INVESTIGATION OF THE HETEROGENEITY AMONG *FLAVOBACTERIUM PSYCHROPHILUM* STRAINS DEVASTATING SALMONID STOCKS IN THE LAURENTIAN GREAT LAKES BASIN

By

Danielle Mary Van Vliet

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ABSTRACT

INVESTIGATION OF THE HETEROGENEITY AMONG *FLAVOBACTERIUM PSYCHROPHILUM* STRAINS DEVASTATING SALMONID STOCKS IN THE LAURENTIAN GREAT LAKES BASIN

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Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD), threatens wild and propagated salmonid populations and is considered one of the most important salmonid pathogens worldwide, yet a substantial lack of epidemiological knowledge exists. In particular, the heterogeneity among *F. psychrophilum* strains in the United States is largely unknown, which has hindered our understanding of the pathogen tracking dynamics as well as development of vaccines based on fish host species and/or highly virulent strains. I have focused my research on understanding the diversity of *F. psychrophilum,* particularly as it pertains to Great Lakes basin (GLB) salmonid populations. Because of the variable fish host susceptibility to *F. psychrophilum* and its vertical transmission, I hypothesized the pathogen prevalence among different salmonid broodstocks in the GLB will vary by species and demonstrate a link to the incidence of BCWD in resultant progeny. To test these hypotheses, a multiyear study was performed involving 7 species spanning 11 broodstock populations and their resultant progeny. Chinook salmon (*Oncorhynchus tshawytscha*) broodstock had the highest infection prevalence (63.2%); however, steelhead (*O. mykiss*) progeny had the highest incidence of BCWD. Building on these results, a thorough understanding of the genetic heterogeneity of *F. psychrophilum* in the GLB may influence the development of vaccines against this pathogen, particularly if dominant, highly virulent, and/or species specific strains are identified. Herein, I hypothesized the use of alternative loci, such as

gyrB, murG, and *tuf* will demonstrate a robust intraspecific phylogeny of GLB *F. psychrophilum*. My results indicated a genetically diverse *F. psychrophilum* population, identified dominant and highly virulent strains, and demonstrated an association between sequence variation and fish host species within the GLB. To further the understanding of this genetic diversity nationwide, and allow for larger, more robust epidemiological conclusions, a multilocus sequence typing approach was used on 96 U.S. *F. psychrophilum* isolates. I hypothesized that some sequence types (STs) would be unique to the GLB, while others would be widespread in their distribution. This study resulted in the identification of 28 novel STs, including ST78 which has been linked to mass mortalities in multiple states. Furthermore, these results confirm the broad distribution of ST10 within the U.S. and lend evidence to the global dissemination of *F. psychrophilum*. Historically, the use of antimicrobials, such as oxytetracycline and florfenicol, has been the main method of *F. psychrophilum* control in the U.S. In this context, I hypothesized that GLB isolates will show resistance to these antimicrobials due to their frequency of use. The phenotypic diversity among these isolates as it pertains to antimicrobial susceptibility was demonstrated using a standardized microbroth dilution assay. My results show the occurrence of oxytetracycline resistance among *F. psychrophilum* isolates in Michigan, although no resistance to florfenicol has been detected at this point. The demonstrated widespread distribution of *F. psychrophilum* and its genetic and phenotypic heterogeneity, along with the frequent BCWD outbreaks within the GLB, identifies the need for improved *F. psychrophilum* control measures. The documentation of strains that are fish host species specific, highly virulent, and/or resistant to commonly used antimicrobials provides a platform for development of targeted control measures including vaccination, biosecurity, and chemotherapeutic strategies.

This dissertation is dedicated to my favorite fishing partner, my dad.

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Lastly, I must recognize the lives of all the fish that were sacrificed for this research, these animals will forever be respected and acknowledged.

TABLE OF CONTENTS

LIST OF TABLES xii

LIST OF FIGURES xiv

KEY TO ABBREVIATIONS xviii

Introduction 1
1. Study objectives 3
REFERENCES 6

Chapter 1. Literature Review 10
1. Introduction 11
2. The disease 12
2.1. Host range and host susceptibility 12
2.2. Geographic distribution 13
2.2.1 Great Lakes basin 13
2.3. Transmission 14
2.4. Pathogenesis 15
2.5. Clinical signs 16
2.6. Factors affecting disease course 17
2.7. Losses and economic impacts 18
2.8. Treatment, control, and prevention 19
2.8.1. Chemotherapy 19
2.8.2. Husbandry and biosecurity 20
2.8.3. Fish diet, dietary supplements, and probiotics 21
2.8.4. Disease-resistant fish strains 21
2.8.5. Vaccination 22
3. The pathogen 23
3.1. Taxonomic classification 23
3.2. Phenotypic characteristics 23
3.3. Bacterial culture conditions 24
3.4. Pathogen diversity 25
3.4.1. Phenotypic diversity 25
3.4.1.1. Antimicrobial susceptibility 26
3.4.2. Serological diversity 27
3.4.3. Molecular and genetic diversity 28
3.4.3.1. Pulsed-field gel electrophoresis and restriction enzyme digestion 29
3.4.3.2. Restriction fragment length polymorphism analysis 30
3.4.3.3. Ribotyping 30
3.4.3.4. Random amplified polymorphic DNA analysis 31

3.4.3.5. Plasmid profiling 31
3.4.3.6. Sequence analysis methods 32
3.4.3.6.1. Multilocus sequence typing 32
REFERENCES 36

Chapter 2. *Flavobacterium psychrophilum* infections in salmonid broodstock and hatchery-propagated stocks of the Great Lakes basin 56
1. Abstract 57
2. Introduction 58
3. Materials and Methods 60
3.1. Collection of broodstock 60
3.2. Progeny fish 62
3.3. Fish sampling 63
3.4. Flavobacterial isolation and identification 63
3.5. *F. psychrophilum* identification 64
3.6. Statistical analyses 65
4. Results 67
4.1. *F. psychrophilum* identification 67
4.2. Broodstock 67
4.2.1. Feral broodstock 68
4.2.2. Captive broodstock 69
4.3. Progeny 70
5. Discussion 71
APPENDIX 77
REFERENCES 87

Chapter 3. Sequence analysis of *gyrB, murG,* and *tuf* genes individually and combined reveals the genetic diversity and fish host species specificity of *Flavobacterium psychrophilum* in the Great Lakes basin 94
1. Abstract 95
2. Introduction 96
3. Materials and Methods 99
3.1. *F. psychrophilum* isolation 99
3.2. DNA extraction and *F. psychrophilum* confirmation 100
3.3. Housekeeping genes: PCR amplification and gene sequencing 101
3.4. Data analysis 102
4. Results 103
4.1. Single gene analyses 103
4.1.1. *gyrB* 103
4.1.2. *murG* 104
4.1.3. *tuf* 106
4.2. Concatenated sequence analyses 107
4.2.1. *gyrB* and *murG* 107
4.2.2. *gyrB* and *tuf* 108

4.2.3. *murG* and *tuf* 108
4.2.4. *gyrB, murG,* and *tuf* 109
5. Discussion 110
APPENDIX 115
REFERENCES 131

Chapter 4. Genetic diversity of *Flavobacterium psychrophilum* isolated from three *Oncorhynchus* spp. in the U.S.A. revealed by multilocus sequence typing 137
1. Abstract 138
2. Introduction 139
3. Materials and Methods 141
3.1. Fish collection and isolation of *F. psychrophilum* 141
3.2. MLST 143
3.3. MLST data analysis 144
4. Results 145
4.1. Genetic diversity of *F. psychrophilum* in the U.S.A. 145
4.2. Identification of clonal complexes 146
4.3. Association between STs and fish host species 147
4.4. Geographical origin of the STs 148
5. Discussion 149
APPENDIX 155
REFERENCES 171

Chapter 5. Antimicrobial susceptibilities of *Flavobacterium psychrophilum* isolates from the Great Lakes basin, Michigan, U.S.A. 178
1. Abstract 179
2. Introduction 180
3. Material and Methods 182
3.1. *F. psychrophilum* collection and identification 182
3.2. Antimicrobial susceptibility testing 183
3.2.1. Antimicrobial agents 183
3.2.2. Broth dilution preparation 184
3.2.3. Epidemiological cut-off values 185
3.3. PCR of tetracycline resistance genes 186
4. Results 188
4.1. Antimicrobial susceptibility profiles and epidemiological cut-off values 188
4.1.1. Oxytetracycline 188
4.1.2. Florfenicol 189
4.1.3. Ormetoprim-sulphadimethoxine 189
4.1.4. Erythromycin 190
4.1.5. Oxolinic acid 190
4.1.6. Flumequine 190
4.1.7. Trimethoprim-sulphamethoxazole 191
4.1.8. Enrofloxacin 191

4.1.9. Gentamicin 191
4.1.10. Ampicillin 192
4.1.11. Comparison between CO_{NRI} and CO_{ECOFF} values 192
4.2. *tet* gene detection 193
5. Discussion 193
APPENDIX 199
REFERENCES 216

Chapter 6. Conclusions and Future Research 222
1. Conclusions 223
2. Future Research 227

LIST OF TABLES

Table 2.1 Feral broodstock found to be infected with *F. psychrophilum*. LMRW, Little Manistee River Weir; SRW, Swan River Weir; PRW, Platte River Weir; SMR, St. Mary's River 78

Table 2.2 Captive broodstock found to be infected with *F. psychrophilum.* MSFH, Marquette State Fish Hatchery; OSFH, Oden State Fish Hatchery 79

Table 2.3 Summary of AIC values for the *Flavobacterium psychrophilum* infection prevalence models..... 80

Table 2.4 Progeny lots positive for *F. psychrophilum* during the course of the study. *Flavobacterium psychrophilum* was detected from all clinical disease outbreak samplings reported. WLFSH, Wolf Lake State Fish Hatchery; TSFH, Thompson State Fish Hatchery; HSFH, Harrietta State Fish Hatchery; OSFH, Oden State Fish Hatchery; PRSFH, Platte River State Fish Hatchery; MSFH, Marquette State Fish Hatchery. [a]All year-classes of all species reared at all appropriate hatcheries were assessed for the presence of *F. psychrophilum* throughout the study, only the lots which were positive for the pathogen are presented in this table 81

Table 3.1 Great Lakes basin *Flavobacterium psychrophilum* isolates compared in this study. Isolates with the same superscript letter in the Isolate ID column were recovered from the same fish. All isolates were recovered from locations within Michigan. SRW, Swan River Weir; LMRW, Little Manistee River Weir; PRW, Platte River Weir; OSFH, Oden State Fish Hatchery; WLSFH, Wolf Lake State Fish Hatchery; TSFH, Thompson State Fish Hatchery 116

Table 3.2 *Flavobacterium psychrophilum*-specific primer sequences used to amplify 3 housekeeping genes and the expected amplicon length for each loci 118

Table 3.3 Nucleotide divergences and the supposed amino acid changes found in the genes of *Flavobacterium psychrophilum* isolates from the Great Lakes basin. Nucleotide positions are according to those of ATCC 49418^T (*gyrB* EU428468.1; *murG* EU428518.1) Supposed amino acid enclosed in parenthesis. *indicates epidemic isolate.. 119

Table 4.1a Characteristics of the 96 United States *Flavobacterium psychrophilum* isolates investigated using multilocus sequence typing. Isolates designated WLSFH, TSFH, LMRW, PRW, and OSFH recovered from Lake Michigan watershed. Isolates designated SRW recovered from Lake Huron watershed. [1]Isolates originating from the same fish denoted by the same superscript letter.[2]United States abbreviations: ID, Idaho; NC, North Carolina; NM, New Mexico; UT, Utah; WV, West Virginia; CO, Colorado; MI, Michigan; OR, Oregon; WA, Washington. [*]Isolates recovered during high levels of morbidity and mortality 156

Table 4.1b Multilocus sequence typing allele and sequence types (STs) and association with clonal complexes (CC) of 96 United States *F. psychrophilum* isolates. Underlined text in Allelic profile and ST column denote novel allele types and STs 161

Table 4.2 Sequence types (STs) identified in this study and have been found in other locations in the world 164

Table 4.3 *Flavobacterium psychrophilum*-specific primer sequences used to amplify 7 loci for multilocus sequence typing analysis 166

Table 4.4 MLST sequence types (STs) in this study summarized by fish host species. Numbers in parentheses indicate the number of isolates identified as that ST in this study 167

Table 5.1 Characteristics of *Flavobacterium psychrophilum* isolates analyzed in this study. ST, sequence type; CC, clonal complex. [1]Isolate IDs with an asterisk indicate *F. psychrophilum* recovery during times of morbidity/mortality characteristic of bacterial coldwater disease [2]Isolates recovered from the same fish marked with the same superscript letter 200

Table 5.2 Primer sequences used to detect tetracycline resistance genes. 202

Table 5.3 Summary of *Flavobacterium psychrophilum* antimicrobial susceptibility testing data by broth microdilution. Epidemiological cut-off values (CO_{WT}) were calculated using both the ECOFFinder analysis (CO_{ECOFF}) and normalized resistance interpretation (CO_{NRI}) method. MIC value reported for trimethoprim-sulphamethoxazole and ormetoprim-sulphadimethoxine is drug concentration that produced ~50% bacterial growth compared to positive control. CO_{WT} could not be calculated for gentamicin or ampillicin data 203

Table 5.4. Characteristics of *Flavobacterium psychrophilum* isolates analyzed in this study. ST, sequence type; MIC, minimum inhibitory concentration; AMP, ampicillin; GEN, gentamicin; ENRO, enrofloxacin; OXO, oxolinic acid; FLUQ, flumequine; SXT, trimethoprim-sulphamethoxazole; PRI, ormetoprim-sulphadimethoxine; ERY, erythromycin; FFN, florfenicol; OXY, oxytetracycline. [1]Isolate IDs with an asterisk indicate *F. psychrophilum* recovery during times of morbidity/mortality characteristic of bacterial coldwater disease. [2]Isolates recovered from the same fish marked with the same superscript letter. [3]OS, off scale = lower drug concentrations required to determine MIC. Trailing = higher drug concentrations required to determine MIC.[4]MIC value reported is drug concentration that produced ~50% bacterial growth compared to positive control 204

LIST OF FIGURES

Figure 2.1 The locations from which all spawning salmonids and resultant progeny were sampled throughout the course of the study. Triangles indicate gamete collection facilities (weirs). Circles indicate hatchery facilities 83

Figure 2.2 The prevalence (%) of *F. psychrophilum* infected salmonid broodstock fish as sampled throughout the course of the study. (A) Feral salmonid broodstocks sampled by year, species, and location. CHS, Chinook salmon; COS, Coho salmon; ATS, Atlantic salmon; STT, steelhead. SRW, Swan River weir; LMRW, Little Manistee River weir; PRW, Platte River weir; SMR, St. Mary's River. (B) Captive broodstocks sampled by year and species and strain. Oden State Fish Hatchery: GC-BNT, Gilchrist Creek strain brown trout; WR-BNT, Wild Rose strain brown trout; EL-RBT, Eagle Lake strain rainbow trout; SR-BNT, Sturgeon River strain brown trout. Marquette State Fish Hatchery: LS-LAT, Lake Superior strain lake trout; BKT, brook trout..... 84

Figure 2.3 The predicted *F. psychrophilum* infection probability of feral salmonid broodstock populations throughout the course of the study, as determined by a least-squares means analysis. CHS, Chinook salmon; COS, Coho salmon; ATS, Atlantic salmon; STT, steelhead. SRW, Swan River weir; LMRW, Little Manistee River weir; PRW, Platte River weir; SMR, St. Mary's River 85

Figure 2.4 The predicted *F. psychrophilum* infection probability of captive salmonid broodstock populations throughout the course of the study, as determined by a least-squares means analysis. GC-BNT, Gilchrist Creek strain brown trout; WR-BNT, Wild Rose strain brown trout; EL-RBT, Eagle Lake strain rainbow trout; SR-BNT, Sturgeon River strain brown trout 86

Figure 3.1 Map of locations within the state of Michigan where *Flavobacterium psychrophilum* isolates were recovered..... 120

Figure 3.2 Neighbor-joining tree obtained from *Flavobacterium psychrophilum gyrB* nucleotide sequences 1,077 bp in length based on Tamura three-parameter model distances. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk. 121

Figure 3.3 Neighbor-joining tree obtained from *Flavobacterium psychrophilum gyrB* amino acid sequences based on the JTT matrix method. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk..... 122

Figure 3.4 Neighbor-joining tree obtained from *Flavobacterium psychrophilum murG* nucleotide sequences 681 bp in length based on Tamura three-parameter model distances. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk 123

Figure 3.5 Neighbor-joining tree obtained from *Flavobacterium psychrophilum murG* amino acid sequences based on the JTT matrix method. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk..... 124

Figure 3.6 Neighbor-joining tree obtained from *Flavobacterium psychrophilum tuf* nucleotide sequences 795 bp in length based on Tamura-Nei model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk 125

