8722) and Naubinway (latitude 4553-4603; longitude 8513-8535), and the two Lake Huron sites, Detour (latitude 4555-4556.97; longitude 8350.5-8419.12) and Cheboygan (latitude 4540.24-4548; longitude 8424-8437.87) were selected for this study due to their association with large spawning aggregations and their accessibility via commercial fishing (Figure 1). A total of 1286 lake whitefish from the four sites were sampled throughout the course of this study.

Fish were captured using commercial trap nets and, when necessary, commercial gill nets, with only live fish being used for the study. The collected fish were transported alive to the Chippewa Ottawa Research Authority (CORA) Fishery Enhancement Facility near Hessel, Michigan. The fish were then chilled on ice and transported to the Aquatic Animal Health Laboratory at Michigan State University, East Lansing, MI, where they were immediately subjected to thorough internal and external clinical examinations.

Bacterial isolation. Bacterial samples retrieved from the whitefish were collected from the kidneys, as well as visible lesions. Tissue samples were struck onto Trypticase Soy Agar (TSA; Remel Inc., Lenexa, KS) and incubated at 22°C for up to 72 hr. Periodic examination of bacterial growth was recorded and individual colonies were

sub-cultured onto TSA and Coomassie Brilliant Blue Agar (CBBA) (Udey 1982), a differential medium for distinguishing strains of *A. salmoncida salmonicida* that possess an additional layer (poly-A layer) associated with the external cellular membrane. Subcultures were then incubated for 24 hours at 22°C. A total of 4 isolates presumptively identified as *A. salmonicida salmonicida* (Table 5) were recovered and examined.

Biochemical characterization. Isolated bacteria were initially identified using a battery of morphological and biochemical tests, including Gram reaction, cytochrome oxidase, catalase reaction (3% H_2O_2), motility, indole production, hydrogen sulfide production, oxidation/fermentation reaction (BD Scientific, Sparks, MD), methyl red, 2,3-butanediol production from glucose (Voges-Proskauer), nitrate reduction, TSI reaction, ONPG (*o*-nitrophenyl-*β*-D-galactopyranoside), gelatinase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, esculin hydrolysis, and production of gas from glucose. Production of acid from the following carbohydrates was examined in phenol red broth base at a final concentration of 1%: arabinose, dextrose, galactose, glycerol, maltose, sucrose, trehalose, and xylose. Tests were incubated for 10 days and read periodically, with the following exceptions: methyl red, Voges-Proskauer, indole production, and TSI reactions were read at 2 days. All materials and reagents were purchased from Remel Inc. (Lenexa, KS) unless specified otherwise.

Confirmation of isolate identification with polymerase chain reaction (PCR). Representative colonies with the typical morphological and biochemical characteristics of *A. salmonicida salmonicida*, as listed in Bergey's Manual of Determinative Bacteriology

(Holt et al. 2000) were selected for this study. Single colonies were resuspended in Trypticase Soy Broth (Remel), and incubated overnight at 22 °C. DNA was extracted using a Qiagen DNeasy tissue kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's protocol for Gram-negative bacteria. PCR amplification was performed using the following *Aeromonas salmonicida salmonicida* primers: MIY1 (5'-AGCCTCCACGCGCTCACAGC-3') and MIY2 (5'-AAGAGGCCCCATAGTGTGGG-3') (Miyata et al. 1996). Each 25µl reaction contained 0.6 U *Taq* polymerase w/ loading buffer (Denville Scientific Inc., Metuchen, NJ), 2.5 µl 10x PCR buffer containing 1.5 mmol $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.5 l dNTP (Invitrogen), and 2.5 µl of primers MIY1 and MIY2. The PCR amplification program used was that of Byers et al (2002), with modifications, which included an initial denaturation step of 94°C for 3 min, then amplified for 30 cycles, with denaturation for 40 s at 94°C, annealing for 40 s at 60°C, and elongation for 40 s at 72°C, and a final extension was performed at 68°C for 3 min. The expected size of the generated amplicon was 512 bp. The amplified products were subsequently run on 1.5% agarose gel for 30 minutes at 50 volts and then stained with ethidium bromide for viewing via UV exposure. A 1 Kb plus DNA Ladder (Invitrogen) was used as a molecular marker.

Detection of *A.salmonicida salmonicida* with polymerase chain reaction (PCR). Kidney tissues of culture negative lake whitefish exhibiting furuncle-like lesions were assayed with the same primers as above. DNA was extracted using a Qiagen DNeasy tissue kit (Qiagen Sciences, MD) according to the manufacturer's protocol for

the appropriate tissue. Assayed tissues included kidney/spleen homogenates. PCR amplification, as well as the PCR protocol, were performed as described above.

Histopathological analyses. Samples of tissue with gross clinical abnormalities were fixed in 10% buffered formalin. The fixed samples were then embedded within paraffin, sectioned, and stained with hematoxylin and eosin (Prophet et al. 1992).

RESULTS

Throughout the course of this study, a number of bacterial species were isolated and characterized based on morphological, biochemical, and molecular attributes. Among these, four isolates were presumptively identified as *Aeromonas salmonicida* subspecies *salmoncida*. Following 24-48 hrs incubation at 22°C on TSA, all isolates produced colonies that measured 1-2 mm in diameter and were opaque, smooth, entire, and convex. The consistency of the colonies was typical of *A. salmonicida salmonicida,* being friable and readily sliding across the culture medium upon attempted colony removal. All isolates produced a brown diffusible pigment that was evident at 48 hrs of incubation at 22°C on TSA. On CBBA, all isolates produced dark blue colonies. Colony counts ranged from 9 to extremely profuse, interconnected, growth that covered the entire inoculum streak per 10µl of kidney inoculum.

Morphologically, these isolates were Gram-negative coccobacilli, arranged in bunches that measured 1.5 µm by 1 µm, although slight pleomorphism was often noticed. Biochemical tests demonstrated that all isolates were non-motile, facultative anerobes that did not produce indole, H_2S, or 2,3-butanediol (Table 6). The lake whitefish isolates utilized the mixed-acid fermentation pathway (methyl red +), hydrolyzed esculin, yielded an alkaline over acid TSI reaction without any gas production, and produced catalase, cytochrome oxidase, and gelatinase. Acid production from fermentation reactions occurred from the following sugars: arabinose, dextrose, galactose, glycerol, and maltose. No acid was produced from sucrose, trehalose, or xylose. *Aeromonas salmonicida salmonicida* isolates recovered from lake whitefish produced two atypical reactions; a

lack of gas production from glucose and no acid production from trehalose (Holt et al. 2000).

Gel electrophoresis of generated PCR amplicons derived from isolates of *A. salmonicida salmonicida* retrieved from lake whitefish were consistent with those reported previously (Byers et al. 2002). Gel electrophoresis of the PCR amplicons generated from culture positive kidney tissue yielded mixed results. Of the 4 tissues assayed from culture positive individuals, three were positive, while one was negative.

The overall prevalence of lake whitefish that were culture positive for *A. salmonicida salmonicida* infections was 0.3% of the 1286 individuals that underwent bacteriological examination (Table 7). Of the four populations that were studied, *A. salmoncida salmoncida* infections were detected only in the Naubinway, Lake Michigan site, and the Detour Village, Lake Huron site. The detected prevalence in these two sites was 0.64% and 0.60%, respectively.

Kidney tissues from 3 lake whitefish exhibiting furuncle-like lesions, yet culture-negative, were assayed with PCR using specific primers for *A. salmonicida salmonicida.* Gel electrophoresis of kidney/spleen homogenate amplicons from these individuals produced negative results.

Clinical signs associated with lake whitefish from which *A. salmonicida salmonicida* was retrieved included combinations of extensive petechial and echymmotic hemorrhaging in the fins, musculature and vent (Figure 13), head, and eyes, exopthalmia, varying degrees of splenomegaly, pallor in the liver and kidney, congestion in the kidney and spleen, friability of the kidney, and fibrinous adhesions on the spleen and liver. In one instance, there was also predominant pathology involving the gastrointestinal tract,

with a large amount of gas present within the stomach, along with severe hemorrhagic enteritis. Additionally, a 5 cm by 5 cm shallow hemorrhagic ulceration was present on the left lateral musculature of one infected individual.

Clinical signs associated with culture negative, furuncle-like-lesion positive lake whitefish included mild-moderate hemorrhaging within the fins, varying degrees of congestion in the liver, spleen, and kidneys, severe splenomegaly, and periodic hemorrhagic enteritis. Furuncle-like lesions were raised, endurate, circular lesions protruding from the dorsal and lateral musculature (Figure 14). The underlying musculature was necrotically liquefied and cavitated (Figure 15). In one instance, previously healed muscular lesions were also evident.

Histopathological analyses of tissues retrieved from culture positive fish demonstrated some microscopic pathological effects. For example, skin and the underlying musculature taken from an individual with extensive severe external hemorrhaging demonstrated multi-focal hemorrhaging in the fat under the skin and seperating muscles, along with mild to moderate infiltration by lymphocytes and histiocytes. The same individual also had massive diffuse congestion within the spleen.

Histopathological analyses of tissues retrieved from culture negative fish with furuncle-like lesions produced a variety of microscopic lesions. One of the "furuncular" lesions involving the skin and underlying musculature exhibited ulceration, necrotizing dermatitis and myositis, along with an infiltration of mixed lymphocytes, macrophages, and infrequent heterophils (Figure 16). There was also predominant hemorrhage and fibrin deposition; however, no bacterial colonies were observed. The kidney and liver from this individual did not exhibit histopathological alterations. Another "furuncular"

lesion exhibited superficial & patchy muscle degeneration, with evident pallor, vacuolation, and separation, as well as necrosis. No inflammation was present. Severe and diffuse congestion, along with scattered macrophages containing brown-to-black cytoplasmic hemosiderin pigment, was observed in the spleen of this individual. Hemosiderosis can be indicative of extravascualr hemolysis (Kumar et al. 2005).