Figure 3.7 Neighbor-joining tree obtained from *Flavobacterium psychrophilum tuf* amino acid sequences based on the JTT matrix method. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk..... 126

Figure 3.8 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum gyrB* and *murG* nucleotide sequences 1,758 bp in length based on Tamura three-parameter model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk. 127

Figure 3.9 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum gyrB* and *tuf* nucleotide sequences 1,872 bp in length based on Tamura-Nei model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk..... 128

Figure 3.10 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum murG* and *tuf* nucleotide sequences 1,476 bp in length based on Tamura three-parameter

model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk 129

Figure 3.11 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum gyrB, murG,* and *tuf* nucleotide sequences 2,553 bp in length based on Tamura-Nei model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk 130

Figure 4.1 The number of *F. psychrophilum* isolates from each location where samples were collected in the United States. Isolates were recovered from three *Oncorhynchus* spp. *O. mykiss* (n = 54), *O. tshawytscha* (n = 26), and *O. kisutch* (n = 16). 168

Figure 4.2 eBURST diagram depicting the relationships of the 96 U.S.A. *F. psychrophilum* isolates of this study and the 10 previously typed U.S.A. isolates (Nicolas et al. 2008). Sequence types (STs) followed by (2008) denote isolates previously typed by Nicolas et al. (2008). STs 9, 10, 13, and 29 were detected in the U.S.A. in both the current U.S.A. study as well as in Nicolas et al. (2008). †: STs found in both the U.S.A. and abroad. All other isolates are unique to the U.S.A. Light gray denotes the predicted founder ST. Clonal complexes (CC; numbers within rectangles) are named after the predicted founding ST. In CCs composed of two STs, the CC is named after the most abundant ST; if both STs are equally represented, then the CC is named for the earliest found ST 169

Figure 4.3 eBURST diagram depicting the relationships among global *F. psychrophilum* isolates, including the isolates of this North American study (n = 1091). †: Sequence types (STs) found in both North America and abroad. Þ: STs currently present only in North America. Light gray denotes predicted founder ST. Clonal complexes (CC; numbers within rectangles) are named after the predicted founding ST. In CCs composed of two STs, the CC is named after the most abundant ST; if both STs are equally represented, then the CC is named for the earliest found ST 170

Figure 5.1 Map of locations where *Flavobacterium psychrophilum* isolates were collected ... 207

Figure 5.2 The distrubution of *Flavobacterium psychrophilum* isolates (%) according to determined minimum inhibitory concentrations (MICs) for 4 antimicrobial compounds frequently used in Great Lakes basin aquaculture. Dotted lines represent epidemiological cut-off values calculated by the national resistance interpretation method (CO_{NRI}), dashed lines represent epidemiological cut-off values calculated by the ECOFFinder analysis (CO_{ECOFF}), and

solid lines represent epidemiological cut-off values that were of the same result from both methods 208

Figure 5.3 Scatter plot comparision of oxytetracycline (y-axis) and A) florfenicol (x-axis), B) ormetoprim-sulphadimethoxine (x-axis), C) erythromycin (x-axis), D) oxolinic acid (x-axis), E) flumequine (x-axis), F) trimethoprim-sulphamethoxazole (x-axis), G) enrofloxacin (x-axis), H) gentamicin (x-axis), and I) amplicillin (x-axis) MIC values for *Flavobacterium psychrophilum.* The number of isolates with the given combination of MIC values is indicated in each square. Dashed lines indicate the epidemiolocigal cut-off (CO_{WT}) values calculated by the normalized resistance interpretation (CO_{NRI}) method, and dotted lines indicate the CO_{WT} values calculated by the ECOFFinder (CO_{ECOFF}) analysis. The bolded solid lines indicate the CO_{WT} values that were of the same result from both methods. No CO_{WT} values could be calculated for the gentamicin or ampicillin MIC data 209

Figure 5.4 The distrubution of *Flavobacterium psychrophilum* isolates (%) according to determined minimum inhibitory concentrations (MICs) for 6 antimicrobial compounds that are not approved for use in United States aquaculture. Dotted lines represent epidemiological cut-off values calculated by the national resistance interpretation method (CO_{NRI}), dashed lines represent epidemiological cut-off values calculated by the ECOFFinder analysis (CO_{ECOFF}), and solid lines represent epidemiological cut-off values that were of the same result from both methods. Epidemiological cut-off values could not be calculated for genetamicin or ampicillin data 215

KEY TO ABBREVIATIONS

AFS-FHS	American Fisheries Society Fish Health Section
AIC	Akaike information criterion
AMP	Ampicillin
ARGs	Antibiotic resistance genes
AT	Allele type
ATCC	American Tissue Culture Collection
atpA	Adenosine triphosphate synthetase, α subunit
ATS	Atlantic salmon
BCWD	Bacterial coldwater disease
BKD	Bacterial Kidney Disease
BKT	Brook trout
bp	Base pairs
CA	Cytophaga agar
CC	Clonal complexes
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
CHS	Chinook salmon
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CO	Colorado

CO_{ECOFF}	Epidemiological cut-off value calculated by the ECOFFinder analysis
CO_{NRI}	Epidemiological cut-off value calculated by normalized resistance interpretation
COS	Coho salmon
CO_{WT}	Epidemiological cut-off value wild-type
DCAMHB	Dilute cation adjusted Mueller Hinton broth
DNA	Deoxyribonucleic acid
dnaK	Chaperone heat shock protein 70
ECOFF	Epidemiological cut-off value accepted by EUCAST
ECV	Epidemiological cut-off value accepted by CLSI
EED	Epizootic epitheliotropic disease
ELISA	Enzyme linked immunosorbent assay
EL-RBT	Eagle Lake strain rainbow trout
ENRO	Enrofloxacin
ERY	Erythromycin
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FFN	Florfenicol
FLUQ	Flumequine
ftsQ	Filamentation, temperature sensitive gene
fumC	Fumarate hydratase class II
GC-BNT	Gilchrist Creek strain brown trout
GEN	Gentamicin
GLB	Great Lakes basin

glyA	Serine hydroxymethyltransferase
gyrB	Topoisomerase II, β subunit
HSFH	Harrietta State Fish Hatchery
ID	Idaho
IHNV	Infectious hematopoietic necrosis virus
INAD	Investigational new animal drug exemption
LMRW	Little Manistee River weir
LS-LAT	Lake Superior strain lake trout
MDNR	Michigan Department of Natural Resources
MI	Michigan
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MS-222	Tricaine methanesulfonate
MSFH	Marquette State Fish Hatchery
MSU-AAHL	Michigan State University Aquatic Animal Health Laboratory
murG	Glycosyltransferase murein G
NaCl	Sodium chloride
NC	North Carolina
NM	New Mexico
NRI	Normalized resistance interpretation
OIE	World Organization of Animal Health
OR	Oregon

OSFH	Oden State Fish Hatchery
OXO	Oxolinic acid
OXY	Oxytetracycline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PNW	Pacific Northwest
ppm	Parts per million
PRI	Ormetoprim-sulphadimethoxine
PRSFH	Platte River State Fish Hatchery
PRW	Platte River weir
RAPD	Random amplified polymorphic DNA analysis
recA	DNA recombination protein A
RFLP	Restriction fragment length polymorphism
rplB	Ribosomal protein, large
rRNA	Ribosomal ribonucleic acid
RTFS	Rainbow trout fry syndrome
SFH	State Fish Hatchery
SLV	Single locus variant
SMR	St. Mary's River
SR-BNT	Sturgeon River strain brown trout
SRW	Swan River weir
ST	Sequence type

STT	Steelhead
SXT	Trimethoprim-sulphamethoxazole
tetA	Tetracycline resistance gene A
tetE	Tetracycline resistance gene E
tetH	Tetracycline resistance gene H
tetM	Tetracycline resistance gene M
trpB	Tryptophane synthetase β subunit
TSFH	Thompson State Fish Hatchery
tuf	Elongation factor Tu
TYES	Tryptone yeast extract salts medium
US-FDA	U.S.A. Food and Drug Administration
USFWS-AADAP	United States Fisheries and Wildlife Services Aquatic Animal Drug Approval Partnership
UT	Utah
UV	Ultraviolet
VNTR	Variable number of tandem repeats
WA	Washington
WLSFH	Wolf Lake State Fish Hatchery
WR-BNT	Wild Rose strain brown trout
WV	West Virginia

Introduction

Introduction

Bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS) are caused by *Flavobacterium psychrophilum*, threaten global wild and propagated salmonid populations, and lead to substantial economic losses (Starliper 2011). Since initial identification of this pathogen in North America (Borg 1948) it has been reported in nearly all areas that practice intense salmonid aquaculture (Bernardet and Bowman 2006), including Europe, South America, Asia, and Australia (Nematollahi et al. 2003). BCWD and RTFS are considered two of the most important salmonid diseases worldwide (Nematollahi et al. 2003). Unfortunately, the exact details regarding the geographic dissemination and global spread of this deadly pathogen are largely unknown. Further complicating the understanding of the spread of *F. psychrophilum* is the dual transmission route within a fish stock. The likely vertical transmission from infected parent to progeny in addition to the transmission from infected fish to fish in a horizontal fashion (Brown et al. 1997) leads to difficulties in controlling *F. psychrophilum*. Moreover, the use of antimicrobial therapy and biosecurity remain the only mechanisms to control *F. psychrophilum,* as no efficacious vaccine has been developed (Gomez et al. 2014). There is a substantial lack of important epidemiological knowledge regarding *F. psychrophilum* such as the fish host species specificity, geographical distribution, antimicrobial resistance, and virulence level of individual strains. This knowledge gap has hindered the efforts to improve current control measures, such as vaccine development based on strains of clinical significance, species specific vaccines, and targeted chemotherapeutic strategies.

Salmonid enhancement efforts are widely employed by natural resource managers across North America to enhance sport-fisheries and associated industries. The Great Lakes

basin (GLB) represents a unique environment whereby the intentional introduction of salmonid species highly susceptible to *F.psychrophilum* has occurred over the last century (Kocik and Jones 1999), complicating the understanding of the dissemination and population structure of *F. psychrophilum* in the region. Indeed *F. psychrophilum* infections are known to occur widely throughout the GLB and are detrimental to its salmonid stocks (Loch and Faisal 2014), however the full extent of the prevalence and severity of these infections are largely unknown. Considering the likelihood of vertical transmission and that both feral and captive salmonids provide gametes for fishery propagation in the GLB, the *F. psychrophilum* infection status of these broodstocks may directly relate to the incidence of disease within their progeny that are reared at State Fish Hatcheries.

Until recently, *F. psychrophilum* was thought to be genetically homogenous (Madsen and Dalsgaard 2000), however more recent studies have hinted at the genetic diversity of this pathogen. Unfortunately, many of these analyses should be taken with caution as they have often not been reproducible, which may stem from the fact that they are based on the 16S rRNA gene, which exists in 6 copies within the *F. psychrophilum* genome (Duchaud et al. 2007). The multiplicity of this gene may have contributed to the variable results of previous genetic analyses. The use of single-copy genes, such as *gyrB* has been successful in providing a robust phylogeny of closely related *Flavobacterium* spp., including *F. psychrophilum* (Izumi et al. 2003; Peeters and Willems 2011).

Improvements upon traditional phylogenetic approaches have resulted in a technique using a number of single-copy genes in combination, known as multilocus sequence typing (MLST). Using 7 single-copy housekeeping genes, an MLST approach has been recently

optimized for use with *F. psychrophilum* (Nicolas et al. 2008; Siekoula-Nguedia et al. 2012). This approach has identified fish host species specific sequence types (STs), in addition to highly virulent STs known to be associated with overt BCWD outbreaks (Nicolas et al. 2008; Siekoula-Nguedia et al. 2012; Apablaza et al. 2013; Fujiwara-Nagata et al. 2013; Strepparava et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014). It is also suggested, largely through this work, that the trade of live fish and their eggs are responsible for the global transmission of this pathogen (Wakabayashi et al. 1994; Kumagai and Takahashi 1997; Avendaño-Herrera et al. 2014). Unfortunately, a major lack of focus on the North American *F. psychrophilum* population precludes the drawing of major conclusions pertaining to the transcontinental *F. psychrophilum* distribution.

Due to the widespread distribution and devastating effects of *F. psychrophilum* infections, as well as limited control options (antimicrobial agents and strict biosecurity), the World Organization of Animal Health (OIE) recommends performing antimicrobial susceptibility testing on these isolates. Alarmingly, reduced susceptibility to commonly used aquaculture drugs, namely oxytetracycline, oxolinic acid, and florfenicol, has been reported (Bruun et al. 2000; Kum et al. 2008; Hesami et al. 2010). Unfortunately, this valuable information does not currently exist for isolates within the GLB of Michigan, a matter of utmost importance considering the widely employed salmonid conservation efforts throughout this region.

1. Study objectives

The overall objective of this study was to examine the heterogeneity among *F. psychrophilum* strains and the infections they cause in the United States, and particularly within

the GLB of Michigan. The infection prevalence as it pertains to multiple salmonid stocks, the genetic diversity and molecular epidemiology, and the phenotypic diversity in regards to antimicrobial susceptibility patterns have thoroughly been investigated in this dissertation.

In Chapter 1, a thorough review of the literature as it pertains to the diseases caused by *F. psychrophilum*, their economic and ecologic impacts, epizootiology, and treatment and control measures is given. A review of the pathogen characteristics and diversity is also provided.

Chapter 2 of this study focused on identifying the salmonid broodstock populations of the GLB most at risk for *F. psychrophilum* infections, as well as investigating the link between these infections and the incidence of BCWD among their progeny. I hypothesized the pathogen prevalence among different salmonid broodstocks in the GLB will vary by species and demonstrate a link to the incidence of BCWD in resultant progeny. Analyses demonstrated the significance of this pathogen in both wild waters and hatchery facilities within the GLB.

Chapter 3 focused on understanding the intraspecific phylogeny of GLB *F. psychrophilum* isolates using traditional phylogenetic approaches based on three alternative loci; *gyrB, murG,* and *tuf* genes. I hypothesized these alternative loci would demonstrate a robust intraspecific phylogeny of GLB *F. psychrophilum.* Results indicated that the GLB *F. psychrophilum* population is genetically diverse, with few trends associated with host species and/or association with morbidity and mortality.

In Chapter 4, the intraspecific genetic diversity of *F. psychrophilum* from the United States was further investigated using an MLST approach, which improves the robustness of the phylogenetic relationships inferred from Chapter 3. It was hypothesized that some *F.*

psychrophilum STs would be specific to the GLB, and others would demonstrate a more widespread distribution. A large number of novel and unique STs were detected in the *F. psychrophilum* population of the United States, and specifically within the GLB region.

Chapter 5 focused on revealing the antimicrobial susceptibility profiles of GLB *F. psychrophilum* isolates, and the species-specific epidemiological cut-off values for each compound were calculated. I hypothesized that resistance to commonly used aquaculture antimicrobials would be seen. The antimicrobial susceptibility data generated herein will contribute to the validation of *F. psychrophilum*-specific epidemiological cut-off values.

Lastly, Chapter 6 summarizes the overall conclusions and provides recommendations for future research. As this research provides valuable knowledge regarding multiple aspects of *F. psychrophilum* heterogeneity and BWCD epidemiology, particularly as it pertains to GLB salmonid stocks, there is still much we need to learn to improve upon management, treatment, and control decisions.

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Chapter 1

Literature Review

1. Introduction

Flavobacterium psychrophilum (Family *Flavobacteriaceae*) is a Gram negative, psychrophilic bacterium that causes bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), which threatens a variety of salmonid fish stocks worldwide and causes significant economic losses in hatchery systems and beyond (Nematollahi et al. 2003). For example, in Chilean fish farms, *F. psychrophilum* infections are responsible for mortality rates of up to 70%, resulting in economic losses that rank second to those caused by *Piscirickettsia salmonis* (Valdebenito and Avendaño-Herrera 2009; Avendaño-Herrera et al. 2014). Furthermore, French fish farms report infections caused by *F. psychrophilum* are the second most prevalent cause of bacterial diseases, after *Aeromonas salmonicida* infections (Siekoula-Nguedia et al. 2012). BCWD also threatens North American fish stocks; for instance, the state of Utah reports 30% of hatchery-reared steelhead trout (*Oncorhynchus mykiss)* are lost to BCWD annually (Oplinger and Wagner et al. 2013). The Great Lakes basin (GLB) is another North American geographical region which has been affected by BCWD. *Flavobacterium psychrophilum* and other fish-pathogenic bacteria within the Family *Flavobacteriaceae* have historically been associated with more fish mortalities in Michigan State Fish Hatcheries than all other fish pathogens combined (Faisal and Hnath 2005; Faisal et al. 2013), however the extent of *F. psychrophilum* infections in GLB salmonid stocks has received little attention. The effects of *F. psychrophilum* infections can be long lasting and exceptionally detrimental considering fish surviving infections are often left with spinal abnormalities and stunted growth (Madsen et al. 2001). Despite nearly 80 years of research, much remains unknown for this bacterium, particularly in terms of epidemiology.