DISCUSSION

Morphological and biochemical analyses performed on four bacterial isolates retrieved from lake whitefish definitively validated their initial presumptive identification as *Aeromonas salmonicida salmonicida.* All four isolates were Gram-negative, non-motile coccobacilli that were facultatively anaerobic, and produced cytochrome oxidase and catalase. Additionally, the production of a brown, diffusible pigment, along with the lack of indole and H_2S production and sucrose fermentation, allows for the differentiation of *A. salmonicida salmonicida* from other *A. salmonicida* subspecies (Popoff 1984). Furthermore, the acid production from mannitol and maltose, as well as the hydrolysis of esculin, further support an identification of *A. salmonicida salmonicida* (Holt et al. 2000; Bohm et al. 1986). The production of an A-layer, as evident by the deep blue appearance of our colonies on CBBA, is a factor associated with virulence due to its role in different binding activities, and is also critical for adherence and invasion of macrophages (Kay and Trust 1997). Moreover, gel electrophoresis of generated PCR amplicons derived from isolates of *A. salmonicida salmonicida* cultured from lake whitefish were consistent with those reported previously (Miyata et al. 1996). According to Byers et al. (2002), MIY PCR is 100% specific to *A. salmonicida salmonicida.*

In all four culture-positive individuals, a significant amount of pathology was apparent, both grossly and microscopically. Clinically, lake whitefish infected with *A. salmonicida salmonicida* exhibited extensive hemorrhaging within the external musculature, concurrent with the splenomegaly and congestion within the kidneys and spleens, which are strongly suggestive of an acute septicemia. Experimental infection

studies performed by Klontz et al. (1966) and Ellis et al. (1981) found splenomegaly and congestion to be the most consistent gross clinical finding in salmonids infected *A. salmonicida salmonicida*. Moreover, Klontz and co workers also found that the site most prominently involved pathologically was the musculature. They found a marked increase of macrophages and small lymphocytes, along with a moderate infiltration of neutrophils and fibroblasts and an increase in extravascular erythrocytes at 32 hours post-injection. These findings correlate well with the histopathological results of this study. Hemorrhagic enteritis and gastrointestinal involvement, similar to reports by Amlacher (1961), were observed in one of the four culture-positive whitefish. Klontz et al. (1966) did not find any pathological abnormalities in the stomach, pancreas, or large intestine of intramuscularly infected fish. However, in fish injected by both intraperitoneal and intramuscular injections, as was done by Ellis et al. (1981), inflammation of the vent and rectum was only observed in I.P. injected individuals. This suggests that the route of *A. salmonicida salmonicida* infection may play an integral role in the site-specific generation of pathology and may help to explain the differences in pathological effects from one individual to the next, at least in experimental infections. A possible difference in mode of infection or infection target may exist for this particular isolate; however, further investigation is required. In addition, it is important to keep in mind that experimental infections are vastly different from natural routes of infection, so caution should be used when making comparisons of the two. When taken together, the clinical and histopathological findings within these infected whitefish are strongly suggestive of the ability of endemic *A. salmonicida salmonicida* isolates to generate morbidity and mortality, although under what conditions this can occur remains to be elucidated.

In acute *A. salmonicida salmonicida* infections, the onset is sudden, with death typically occurring within 2-3 days (McCarthy 1975). Thus, a relatively small "window of opportunity" is available for detecting acute *A. salmonicida salmonicida* infections, a fact that most likely has an effect on studies attempting to quantify the prevalence of *A. salmonicida salmonicida* within fish populations. As such, the overall 0.31% prevalence that was observed in this study may not be representative of the true prevalence.

With regard to the 3 individual lake whitefish that were culture and PCR negative, yet displayed furuncle-like lesion that histologically were very similar to *A. salmonicida*-induced furuncles, there are a number of possible explanations. First, the fish may have recovered from the infection, yet the affected tissues were not fully healed. Second, inhibitory substances may have inhibited the PCR reaction, a problem that has been reported previously (Byers et al. 2002; Høie et al. 1997; Gustafson et al. 1992). These inhibitive properties may also explain why the PCR was negative in one of the four cases presented in this study, despite the successful isolation of *A. salmonicida salmonicida* from the same tissue and confirmation of the colony identity by PCR. Alternatively, these lesions may have been caused other organisms that were not detected. Lastly, the transient nature of *A. salmonicida salmonicida* infections (Scallan et al. 1993), as well as its presence in varying sites depending on the stage of infection (Klontz et al. 1966) also can be a source of "false negative" detection results via culture and non-culture based 'proxy' methods. Histopathological observations of the "furuncular" lesions were similar to those seen by Klontz et al. (1966), although no bacterial colonies could be found. In addition, splenomegaly was observed in these culture negative individuals. Therefore,

the possibility that the detection of *A. salmonicida salmonicida* was missed in these individuals exists.

Although reported from other *Coregonus* spp. from Finland (Rintamäki & Koski, 1987), the isolation of *A. salmonicida salmonicida* from lake whitefish (*C. clupeaformis*) has never been reported. This occurrence is not considered surprising, as *A. salmonicida salmonicida* is known to infect a plethora of salmonids (Cipriano and Bullock 2001) and studies focusing on bacterial diseases of *C. clupeaformis* are few and far between. Furthermore, *A. salmonicida salmonicida* has been isolated from a number of salmonids and non-salmonid fish species in the Great Lakes basin (Faisal and Hnath, 2005; Faisal and Loch, unpublished data; McCraw 1952).

In conclusion, *Aeromonas salmonicida salmonicida* has been isolated from lake whitefish inhabiting Lakes Michigan and Huron. These infections were detected at a relatively low overall prevalence of 0.3% throughout the course of this three-year study. As such, the potential impact that *A. salmonicida salmonicida* derived infections may currently have on lake whitefish within the study sites would seem to be low. Additionally, a number of lake whitefish with furuncle-like lesions were observed, yet this bacterium could not be detected histologically, by culture, or via PCR. These individuals could potentially represent those on their way to recovery, thus suggesting that clearing of the infection can occur, or alternatively, these lesions may have been unrelated to *A. salmonicida salmonicida*. However, the pathological effects that were observed in individuals from which *A. salmonicida salmonicida* was isolated, both grossly and histologically, may indicate that severe disease can ensue upon infection. Thus, while this disease was not extremely predominant in sampled lake whitefish, its

presence could potentially affect lake whitefish at the population level if conditions become more favorable for disease causation through habitat alteration, nutritional stress, depressed condition factors, and/or other environmental/physiological stressors. Additionally, if lake whitefish are somewhat resistant to this disease but are able harbor the bacterium at certain times or under certain conditions, they may represent a possible reservoir for *A. salmonicida salmonicida* derived infections in other endemic fish species.

Sampling Date	Collection Site	Organ	Isolate ID #
8/28/04	Detour Village	Kidney	DV-AS-1
9/11/04	Naubinway	Kidney	NB-AS-1
1/07/05	Detour Village	Kidney	DV-AS-2
6/28/06	Naubinway	Kidney	NB-AS-2

Table 5. Sampling dates and sites from which *A. salmonicida* subspecies *salmonicida* was recovered from lake whitefish using culture techniques during this three-year study (Nov. 2003-Aug. 2006).

Pigment Production on TSA	+	H_2S	-
Triple Sugar Iron	K/A/-/-	Indole	-
Gas from Glucose	-	Motility	-
ONPG	+	Methyl Red	+
Arabinose	(+)	Voges Proskauer	-
Galactose	+	Bile Esculin	+
Glycerol	+	Catalase	+
Maltose	+	Cytochrome Oxidase	+
Sucrose	-	Lysine Decarboxylase	(+)
Trehalose	-	Ornithine Decarboxylase	-
Xylose	-	Arginine Dihydrolase	+
Nitrate Reduction	+	Gelatinase	+

Table 6. Biochemical phenotypes of *A. salmonicida salmonicida* isolates retrieved from lake whitefish. +, positive reaction; -, negative reaction; (+) weak and/or delayed positive reaction. All tests were incubated at 22°C, with results being recorded at up to 7 days post-inoculation with the following exceptions: methyl red, Voges-Proskauer, indole production, and TSI reactions were read at 2 days. Production of acid from carbohydrates was examined in phenol red broth base at a final concentration of 1%. All materials and reagents were purchased from Remel Inc. (Lenexa, KS). All strains were Gram-negative, non-spore forming, nonmotile, faculatative anaerobes that produced an alkaline over acid TSI reaction without any gas or H_2S production.

Collection Site	Season				Site Total
	Fall	Winter	Spring	Summer	
Big Bay de Noc	0% (0/91)	0% (0/75)	0.0% (0/90)	0% (0/82)	**0% (0/338)**
Naubinway	0% (0/90)	0% (0/53)	1.1% (1/90)	1.3% (1/80)	**0.6% (2/313)**
Cheboygan	0% (0/56)	0% (0/77)	0.0% (0/80)	0% (0/88)	**0% (0/301)**
Detour Village	0% (0/94)	1.4% (1/70)	0% (0/90)	1.3% (1/80)	**0.6% (2/334)**
SeasonTotal	**0% (0/331)**	**0.4% (1/275)**	**0.3% (1/350)**	**0.6% (2/330)**	**0.3% (4/1286)**

Table 7. Percent prevalence of *Aeromonas salmonicida* subspecies *salmonicida* infections in lake whitefish by site and season, as well as the total prevalence, throughout the course of the study (Nov. 2003-Aug. 2006).