2. The disease

2.1. Host range and host susceptibility

Flavobacterium psychrophilum infects a variety of fish hosts, with members of the family Salmonidae being the primary target. Notably, Coho salmon (*O. kisutch)* and rainbow trout (*O. mykiss)* are considered the most susceptible to this infection (Davis 1946; Taylor 2004; Cipriano and Holt 2005), followed by Atlantic salmon (*Salmo* salar; Ekman et al. 1999; Cipriano 2005). Many other salmonid species have been reported to harbor *F. psychrophilum,* including sockeye salmon (*O. nerka)*, Chinook salmon (*O. tshawytscha*), chum salmon (*O. keta*), cutthroat trout *(O. clarki),* brook trout (*Salvelinus fontinalis*), masou salmon (*O. masou*), lake trout (*S. namaycush*), brown trout (*S. trutta*), Arctic char (*S. alpinus*), and grayling (*Thymallus thymallus*; Rucker et al. 1953; Schachte 1983; Holt et al. 1993; Amita et al. 2000; Madetoja et al. 2001). Additionally, the pathogen has been reported from non-salmonid hosts, including the cyprinid species common carp (*Carassius carpio*), crucian carp (*C. carassius*), tench (*Tinca tinca*), Indian catfish (*Clarias batrachus*), pale chub (*Zacco platypus*), and silver crucian carp (*Carassius auratus langsdorfi*; Lehmann et al. 1991; Austin and Austin 1999; Nagai and Nakai 2011; Verma and Prasad 2014). Other non-salmonid hosts include Japanese eel (*Anguilla japonica*), European eel (*A. anguilla*), roach (*Rutilis rutilis*), perch (*Perca fluviatilis*), ayu (*Plecoglossus altivelis*) and sea lamprey (*Petromyzon marinus*; Lehmann et al. 1991; Wakabayashi et al. 1994; Madetoja et al. 2002; Izumi et al. 2003; Elsayed et al. 2006). *F. psychrophilum* has even been detected from multiple non-fish sources, including algae (Amita et al. 2000), newts (Brown et al. 1997), and a common freshwater leech (*Myzobdella lugubris*; Schulz and Faisal 2010). However, the role that these play in the maintenance and transmission of *F. psychrophilum* is unclear, however they may act

as reservoirs or vectors for the disease (Brown et al. 1997; Elsayed et al. 2006; Nilsen et al. 2011).

2.2. Geographic distribution

Although first observed in West Virginia and the Pacific Northwestern United States in the 1940s, *F. psychrophilum* has now been reported worldwide and particularly where salmonids are aquacultured. In North America, *F. psychrophilum* can be found across the United States (Holt 1987; LaFrentz and Cain 2004), including the Great Lakes region (Loch et al. 2013), as well as in Canada (Allen et al. 2008). The disease can be found in nearly every country in Europe, including France (Bernardet et al. 1988; Siekoula-Nguedia et al. 2012), Germany (Nilz et al. 2009), Denmark (Lorenzen et al. 1991), United Kingdom (Austin and Stobie 1991), Spain (Toranzo and Barja 1993), Italy (Sarti et al. 1992), Finland (Dalsgaard and Madsen 2000), Norway (Nilsen et al. 2011), Switzerland (Strepparava et al. 2013), Belgium (Nematollahi et al. 2003), and Turkey (Kum et al. 2008). With the expansion of salmonid aquaculture in South America, BCWD is now frequently reported in salmonid farms of Chile (Valdebenito and Avendaño-Herrera 2009) and Peru (Leon et al. 2009). *F. psychrophilum* has also been reported in Asian countries, such as Japan (Wakabayashi et al. 1994) and Korea (Lee and Heo 1998), and has also been detected in Australia (Schmidtke and Carson 1995).

2.2.1. Great Lakes basin

F. psychrophilum infections are indeed present throughout the GLB, in fact, bacteria within the Family *Flavobacteriaceae* have historically been associated with more fish mortalities

in state fish hatcheries than all other fish pathogens combined (Faisal and Hnath 2005; Faisal et al. 2013). Although recent studies demonstrated the multitude of flavobacterial infections among GLB salmonid stocks (Loch et al. 2013; Loch and Faisal 2014), thorough investigation of specifically *F. psychrophilum* infections are lacking, a matter of importance considering the economic and ecological role of *F. psychrophilum*-susceptible salmonids residing throughout the GLB. Furthermore, the GLB has been the recipient of intentionally introduced salmonid species, providing a unique system to investigate the pathogen diversity in the area as it relates to the native range and founding stocks of the salmonid hosts.

2.3. Transmission

With the propensity to readily detect this pathogen in water sources and the high shedding rates of infected fish, waterborne transmission likely plays a role in *F. psychrophilum* infections (Nematollahi et al. 2003). Additionally, *F. psychrophilum* has been found in the stomach lumen of naturally infected rainbow trout, suggesting the gastrointestinal tract as an important route of pathogen entry and exit (Lorenzen 1994; Liu et al. 2001). Horizontal transmission of the pathogen can be demonstrated by successful experimental immersion or contact and cohabitation challenges (Rangdale 1995; Madsen and Dalsgaard 1999).

Multiple studies have demonstrated that *F. psychrophilum* is frequently present in the reproductive fluids of spawning fish (Brown et al. 1997; Taylor 2004; Kumagai and Nawata 2011; Long et al. 2012; Long et al. 2014), as well as on the egg surfaces (Ekman et al. 1999; Kumagai et al. 2000), and others have demonstrated that the bacterium can be present within the egg (Brown et al. 1997; Cipriano 2005). However, intra-ova transmission of *F.*

psychrophilum is still debated as some studies were unable to reproduce the results (Ekman et al. 1999; Madsen et al. 2005). Nevertheless, *F. psychrophilum* has been detected intra-ovum through aseptically aspirated egg contents (Brown et al. 1997; Taylor 2004; Cipriano 2005). It has been suggested that *F. psychrophilum* invades the perivitelline space by way of the micropyle prior to or during water hardening (Kumagai et al. 2000). Many bacterial species are susceptible to lysozyme; however *F. psychrophilum* can survive in lysozyme concentrations greater than that found in fish eggs (Brown et al. 1997). Collectively, *F. psychrophilum* is suggested to be both vertically and horizontally transmitted.

2.4. Pathogenesis

After adhering to external surfaces of fish hosts, *F. psychrophilum* facilitates its invasion through the production of proteases. Multiple proteases have been identified that degrade casein, gelatin, actin, and myosin (Bertolini et al. 1994). These proteases make it possible for degradation and migration through host connective tissue and muscle (Evensen and Lorenzen 1996). Because of these characteristics, *F. psychrophilum* has as affinity for collagenous connective tissue, including the lower jaw, fins, caudal peduncle, and immature bone tissues of the cranium, and gill (Kondo et al. 2002; Martinez et al. 2004). After migration through connective tissues and musculature, the bacterium may eventually invade internal organs by way of blood vessels (Martinez et al. 2004). *F. psychrophilum* suppresses the nonspecific humoral defense mechanisms of the host, which allows it to survive within the bloodstream and migrate through internal organs (Barnes and Brown 2011).

Furthermore, both live and dead fish have been shown to shed the pathogen into the environment, with live fish shedding up to 10^7 colony forming units (CFU) $fish^{-1}$ $hour^{-1}$, and dead fish shedding even greater numbers for up to 80 days after death (Madetoja et al. 2000). Because *F. psychrophilum* is commonly found in water sources (Madetoja et al. 2003), it is not surprising that *F. psychrophilum* is also commonly found in external fish tissues, including mucus, fins, and gills (Holt et al. 1993; Lorenzen 1994; Madetoja et al. 2002).

2.5. Clinical signs

F. psychrophilum infections cause BCWD and RTFS in salmonid populations worldwide (Starliper 2011) and cause very characteristic disease signs. The classic clinical sign of BCWD is a "saddle-like" lesion on the caudal peduncle region, which often progresses to muscle necrosis and exposure of the spinal cord in severe infections (Davis 1946). Erosion of all fins often resulting in exposure of fin rays is also frequently observed (Borg 1948; Holt et al. 1993). Other external signs of disease include ulcerations near the eye, lower jaw, and vent (Nematollahi et al. 2003). Exophthalmia, anemia, increased mucus production, and melanosis, along with gill necrosis and hemorrhage are also common among fish suffering from BCWD (Nematollahi et al. 2003). Internal signs include ascites, pale and/or swollen liver, spleen, and kidneys, and intestinal inflammation (as reviewed in Barnes and Brown 2011). Histologically, most internal organs show signs of necrosis, with renal tubular epithelium and hematopoietic tissue most compromised (Bruno 1992; Evensen and Lorenzen 1996). Epithelial damage can be seen by severe necrosis and hyperplasia (as reviewed in Barnes and Brown 2011). Fish surviving

infections often exhibit spinal deformities, abnormal swimming, and stunted growth due to compromised connective and cartilaginous tissues (Holt et al. 1993; Madsen et al. 2001).

Rainbow trout fry infected with *F. psychrophilum* suffer from RTFS, which presents slightly differently than BCWD in hosts of other species and ages. Clinically, the fry will appear anemic with bilateral exophthalmia, however muscle lesions are usually only present in fish larger than 10 grams (Cipriano 2005).

2.6. Factors affecting disease course

Many environmental parameters affect the occurrence and severity of *F. psychrophilum* infections, including water temperature and quality. The disease commonly occurs at water temperatures of 3 to 20°C (Borg 1948; Austin and Austin 1999); however the outbreaks are most severe when water temperatures are between 3 and 15°C (Holt et al. 1987). Additionally, poor water quality in the form of high organic loads and elevated nitrite concentrations can increase the severity of infection (Garcia et al. 2000).

Furthermore, the presence of other pathogens can also influence the progression of *F. psychrophilum* infections (Decostere et al. 2000). Busch et al. (2003) showed that the presence of ectoparasites (*Gyrodactylus derjavini*) during an *F. psychrophilum* outbreak enhanced the invasiveness of the bacterium and lead to higher mortality. Additionally, BCWD outbreaks are associated with coinfections of *F. psychrophilum* and infectious hematopoietic necrosis virus (IHNV; LaFrentz and Cain 2004; Long et al. 2012), as well as other flavobacteria (Loch et al. 2013).

Bacterial virulence also significantly influences the course of infection and there is evidence of variable virulence amongst *F. psychrophilum* strains (Madetoja et al. 2002; Nicolas et al. 2008; Stenholm et al. 2008). Particularly virulent strains have been identified using a variety of methods. A relationship between certain serotypes and particular ribotypes and increased virulence was reported among Danish isolates (Madsen and Dalsgaard 2000), and molecular methods have identified sequence types that group in clonal complexes that are associated with BCWD outbreaks around the world (Fujiwara-Nagata et al. 2013; Strepparava et al. 2013; Nilsen et al. 2014; Sundell and Wiklund 2015).

2.7. Losses and economic impacts

The losses associated with *F. psychrophilum* infections vary by species, with the highest mortality frequently occurring in rainbow trout, ranging from 10% -90% in various locations (Brown et al. 1997; Nilsen et al. 2011; Oplinger and Wagner 2013). Average Coho salmon mortalities are reported between 30 and 50% (Holt 1987; Cipriano and Holt 2005). Cutthroat trout have reported mortalities of 30 to 45% (Pravacek and Barnes 2003; Ryce and Zale 2004). Typically lake trout mortality averages 25% (Schachte et al. 1983). However, within species, mortality can vary substantially as a result of many factors, including of the age of the host, water quality and temperature, and presence of other pathogens. The economic losses associated with the high mortality rates can be overwhelming, exceeding millions of dollars in annual losses. For example, Chilean fish farms report *F. psychrophilum* infections in salmonid fingerlings cause losses that are second only to *P. salmonis* (Valdebenito and Avendaño-Herrera

2009; Avendaño-Herrera et al. 2014). In the United States, the trout industry of Idaho reports $9-10 million losses due to BCWD annually (Ken Cain, personal communication).

2.8. Treatment, control, and prevention

2.8.1. Chemotherapy

Currently, the most widely used control method is antimicrobial or chemotherapeutic treatments (Nematollahi et al. 2003; Barnes and Brown 2011). Despite the large amount of antibiotics used in aquaculture, there is limited approval of antibiotic compounds for treatment of *F. psychrophilum* worldwide. Oxytetracycline and florfenicol are used worldwide (Bruun et al. 2000; LaFrentz and Cain 2004), and remain the only two antibiotics fully approved for fully approved by the U.S.A. Food and Drug Administration (US-FDA) to treat *F. psychrophilum* infections in the United States (USFWS-AADAP). Other major aquaculture countries have only approved the additional use of trimethoprim-sulphamethoxazole, oxolinic acid, and flumequine to treat these infections (Bruun et al. 2000; Hesami et al. 2010; Shah et al. 2012). Unfortunately, increased use of some of the aforementioned agents have led to the development of antibiotic resistant *F. psychrophilum* strains, including acquired resistance to oxytetracycline, amoxicillin, and oxolinic acid (Bruun et al. 2000; Schmidt et al. 2000; Kum et al. 2008; Durmaz et al. 2012; Henriquez-Nunez et al. 2012). Currently, florfenicol seems to be associated with little to no resistance (Rangdale et al. 1997; Schmidt et al. 2000; Durmaz et al. 2012), probably due to the more recent approval and use of this drug.

To improve the efficacy of antibiotic treatments, many researchers have advocated for the use of antibiotics in combination with alternative options. For example, external

disinfectants, such as hydrogen peroxide, quaternary ammonium, potassium permanganate, and chloramine-T, administered prior to treatment with oral antibiotics, have been reported as useful treatment options (Schachte 1983; LaFrentz and Cain 2004; Gultepe and Tanrikul 2006).

2.8.2. Husbandry and biosecurity

Improved husbandry and biosecurity can also greatly reduce the risk of BCWD outbreaks (Bebak et al. 2007; Madsen and Dalsgaard 2008). It has been demonstrated that diligent removal of dead and moribund fish from the system, as well as reducing the transfer of pathogen between tanks, and intense cleaning and disinfection has helped reduce the losses associated with BCWD outbreaks (Bebak et al. 2007). Reducing the influx of pathogen into the rearing units can also be accomplished by using pathogen-free source water, or treating source water with ultraviolet light (Cipriano and Holt 2005). Finally, reducing any stress and physical handling, as well as reduced rearing densities and improved water quality can also help to prevent BCWD outbreaks (LaFrentz and Cain 2004; Cipriano and Holt 2005).

Another promising method involves screening broodstock and gametes for the presence of the *F. psychrophilum* and culling those individuals with high pathogen loads, which has shown promise in reducing the incidence of disease in the progeny as these infections are thought to be vertically transmitted (Lindstrom et al. 2009; Long et al. 2012; Long et al. 2014). In a similar attempt to reduce vertical transmission, egg disinfection methods using povidone-iodine prior to fertilization and erythromycin baths during water hardening have been suggested to help control *F. psychrophilum* (Kumagai and Nawata 2010).

2.8.3. Fish diet, dietary supplements, and probiotics

It has been long suggested that malnutrition plays a role in the onset of BCWD and that the use of a high quality diets can be beneficial in reducing disease outbreaks (Post 1987). However, the use of feed-additives (i.e., extracts and probiotics) as a preventative tool against *F. psychrophilum* has only been recently investigated. A variety of diet additives including humus, salmon testes meal, and β-glucans have shown some protection to fish against BCWD challenges, however the efficacy of these methods is largely unknown (Nakagawa et al. 2009; Ringo et al. 2011; Fehringer et al. 2014). Probiotics are microbial feed supplements which beneficially affect the host by improving intestinal balance (Fuller 1987), and are increasingly being used and investigated in aquaculture. Some probiotics have shown promise during both *in vitro* and *in vivo* studies (Burbank et al. 2012; Boutin et al. 2013; LaPatra et al. 2014), however large production and use of these strains are still under investigation.

2.8.4. Disease-resistant fish strains

Naturally existing fish stocks have differential susceptibility to BCWD (Kageyama et al. 2013), and researchers and managers can artificially select for BCWD-resistant strains. Leeds et al. (2010) initiated a selection program that proved effective at improving BCWD resistance through multiple generations. Building upon this program, Wiens et al. (2013) has demonstrated on-farm performance with higher percent survival in the BCWD-resistant line than control and susceptible lines. Fortunately, Silverstein et al. (2009) demonstrated that growth characteristics were not compromised when selecting for BCWD resistance. Despite the

possible side effects (e.g. differential selection, loss of genetic variation), selective breeding programs are becoming increasingly important in combatting BCWD issues.