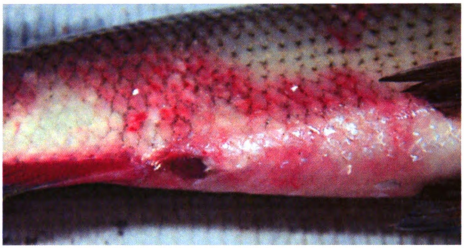

Figure 13. Extensive severe hemorrhaging within the musculature and vent of a lake whitefish infected with *A. salmoncida salmonicida*.

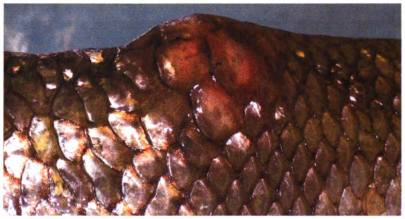

Figure 14. Furuncule-like lesion on the dorsal musculature of a lake whitefish that was both culture and PCR negative for *A. salmonicida salmonicida*.

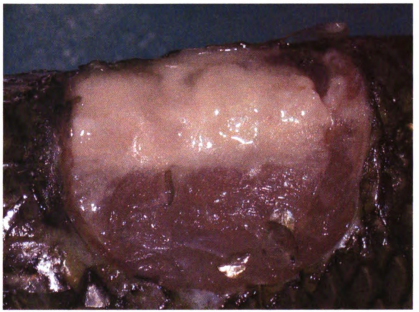

Figure 15. Musculature underlying a furuncule-like lesion, exhibiting severe muscular degeneration and necrosis in a lake whitefish.

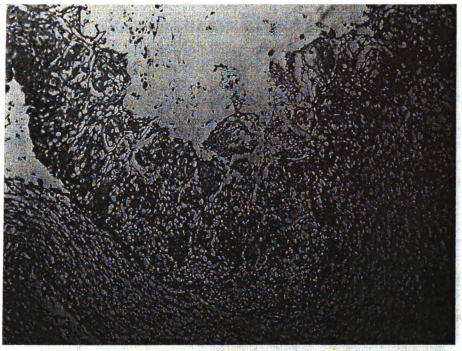

Figure 16. Skin and underlying musculature from a furuncle-like lesion found in lake whitefish exhibiting ulceration, necrotizing dermatitis & myositis, mixed lymphocytes, macrophages, rare heterophils, fibrin, and hemorrhage. No bacterial colonies were observed (H&E stain, 10X magnification).

CHAPTER FOUR

ISOLATION OF MOTILE *AEROMONAS* SPP. FORM LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*) IN LAKES MICHIGAN AND HURON

ABSTRACT

A number of motile *Aeromonas* species have been isolated from lake whitefish (*Coregonus clupeaformis*) collected from four stocks within Lakes Michigan and Huron, USA. Sixty-nine isolates from the *A. hydrophila*, *A. sobria*, or *A. caviae* complexes were recovered from the kidneys of infected fish, some in pure, and some in mixed culture. Representative isolates were cytochrome oxidase positive, faculatatively anaerobic Gram-negative bacilli. Using biochemical identification schemes to speciate representative isolates, twenty-two were identified as *A. hydrophila sensu stricto*, nine as *A. jandaei*, three as *A. veronii* bv *sobria*, and two as *A. eucrenophila*. The prevalence of infection by motile aeromonads varied by site and season, with lake whitefish sampled in the summer having the highest prevalence. External clinical signs of disease in lake whitefish included congestion and or hemorrhaging in the fins, general pallor, and petechial/ecchymotic hemorrhagic foci within the musculature. Internal clinical signs included varying combinations of congestion, mottling, hemorrhaging, pallor, and multifocal necrotic foci within the liver; moderate to severe splenomegaly; congestion, friability, mottling, and/or swelling in the kidneys; pronounced erythema within the

peritoneal cavity; and mild hemorrhagic enteritis. This study provided evidence on the wide spread motile aeromonad infections present within the Great Lakes Basin.

INTRODUCTION

Lake whitefish is an indigenous species within the Great Lakes basin that comprises one of the most economically important fisheries (Bronte et al. 2003; Hoyle 2005; Schneeberger et al. 2005; Cook et al. 2005; Mohr and Ebener 2005). The lake whitefish plays an integral role in the benthic food-web by efficiently cycling energy harnessed from primary producers by benthic organisms into the upper trophic levels, hence leading to a harvestable resource (Mohr and Nalepa 2005). Recent declines in abundance, condition, and size at age in harvested lake whitefish have been observed in a number of the Great Lakes (Hoyle et al. 1999; Pothaven et al. 2001; Madenjian et al. 2002; Mohr and Ebener 2005), spurring much research on the factors that may be contributing to these declinations.

Many diseases are well known to affect animal survival at the population and community levels (Holmes 1996). One of the many important bacterial genera that are known to cause disease in fish is the genus *Aeromonas.* These oxidase-positive, facultatively anaerobic, glucose-fermenting, Gram-negative bacilli are ubiquitous to the aquatic environment and have been found in both healthy (Trust et al. 1974) and diseased poikilothermic and homeothermic organisms, including humans (Austin and Adams 1996; Janda and Abbott 1996). The motile, mesophilic members of the genus *Aeromonas* often cause disease in association with various environmental or physiological stressors, such as spawning, warm water temperatures, poor water quality, poor nutritional status, parasitic infections, or poor over-wintering conditions (Aoki 1999; Noga 2000; Austin and Adams 1996). Disease in fish, often termed motile aeromonad septicemia (MAS),

typically manifests in an acute, chronic, or sub-clinical form (Cipriano 2001), and can cause a variety of clinical signs, such as fin/tail rot, dermal ulceration, and hemorrhagic septicemia (Aoki 1999). MAS is often characterized by small surface lesions that may lead to sloughing of scales, hemorrhaging in the gills and anus, ulcers, abcesses, exopthalmia, anemia, renal and hepatic swelling, and abdominal swelling due to presence of ascites (Austin and Adams 1996).

The *Aeromonas* species that are most connected to diseases in fish are *A. caviae* (Paniagua et al. 1990), *A. allosaccharophila* (Martinez-Murcia et al. 1992), *A. bestiarum* (Ali et al. 1996), *A. veronii bv veronii* (Sugita et al. 1995), *A. veronii* biovar *sobria* (Rahman et al. 2002), *A. jandaei* (Esteve et al. 1994), *A. encheleia* (Esteve et al. 1995), *A. eucrenophila* (Huys et al. 1997), *A. hydrophila* (Santos et al. 1988; McGarey et al. 1991; Monette et al. 2006), and phenospecies *A. sobria* (Toranzo et al. 1989; McGarey et al. 1991; Wahli et al 2005). Herein, we report on the isolation of multiple motile *Aeromonas* species from systemically infected lake whitefish stocks collected from lakes Michigan and Huron, U.S.A. Characterization of the various isolates using morphologic and biochemical criteria were conducted.

MATERIALS AND METHODS

Fish and Sampling. Between October of 2005 and August of 2006, lake whitefish were collected for bacteriological analyses from 4 representative stocks on four sampling occasions (~30 fish/site/season, Table 1). The two Lake Michigan sites, Big Bay de Noc (latitude 4527-4548.20; longitude 8535-8722) and Naubinway (latitude 4553-4603; longitude 8513-8535), and the two Lake Huron sites, Detour (latitude 4555-4556.97; longitude 8350.5-8419.12) and Cheboygan (latitude 4540.24-4548; longitude 8424-8437.87) were selected for this study due to their association with large spawning aggregations and their accessibility via commercial fishing (Figure 1). A total of 443 lake whitefish from the four sites were analyzed in this study (Table 8).

Fish were captured using commercial trap nets and, when necessary, commercial gill nets, with only live fish being used for the study. The collected fish were transported alive to the Chippewa Ottawa Research Authority (CORA) Fishery Enhancement Facility near Hessel, Michigan. The fish were then chilled on ice and transported to the Aquatic Animal Health Laboratory at Michigan State University, East Lansing, MI, (approximately 4 hours) where they were immediately subjected to thorough internal and external clinical examinations.