2.8.5. Vaccination

Vaccines are of the most effective preventative measures against a number of fish pathogens because they easily administered to young fish in mass quantities, can protect fish in early life stages when antibiotics may not be a viable option, and can provide lasting immunity (Sommerset et al. 2005). However, despite intense efforts there is still an absence of an efficacious vaccine available for BCWD. Vaccine development has been a difficult task, as Gomez et al. (2014) suggests could be due to the lack of knowledge on multiple bacterial characteristics, such as key virulence factors, putative antigens, route of entry, serotype variability, and host immune response. A variety of vaccine preparations have been attempted, and while initially bacterin preparations showed mixed results, they were never tested on a large scale basis (Holt 1987; Rahman et al. 2000; Madetoja et al. 2005). Vaccines based on part, or all, of the antigenic outer layer have been hypothesized to offer better protection than vaccines based on the whole *F. psychrophilum* cell (Rahman et al. 2002), however despite the identification of many potential target molecules, no efficacious subunit or recombinant vaccine has been developed (Crump et al. 2001; Crump et al. 2005; Dumetz et al. 2007; Plant et al. 2009; LaFrentz et al. 2011). Although live attenuated vaccine preparations may offer longer lasting protection relative to bacterins or subunit preparations, few attempts have been made in this regard considering the risks associated with these (i.e., virulence reversion and antibiotic resistant gene spread) as well as the difficultly in the approval process (Gomez et al. 2014). Two

mutant strains have recently shown promise, including a rifamicipin-attenuated strain and a strain exhibiting a mutation associated with the *ExbD2* protein (Alvarez et al. 2008; LaFrentz et al. 2008).

3. The pathogen

3.1. Taxonomic classification

Since the initial description, this bacterium has undergone multiple taxonomic changes due in large part to advances in molecular techniques. The pathogen was originally described as *Cytophaga psychrophila* and placed in the order Myxobacterales based upon the biochemical analysis of Borg (1960). Using DNA homology, the bacterium was reclassified under the genus *Flexibacter*, and thus renamed *Flexibacter psychrophilus* (Bernardet and Grimont 1989). However, another reclassification occurred when DNA-rRNA hybridization revealed that the bacterium belonged to the genus *Flavobacterium* (Phylum Bacteroidetes; Class Flavobacteriia; Order Flavobacteriales; Family Flavobacteriaceae), and was thus designated *F. psychrophilum* (Bernardet et al. 1996; Bernardet 2011) and currently remains as such.

3.2. Phenotypic characteristics

F. psychrophilum is Gram negative and takes the form of filamentous rods (0.75 µm in diameter and 1.5 to 7.5 µm in length; Pacha 1968) that are flexible, weakly refractile, and do not produce spores (Holt 1987; Bernardet 2011). *F.psychrophilum* colonies contain a non-diffusible yellow pigment due to the presence of flexirubin-type components (Bernardet and Bowman 2006; Bernardet 2011). Colony morphology is often described as “fried egg-like” in

appearance, with a bright yellow, slightly raised center and thin, spreading edges (Nematollahi et al. 2003). Growth of *F. psychrophilum* is strictly aerobic and does not occur at temperatures greater than 25°C (Bernardet and Kerouault 1989). *F. psychrophilum* is highly proteolytic and can degrade casein, tributyrin, collagen, fibrinogen, gelatin, elastin, chondroitin sulfate, and fish muscle extract (Bernardet and Grimont 1989; Holt et al. 1993; Bertolini et al. 1994); however it cannot hydrolyze starch, esculin, chitin, or xanthine (Pacha 1968; Bernardet and Kerouault 1989; Nematollahi et al. 2003), and does not utilize carbohydrates (Bernardet and Kerouault 1989). This bacterium does not reduce nitrite to nitrate, nor produce hydrogen sulfide, indole, or lysine and ornithine decarboxylase (Pacha 1968; Nematollahi et al. 2003; Barnes and Brown 2011). Salinity tolerance varies by strain, as Pacha (1968) reported *F. psychrophilum* growth in 0.8% NaCl, with growth inhibition at concentrations of 2.0%, while Bernardet and Kerouault (1989) observed no growth at NaCl concentrations greater than 0.5%.

3.3. Bacterial culture conditions

The isolation and culture of *F. psychrophilum* can be problematic due to the fastidious nature of the bacterium and its propensity to be outcompeted or inhibited by other bacterial or fungal species (Wiklund et al. 2000). However, the use of selective growth media has greatly improved the culturing abilities of this bacterium. The use of specific nutrient-low growth media such as cytophaga agar (CA; Anacker and Ordal 1959), modified CA medium (Daskalov et al. 1999), Shieh's medium (Shieh 1980), Hsu-Shotts medium (Bullock et al. 1986), tryptone yeast extract salts medium (TYES; Holt 1987), and medium #2 (Starliper et al. 2007) have all been successful. The inclusion of antibiotic and antimycotic compounds in some of these media and

their derivatives has improved the selectivity towards *F. psychrophilum* and helps significantly to inhibit growth of less fastidious bacteria and fungus that is often present in external lesions of fish (Schmidt et al. 2000; Nematollahi et al. 2003). The temperature range at which *F. psychrophilum* grows is typically between 4 and 23°C (Holt 1987; Bernardet and Kerouault 1989), with an optimal incubation temperature of 15°C producing a 2 hour generation time (Holt et al. 1993).

3.4. Pathogen diversity

A number of studies have demonstrated that *F. psychrophilum* is a phenotypically, serologically, and genotypically diverse species despite initial thoughts to the contrary. However, efforts to connect this variability to host predilection, geographic origin, or strain virulence have produced mixed results. Nevertheless, the diversity of *F. psychrophilum* strains is becoming more evident.

3.4.1. Phenotypic diversity

In terms of phenotype, some *F. psychrophilum* isolates vary in their ability to grow at 25°C or in tryptone soya broth, as well as their ability to hydrolyze elastin and yeast cells (Lorenzen et al. 1997; Madetoja et al. 2001). Gliding motility can be detected in some isolates, as well as H_2S production (Lorenzen et al. 1997; Madetoja et al. 2001). Furthermore, variability of enzymatic activity of α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and *N*-acetyl-β-glucosaminidase have been documented (Hesami et al. 2008). Additionally, two distinct colony morphologies (i.e., rough and smooth) have been demonstrated (Hogfors-

Ronnholm and Wiklund 2010), and cells of both morphotypes can be simultaneously isolated during epizootic events (Sundell et al. 2013).

3.4.1.1. Antimicrobial susceptibility

Various antimicrobial compounds have been used to treat *F. psychrophilum* infections, which have previously been discussed. *F. psychrophilum* isolates can exhibit a wide range of susceptibility profiles to these compounds. Particularly in response to drugs commonly used in aquaculture *F. psychrophilum* isolates seem to vary amongst their antibiograms (Rangdale et al. 1997; Bruun et al. 2000; Dalsgaard and Madsen 2000; Kum et al. 2008; Hesami et al. 2010). Bruun et al. (2000) reported 100% resistance to oxolinic acid in Denmark in just 14 years of use. The resistance to oxolinic acid seems to vary by geographic location, whereby approximately 80% of Canadian isolates (Hesami et al. 2010) and 50% of Japanese (Izumi and Aranishi 2004) isolates are resistant. Increased resistance to oxytetracycline has also been reported, for example 90% of Chilean isolates (Henriquez-Nunez et al. 2012), >75% of Danish isolates (Bruun et al. 2000; Schmidt et al. 2000), approximately 60% of Canadian isolates (Hesami et al. 2010), and 20% of Turkish isolates are resistant to oxytetracycline (Kum et al. 2008). Varied susceptibility to florfenicol has also been demonstrated, >90% of Chilean isolates (Henriquez-Nunez et al. 2012), >50% of Canadian isolates (Hesami et al. 2010), 25% of Turkish isolates (Kum et al. 2008), and 0% of Danish isolates (Schmidt et al. 2000) are resistant to florfenicol. Additionally, *in vitro* studies have shown resistance to ampicillin, kanamycin, polymyxin B, gentamicin, neomycin, erythromycin, phosphomycin, penicillin, and sulfamethoxazole-trimethoprim (Lorenzen 1994; Bustos et al. 1995; Rangdale 1995; Kum et al. 2008; Durmaz et al.

2012). Currently, enrofloxacin, doxycycline, and sarafloxacin seem to be associated with little to no resistance (Rangdale et al. 1997; Schmidt et al. 2000; Kum et al. 2008; Durmaz et al. 2012). Unfortunately, the lack of standardized methods of *in vitro* antibiotic susceptibility for *F. psychrophilum* makes comparisons of resistance profiles from various studies quite difficult. In fact, the World Organization for Animal Health (OIE) has strongly urged the monitoring and surveillance of antimicrobial susceptibility patterns of bacteria isolated from aquatic animals using a standardized protocol (OIE-Aquatic Animal Health Code). In this context, a recent validation and approval of a microbroth dilution assay specific for *F.* psychrophilum, including quality control ranges (Gieseker et al. 2012; CLSI 2014a; CLSI 2014b), has allowed for investigators to comply with the OIE recommendations. The approval of this standardized protocol will vastly improve our global understanding of *F. psychrophilum* antibiotic resistance. In an attempt to further interpret and compare the data generated among investigators using this standardized protocol, the calculation and validation of epidemiological cut-off values specific to *F. psychrophilum* and each individual antimicrobial agent has recently been a point of focus (Smith et al. 2016). The use of these values allow reliable comparisons between studies, often performed from different geographic regions and provide consistent knowledge regarding emergence of resistant strains.

3.4.2. Serological diversity

Multiple serotyping studies have been attempted, however methods were not consistent among studies making interpretation difficult. Nevertheless, trends among serotypes within studies have been observed. For example, Madsen and Dalsgaard (2000) reported

isolates identified as serotype Fp^T (and ribotype B) to be less virulent than other isolates. Additionally, a serotyping method developed by Mata et al. (2002), using slide agglutination and ELISA assays identified 7 host-depended serotypes (Mata et al. 2002). Rainbow trout hosts were infected with multiple serovars (i.e., 21, 2b, and 3), whereas Coho salmon (serotype 1), European eel (serotype 4), carp (serotype 5), tench (serotype 6), and ayu (serotype *7)* all were infected by only one serotype each (Mata et al. 2002). Similarly, Izumi and Wakabayashi (1999) reported a particularly strong association between serotype O-2 and ayu hosts. Using slide agglutination tests and heat stable O-antigens of *F. psychrophilum* isolates, Valdebenito and Avendaño-Herrera (2009) did not find host-specific serotypes, whereby both Atlantic salmon and rainbow trout isolates were classified as group 1 serovars, and Coho salmon and rainbow trout isolates were classified as group 4 serovars. Additional rainbow trout isolates were classified as group 2 serovar, and a single Atlantic salmon isolate was classified as group 3 serovar (Valdebenito and Avendaño-Herrera 2009). However, there are problems associated with this technique, Madetoja et al. (2002) reported that different morphotypes (i.e., smooth versus rough) could not be serotyped due to their autoagglutinating traits. Furthermore, Lorenzen and Olesen (1997), and Izumi and Wakabayashi (1999) reported few isolates that were unable to be serotyped due to unknown mechanisms.

3.4.3. Molecular and genetic diversity

Molecular and genetic investigations are the most common ways for differentiating between *F. psychrophilum* strains and a wide variety of methods have been used with mixed results. With more recent knowledge that *F. psychrophilum* contains 6 copies of the 16S rRNA

gene (Duchaud et al. 2007), a gene commonly used in molecular investigations of bacterial species, data generated using these methods should be taken with caution. Nevertheless, these studies hint at the heterogeneity among *F. psychrophilum* isolates, and few of these studies have linked certain strains with fish host specificity, geographical location, and/or association with disease. These studies, although rarely reproducible and comparable, provide a basis for further, more thorough molecular epidemiological investigations.

3.4.3.1. Pulsed-field gel electrophoresis and restriction enzyme digestion

Pulsed-field gel electrophoresis (PFGE) has been used to distinguish between *F. psychrophilum* isolates, although the use of multiple restriction enzymes makes comparisons difficult. When using *Bln*I and *Xho*I restriction enzymes a total of 42 PFGE banding patterns were identified, which resulted in the conclusion that strains isolated from ayu were genetically different than strains isolated from other species (Arai et al. 2007). Using the restriction enzyme *Sac*I, Chen et al. (2008) reported multiple PFGE band patterns from captive rainbow trout and feral Coho salmon, whereby the spawning Coho salmon had a much more genetically diverse population of *F. psychrophilum* than did the aquacultured rainbow trout. Although unable to demonstrate a correlation between fish host species and PFGE banding pattern, the use of the restriction enzyme *Stu*I resulted in a total of 17 PFGE band patterns that correlated with the isolate origin in a study in Spain (del Cerro et al. 2010).

3.4.3.2. Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism methods have also demonstrated at least some host-specific strains, whereby Izumi et al. (2003) found that isolates from ayu correspond to a unique genotype (i.e., genotype A) when compared to isolates from other host species. Furthermore, this method has also helped distinguish between isolates associated with quinolone resistance (i.e., genotypes QR and QS; Izumi et al. 2007), and tetracycline resistance (Lineage II; Soule et al. 2005). However, other studies failed to produce results with strong associations between genotypes and host species, location, or association with disease (Hesami et al. 2008; Valdebenito and Avendaño-Herrera 2009).

3.4.3.3. Ribotyping

By means of ribotyping, a correlation among dominant ribotypes and the fish farm of origin was found (Madetoja et al. 2002). Furthermore, host association between ribotypes and fish host species was also demonstrated, although not always completely strict. For example, an isolate from European eel grouped with the majority of rainbow trout isolates, and multiple tench isolates grouped with Atlantic salmon isolates (Chakroun 1998). However, Madsen and Dalsgaard (2000) were unable to generate trends among ribotypes and fish host species or location, and suggest this method using the restriction enzyme *Eco*RI may not be useful for epidemiological studies.

3.4.3.4. Random amplified polymorphic DNA analysis

Random amplified polymorphic DNA (RAPD) analysis has also been used to differentiate among *F. psychrophilum* strains. Similar to previously mentioned methods, trends between RAPD profiles and fish host species has also been demonstrated, for example ayu and tench isolates seem to provide unique RAPD profiles (Chakroun et al. 1997). Conversely, no association between fish host species and RAPD profile was demonstrated among Chilean isolates of Atlantic salmon and rainbow trout origin (Valdebenito and Avendaño-Herrera 2009). Furthermore, there is evidence of strains with identical RAPD profiles occurring in broodstock as well as in associated progeny, furthering the hypothesis of vertical transmission of this pathogen (Hatakeyama et al. 2013).

3.4.3.5. Plasmid profiling

Plasmids of various sizes have frequently been detected among *F. psychrophilum* isolates, however there seems to be high homogeneity and this method has been suggested to offer limited epidemiological value (Chakroun et al. 1998). Madsen and Dalsgaard (2000) reported 95% of isolates tested had a single 3.3 kb plasmid, which is similar to the results of Lorenzen et al. (1997), where most isolates contained a plasmid of approximately the same size. Plasmid profiles in association with virulence has been suggested, whereby isolates originating from disease outbreaks contained one small plasmid (Rangdale 1995), however this warrants further investigation as plasmids of the same size have been found in both virulent and avirulent isolates (Chakroun et al. 1998; Madsen and Dalsgaard 2000).

3.4.3.6. Sequence analysis methods

Combining suppression subtractive hybridization and microarrays has allowed for demonstration of two genetic lineages with a likelihood of fish host species specificity (Soule et al. 2005). Furthermore, variable number of tandem repeats (VNTR) analysis has provided delineation among *F. psychrophilum* strains with moderate success, however this method could not discriminate among strains from different fish host species, with the exception of Atlantic salmon (Apablaza et al. 2015). Sequence analyses based on the *gyrB* gene have provided a clear phylogeny of closely related *Flavobacterium* species, and have in fact showed this gene to have been discriminatory power when compared side by side with the 16S rRNA gene (Izumi et al. 2005; Peeters and Willems 2011). The *gyrB* gene is recommended as an epidemiological marker and has been urged to be investigated among all known *Flavobacterium* species (Peeters and Willems 2011). Similarly, the *murG* gene has also been shown side by side to outperform the 16S rRNA gene among closely related *Flavobacterium* strains (Mun et al. 2013). Moreover, a recent study on *F. branchiophilum*, another fish-pathogenic flavobacterium, has identified the use of the *tuf* gene to demonstrate a relationship between sequence variation and location of isolate recovery (Skulska 2014). These studies highlight the importance of investigating alternative loci when performing molecular epidemiological studies.