Bacterial Isolation. Bacterial samples retrieved from the whitefish were collected from the kidneys, as well as visible lesions. Tissue samples were inoculated directly onto trypticase soy agar (TSA) (Remel Inc., Lenexa, KS) and incubated at 22°C

for up to 72 hr. Periodic examination of bacterial growth was recorded and individual colonies were sub-cultured onto TSA and incubated for 24 hours at 22°C

Biochemical characterization. Isolated bacteria were initially identified as presumptive *Aeromonas* spp. based on colony morphology, Gram reaction, cytochrome oxidase, and oxidation/fermentation (BD Scientific, Sparks, MD) reactions, as well as resistance to the vibriostatic agent 0/129 (2,4-diamino,6,7-di-isopropyl pteridine; Holt et al. 2000; MacFaddin 2000). Following these criteria, representative isolates were then subjected to additional biochemical analyses following the scheme and recommendations of Abbott et al. (2003). Using tests for the hydrolysis of esculin, production of acetoin (Voges-Proskauer), gas production from glucose, and acid production from arabinose, isolates were initially dichotomized into one of the three complexes (*A. hydrophila* complex, *A. caviae* complex, and *A. sobria* complex). Isolates that were esculin, Voges-Proskauer, and gas from glucose positive (one exception), but arabinose variable were placed into the *A. hydrophila* complex. Assignation to the *A. sobria* complex occurred when isolates were esculin negative, Voges-Proskauer positive (one exception), gas from glucose positive, and arabinose negative. Esculin variable, Voges-Proskauer negative, gas from glucose variable, and arabinose negative isolates were assigned to the *A. caviae* complex. In cases where results of these four tests were ambiguous for complex assignation and/or speciation was possible, the Moeller Reactions (lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase), as well as other assays, including the catalase test (3% H_2O_2), motility, indole production, hydrogen sulfide production, methyl red, nitrate reduction, phenylalanine deaminase, gelatinase, and Simmon's citrate were

used. Additionally, production of acid from the following carbohydrates was examined in phenol red broth base at a final concentration of 1%: arabinose, adonitol, cellobiose, galactose, glycerol, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Tests were incubated for 10 days and read periodically, with the following exceptions: tests for susceptibility to O/129 were read at 1 day; methyl red, Voges-Proskauer, indole production, and Simmons citrate reactions were read at 2 days. All materials and reagents were purchased from Remel Inc. (Lenexa, KS) unless specified otherwise.

Analysis of isolates by species, site, and season. Upon identification to the species level using morphological and biochemical profiles, an analysis of the predominant species found in association with each season and/or site was conducted.

Statistical analysis. Comparisons between the fish stocks for differences in the prevalence of motile *Aeromonas* spp. by site or season were carried by the Pearson Chi square (χ^2) analysis of contingency tables. Yates continuity correction was applied in the calculation of values when the degrees of freedom (DF)=1. All calculations were performed using the SigmaStat software package (Jandel Scientific Inc, San Rafael, CA).

RESULTS

Throughout this study, a number of bacterial species were isolated and characterized based on their morphological and biochemical characteristics. Among these, 69 isolates were presumptively identified as motile *Aeromonas* spp. Twenty-two *Aeromonas* spp. isolates were obtained in pure culture, while the remaining forty-seven isolates were present in mixed cultures. Colony counts of primary cultures varied, with a range of 1 colony forming unit (CFU) per 10 µl of kidney tissue inoculum to heavy interconnected growth covering the inoculum streaks. Other bacterial species recovered along with the motile aeromonads using standard culture techniques in this study included nine isolates of *Shewanella* spp., one isolate of *Carnobacterium maltaromaticum*, and rarely, members of the enteric bacteria (Family Enterobacteriaceae). These bacteria will not be discussed further in this chapter.

Bacterial colonies suspected of being an *Aeromonas* spp. were subcultured onto TSA and incubated for 18-24hrs at 22°C. Upon subculture, individual colonies ranged from 0.75 mm to 1.25 mm in diameter, were cream to buff-colored, and were convex with entire margins. Gram-stains performed on 18-24 hr old sub-cultures of suspect *Aeromonas* isolates demonstrated Gram-negative bacilli (length of 0.75-2.5µm; width of 0.3-0.5 µm) that were sometimes found in short chains. In some cases, evident pleomorphism was present. Forty-eight *Aeromonas* isolates were selected for additional analysis. The selected isolates, which were cytochrome oxidase positive, facultatively anaerobic, and resistant to the vibriostatic agent 0/129, were further analyzed using biochemical means, following the scheme of Abbott et al. (2003).

Results from the performed biochemical tests enabled the majority of isolates to be grouped within the *Aeromonas* complexes (*A. hydrophila, A. sobria, A. caviae*), however, six isolates could not be grouped in this manner (Table 9). Following the *Aeromonas* identification schemes of Abbott et al. (2003), of the 48 isolates, 25 belonged to the *A. hydrophila* complex, 14 to the *A. sobria* complex, and 3 to the *A. caviae* complex. When the 25 isolates within the *A. hydrophila* complex were speciated according to Abbott et al. (2003), 22 were identified as *A. hydrophila sensu stricto*, while three could not be speciated. Within the *A. sobria* complex, nine were identified as *A. jandaei*, three as *A. veronii* bv *sobria*, and two could not be speciated. Of the three isolates within the *A. caviae* complex, two were identified as *A. eucrenophila,* while one could not be speciated.

As displayed in Table 10, the twenty-two *A. hydrophila sensu stricto* isolates were positive for the Voges-Proskauer reaction, esculin hydrolysis, presence of lysine decarboxylase and phenylalanine deaminase, and acid production from cellobiose, galactose, glycerol, maltose, mannitol, mannose, and trehalose. All *A. hydrophila* isolates were negative for H_2S production, as well as acid production from adonitol, inositol, lactose, sorbitol, raffinose, rhamnose, and xylose. The remaining tests that were examined yielded variable results.

Among the recovered isolates belonging to the *A. sobria* complex, nine were identified as *A. jandaei*. Biochemical evaluation of these isolates produced uniformly positive results for catalase and indole production, motility, gas production from glucose, and acid production from galactose, glycerol, mannitol, mannose, and trehalose (Table 10). Negative results were observed for H_2S production, esculin hydrolysis, and acid

production from adonitol, arabinose, salicin, sorbitol, sucrose, raffinose, rhamnose, and xylose for all tested isolates. The remaining tests gave variable results

Biochemical examination results for the three isolates identified as *A. veronii* bv *sobria* (Table 10) are as follows: All isolates were positive for indole production, motility, Voges-Proskauer reaction, gas production from glucose, presence of lysine decarboxylase and arginine dihydrolase, and acid production from sucrose. Moreover, all 3 isolates were negative for esculin hydrolysis, presence of ornithine decarboxylase, production of H_2S, and acid production from arabinose. The only examined tests that gave variable results for these isolates were Simmons citrate (33% positive) and mixed acid production (33% methyl red positive).

The two isolates within the *A. caviae* complex that were identified as *A. eucrenophila* yielded uniform positive results for mixed acid production (MR+), motility, catalase and indole production, esculin hydrolysis, gas production from glucose, and presence of lysine decarboxylase and arginine dihydrolase. Negative results for H_2S production, utilization of citrate, presence of ornithine decarboxylase, Voges-Proskauer reaction, and acid production from arabinose were obtained for these isolates.

Among the 48 isolates selected for further analysis, 11 were retrieved both in pure culture (in one instance, two distinct *Aeromonas* spp. isolated from one lake whitefish) and were all associated with varying signs of clinical disease (Table 11). Among the fish from which these isolates were recovered, a portion were normal externally (5/10), while a number of infected individuals presented with congestion and or hemorrhaging in the fins (3/10), pale coloration (2/10), and/or petechial and ecchymotic hemorrhagic foci within the ventral musculature (1/10). Internally, some of the infected individuals

exhibited hepatic abnormalities such as congestion (1/10), mottling (2/10), hemorrhaging (1/10), pallor (1/10), and multifocal necrotic foci (1/10). Additionally, moderate to severe splenomegaly, with or without darkening, was apparent in 6/10 individuals. Observed renal abnormalities included congestion (5/10), friability (5/10), mottling (3/10), and/or swelling (1/10). In one case, pronounced erythema within the peritoneal cavity was evident. Moreover, mild hemorrhagic enteritis was apparent in 3/10 individuals. Of the 11 motile aeromonad species that were retrieved in pure culture, six belonged to the *A. hydrophila* complex, four belonged to the *A. sobria* complex, and one belonged to the *A. caviae* complex.

The overall detected prevalence of motile aeromonads infecting lake whitefish during the period of this study was 14.45%. The prevalence of lake whitefish infected with motile *Aeromonas* spp. was higher in Lake Huron, at 15.71%, than in Lake Michigan, 13.30%, but these differences were not statistically significant. The prevalence of lake whitefish infected with motile aeromonads did vary by site (15.00%, 11.50%, 17.78%, and 14.17% for the Big Bay de Noc, Naubinway, Cheboygan, and Detour sites, respectively), but differences were not statistically significant (Figure 17). However, differences in motile aeromonad prevalence among seasons were statistically significant (χ^2=22.098; DF=3; P=<0.001), with the highest prevalence occurring in summer samples (Figure 18). The overall seasonal prevalence of motile aeromonad infections in lake whitefish by site is depicted in Figure 19.

The number of motile *Aeromonas* isolates identified to the complex level, and the sites and seasons from which they were recovered can be found in Table 12, while the prevalence of motile aeromonad derived infections can be found in Table 13.

Statistically significant differences in the prevalence of infection caused by members of the *A. hydrophila, A. sobria, and A. caviae* complexes were observed in this study (χ^2=17.85; DF=2; P=<0.001), with *A. hydrophila* complex members causing the highest number of detected infections, followed by *A. sobria* complex. The proportion of isolates belonging to each of the three complexes that were isolated from lake whitefish at each of the studied sites can be seen in Figure 20. Additionally, the proportion of isolates recovered in the four sampled seasons belonging to each of the three complexes can be found in Figure 21.

Of the 25 isolates belonging to the *A. hydrophila* complex, eight were recovered from lake whitefish caught from the Naubinway site, six from Cheboygan, seven from Big Bay de Noc, and four from Detour. This corresponded to an overall prevalence of 5.64% in all sampled sites, with 7.08% prevalence in Naubinway lake whitefish, 6.67% in Cheboygan, 5.83% in Big Bay de Noc, and 3.33% in Detour. *A. hydrophila* complex infections in lake whitefish were most prevalent during the Summer sampling period (8.33%), followed by the Spring (5.83%), Winter (5.31%), and Fall (2.22%) (Table 13). Differences in the prevalence of infection by members of the *A. hydrophila* complex were not statistically significant among sites or seasons.