3.4.3.6.1. Multilocus sequence typing

A specific sequence analysis method, known at multilocus sequence typing (MLST) has recently been developed and optimized specifically for the use of *F. psychrophilum* molecular epidemiology investigations (Nicolas et al. 2008). Maiden et al. (1998) first proposed a MLST

method to explore the strain diversity of *Neisseria meningitides,* briefly, where the sequence diversity of 5-10 housekeeping genes was used to differentiate individual strains within a species (van Belkum et al. 2007) by way of identifying different sequence types (STs). Groupings of closely related STs can then form clonal complexes (CCs) and be used to infer the population structure of the organism. Adaptation of this method to many human and animal pathogens has been very successful (Maiden et al*.* 1998). In fact, MLST has been used to analyze the epidemiology of a number of common fish pathogens, including *Aeromonas* spp. (Martino et al. 2011; Zhang et al. 2014), *Edwardsiella tarda* (Yang et al. 2013), *Tenacibaculum* spp. (Habib et al. 2014), and *Yersinia ruckeri* (Bastardo et al. 2012). Success in developing a *F. psychrophilum*-specific MLST protocol was achieved by Nicolas et al. (2008), whereby the use of 11 protein-coding loci (*trpB, gyrB, glyA, dnaK, tuf, rplB, fumC ,ftsQ, murG, recA, atpA*) showed a strong relationship between certain STs and their host fish species, particularly among rainbow trout hosts (Nicolas et al. 2008). Furthermore, this study identified the population as very prone to intraspecific homologous recombination (Nicolas et al. 2008). However, this study only analyzed 50 isolates from 6 geographic areas (North America, Europe, Israel, Chile, Tasmania, and Japan), and only 10 isolates from the United States were included. Since the pioneer study, 7 loci have been determined sufficient to capture the heterogeneity of *F. psychrophilum* and have been reduced to *trpB, gyrB, dnaK, tuf, fumC, murG, and atpA* (Siekoula-Nguedia et al. 2012). Using this MLST scheme, and with a focus on isolates from France, Siekoula-Nguedia et al. (2012) demonstrated isolates recovered from rainbow trout are strongly associated with particular CCs, whereby the founding STs of these CCs have given rise to multiple closely related strains that seem to be rainbow trout specific. Apablaza et al. (2013) followed up with a study including

isolates from Norway, Chile, North America, and Europe, but found no distinct geographical or host-specific associations with the isolates examined. However, the results from Apablaza et al. (2013) combined with information gleaned from *F. psychrophilum* MLST studies performed afterwards helps identify further host specific trends and formation of CCs. Fujiwara-Nagata et al. (2013) applied the MLST scheme to isolates originating from Japan and found high genetic diversity associated with these isolates (pairwise diversity measure of 0.68, which is the highest diversity measure of *F. psychrophilum* MLST studies to date). Furthermore, host-specific trends were also observed, particularly among rainbow trout (ST10), Coho salmon (STs 13 and 30) and ayu (STs 5, 45, 48, 49, 53, 56, 65, and 67). The next study focused on isolates recovered from Swiss fish farms and demonstrated dominant STs circulating between rainbow trout populations, particularly STs within CC-ST2/10 (Strepparava et al. 2013). Furthermore, isolates belonging to CC-ST2/10 frequently cause disease outbreaks and are considered to be highly virulent, as well as having a worldwide distribution (Strepparava et al. 2013). The population structure of *F. psychrophilum* in Chilean fish farms has been surveyed with results suggesting an influence of fish farming practices on the distribution of *F. psychrophilum* across the country, and beyond, as evidenced by the dominant STs also being the predominant STs in Europe and North America (i.e., CC-ST2/10; Avendaño-Herrera et al. 2014). Finally, an extensive MLST study conducted in Nordic countries strengthens the idea of recombination driving *F. psychrophilum* evolution (Nilsen et al. 2014). This large MLST study also supports the hypothesis that isolates belonging to CC-ST2/10 are seemingly specific to rainbow trout and frequently cause disease outbreaks. Using this MLST protocol, the typing of isolates from three of the main salmonid farming areas has been conducted: Europe (France, Switzerland, Nordic countries; Siekoula-

Nguedia et al. 2012; Apablaza et al. 2013; Strepparava et al. 2013; Nilsen et al. 2014), South America (Chile; Apablaza et al. 2013; Avendaño-Herrera et al. 2014), and Asia (Japan; Fujiwara-Nagata et al. 2013). *F. psychrophilum* isolates from the United States have rarely been examined, knowledge which is extremely important considering the economic and ecological role salmonids play in the U.S, whereby they have been artificially propagated since the end of the nineteenth century (Parker 1989). Through these MLST studies, dominant sequence types have been identified worldwide, suggesting the influence of human activities in the spread of *F. psychrophilum,* including the trade of live fish and their eggs, as well as expansion of hosts beyond their native ranges (Nicolas et al. 2008; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014). Similarly, captive and feral salmonid populations that were intentionally introduced into the GLB over the last century (Kocik and Jones 1999) continue to suffer from *F. psychrophilum* infections (Van Vliet et al. 2015), yet little is known about the pathogen population structure there.

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Chapter 2

Flavobacterium psychrophilum infections in salmonid broodstock and hatchery-propagated stocks of the Great Lakes basin

Van Vliet, D., T.P. Loch, and M. Faisal. 2015. *Flavobacterium psychrophilum* infections in salmonid broodstock and hatchery-propagated stocks of the Great Lakes basin. Journal of Aquatic Animal Health 27:192-202.

1. Abstract

Bacterial coldwater disease (BCWD), caused by *Flavobacterium psychrophilum*, threatens wild and propagated salmonids worldwide, and leads to substantial economic losses. In addition to being horizontally transmitted, *F. psychrophilum* can be passed from infected parents to progeny, furthering its negative impacts. In Michigan, both feral and captive salmonid broodstocks are the gamete sources used in fishery propagation efforts. A 5 year study was initiated to follow the prevalence of systemic *F. psychrophilum* infections in feral steelhead (*Oncorhynchus mykiss*; potadromous rainbow trout), Coho salmon (*O. kisutch)*, Chinook salmon (*O. tshawytscha)*, and Atlantic salmon (*Salmo salar)* broodstock residing in three Great Lakes watersheds. Additionally, captive rainbow trout, brown trout (*Salmo trutta)*, lake trout (*Salvelinus namaycush)*, and brook trout (*Salvelinus fontinalis)* broodstock maintained at two facilities were assessed for the presence of *F. psychrophilum*. The resultant offspring from each broodstock population were sampled for *F. psychrophilum* infections multiple times throughout their residency in the hatchery. Using selective flavobacterial culture and PCR confirmation, *F. psychrophilum* was detected in all broodstock populations except the captive lake trout and brook trout stocks. Logistic regression analysis demonstrated that Chinook salmon from the Lake Michigan watershed had the highest systemic *F. psychrophilum* infection prevalence among infected feral broodstocks, with a mean infection prevalence of 63.2%. Among captive stocks, Gilchrist Creek strain of brown trout had the highest infection prevalence, with a mean infection prevalence of 5%. Collectively, captive broodstocks were found to have significantly lower infection prevalence than feral broodstocks. Despite the high prevalence of systemic *F. psychrophilum* infections in many broodstock populations, the

bacterium was rarely detected in their progeny while in the hatchery system. However, clinical BCWD outbreaks associated with heavy losses did occur. Collectively, these results reinforce that BCWD continues to threaten salmonids of the Great Lakes basin.

2. Introduction

Flavobacterium psychrophilum (Family *Flavobacteriaceae*), the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), is a Gram-negative bacterium that devastates wild and propagated salmonid stocks worldwide (Starliper 2011). In addition to being readily transmitted from fish to fish in a horizontal fashion, *F. psychrophilum* can be vertically transmitted from infected parents to offspring (Brown et al. 1997; Taylor 2004; Cipriano 2005), thus making control efforts particularly problematic. Furthering the challenges associated with *F. psychrophilum* infections is the failure of all known control strategies, including vaccination, to limit the spread of this pathogen or minimize its associated economic losses (Gomez et al. 2014). For example, the state of Utah reports that 25-30% of the hatchery reared steelhead (*Oncorhynchus mykiss*; anadromous rainbow trout) are lost to *F. psychrophilum* infections annually (Oplinger and Wagner 2013). Furthermore, Chilean fish farms report *F. psychrophilum* infections result in economic losses that rank second only to those caused by *Piscirickettsia salmonis* (Valdebenito and Avendaño-Herrera 2009; Avendaño-Herrera et al. 2014). Feral salmonid broodstocks are also affected by these infections, for example nearly 25% of the returning spawning Atlantic salmon (*Salmo salar*) in the Baltic Sea have been shown to harbor this bacterium (Ekman et al. 1999). Indeed, BCWD is considered one of the most significant freshwater fish diseases worldwide (Michel et al. 1999).

Specifically in Michigan, fish-pathogenic bacteria within the Family *Flavobacteriaceae* have historically been associated with more fish mortalities in state fish hatcheries (SFHs) than all other fish pathogens combined (Faisal and Hnath 2005; Faisal et al. 2013). A recent study conducted in the MSU-AAHL demonstrated that a multitude of flavobacterial species were associated with systemic disease and mortality in feral and hatchery salmonid stocks, among which *F. psychrophilum* played a major role (Loch and Faisal 2014; Loch and Faisal 2015). Despite the presence of many novel *Flavobacterium* spp., *F. psychrophilum* continues to be the most common cause of disease outbreaks in Michigan SFH-reared salmonid fry and fingerlings (Loch et al. 2013). Unfortunately, there have been no concrete reports documenting the prevalence and severity of *F. psychrophilum* infections in salmonid broodstock populations of the Great Lakes basin (GLB), a matter that may directly influence the risk of BCWD outbreaks in the resultant hatchery-reared progeny.

In the GLB of North America, salmonid enhancement efforts are widely employed by natural resource agencies to enhance the sport-fishery and associated industries. Gametes from both feral and captive broodstock populations are used in fish propagation efforts; feral and captive broodstocks experience different environmental conditions that may influence their infection rates as well as the potential for transmitting *F. psychrophilum* vertically to progeny. Gametes are collected from either feral or captive broodstock fish, and the resultant offspring are raised in SFHs and eventually stocked into GLB waterways. Throughout this process, *F. psychrophilum* may be transmitted within the gametes and has the potential to negatively impact the growing fry and fingerlings that are maintained under artificial conditions. Therefore, this study was initiated to determine the prevalence of systemic *F.*

psychrophilum infections in feral salmonid broodstock populations returning to spawn at gamete collection facilities (weirs) located in rivers within the watersheds of Lake Huron, Lake Michigan, and Lake Superior. Furthermore, the captive salmonid broodstock populations maintained at two Michigan SFHs were also assessed for the prevalence of systemic *F. psychrophilum* infections, thereby providing managing authorities with the knowledge of where control efforts should be focused.

3. Materials and Methods

3.1. Collection of broodstock

Although *F. psychrophilum* can readily be isolated from the gills and skin of fish, I limited the isolation attempts to the kidney tissue so as to determine the prevalence of systemic infections. Bacterial isolation was attempted from the kidneys of 1,620 feral salmonid broodstock returning to spawn in four Michigan weirs (Fig. 2.1) over a 5 year period (2010-2014). The fish collected during fall spawning runs included 340 Chinook salmon (*O. tshawytscha;* CHS) from the Little Manistee River Weir (LMRW, Manistee County, Lake Michigan watershed; Table 2.1), 340 Chinook salmon from the Swan River Weir (SRW, Presque Isle County, Lake Huron watershed; Table 2.1), 340 Coho salmon (*O. kisutch;* COS) from the Platte River Weir (PRW, Benzie County, Lake Michigan watershed; Table 2.1), and 300 Atlantic salmon (ATS) from the St. Mary's River (SMR, Chippewa County, Lake Superior watershed; Table 2.1). Additionally, 300 steelhead (STT) were collected from the LMRW during spring spawning runs (Table 2.1). A total of 713 captive salmonid broodstock fish were sampled prior to artificial spawning during the fall at two Michigan SFHs (Fig. 2.1) from 2010-2014. The fish sampled from

Marquette State Fish Hatchery (MSFH; Marquette County, Lake Superior watershed) included 155 Lake Superior strain lake trout (*Salvelinus namaycush;* LS-LAT; Table 2.2), and 141 brook trout (*Salvelinus fontinalis;* BKT; Table 2.2) and were all maintained at the facility that uses a local stream and deep wells as source water. The fish sampled from Oden State Fish Hatchery (OSFH, Emmett County, Lake Michigan watershed) included 100 Eagle Lake strain rainbow trout (EL-RBT; Table 2.2), 117 Sturgeon River strain brown trout (*Salmo trutta*; SR-BNT; Table 2.2), 120 Wild Rose strain brown trout (WR-BNT), and 80 Gilchrist Creek strain brown trout (GC-BNT; Table 2.2) and were all maintained on fish-free, deep well water. Gametes from both feral and captive broodstock populations were then used for artificial fertilization by Michigan SFH staff. Gametes were collected on site at the locations where feral broodstock return or hatcheries where captive broodstock are maintained. Eggs and milt from mature fish were gently mixed with a 0.75% saline solution to improve fertilization rates and left to sit for approximately 1 min. The excess saline solution was poured off and fertilized eggs were then transferred to an erythromycin (2 ppm) bath to water harden for 1 h. The erythromycin solution was then poured off, and the water-hardened fertilized eggs were rinsed with fresh water. An iodophor solution (50 ppm) was then added to the eggs for surface disinfection for 30 min. The eggs were then rinsed with fresh water and packed for shipment to the hatchery. Upon arrival to each respective hatching unit at the six SFHs the eggs were surface disinfected again with an iodophor solution (100 ppm, 10 min).

3.2. Progeny fish

The offspring of the aforementioned broodstock populations were hatched and maintained at six Michigan SFHs (Fig. 2.1) supplied by varying types of water sources. Platte River State Fish Hatchery (PRSFH; PRW, Benzie County, Lake Michigan watershed) relies on a combination of spring and stream water to feed the facility, and rears Coho salmon, Atlantic salmon, and Chinook salmon. Marquette SFH uses local stream water supplemented with deep well water and rears brook trout, Lake Superior strain lake trout, and Seneca Lake strain lake trout originating from broodstock maintained at a national fish hatchery. Wolf Lake State Fish Hatchery (WLSFH; Van Buren County; Lake Michigan watershed), Harrietta State Fish Hatchery (HSFH; Wexford County; Lake Michigan watershed), and OSFH are all fed entirely by fish-free well water. Wolf Lake SFH rears both Chinook salmon and steelhead, while HSFH and OSFH rear Eagle Lake strain rainbow trout, and Wild Rose and Sturgeon River strain brown trout; additionally HSFH also rears Gilchrist Creek strain brown trout. Thompson State Fish Hatchery (TSFH; Schoolcraft County; Lake Michigan watershed) utilizes both fish-free well and spring water and rears Chinook salmon, steelhead, and Gilchrist Creek strain brown trout. The length of time spent in the hatchery before being stocked into GLB waters varies by species; Chinook salmon are stocked approximately six months post-hatch; steelhead approximately 12 months post-hatch; and Coho salmon, Atlantic salmon, lake trout, Eagle Lake strain rainbow trout, and all strains of brown trout approximately 18 months post-hatch. During the rearing cycle, fish were sampled when a clinical disease outbreak and/or elevated mortality was observed. However, all progeny lots were assessed for systemic *F. psychrophilum* infections during health assessments approximately six weeks before stocking.

3.3. Fish sampling

All broodstock fish and annual health assessment fingerlings were randomly collected (equal number of males and females for feral population sampling), whereas moribund fish were targeted for collection during clinical disease outbreak samplings. All fish were collected by Michigan Department of Natural Resources (MDNR) personnel and euthanized on-site using a pneumatic stunner (Seafood Innovations, Australia), or in the laboratory by an overdose of tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington; Western Chemical, Ferndale, Washington). Immediately thereafter, all fish were grossly examined and necropsied, whereby all fish were surface disinfected with 70% ethanol and opened with sterile dissecting tools, as previously described (Loch et al. 2012; Diamanka et al. 2013).

3.4. Flavobacterial isolation and identification

For flavobacterial isolation and identification, all reagents were purchased from Remel Inc. (Lenexa, Kansas) unless noted otherwise. Kidneys were made accessible by an incision in the surrounding connective membranous tissues, and tissues were then collected using sterile disposable 1 or 10 µL loops (Sigma -Aldrich Corp., St. Louis, Missouri), depending upon the size of the fish. Particularly during clinical disease outbreaks, external lesions were often present, whereby 70% ethanol was used to first surface disinfect the area before the leading edge of the lesion was cultured from using sterile disposable 1 µL loops. All collected tissues were immediately streaked directly onto cytophaga agar (CA; Anacker and Ordal 1959),

supplemented with neomycin sulfate at 4 mg L^{-1}, and incubated at 15°C for 72-144 h, as per the guidelines of the American Fisheries Society – Fish Health Section Blue Book (AFS-FHS 2012). Bacterial growth was recorded and individual colonies were subcultured onto fresh CA plates and incubated for 48-72 h at 15°C for further phenotypic and molecular characterization. For cryopreservation, bacterial isolates were grown in CA broth for 48-96 h, after which glycerol was added (20% v/v) and the bacterial suspension immediately frozen at -80°C.