The 14 isolates identified as members of the *A. sobria* complex were recovered from all four sites; six from Big Bay de Noc, four from Cheboygan, three from Naubinway, and one from Detour Village. This complex was detected at an overall prevalence of 3.16%, and were most prevalent in the Big Bay de Noc samples (5.00%), followed by Cheboygan (4.44%), Naubinway (2.65%), and Detour (0.83%). Seasonally, the prevalence of the *A. sobria* complex was most prevalent in Summer (6.67%),

followed by Winter (3.54%), Fall (1.11%) and Spring (0.83%) sampling periods. Differences in infection prevalence by *A. sobria* complex were not statistically significant among sites and seasons. Infections with *A. caviae* complex members were detected at a prevalence of 0.68% from all of the sampled sites. Big Bay de Noc (1.67%) and Detour (0.83%) were the only sites where this complex was detected, with summer having the highest prevalence (1.67%), followed by the spring (0.83%); however, these differences were not statistically significant.

When broken down by species, *A. hydrophila sensu stricto* was the highest in prevalence among infected fish (22/443; χ^2=41.9; DF=4; P=<0.001;Table 14). The prevalence of individuals infected with *A. hydrophila* was greatest in Naubinway samples (6.19%), followed by Cheboygan (5.56%), Big Bay de Noc (5.00%), and Detour (3.33%). The percent of infected individuals was greatest in the summer (6.67%), followed by winter (5.31%), spring (5.00%), and fall (2.22%).

The next most common motile aeromonad species isolated in this study was *A. jandaei* (*A. sobria* complex), with an overall prevalence of 2.03%. Prevalence of infection was highest in Naubinway samples (2.65%), followed by Big Bay de Noc (2.5%), Cheboygan (2.22%), and Detour (0.83%). The season of highest prevalence was winter (3.54%), followed by summer (2.50%), fall (1.11%), and spring (0.83%). *A. veronii* bv. *sobria* was the next most predominant motile aeromonad, with an overall prevalence of 0.68%. This bacterium was prevalent only in Big Bay de Noc lake whitefish sampled within the summer (summer prevalence from BDN at 10.00%). Thus, a prevalence of 2.50% was detected in Big Bay de Noc and summer samples, while all other seasons and sites were negative. *A. eucrenophila* (overall prevalence of 0.45%)

was also isolated from individual lake whitefish from both Big Bay de Noc and Detour samples, with a prevalence of .83% in both sites. The seasonal prevalence was 1.67% within summer samples, but negative in all other seasons.

DISCUSSION

Throughout the course of this study, numerous isolates identified as *Aeromonas* spp. were recovered from systemically infected lake whitefish. While a portion of these isolates were found in mixed culture or in the presence of other pathogenic organisms, many isolates were recovered in pure culture and were associated with obvious signs of disease. For instance, fish infected with *A. hydrophila* are known to exhibit hemorrhage in the trunk and fins (Hoshina 1962). Similar observations were made in lake whitefish infected with motile aeromonads. Additionally, the degenerative changes observed in the livers and kidneys of many lake whitefish infected with motile aeromonads have also been reported from other fish species systemically infected with *Aeromonas* spp. spontaneously (Huizinga et al. 1979, Miyazaki and Kaige 1985) and experimentally (Bach et al. 1978). Furthermore, through rapid proliferation within the intestine, some motile aeromonads can elicit a toxemia via adsorption of toxic metabolites through the intestinal epithelium (Miyazaki and Jo 1985; Miyazaki and Kaige 1985), which among other things, generates significant pathology within the gastrointestinal tract.

A number of isolates were also recovered from individuals with marked external ulcerations, along with a multitude of internal clinical signs, but were present along with other pathogens. These include *R. salmoninarum*, the etiological agent of bacterial kidney disease, *C. maltaromaticum*, the causative agent of pseudokidney disease, and *Shewanella* spp., which cause shewanellosis, as well as a number of parasites, such as *Cystidicola farionis,* encysted metacercariae, and acanthocephalans. Interactions among and between these pathogens in this host species are currently unknown.

Of the four species of motile aeromonads that were isolated from infected lake whitefish and identified according to Abbott et al. (2003), *A. hydrophila sensu stricto* was the most prevalent, accounting for the highest proportion of infections in all sampled seasons and sites. The prevalence of this bacterium was at least double that of the next most predominant species in all sampled sites. The role of *A. hydrophila* in fish infections is well known and, among the mesophilic aeromonads, is considered to be the most important fish pathogen (Noga 2000). The next most prevalent motile aeromonad species was *A. jandaei*, which was detected in over 2.00% of the lake whitefish sampled from 3 of the 4 sites. An interesting aspect of these infections was that they were most prevalent in the winter sampling period. This in contrast to all other detected motile aeromonad species, which were without exception, most prevalent in lake whitefish during the summer. Reasons for this seasonal difference in *A. jandaei* prevalence are currently unknown. It is also interesting to note that this bacterium was always recovered from organisms concurrently infected with another bacterial species, although the mixed flora was not consistent. In a previous study by Esteve and Garay (1991), *A. jandaei* , along with a number of other bacterial species (i.e. *A. hydrophila, Pseudomonas fluorescens, Shewanella putrifaciens*) were recovered from 3 disease epizootics in the European eel (*Anguilla anguilla*), during which mortality rates of up to 80% were observed. These isolates were later analyzed for their virulence via experimental exposure, and only the *A. hydrophila* and *A. jandaei* isolates were able to reproduce the clinical signs observed in the natural infections (Esteve et al. 1993).

Aeromonas veronii bv *sobria* was also isolated in this study, although its presence was limited to Big Bay de Noc fish and only in the summer. Additionally, two of the

three *A. veronii* bv *sobria* isolates were recovered in pure culture, and the infected individuals presented with clinical signs of disease, including renal mottling and pallor, multi-focal necrotic foci within the liver, and mild hemorrhagic enteritis in the posterior portion of the intestine. As such, the relatively high prevalence of this bacterium in lake whitefish collected from Big Bay de Noc in the summer, along with the observed gross pathological effects, suggested that an *A. veronii* bv *sobria*-derived widespread infection may have occurred. In a study by Rahman et al. (2002), virulence factors of *A. veronii* bv *sobria* isolates recovered from deep hemorrhagic ulcers in a multitude of fish species in Bangladesh, exhibited cytotoxic and hemolytic properties , in contrast to other *Aeromonas* spp. recovered from environmental sources (Rahman et al. 2002).

The two isolates identified as *A. eucrenophila* were recovered from Big Bay de Noc and Detour whitefish in the summer. These isolates were recovered from individuals with mixed infections exhibiting hemorrhagic and prolapsed vents, generalized erythema within the peritoneum, including the internal lateral and ventral musculature, a small amount of serosanguinous fluid within the peritoneum, multi-focal hemorrhage within the liver, moderate splenomegaly, a friable kidney, and gastroeneteritis/hemorrhagic enteritis. In a study by Santos et al. (1999), the presence of a number of virulence factors implicated in disease causation, such as aerolysin, siderophores, and other hemolytic toxins, were detected in strains of *A. eucrenophila* recovered from a variety of freshwater fish.

A number of atypical biochemical reactions were observed for the motile aeromonad isolates recovered in this study. For example, 13.64% of the *A. hydrophila* isolates recovered from lake whitefish were ornithine decarboxylase positive, 9.10% were

arginine dihydrolase negative, and 4.55% were catalase negative, none of which was an observed occurrence in *A. hydrophila* isolates studied by Abbott et al. (2003). Acid production from cellobiose was a rare occurrence for *A. hydrophila* isolates analyzed by Abbott, yet all of our *A. hydrophila* isolates were positive. Additionally, acid production from melibiose has been rarely reported in *Aeromonas* spp., let alone in *A. hydrophila*, but 66.67% of the isolates tested for this characteristic in this study were positive. Additional atypical reactions recorded in this study include: lysine decarboxylase negative (11.11%), ornithine decarboxylase positive (11.11%), arginine dihydrolase negative (11.11%), and acid production from lactose positive (25.00%) strains of *A. jandaei*, as well as lysine decarboxylase in *A. eucrenophila* (100%)

One possible explanation for these atypical reactions is the extremely heterogenous nature of *Aeromonas* spp, which can account for large discrepancies in phenotypic characteristics. Moreover, extrachromosomal elements can encode for various metabolic activities, as well as antibiotic resistance, further perpetuating the already diverse phenotypes associated with many *Aeromonas* strains (Abbott et al. 2003; Walsh et al. 1995).

The diversity of recovered motile *Aeromonas* spp. was the highest in lake whitefish collected from Big Bay de Noc, with all four of the species isolated in this study being represented. Conversely, motile aeromonad infections in Naubinway and Cheboygan were only by *A. hydrophila* and *A. jandaei*. Detour *Aeromonas* isolates were only slightly more diverse, with *A. hydrophila*, *A. jandaei*, and *A. eucrenophila* being detected here. Reasons for the diversity in Big Bay de Noc, or lack thereof in the other sites, are currently unknown, but may be explained by differences in migration rates,

increased reservoir abundance in particular sites, and/or increased susceptibility to motile aeromonad infections in particular lake whitefish populations, though further investigation is required.

At the complex level, statistically significant differences in motile aeromonad prevalence were detected. *Aeromonas hydrophila* complex members were responsible for the most infections caused by a motile aeromonad in this study, followed by *A. sobria* and *A. caviae* complex members. In other studies, such as the one conducted by Paniagua et al. (1990), *A. hydrophila* was the most common group of aeromonads isolated from river water, and these isolates were found to be virulent more often than the other less frequently isolated motile aeromonads.