3.5. *F. psychrophilum* identification

Purified cultures of yellow-pigmented bacteria were initially tested for catalase activities (3% hydrogen peroxide), the presence of flexirubin-type pigments (3% potassium hydroxide), and Gram reaction using the string test (Whitman 2004), and the Gram stain assay (Bartholomew and Mittwer 1952). Bacterial isolates possessing the morphological characteristics of *F. psychrophilum* (e.g., bright yellow, slightly raised, often with thin spreading edges; Nematollahi et al. 2003) and that were catalase, and flexirubin-type pigment positive were further examined using a *F. psychrophilum*-specific polymerase chain reaction (PCR) assay targeting a partial stretch of the 16S rRNA gene (Toyama et al. 1994). Prior to DNA extraction, bacteria were harvested, suspended in 1,000 µL of sterile 0.9% saline, briefly vortexed, then centrifuged for 10 min at 20,000 *g.* Extraction of DNA from bacterial pellets was conducted using the DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, California), according to the manufacturer's Gram-negative bacterium protocol, with the exception that 50 µL of elution buffer was used in the final step. Next, DNA was quantified using a Quant-iT® DS DNA Assay Kit and a Qubit® fluorometer (Life Technologies, Grand Island, New York). Dilution of extracted

DNA to a standard concentration of 10 ng of DNA μL^{-1} of water was then performed using sterile nuclease-free water. All PCR reactions were carried out in a Mastercyler® Gradient Thermalcycler (Eppendorf, Hauppauge, New York) with a total reaction volume of 20 µL for each sample. Each reaction included 10 µL of 2x Go-Taq Green master mix (Promega, Madison, Wisconsin), 10 ng of DNA template, 5 ng of each primer, and nuclease-free water comprising the remainder of the reaction. PCR specifications included an initial denaturation step at 94°C for 2 min, followed by 30 cycles of amplification, which included denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min 30 sec. A final elongation step was performed at 72°C for 7 min. A previously sequenced *F. psychrophilum* strain (Loch et al. 2013) was used as a positive control, and nuclease free water served as the negative control. SYBR® Green gel stain (Cambrex Bio Science Rockland, Inc., Rockland, Maine) was combined with each amplicon and run on a 1.5% agarose gel at 100 V for 45 min. A 1-kb Plus DNA ladder (Life Technologies, Grand Island, New York) was used as a molecular marker. Amplicons were then visualized under UV exposure (UVP, LCC, Upland, California), whereby the presence of an amplicon of approximately 1,100 bp was considered confirmatory for *F. psychrophilum* (Toyama et al. 1994).

3.6. Statistical analyses

The prevalence of infection was calculated by dividing the number of fish infected with *F. psychrophilum* by the total number of fish sampled during that event. To investigate any potential difference between feral broodstock infection prevalence and captive broodstock infection prevalence the grand means and 95% confidence intervals of both groups were

calculated and compared. For logistic regression analyses, two separate groups of models were constructed to assess the differences in *F. psychrophilum* infections among feral and captive salmonid broodstock populations. *Flavobacterium psychrophilum* infection scores were analyzed using a binary method (*F. psychrophilum* absence = 0 and presence = 1). Feral broodstock models ranged in complexity and were constructed to test the main effect of species/location combination, sex, and year as well as their first order interactions. Because some of the variables to be compared were not mutually exclusive (i.e., CHS were sampled from two locations, and two fish species were sampled from LMRW), a unique indicator variable was used for each species/location combination (e.g., CHS-LMRW, CHS-SRW, COS-PRW, ATS-SMR, STT-LMRW). Model goodness of fit was assessed by the calculation of Akaike information criterion (AIC) scores, whereby lower AIC scores indicate a more parsimonious fit of the model to the data (Burnham and Anderson 2002). In addition, an analysis of deviance was conducted to determine the model of best fit. To determine the effect of species/strain and year as well as their interactions on captive broodstock infection prevalence, a set of generalized linear models were constructed. The year variable in all models was treated as categorical. To predict the probability of infection of each species/location combination throughout the five years of the study, a least-squares means prediction analysis was conducted on both the feral and captive models. All analyses were performed using R: A Language and Environment for Statistical Computing (R Core Team 2013).

4. Results

4.1. *F. psychrophilum* identification

Bacterial cultures on CA from the kidneys and lesions of the fish examined in this study yielded presumptive *F. psychrophilum* isolates that were bright yellow, slightly raised colonies with often thin spreading edges. The intensity of infection ranged from one colony-forming unit to heavy interconnected growth per 1 or 10 µL of inoculum. Representative suspect *F. psychrophilum* colonies were further examined; whereby presumptive *F. psychrophilum* isolates were Gram-negative filamentous bacilli that were positive for flexirubin-like pigments and catalase activity. Polymerase chain reaction amplification with the primers described by Toyama et al. (1994) yielded amplicons of approximately 1,100 bp in length for *F. psychrophilum* confirmation.

4.2. Broodstock

The overall number of *F. psychrophilum* infected broodstock fish throughout the course of the study was 595 out of a total of 2,333 fish sampled (25.5%; Tables 2.1-2.2). Collectively, feral broodstock populations experienced significantly greater infection prevalence when compared to captive broodstock populations; e.g., 566 of a total of 1,620 feral broodstock fish sampled (34.9%; 95% CI: 33.6-38.3%; Table 2.1) were infected with *F. psychrophilum,* compared to only 15 of 713 (2.1%; 95% CI: 1.9-2.2%; Table 2.2) captive broodstock.

4.2.1. Feral broodstock

The highest average infection prevalence was observed in Chinook salmon from the LMRW (63.2%; Lake Michigan watershed; Table 2.1). Overall, the Atlantic salmon sampled from the SMR (Lake Superior watershed) had the second highest infection prevalence (53.0%; Table 2.1). The Chinook salmon sampled from the SRW (Lake Huron watershed) had an average infection prevalence of 25.0% (Table 2.1). The next highest average infection prevalence was observed in Coho salmon sampled at the PRW (22.6%; Lake Michigan watershed; Table 2.1). Lastly, the group with the lowest average *F. psychrophilum* infection prevalence was the LMRW-steelhead (10.0%; Lake Michigan watershed; Table 2.1). The best performing model included the main effect of species/location combination and year as well as their interactions (Table 2.3).

Chinook salmon collected from the LMRW had an infection prevalence ranging from 50.0-86.7%, in comparison, the infection prevalence ranged from 10.0-51.7% in Chinook salmon from the SRW (Fig. 2.2). Atlantic salmon from the SMR had an infection prevalence ranging from 40.0-63.3%, Coho salmon from the PRW infection prevalence ranged from 0.0-43.3%, and steelhead from the LMRW infection prevalence ranged from 0.0-26% (Fig. 2.2). According to the least-squares means analysis, the predicted probability of infection varied substantially between species/location combination, as well as within species/location combination (Fig. 2.3). For example, the predicted probability of infection among Chinook salmon from the LMRW increased linearly from 2010-2012 (0.5-0.88), but then was lower in 2013 and 2014 (0.52 and 0.56; Fig. 2.3). In comparison, the predicted probability of infection among steelhead from

the LMRW decreased linearly from 2010-2012 (0.25 to < 0.001), but then increased in 2013 (0.15) followed by a slight decrease in 2014 (0.06; Fig. 2.3).

4.2.2. Captive broodstock

The highest average infection prevalence in the captive broodstock populations were observed in the Gilchrist Creek strain brown trout (5.0%; Table 2.2), followed by the Wild Rose strain brown trout (4.2%; Table 2.2). The captive rainbow trout (Eagle Lake strain) had an average infection prevalence of 4% (Table 2.2), while the Sturgeon River strain brown trout had an average infection prevalence of all 1.7% (Table 2.2). Over the course of the study, no *F. psychrophilum* was detected from any captive lake trout (0.0%; Table 2.2), or brook trout (0.0%; Table 2.2). The best performing model included the main effect of species/strain combination and year (Table 2.3).

The *F. psychrophilum* infection prevalence in the Gilchrist Creek strain brown trout ranged from 0.0-20.0%, Wild Rose strain brown trout infection prevalence ranged from 0.0-10.0%, and Sturgeon River strain brown trout infection prevalence ranged from 0.0-6.25% (Fig. 2.2). The infection prevalence in the Eagle Lake rainbow trout ranged from 0.0-15.0% (Fig. 2.2). The full model (i.e., main effects of species/strain combination and year, and their interactions) was found less parsimonious and thus the reduced model was used for further analyses. The reduced model produced identical trends of predicted probability of infection between species/strain combination and year (Fig. 2.4). Overall, there was an initial increase in the predicted probability of infection from 2010 to 2011, but then the infection probability decreased linearly through the rest of the study (Fig. 2.4).

4.3. Progeny

To determine infection status prior to stocking, a total of 110 sampling events (n = 60-65 fish/event) of resultant progeny were completed, 10 of which revealed systemic *F. psychrophilum* infections (Table 2.4). *Flavobacterium psychrophilum*-infected production lots that originated from broodstock that were also systemically infected included steelhead, all strains of brown trout, Atlantic salmon, Eagle Lake strain rainbow trout, LMRW-Chinook salmon, and Coho salmon (Table 2.4). In three instances, however, *F. psychrophilum* was detected just prior to fish stocking in production lots that originated from broodstock that were not systemically infected with *F. psychrophilum* (Table 2.4). In addition to *F. psychrophilum* infections being detected in fish just prior to stocking, occasional clinical disease outbreaks occurred during the rearing cycle, in which fish were submitted to the laboratory for diagnostic analysis. Over a 5 year period, a total of 48 diagnostic events occurred in the studied salmonid lots, whereby *F. psychrophilum* was detected on 27 occasions and determined to be the primary etiological agent in 25 (Table 2.4). In the majority of cases (n = 19), these disease outbreaks occurred in production lots that originated from broodstock lots that were found to be infected with *F. psychrophilum* (Table 2.4). Among these, four production lots (e.g., 2010 year-class of steelhead raised at WLSF, 2010 year-class of Atlantic salmon raised at PRSFW, 2012 year-class of Wild Rose strain and Sturgeon River strain brown trout raised at OSFH) experienced multiple mortality events during their rearing cycle that were attributed to *F. psychrophilum* (Table 2.4). However, five outbreaks occurred in production lots that originated from *F. psychrophilum*-negative broodstock lots (Table 2.4), two lots of which experienced

repeated mortality events (e.g., 2012 year-class steelhead raised at WLSFH, and 2011 year-class Lake Superior strain lake trout raised at MSFH; Table 2.4). Interestingly, 13 production lots that experienced *F. psychrophilum*-related mortality events were negative for the bacterium just prior to stocking (Table 2.4).

5. Discussion

Data presented herein summarizes the first comprehensive analysis of *F. psychrophilum* prevalence in the Great Lakes basin. Thus, the findings of this study are of particular importance to fishery managers because they illustrate how widespread systemic *F. psychrophilum* infections are in multiple salmonid stocks of the GLB. In fact, *F. psychrophilum* infections were responsible for more than half of all the disease outbreaks in the studied progeny lots, illustrating a serious risk to GLB hatchery reared salmonids.

A major conclusion of this study was that captive salmonid broodstock populations maintained at Michigan SFHs had a significantly lower *F. psychrophilum* infection prevalence than feral broodstock populations of the GLB. This is somewhat of a surprise given that captive broodstock are maintained under artificial conditions and at higher densities than their free-ranging counterparts. Although captive broodstock populations are often held in high-density raceways, a factor which increases stress among the fish, a well-balanced diet, good husbandry, and strict biosecurity practices seem to help minimize the effects of captivity-related stress. Additionally, in the case of the captive broodstock in this study, the possibilities of *F. psychrophilum* invading the hatchery system with the incoming water supply are minimized with the use of fish-free well water (i.e., OSFH), or UV light treated stream water (i.e., MSFH).

Moreover, these stocks are also continuously monitored for various pathogens and abnormal behaviors, and chemotherapeutically treated when necessary (Faisal et al. 2013), a practice that is unavailable to feral fish. Collectively, these results suggest that the husbandry and biosecurity efforts employed by the MDNR are proving efficacious at reducing the risk of *F. psychrophilum* infections among captive broodstock populations maintained at Michigan SFHs (i.e. OSFH and MSFH), as well as reducing the risk in the progeny reared at those facilities. This potentially explains the absence of variation in the predicted probability of infection among captive salmonid broodstock populations as opposed to the highly variable infection probability that was demonstrated among feral salmonid broodstock populations.

Another important observation of this study was that feral Chinook salmon had a higher *F. psychrophilum* infection prevalence than either feral Coho salmon or feral steelhead, which are both considered to be highly susceptible to BCWD (Borg 1948; Rucker et al. 1954; Holt 1987; Taylor 2004; Cipriano and Holt 2005). Indeed, Chinook salmon are believed to be one of the less *F. psychrophilum*-susceptible salmonids (Rucker et al. 1954; Taylor 2004; Chen et al. 2008). However, our findings indicate feral Chinook salmon broodstock as being most at risk for *F. psychrophilum* infections in the GLB, as these stocks consistently had the highest *F. psychrophilum* infection prevalence over the course of the study. On the other hand, steelhead, which are a migratory strain of rainbow trout and are considered highly susceptible to BCWD, were found to have the lowest infection prevalence amongst the four feral salmonid broodstock species that were examined in this study. An unexpected finding of this study was the apparent inverse trend in *F. psychrophilum* infection probability that was observed between

Chinook salmon and steelhead at LMRW across all five years of the study (Fig 2.3), a matter that warrants further investigation.

Many different factors may be influencing the observed differences of *F. psychrophilum* infections between Great Lakes salmonid populations and those observed in regions elsewhere. The majority of the Great Lakes salmonid species were introduced to the region throughout the last century (Kocik and Jones 1999). Over time, the genetic variability between the Pacific Northwest (PNW) stocks and the GLB stocks has increased (Weeder et al. 2005), which could account for the apparent differences in *F. psychrophilum* susceptibility between these salmonid populations. Indeed, differences in susceptibility to *Renibacterium salmoninarum*, the causative agent of Bacterial Kidney Disease (BKD), has been observed between PNW founder and GLB stocked Chinook salmon populations (Purcell et al. 2008; Metzger et al. 2010; Purcell et al. 2014). Thus, it is possible that genetic differences may be playing a role in the apparently different *F. psychrophilum* susceptibilities. Furthermore, Purcell et al. (2008) has suggested phenotypic divergence between the founder and GLB stocks of salmonids may be related to disease epizootics or other conditions faced in the unique environment of the Great Lakes. Indeed, differential susceptibly to *F. psychrophilum* infections due to genetic differences has been demonstrated by the development of *F. psychrophilum*-resistant and susceptible strains of rainbow trout (Marancik et al. 2014a; Marancik et al. 2014b).

Interestingly, despite the fact that feral Chinook salmon broodstock had the highest *F. psychrophilum* infection prevalence, Chinook salmon progeny only experienced one overt BCWD outbreak during the course of the study and systemic infections were never detected during sampling events just prior to stocking (Table 2.4). In contrast, feral steelhead broodstock

had the lowest infection prevalence, but clinical BCWD outbreaks were most common in the resulting progeny (Table 2.4). The same biosecurity and handling measures are employed during artificial spawning of both species. Briefly, the gametes are collected from mature fish, fertilized, water hardened in erythromycin, and surface disinfected with iodophor solution before being sent to the hatcheries for rearing. Erythromycin has been allowed as a chemotherapeutic drug with limited use as an investigational new animal drug exemption (INAD) to combat BKD; specifically by the MDNR it is used under the INAD exemption during water hardening of salmonid eggs to minimize the vertical transmission of the disease (Faisal et al. 2012). Although the MDNR uses erythromycin to reduce the *R. salmoninarum* bacterial load in and around the fertilized eggs, some studies have shown that *F. psychrophilum* is susceptible to erythromycin exposure as well (Brown et al. 1997; Kum et al. 2008; Hesami et al. 2010). However, others have found that erythromycin is ineffective for the control of *F. psychrophilum* (Oplinger and Wagner 2013) and that even if iodophor and erythromycin treatments reduce bacterial loads, BCWD may still subsequently occur. Nevertheless, the current practices are seemingly efficacious in mitigating the threat of transmission in species such as Chinook salmon, Coho salmon, and Atlantic salmon whereby the broodstocks exhibited moderate to high *F. psychrophilum* infection prevalence, but the resultant progeny rarely experienced epizootics. Alternatively, another potential influence on vertical transmission of *F. psychrophilum* is the intensity of infection in broodstock. Although prevalence of infection was high in multiple broodstocks of this study, the intensity of infection was not investigated; it may be possible that although the broodstock fish were infected with *F. psychrophilum* they harbored too few bacteria to cause clinical outbreaks after vertical transmission to progeny. In

contrast, the feral steelhead broodstock population had low *F. psychrophilum*-infection prevalence, but the frequently observed outbreaks in the progeny may have resulted from the remarkable susceptibility of this species to BCWD and/or the presence of predisposing environmental factors.