Other studies have reported motile aeromonad infection prevalences of 7.7% in wild fish populations in Croatia (Popovic et al. 2000), 2.5% in wild Nile tilapia (*Oreochromis niloticus*), and 6.25% in wild Karmout catfish (Eissa et al. 1994). Given that motile aeromonads are extremely widespread in aquatic environments (Hazen et al. 1978; Alonso et al. 1994), and that the Great Lakes are currently in a state of flux (see literature review), the relatively high overall prevalence of motile *Aeromonas* infection (14.45%) is not a complete surprise. By site, the detected prevalence of motile aeromonads infection in lake whitefish was highest in lake whitefish from Cheboygan, followed by Big Bay de Noc, Detour, and Naubinway; however these differences were not statistically significant. In preliminary results from other aspects of the whitefish Natural Mortality Project, lake whitefish from Cheboygan have been demonstrated to have the lowest lipid levels among all four sites, and thus may be under the most nutritional stress, putting them at a higher risk for infection. However, many factors are

known to play a role in disease processes, and as such, further research will be necessary to further elucidate differences among sites (Dr. Michael Jones and Dr. Michael Arts, pers. comm.).

Seasonally, overall motile aeromonad infections in lake whitefish were most prevalent in the summer. The observed seasonal pattern in this study is consistent with what is considered typical in a number of other studies. High temperatures are frequently observed in motile aeromonad infections (Aoki 1999). For example, Meyer (1970) reported epizootics caused by motile aeromonads among warm water fishes in the southeastern U.S. to be most common in spring and early summer when water temperatures are high. Moreover, mesophilic *Aeromonas* spp. present within the environment have been detected in high densities when water temperatures are at their highest point (Osborne et al. 1989). Additionally, pathogen surveys in wild and feral populations of fish conducted at the Aquatic Animal Health Laboratory (AAHL) (Michigan State University) have found a higher prevalence of motile aeromonad infections in warmer months of the year (Loch and Faisal unpublished data). Concurrently, clinical cases submitted to the AAHL involving motile aeromonad infections in captive stocks are more frequent in the warmer months (Loch and Faisal unpublished data).

In conclusion, the clinical findings associated with those *Aeromonas* species that were recovered in pure culture, along with the relatively high prevalence of infections detected in a portion of the sampled sites or seasons (i.e Cheboygan Summer samples greater than 40% prevalence) suggest that this complex of bacteria may be capable of causing significant amounts of disease in lake whitefish populations, especially during

periods of high water temperatures. Furthermore, the high proportion of *Aeromonas* infections detected in the presence of additional pathogens (>80%) suggests that even if many of the *Aeromonas* infections are occurring secondarily to other infections, the possible augmentation of disease processes through the synergistic effects of multiple pathogens may have serious implications on infected individuals. For instance, numerous reports of infestations with parasites and fungi have been shown to predispose fish to infections with motile aeromonads (Noga 1986; Esch and Hazen 1980; Egusa 1978). In addition, immuno-supression brought about by physiologic (i.e., spawning) and environmental (i.e. high temperatures and low water levels) stressors can predispose individuals to significant infections with motile aeromonads, as illustrated by the recent massive fish kill in common carp (*Cyprinus carpio*) residing in the St. Lawrence River (Monette et al. 2006), which was partially attributed to infection by *A. hydrophila*.

Sampling Period	BD	NB	C	DV	Season Total
Fall 05	30	30	0	30	**90**
Winter 06	30	23	30	30	**113**
Spring 06	30	30	30	30	**120**
Summer 06	30	30	30	30	**120**
Site Total	**120**	**113**	**90**	**120**	**443**

Table 8. Representative seasons with number of lake whitefish sampled on each occasion throughout the course of this study.

Test	All Isolates (n=48)	*A. hydrophila* complex (n=25)	*A. sobria* complex (n=14)	*A. caviae* complex (n=3)
Catalase	97.92% (48)	96.00% (25)	100.00% (14)	100.00% (3)
Simmon's Citrate	20.83% (48)	24.00% (25)	21.43% (14)	0.00% (3)
Indole	91.67% (48)	96.00% (25)	100.00% (14)	66.67% (3)
Motility	95.83% (48)	96.00% (25)	100.00% (14)	100.00% (3)
Methyl Red	54.17% (48)	52.00% (25)	50.00% (14)	100.00% (3)
Voges-Proskauer	83.33% (48)	100.00% (25)	93.33% (14)	0.00% (3)
Esculin	59.57% (47)	100.00% (25)	0.00% (14)	66.67% (3)
Gas from Glucose	91.67% (48)	96.00% (25)	100.00% (14)	66.67% (3)
Lysine Decarboxlyase	94.74% (38)	100.00% (22)	91.67% (12)	66.67% (3)
Ornithine Decarboxylase	13.64% (44)	12.00% (25)	14.29% (14)	0.00% (3)
Arginine Dihydrolase	86.36% (44)	92.00% (25)	85.71% (14)	66.67% (3)
Phenylalanine Deaminase	88.89% (18)	100.00% (8)	80.00% (5)	0.00% (1)
Arabinose	4.65% (43)	8.00% (25)	0.00% (13)	0.00% (3)
Adonitol	0% (8)	0% (5)	0.00% (3)	ND
Cellobiose	84.62% (13)	100.00% (6)	66.67% (6)	0.00% (1)
Galactose	100% (8)	100.00% (5)	100.00% (3)	ND
Glycerol	87.50% (8)	100.00% (3)	100.00% (4)	0.00% (1)
Inositol	20.00% (5)	0.00% (3)	50.00% (2)	ND
Lactose	10.00% (10)	0.00% (6)	25.00% (4)	ND
Maltose	100% (5)	100.00% (3)	100.00% (2)	ND
Mannitol	90.90% (11)	100.00% (5)	100.00% (5)	0.00% (1)
Mannose	90.00% (10)	100.00% (5)	100.00% (4)	0.00% (1)
Melibiose	42.86% (7)	66.66% (3)	25.00% (4)	ND
Salicin	20.00% (10)	40.00% (5)	0.00% (5)	ND
Sorbitol	9.09% (11)	12.50% (8)	0.00% (3)	ND
Sucrose	23.53% (17)	20.00% (5)	27.27% (11)	0.00% (1)
Raffinose	0% (8)	0% (5)	0% (3)	ND
Rhamnose	0% (12)	0% (8)	0% (4)	ND
Trehalose	100% (8)	100.00% (5)	100.00% (3)	ND
Xylose	0% (8)	0% (5)	0% (3)	ND

Table 9. Percentage of *Aeromonas* isolates (by complex) in this study that were positive for the performed biochemical tests. Number in parenthesis= number of isolates tested. All isolates were cytochrome oxidase positive, facultative anaerobes that were resistant to the vibriostatic agent O/129 and did not produce H_2S in Sulfur-Indole-Motility medium.

Test	*A. hydrophila* % Positive (n=22)	*A. jandaei* %Positive (n=9)	*A. veronii* bv *sobria* % Positive (n=3)	*A. eucrenophila* % Positive (n=2)
Catalase	95.45% (22)	100.00% (9)	100.00% (3)	100.00% (2)
Simmon's Citrate	27.27% (22)	22.22% (9)	33.33% (3)	0.00% (2)
Indole	95.45% (22)	100.00% (9)	100.00% (3)	100.00% (2)
Motility	95.45% (22)	100.00% (9)	100.00% (3)	100.00% (2)
Methyl Red	54.54% (22)	66.67% (9)	33.33% (3)	100.00% (2)
Voges-Proskauer	100.00% (22)	88.89% (9)	100.00% (3)	0.00% (2)
Esculin	100.00% (22)	0.00% (9)	0.00% (3)	100.00% (2)
Gas from Glucose	95.45% (22)	100.00% (9)	100.00% (3)	100.00% (2)
Lysine Decarboxlyase	100.00% (20)	88.89% (9)	100.00% (3)	100.00% (2)
Ornithine Decarboxylase	13.64% (22)	11.11% (9)	0.00% (3)	0.00% (2)
Arginine Dihydrolase	90.90% (22)	88.89% (9)	100.00% (3)	100.00% (2)
Phenylalanine Deaminase	100.00% (8)	80.00% (5)	ND	ND
Arabinose	4.55% (22)	0.00% (8)	0.00% (3)	0.00% (2)
Adonitol	0.00% (5)	0.00% (3)	ND	ND
Cellobiose	100.00% (6)	66.67% (6)	ND	ND
Galactose	100.00% (5)	100.00% (3)	ND	ND
Glycerol	100.00% (3)	100.00% (4)	ND	ND
Inositol	0.00% (3)	50.00% (2)	ND	ND
Lactose	0.00% (6)	25.00% (4)	ND	ND
Maltose	100.00% (3)	100.00% (2)	ND	ND
Mannitol	100.00% (5)	100.00% (5)	ND	ND
Mannose	100.00% (5)	100.00% (4)	ND	ND
Melibiose	66.67% (3)	25.00% (4)	ND	ND
Salicin	40.00% (5)	0.00% (5)	ND	ND
Sorbitol	0.00% (7)	0.00% (3)	ND	ND
Sucrose	20.00% (5)	0.00% (8)	100.00% (3)	ND
Raffinose	0.00% (5)	0.00% (3)	ND	ND
Rhamnose	0.00% (7)	0.00% (4)	ND	ND
Trehalose	100.00% (5)	100.00% (3)	ND	ND
Xylose	0.00% (5)	0.00% (3)	ND	ND

Table 10. Percentage of *Aeromonas* isolates (by species) in this study that were positive for the performed biochemical tests. Number in parenthesis= number of isolates tested. All isolates were cytochrome oxidase positive, facultative anaerobes that were resistant to the vibriostatic agent O/129 and did not produce H_2S in Sulfur-Indole-Motility medium.