In a similar context, the BCWD outbreaks during October and November 2012 in both strains of lake trout fingerlings at MSFH (Table 2.4) were influenced by perturbations in water quality; the facility experienced heavy rains just prior to overt disease outbreaks, which lead to greatly elevated suspended solids. The Lake Superior strain lake trout fingerlings were reared upstream of the Seneca Lake strain lake trout, and began showing disease signs and increased mortality approximately one week prior to the Seneca Lake strain lake trout. Fish of both strains were heavily infected with *F. psychrophilum* and were later found to be co-infected with epizootic epitheliotropic disease (EED) virus (records of the MSU-AAHL). Although it is unclear which pathogen preceded the other, BCWD outbreaks in lake trout reared at MSFH only occurred on one other occasion in the absence of detectable EED virus. Similarly, Atlantic salmon reared at PRSFH only experienced overt BCWD outbreaks when they were found to be co-infected with *Aeromonas salmonicida* (Records of the MSU-AAHL). Indeed, other studies have shown the presence of other pathogens can influence the progression of *F. psychrophilum* infections (Decostere et al. 2000; Busch et al. 2003; LaFrentz and Cain 2004; Long et al. 2012). Furthermore, other aquaculture practices, including egg-rearing techniques, feeding practices, water quality, and raceway densities have also been suggested to influence the occurrence and/or progression of BCWD (Nematollahi et al. 2003).

Currently, there are no vaccines against *F. psychrophilum* that are licensed for use in the U.S.A.; thus, prevention by other mechanisms increases in significance. Improved biosecurity measures, such as equipment and infrastructure disinfection, as well as air-drying protocols, have been shown to help minimize and prevent the horizontal spread of the disease within fish rearing facilities (Hesami et al. 2010; Oplinger and Wagner 2010). Furthermore, the usage of antibiotic treatment during fertilization may help control vertical transmission of *F. psychrophilum* (Oplinger et al. 2015). The U.S. Food and Drug Administration has approved several antibiotics for the treatment of BCWD outbreaks, including florfenicol and oxytetracycline (USFWS AADAP 2010). However, reports of antibiotic resistance in *F. psychrophilum* isolates from around the world (Bruun et al. 2000; Dalsgaard and Madsen 2000; Durmaz et al. 2012) are of particular concern.

In conclusion, this study confirms how widespread *F. psychrophilum* infections are in Great Lakes salmonid broodstock populations, as well as how frequent epizootics occur in Michigan SFH-reared progeny. It suggests that biosecurity measures continue to be employed until an effective vaccine has been developed.

APPENDIX

Table 2.1 Feral broodstock found to be infected with *F. psychrophilum*. LMRW, Little Manistee River Weir; SRW, Swan River Weir; PRW, Platte River Weir; SMR, St. Mary's River.

Fish species and strain	Collection site	Date of sampling	Total sample number	Males infected with *F. psychrophilum*	Females infected with *F. psychrophilum*
O. tshawytscha	LMRW	Oct. 2010	60	15/30	15/30
		Oct. 2011	100	22/50	32/50
		Oct. 2012	60	23/30	29/30
		Oct. 2013	60	23/30	22/30
		Oct. 2014	60	17/30	17/30
O. tshawytscha	SRW	Oct. 2010	60	7/30	8/30
		Oct. 2011	100	6/50	4/50
		Oct. 2012	60	11/30	8/30
		Oct. 2013	60	18/30	13/30
		Oct. 2014	60	5/30	5/30
O. kisutch	PRW	Oct. 2010	60	6/30	2/30
		Oct. 2011	100	8/50	16/50
		Oct. 2012	60	7/30	12/30
		Oct. 2013	60	9/30	17/30
		Oct. 2014	60	0/30	0/30
S. salar	SMR	Nov. 2010	60	14/30	22/30
		Nov. 2011	60	17/30	7/30
		Nov. 2012	60	18/30	17/30
		Nov. 2013	60	15/30	11/30
		Nov. 2014	60	20/30	18/30
O. mykiss- Lake Michigan	LMRW	April 2010	60	11/30	4/30
		April 2011	60	0/30	2/30
		Mar. 2012	60	0/30	0/30
		April 2013	60	5/30	4/30
		April 2014	60	4/30	0/30

Table 2.2 Captive broodstock found to be infected with *F. psychrophilum.* MSFH, Marquette State Fish Hatchery; OSFH, Oden State Fish Hatchery.

Fish species and strain	Collection site	Date of sampling	Fish infected with *F. psychrophilum*
S. namaycush- Lake Superior	MSFH	Aug. 2010	0/30
		Aug. 2011	0/45
		Aug. 2012	0/30
		Aug. 2013	0/30
		Aug. 2014	0/20
S. fontinalis	MSFH	Aug. 2010	0/51
		Aug. 2011	0/30
		Aug. 2012	0/20
		Aug. 2013	0/20
		Aug. 2014	0/20
O. mykiss- Eagle Lake	OSFH	Nov. 2010	1/20
		Nov. 2011	3/20
		Nov. 2012	0/20
		Nov. 2013	0/20
		Sept. 2014	0/20
S. trutta- Sturgeon River	OSFH	Nov. 2010	1/16
		Nov. 2011	0/21
		Nov. 2012	0/30
		Nov. 2013	1/20
		Sept. 2014	0/30
S. trutta- Wild Rose	OSFH	Nov. 2010	1/20
		Nov. 2011	1/10
		Nov. 2012	3/30
		Nov. 2013	0/30
		Sept. 2014	0/30
S. trutta- Gilchrist Creek	OSFH	Nov. 2010	1/20
		Nov. 2011	2/10
		Nov. 2012	0/20
		Nov. 2013	1/10
		Sept. 2014	0/20

Table 2.3 Summary of AIC values for the *Flavobacterium psychrophilum* infection prevalence models. AIC, Akaike information criterion.

Broodstock Group	Main effects	First order interactions	AIC
Feral	Year, species/location combination	Year, species/location combination	1691.7
	Sex, year, species/location combination	Year, species/location combination	1693.0
	Sex, year, species/location combination	Sex, species/location combination	1820.6
	Year, species/location combination		1823.9
	Sex, year, species/location combination		1825
	Sex, year, species/location combination	Sex, year	1826.6
	Sex, species/location combination	Sex, species/location combination	1849.8
	Species/location combination		1852.7
	Sex, species/location combination		1853.9
	Year		2096.6
	Sex, year		2097.9
	Sex		2119
	Sex, year	Sex, year	2145893
Captive	Year, species/strain		129.64
	Year, species/strain	Year, species/strain	138.67

Table 2.4 Progeny lots positive for *F. psychrophilum* during the course of the study. *Flavobacterium psychrophilum* was detected from all clinical disease outbreak samplings reported. WLFSH, Wolf Lake State Fish Hatchery; TSFH, Thompson State Fish Hatchery; HSFH, Harrietta State Fish Hatchery; OSFH, Oden State Fish Hatchery; PRSFH, Platte River State Fish Hatchery; MSFH, Marquette State Fish Hatchery; NA, not available. [a]All year-classes of all species reared at all appropriate hatcheries were assessed for the presence of *F. psychrophilum* throughout the study, only the lots which were positive for the pathogen are presented in this table.

Fish species and strain	Year-class[a]	Broodstock infection prevalence	Hatchery	Date(s) of ***F. psychrophilum*** positive clinical outbreak(s)	Pre-stocking sampling date(s)	***F. psychrophilum*** results from pre-stocking sampling
O. mykiss-Lake Michigan	2010	25%	WLSFH	Aug. 2010; Oct. 2010	July 2010/Jan. 2011	-/-
			TSFH	Jan. 2011	July 2010/Feb. 2011	-/+
	2011	3.3%	WLSFH	Nov. 2011	July 2011/Feb. 2012	-/-
			TSFH	Dec. 2011	Aug. 2011/Feb. 2012	-/-
	2012	0%	WLSFH	June 2012; Nov. 2012	July 2012/Jan 2013	+/+
	2013	15%	WLSFH	none	July 2013/Jan 2014	+/-
S. trutta-Wild Rose	2010	5%	HSFH	Aug. 2011	Sept. 2011/Feb. 2012	-/+
	2011	10%	HSFH	June 2012	Aug. 2012/Jan. 2013	-/+
			OSFH	Oct. 2012	Sept. 2012/Feb. 2013	-/+
	2012	10%	OSFH	May 2013; July 2013	Feb. 2014	-
S. trutta-Sturgeon River	2010	6.25%	OSFH	Dec. 2011	Feb. 2012	-
	2012	3.2%	OSFH	April 2013; June 2013	Feb. 2014	-
			HSFH	Aug. 2013	Feb. 2014	-

Table 2.4 (cont'd)

Fish species and strain	Year-class[a]	Broodstock infection prevalence	Hatchery	Date(s) of *F. psychrophilum* positive clinical outbreak(s)	Pre-stocking sampling date(s)	*F. psychrophilum* results from pre-stocking sampling
S. trutta-Gilchrist Creek	2011	20%	TSFH	none	July 2012	+
	2012	0%	HSFH	Aug. 2013	Feb. 2014	-
S. namaycush-Seneca Lake	2010	NA	MSFH	Nov. 2011	Jan. 2012	-
	2011	NA	MSFH	Oct. 2012; Nov. 2012	May 2012/Jan. 2013	-/-
S. namaycush-Lake Superior	2011	0%	MSFH	Oct. 2012; Nov. 2012	Jan. 2013	-
S. salar	2010	60%	PRSFH	Oct. 2011; Dec. 2011; April 2012	March 2012	+
O. mykiss-Eagle Lake	2011	15%	HSFH	June 2012	Jan. 2013	-
O. tshawytscha-LMRW	2011	54%	WLSFH	March 2012	Feb. 2012	-
O. kisutch	2011	27%	PRSFH	none	Feb. 2013	+

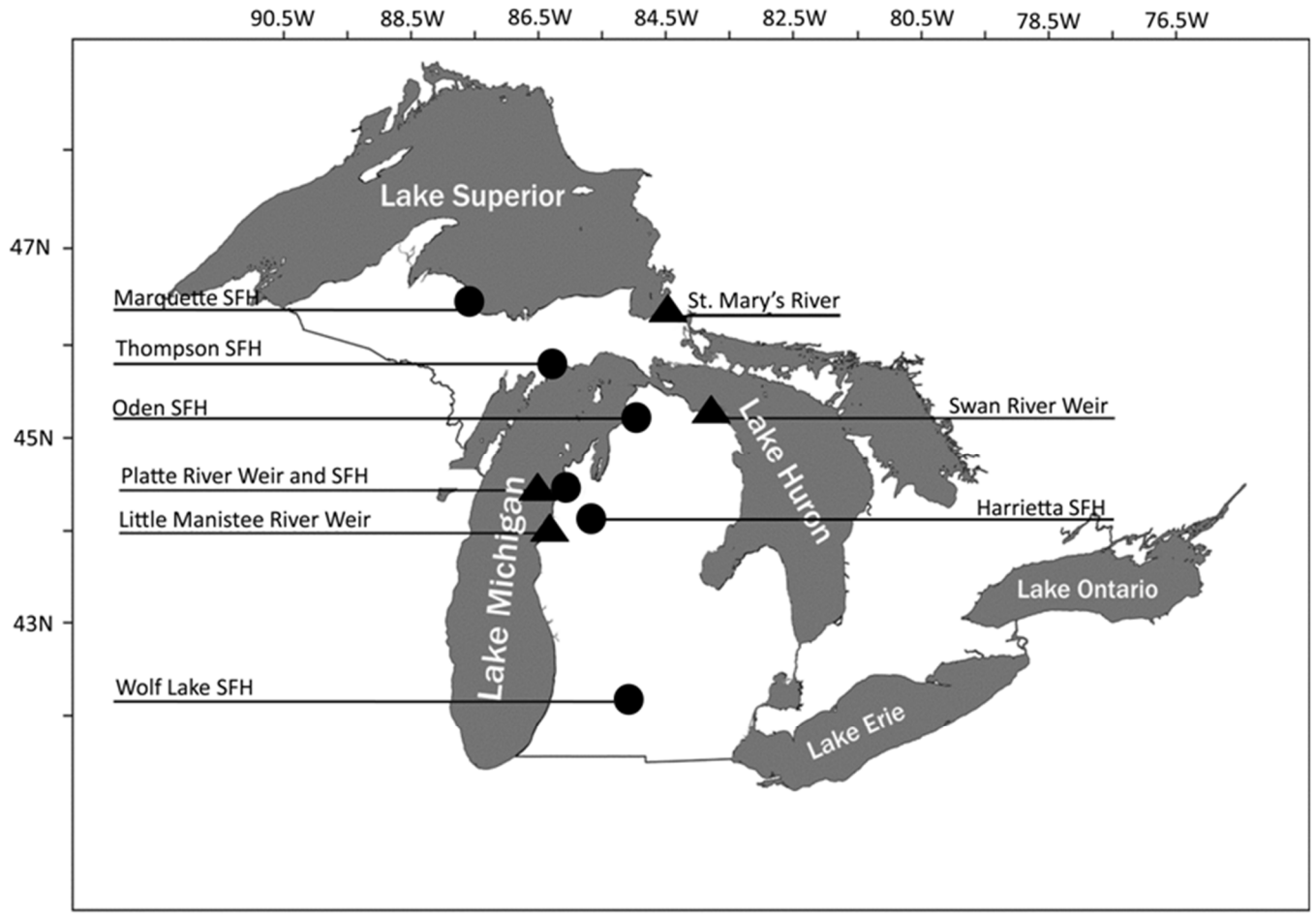

Figure 2.1 The locations from which all spawning salmonids and resultant progeny were sampled throughout the course of the study. Triangles indicate gamete collection facilities (weirs). Circles indicate hatchery facilities.

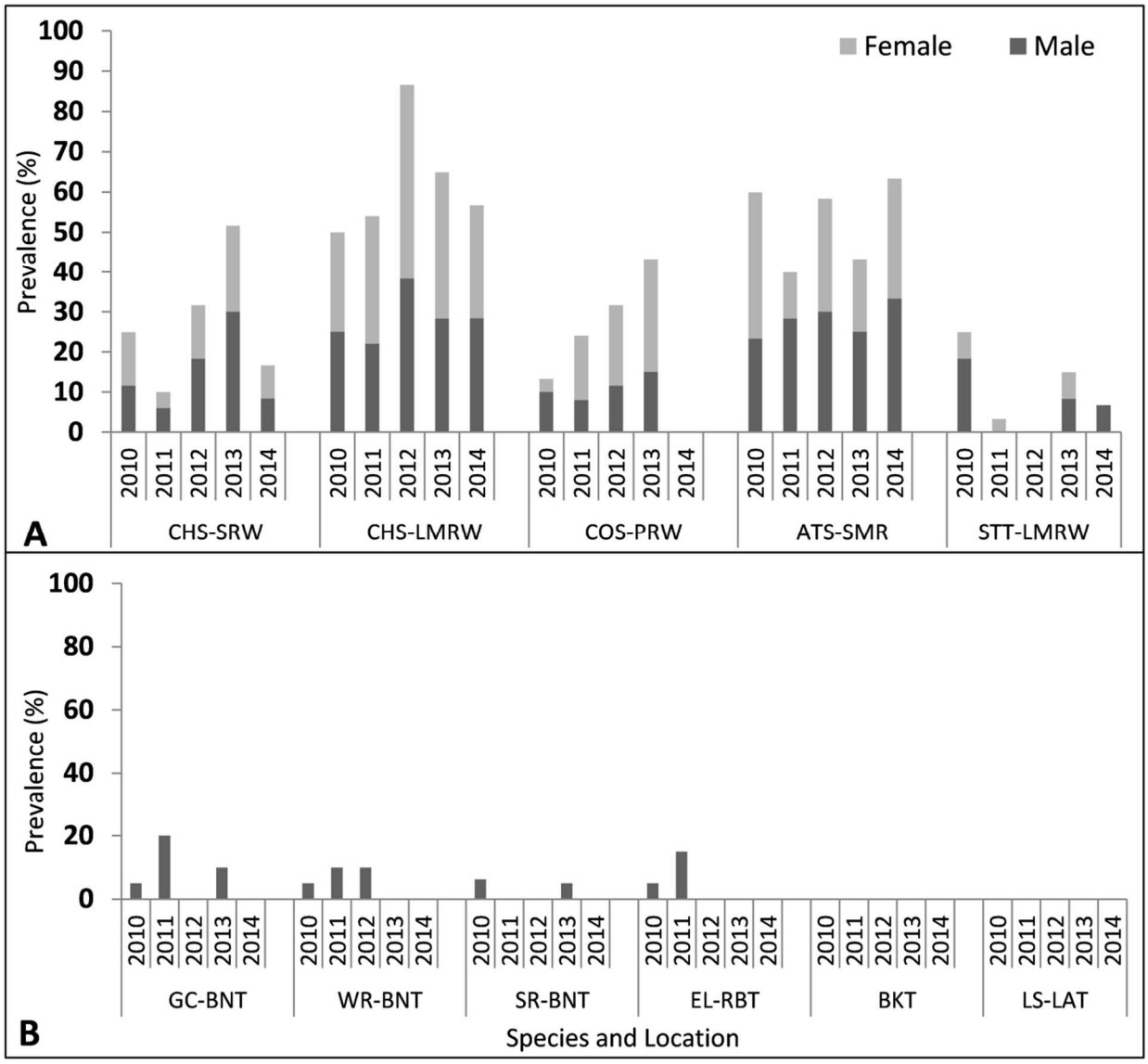

Figure 2.2 The prevalence (%) of *F. psychrophilum* infected salmonid broodstock fish as sampled throughout the course of the study. (A) Feral salmonid broodstocks sampled by year, species, and location. CHS, Chinook salmon; COS, Coho salmon; ATS, Atlantic salmon; STT, steelhead. SRW, Swan River weir; LMRW, Little Manistee River weir; PRW, Platte River weir; SMR, St. Mary's River. (B) Captive broodstocks sampled by year and species and strain. Oden State Fish Hatchery: GC-BNT, Gilchrist Creek strain brown trout; WR-BNT, Wild Rose strain brown trout; EL-RBT, Eagle Lake strain rainbow trout; SR-BNT, Sturgeon River strain brown trout. Marquette State Fish Hatchery: LS-LAT, Lake Superior strain lake trout; BKT, brook trout.