Bacterial Identification	Normal External	Congestion/ Hemorrhage Fins	Hemorrhage Musculature	External Pallor	Hepatic Abnormalities	Splenomegaly	Renal Abnormalities	Enteritis
A. hydrophila sensu stricto	+	-	-	-	+	+	+	-
A. hydrophila complex	-	+	+	-	-	+	+	+
A. hydrophila sensu stricto	-	+	-	-	+	+	+	-
A. hydrophila sensu stricto	-	-	-	+	+	+	+	-
A. hydrophila complex	+	-	-	-	+	-	+	-
A. hydrophila complex	-	-	-	+	+	+	+	-
A. janadei & *A. eucrenophila*	-	+	-	-	+	+	+	+
A. veronii bv *sobria*	+	-	-	-	-	-	+	-
A. veronii bv *sobria*	+	-	-	-	+	-	-	+
A. sobria complex	+	-	-	-	-	-	+	-

Table 11. Gross clinical observations in lake whitefish collected from Lakes Michigan and Huron in which *Aeromonas* spp. were the only detected bacterial species.

Site	*A. hydrophila* complex	*A. sobria* complex	*A. caviae* complex
Big Bay de Noc	7	6	2
Naubinway	8	3	0
Cheboygan	6	4	0
Detour Village	4	1	1
Season			
Fall	2	1	0
Winter	6	4	0
Spring	7	1	1
Summer	10	8	2

Table 12. Number of isolates belonging to each complex that were recovered from lake whitefish collected from Lakes Michigan and Huron between October 2005-August 2006.

Complex Identity	Site				Season			
	BDN	NB	CHE	DV	Fall	Winter	Spring	Summer
***A. hydrophila* complex**	5.83% (7/120)	7.08% (8/113)	6.67% (6/90)	3.33% (4/120)	2.22% (2/90)	5.31% (6/113)	5.83% (7/120)	8.33% (10/120)
***A. sobria* complex**	5.00% (6/120)	2.65% (3/113)	4.44% (4/90)	0.83% (1/120)	1.11% (1/90)	3.54% (4/113)	0.83% (1/120)	6.67% (8/120)
***A. caviae* complex**	1.67% (2/120)	0.00% (0/113)	0.00% (0/90)	0.83% (1/120)	0.00% (0/90)	0.00% (0/113)	0.83% (1/120)	1.67% (2/120)

Table 13. Detected prevalence of the three *Aeromonas* complexes infecting lake whitefish collected from Lakes Michigan and Huron between October 2005-August 2006 (BDN=Big Bay de Noc; NB=Naubinway; CHE=Cheboygan; DV=Detour Village).

Species Identification	Site				Season			
	BDN	NB	CHE	DV	Fall	Winter	Spring	Summer
A. hydrophila	5.00% (6/120)	6.19% (7/113)	5.56% (5/90)	3.33% (4/120)	2.22% (2/90)	5.31% (6/113)	5.83% (7/120)	8.33% (10/120)
A. veronii bv *sobria*	2.50% (3/120)	0.00% (0/113)	0.00% (0/90)	0.00% (0/120)	0.00% (0/90)	0.00% (0/113)	0.00% (0/120)	2.50% (3/120)
A. jandaei	2.50% (3/120)	2.65% (3/113)	2.22% (2/90)	0.83% (1/120)	1.11% (1/90)	3.54% (4/113)	0.83% (1/120)	2.50% (3/120)
A. eucrenophila	0.83% (1/120)	0.00% (0/113)	0.00% (0/90)	0.83% (1/120)	0.00% (0/90)	0.00% (0/113)	0.00% (0/120)	1.67%% (2/120)

Table 14. Detected prevalence of the four *Aeromonas* species infecting lake whitefish collected from Lakes Michigan and Huron between October 2005-August 2006 (BDN=Big Bay de Noc; NB=Naubinway; CHE=Cheboygan; DV=Detour Village).

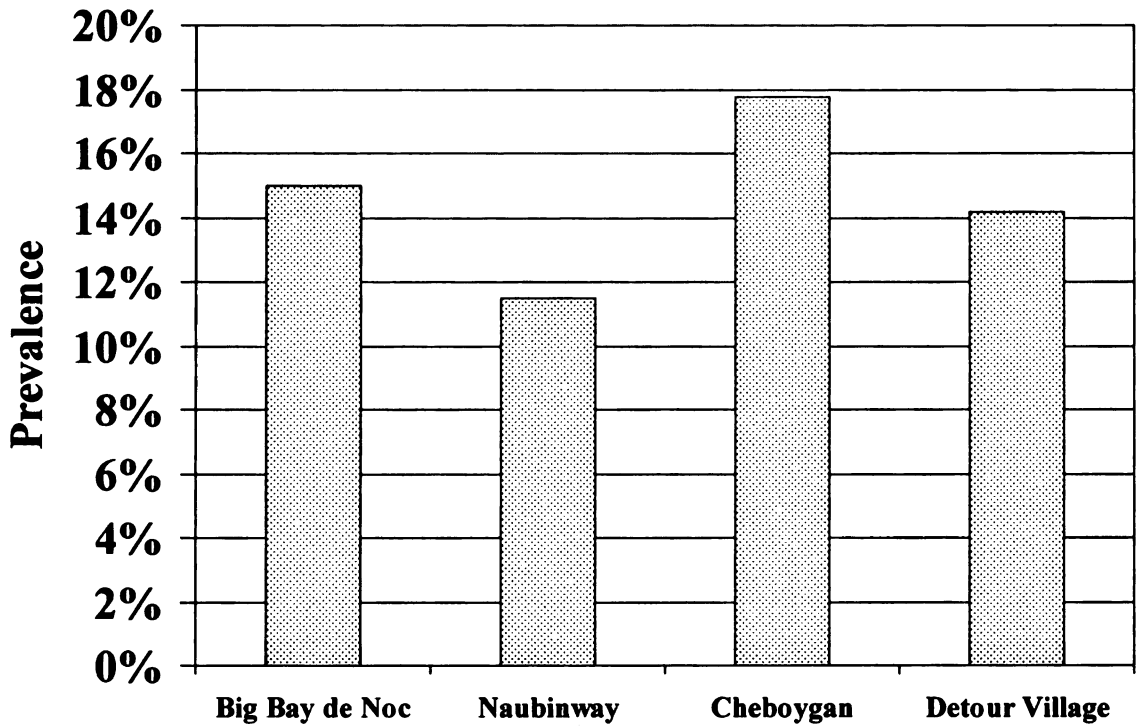

Figure 17. Prevalence of infection by motile *Aeromonas* spp. in lake whitefish from four sites within Lakes Michigan and Huron between October 2005-August 2006.

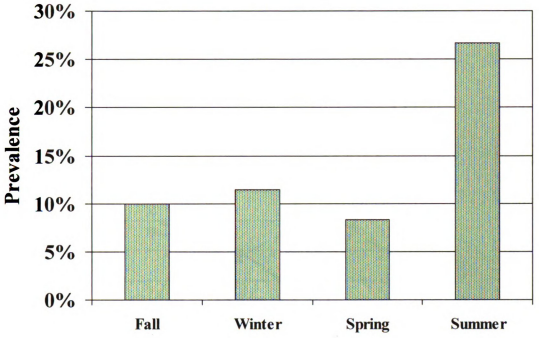

Figure 18. Seasonal prevalence of motile *Aeromonas* spp. infections in lake whitefish collected from four sites within Lakes Michigan and Huron between October 2005-August 2006.

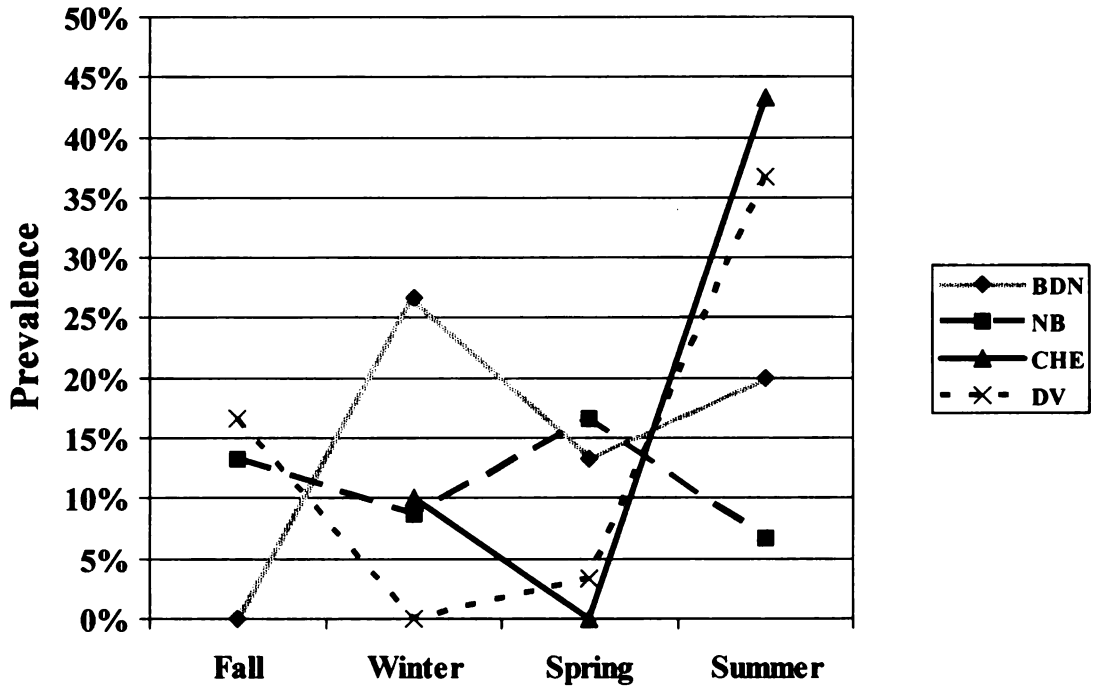

Figure 19. Overall prevalence of lake whitefish infected with motile *Aeromonas* spp. by season and site between October 2005-August 2006.

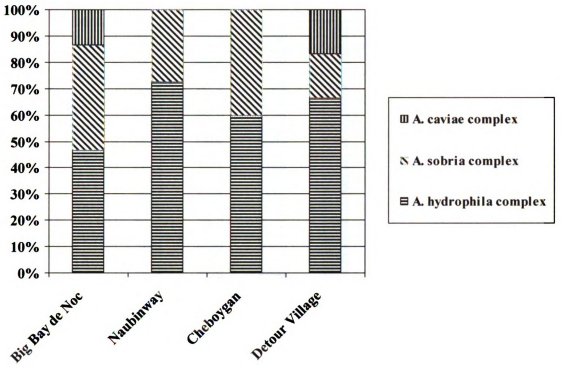

Figure 20. Proportion of the total detected infections in lake whitefish caused by members of the three *Aeromonas* complexes by site.

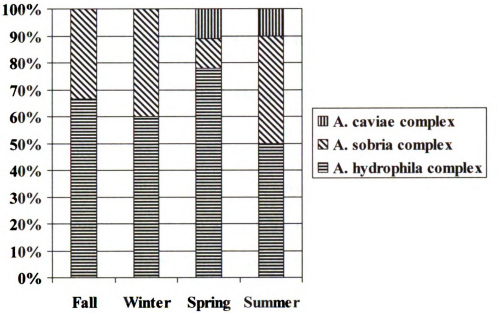

Figure 21. Proportion of the total detected infections in lake whitefish caused by members of the three *Aeromonas* complexes by season.

CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS

Despite the ecological and economic importance of indigenous lake whitefish inhabiting the GLB, extremely little is known of the infectious diseases that afflict them. It is well established that diseases can play a significant role in the dynamics of fish populations, and that there may be an increased risk of diseases in disturbed environments. Disturbances in the Great Lakes, such as habitat alterations, invasions by exotic organisms, and climate/temperature changes, may increase the susceptibility of lake whitefish to disease. To ascertain whether this is truly a problem, one must have some idea as to what pathogens are present within lake whitefish populations and whether these organisms are capable of generating disease in this specific host. The potential of these pathogens to generate mortality is also important. Although the base of knowledge regarding diseases of fish within the GLB is ever expanding, a lack of research on lake whitefish diseases is still a major impediment to understanding what may or may not be playing a role in their population dynamics.

Through this study, I was able to isolate a number of bacterial species infecting lake whitefish inhabiting the GLB, and thus gain some understanding as to what effects these infections may have on this particular host species. In Chapter two, I found that *C. maltaromaticum*, the causative agent of PseudoKidney Disease, which in itself is not extremely well-studied, is present within populations of lake whitefish, and that infections caused by this bacterium can potentially be associated with varying degrees of

pathology. Some of the lake whitefish from which *C. maltaromaticum* was recovered exhibited significant degenerative changes within their livers, spleens, and most dramatically, swim-bladders, thus providing evidence that this bacterium may indeed be capable of causing disease in lake whitefish. Moreover, through the use of biochemical analyses, molecular assays, and genetic analyses, I was able to definitively identify these isolates as *C. maltaromaticum*, yet demonstrate unique phenotypic characteristics and some degree of phylogenetic variability was present among lake whitefish strains and among previously isolated strains. The prevalence of this pathogen was not uniform across space and time, with the majority of infected individuals coming from a single site and sampling occasion (Naubinway, winter). The 20.8% prevalence that was detected in the winter Naubinway samples is considered quite high for a wild fish population and illustrates the potential of this bacterium to generate large-scale epizootics under conditions favorable to disease causation. This was also considered the first report of *C. maltaromaticum* from this host species and within this geographic locale.

Within Chapter 3, I describe the isolation of *Aeromonas salmonicida* subspecies *salmonicida,* the etiological agent of furunculosis, from lake whitefish within the GLB, an occurrence that has never been previously reported. Thorough clinical examination and histopathological assessment, I observed the destructive capability that this bacterium has on lake whitefish that may succumb to infection, thus validating the fact that a serious bacterial pathogen is endemic within populations of GLB coregonines. However, the low overall prevalence of infection that was detected throughout this study may indicate that this bacterium may not currently be a significant threat to lake whitefish populations within Lakes Michigan and Huron. However, if conditions more conducive to disease

generation by *A. salmonicida salmonicida* should arise, the impact on lake whitefish populations could be potentially devastating. Through the use of biochemical assays and PCR, I was able to definitively identify this bacterium as *A. salmonicida salmonicida.* I also attempted to detect *A. salmonicida salmonicida* in lake whitefish that presented with furuncle-like lesions, yet were culture negative. While unable to detect the presence of *A. salmonicida salmonicida* molecularly in these individuals, histopathological examination of a portion of the furuncle-like lesions demonstrated nearly identical tissue alterations to what has been previously reported in fish experimentally infected with this bacterium. Moreover, infections in lake whitefish caused by *A. salmonicida salmonicida* were detected in only two of the four sampled sites.

Finally, in Chapter 4, I describe the isolation of motile *Aeromonas* spp. from lake whitefish within the GLB. This heterogenous group of bacteria was present in all four sampled sites and seasons, with a tendency towards higher prevalence in summer, as might be expected given the higher water temperatures at this time. When assigned to their respective complexes, members of the *A. hydrophila* complex were the most prevalent group, followed by members of the *A. sobria* complex. A total of four distinct *Aeromonas* spp. were detected, including *A. hydrophila sensu stricto, A. jandaei, A. veronii* bv *sobria,* and *A. eucrenophila. A. hydrophila sensu stricto* was the predominant species, a common occurrence among studies looking at motile aeromonad infections in fish, followed by *A. jandaei.* Some of the sampled sites had a more "diverse" population of *Aeromonas* spp. that caused infections than others. Moreover, a large percentage of the isolated motile aeromonads were found in the presence of an array of other pathogens, thus suggesting that often, these bacteria may infect the host secondarily.

Clinical findings in lake whitefish infected only with *Aeromonas* spp. were quite varied, but in general, demonstrated the ability of some of the isolated strains to cause pathological changes in infected lake whitefish.

FUTURE RESEARCH

The data presented within this Thesis established a solid base for future research that may involve bacterial pathogens of lake whitefish within the GLB. However, much more work is needed if one wants to determine what bacteria are capable of affecting lake whitefish at the population level. This Thesis focused on non-fastidious bacterial pathogens of lake whitefish, but many other bacterial species, such as the facultatively intracellular *Mycobacterium* spp., the obligately intracellular *Rickettsia*-like organisms, obligate anaerobic *Clostridium* spp., and many others cannot be detected via the methodologies used in this study. Studies specifically targeting these organisms are necessary to determine their presence and/or rule out any impact, regardless how small, they may have on lake whitefish.

Moreover, studies focusing on the interactions between multiple pathogens that were found to infect a single host concurrently could aid in the elucidation of disease processes in these organisms. It is likely that these pathogens may impact one another negatively in some situations, while in others they may act synergistically in the generation of disease.

Further research on *C. maltaromaticum* isolates recovered in this study could be fruitful, as the presence of a piscicolin precursor gene was detected in our isolates. This bacteriocin is known to have antibacterial properties; thus by further investigating its role *in vivo*, one may be able to elucidate a novel role this protein may play in many processes involving bacterial infections. In addition, an assessment of possible virulence factors

that this bacterium may have could shed light onto the pathogenesis of *C. maltaromaticum.*

Additional studies attempting to quantify the prevalence of *A. salmonicida salmonicida* infections in lake whitefish through the use of serological assays, such as the Quantitative Enzyme Linked Immunosorbent Assay (Q-ELISA), could help in the determination of whether whitefish are able to recover from these infections or if they are always fatal. As was seen in this study, this bacterium is capable of causing severe disease in an infected host, and as such, knowledge of how lake whitefish, as a now known susceptible host species, are able to manage these infections would be useful. Moreover, further molecular characterization of the isolates retrieved in this study might allow one to determine how closely related these strains are to strains recovered in other areas and host species within the GLB.

Further characterization of the motile *Aeromonas* spp. isolated within this study via molecular techniques would allow for a definitive species identification of all isolates. Through the sequencing of housekeeping genes within the *Aeromonas* genome, relatedness of isolates to one another can be determined and sources of these infections can sometimes be traced. In addition, comparison of strain variation among the four sampled stocks could tell us much about migration of lake whitefish, sources of infection, etc. Analysis of the virulence factors associated with the *Aeromonas* isolates recovered in this study could also aid in the determination of how likely these bacteria are to cause disease in this host species and where they may tend to localize in the body upon infection.

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