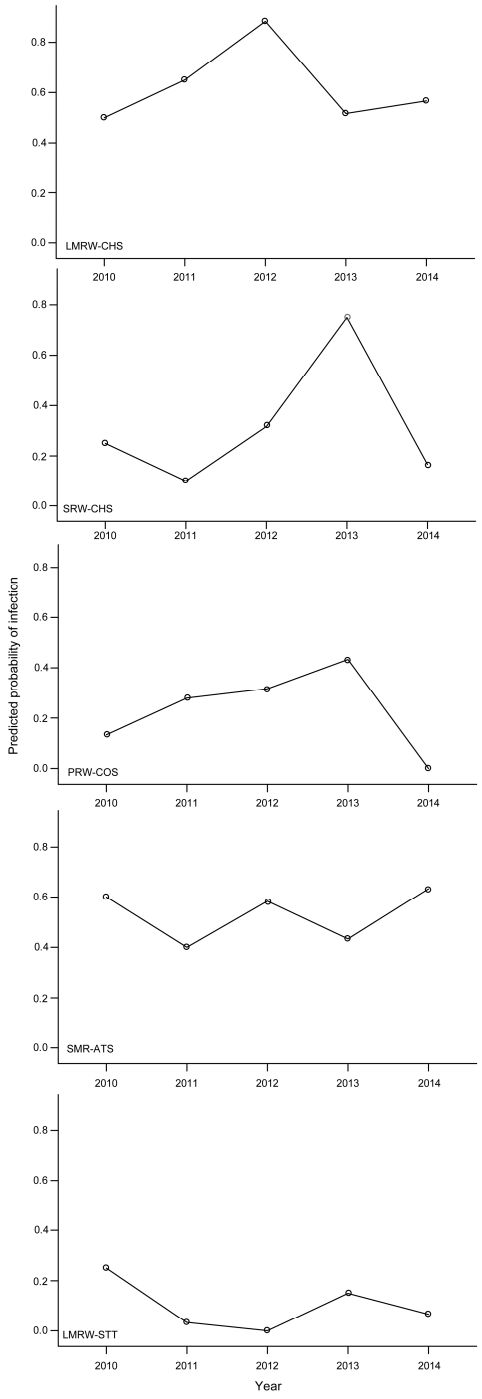

Figure 2.3 The predicted *F. psychrophilum* infection probability of feral salmonid broodstock populations throughout the course of the study, as determined by a least-squares means analysis. CHS, Chinook salmon; COS, Coho salmon; ATS, Atlantic salmon; STT, steelhead. SRW, Swan River weir; LMRW, Little Manistee River weir; PRW, Platte River weir; SMR, St. Mary's River.

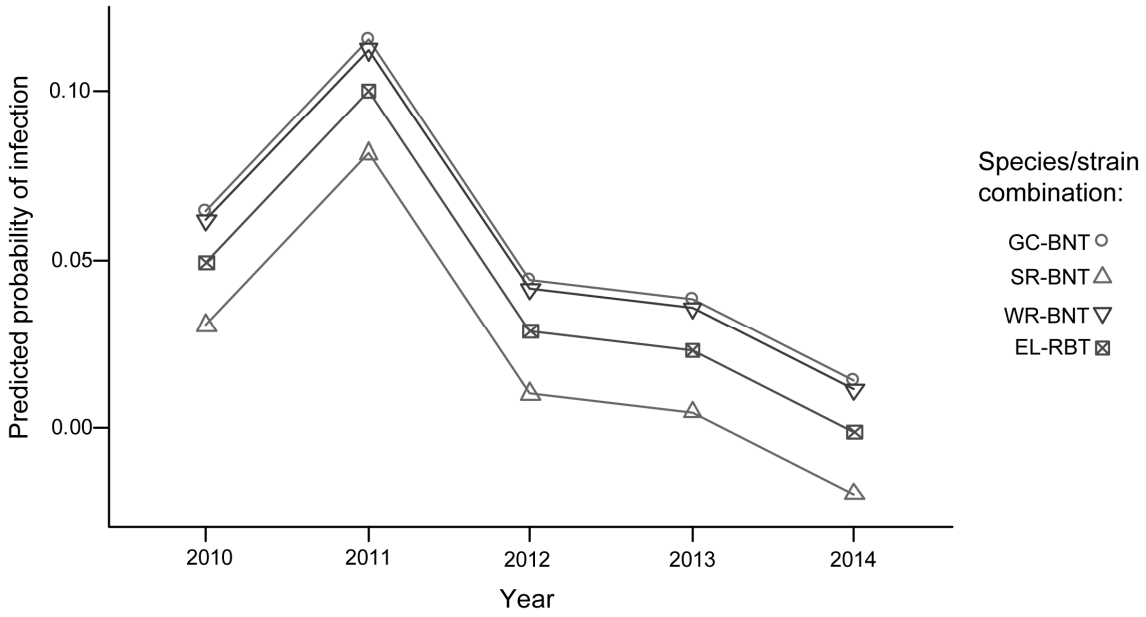

Figure 2.4 The predicted *F. psychrophilum* infection probability of captive salmonid broodstock populations throughout the course of the study, as determined by a least-squares means analysis. GC-BNT, Gilchrist Creek strain brown trout; WR-BNT, Wild Rose strain brown trout; EL-RBT, Eagle Lake strain rainbow trout; SR-BNT, Sturgeon River strain brown trout.

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Chapter 3

Sequence analysis of *gyrB, murG,* and *tuf* genes individually and combined reveals the genetic diversity and fish host species specificity of *Flavobacterium psychrophilum* in the Great Lakes basin

1. Abstract

Flavobacterium psychrophilum is a Gram-negative bacterial pathogen of salmonids worldwide. As the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), *F. psychrophilum* infections lead to substantial economic and ecologic losses. There are large knowledge gaps regarding the genetic diversity of *F. psychrophilum* worldwide, particularly as it pertains to fish host species specific and/or highly virulent strains, matters that may help our understanding of the epidemiology of this deadly pathogen. In this context, the objective of this study was to examine the genetic diversity of 50 *F. psychrophilum* strains from the Great Lakes basin (GLB) using three alternative loci: *gyrB, murG,* and *tuf*. Strains examined were recovered from three *Oncorhynchus* spp., namely *O. tshawytscha, O. mykiss,* and, *O. kisutch*, and were recovered from both apparently healthy fish and fish exhibiting clinical signs associated with BCWD. Individual neighbor-joining trees constructed on the *gyrB, murG,* and *tuf* gene nucleotide sequences varied in their discriminatory power to estimate the *F. psychrophilum* phylogeny. The tree based on the *gyrB* nucleotide sequences offered the most robust estimation when considering all genes individually, however all genes were able to group isolates based on the fish host species upon which they were recovered, as well as group the majority of epidemic isolates together. Upon concatenation of the gene sequences, similar topologies based on the concatenated sequences to the trees based on the individual genes were seen, however the robustness was greatly improved as evidenced by the higher bootstrap support when concatenation of all three genes occurred. Collectively, these results indicate the genetic diversity of GLB *F. psychrophilum* and identify sequence variations of clinical significance, in addition to highlighting the tendency of host specificity.

2. Introduction

Flavobacterium psychrophilum is a Gram-negative bacterial pathogen of salmonid species worldwide (Starliper 2011). This lethal pathogen causes bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), which frequently lead to large economic and ecologic losses in both captive and wild salmonid populations. Since the initial isolation of this bacterium over six decades ago (Borg 1948), its taxonomic status has undergone multiple changes. Originally the pathogen was described as *Cytophaga psychrophila* (Borg 1960), it was then reclassified as *Flexibacter psychrophilus* (Bernardet and Grimont 1989), and now it is accepted to belong to the genus *Flavobacterium* (Bernardet et al. 1996; Bernardet 2011). These reclassifications occurred largely in part due to advances in molecular techniques and gene sequencing (Bernardet 2011).

Over the last six decades, investigators have tried to better understand the intraspecific phylogeny of *F. psychrophilum* (Chakroun et al. 1997; Izumi et al. 2003; Izumi et al. 2007). Initially, this bacterium was thought to be genotypically and phenotypically homogenous (Lorenzen et al. 1997; Madetoja et al. 2001; Valdebenito and Avendaño-Herrera 2009), however, more recent analyses revealed clear diversity among strains collected from different geographical regions. For example, two distinct colony morphologies (i.e., rough and smooth) were identified with the rough morphotype exhibiting more cytotoxicity to rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages *in vitro* (Hogfors-Ronnholm and Wiklund 2012). In the same context, biochemical analysis revealed the presence of multiple biovars, whereby α-galactosidase, β-galactosidase, α -glucosidase, β-glucosidase, and *N*-acetyl-β-

glucosaminidase enzymatic activities varied among isolates from different geographic regions (Hesami et al. 2008). Likewise, based on serological studies, it was found that *F. psychrophilum* isolates span over 10 host-dependent serovars, however, difficulties were reported in reproducing these findings (Izumi and Wakabayashi 1999; Mata et al. 2002; Valdebenito and Avendaño-Herrera 2009).

Studies dealing with genetic diversity focused primarily on the 16S rRNA gene often followed by restriction fragment length polymorphism (RFLP) analysis, ribotyping, suppression subtractive hybridization, and microarrays. Although the results hinted to the presence of genetic heterogeneity among isolates, they were often inconclusive (Izumi et al. 2003; Hesami et al. 2008; Nicolas et al. 2008). For example, initially a strong association between ribotypes and fish host species was demonstrated from isolates recovered around the world (Chakroun et al. 1998), however a follow up study focusing on Danish isolates was unable to establish the same correlation (Madsen and Dalsgaard 2000). Indeed, it was concluded that data generated on 16S rRNA gene sequence should be taken with caution as it is now known that *F. psychrophilum* harbors 6 copies of this gene (Duchaud et al. 2007).

Recent *Flavobacterium* studies have identified the protein-encoding housekeeping gene *gyrB* as a useful molecular marker for epidemiological studies (Izumi et al. 2003; Arai et al. 2004; Izumi et al. 2007; Peeters and Willems 2011). Studies using this gene side by side with the 16S rRNA gene demonstrated that the *gyr*B has better discriminatory power in differentiating between closely related *Flavobacterium* spp. including *F. psychrophilum*. (Arai et al. 2004; Peeters and Willems 2011).

Similarly, although not currently as fully investigated as the *gyrB* gene, the protein-encoding housekeeping gene *murG* has been shown to also be highly polymorphic among *Flavobacterium* spp. and has been recommended as a good candidate for genetic analysis for *F. psychrophilum* (Nicolas et al. 2008). Similarly, this gene exhibited more discriminatory power when compared to the 16S rRNA among closely related *Flavobacterium* strains in Korea (Mun et al. 2013). In the same context, the protein-encoding housekeeping *tuf* gene has allowed for phylogenetic estimation of lactobacilli and streptococcal species and was shown to outperform the 16S rRNA gene (Chavagnat et al. 2002; Picard et al. 2004). When *F. branchiophilum* isolates from Ontario were compared using the *tuf* gene, certain *F. branchiophilum* strains were associated more frequently from one location or another (Skulska 2014).

Most recently, it has been demonstrated that *F. psychrophilum* infections are widespread and prevalent among both feral and captive salmonid stocks of the Great Lakes basin (GLB; Van Vliet et al. 2015). However, little is known regarding the intraspecific phylogeny of GLB *F. psychrophilum,* a matter that could be hindering the efforts to develop efficacious control measures, such as basing vaccination development on fish host species specific and/or highly virulent strains. A thorough understanding of the molecular epidemiology of *F. psychrophilum* will also help to unveil pathogen trafficking dynamics and if there is indeed a high risk of vertical transmission from infected broodstocks to progeny populations, as well as how the distribution of certain strains of this pathogen may exist in the GLB. To this end, the main objective of this study was to use three protein-encoding housekeeping genes, *gyrB, murG,* and *tuf* to infer the intraspecific phylogeny of *F. psychrophilum* to help identify trends associated with host species and/or increased virulence, as this epidemiological knowledge

directly relates to the need for developing better targeted control measures. With a focus on the GLB, this information will help identify the genetic diversity of this pathogen in a very unique aquatic environment with hopes of comparison to other geographical areas.

3. Materials and Methods

3.1. *F. psychrophilum* isolation

A total of 50 isolates originating from the Great Lakes basin region of the U.S.A. were analyzed in this study (Table 3.1). Initial isolation either occurred in the field or in our laboratory when live fish were sent for diagnostic necropsies. Some fish appeared apparently healthy, while others exhibited clinical disease signs commonly associated with BCWD (e.g., fin erosion, muscle ulceration, and/or exophthalmia). Isolates recovered during a BCWD outbreak (i.e., high morbidity and mortality typically exceeding 0.05% of the population in the raceway) are noted as epidemic isolates. Non-epidemic isolates were not associated with apparent disease. All tissues were collected aseptically and cultured immediately and directly onto cytophaga agar (CA; Anacker and Ordal 1959) supplemented with neomycin sulfate at 4 mg l^{-1}, and incubated at 15°C for 72-144 hours, as per the guidelines of the American Fisheries Society – Fish Health Section Blue Book (AFS-FHS 2012). Individual yellow-pigmented colonies were streaked for isolation onto fresh CA plates and incubated for 48-72 hours at 15°C for further characterization. Isolates were then cryopreserved in CA broth supplemented with glycerol (20% v/v) and then immediately frozen at -80°C for future analyses. Isolates were recovered from both feral (n = 36) and captive (n = 14) salmonid populations from six Great Lakes basin locations (Fig. 3.1). Due to their propensity to become infected with *F. psychrophilum* in the

GLB (Van Vliet et al. 2015), three fish host species were targeted for *F. psychrophilum* isolation, Chinook salmon (*Oncorhynchus tshawytscha*; n = 24), steelhead (*O. mykiss*; n = 18), and Coho salmon (*O. kisutch*; n = 8). All isolates were recovered during the time period of 2008-2013, and were collected from multiple tissues including kidneys (n = 41), gametes/eggs (n = 4), brains (n = 3), and external lesions (n = 2).

3.2. DNA extraction and *F. psychrophilum* confirmation

Each suspected *F. psychrophilum* isolate underwent DNA extraction using the DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, California), according to the manufacturers protocol for Gram-negative bacteria. For the majority of isolates, *F. psychrophilum* confirmation was achieved by using *F.* psychrophilum-specific primers (Toyama et al. 1994) and PCR assay to amplify a partial stretch of the 16S rRNA gene. All reactions had 10 µL of 2x Go-Taq Green master mix (Promega, Madison, WI), 10 ng of DNA template, 5 ng of each primer, and nuclease-free water for a total reaction volume of 20 µL for each sample. The PCR parameters were: initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec. A final elongation step was performed at 72°C for 7 min. Sterile nuclease free water served as a negative control in all assays, and *F. psychrophilum* ATCC 49418^T was used as a positive control. Amplicons were then loaded in a 1.5% agarose gel and electrophoresed for 45 min at 100 V, upon which visualization under UV exposure with an amplicon present at approximately 1100 bp was considered confirmatory for *F. psychrophilum* (Toyama et al. 1994). The remaining isolates were confirmed as *F. psychrophilum* by Sanger sequencing. Briefly, a partial 16S rRNA gene stretch was amplified using the universal

degenerate primers 27F (5'-AGAGTTTGATCMTGGCTGAG-3') and 1387R (5'-GGGCGCWGTGTACAAGGC-3'; Marchesi et al. 1998). Amplification was performed with the following PCR parameters: initial denaturation at 95°C for 5 min, followed by 32 cycles of 30 s at 95°C, 30 s at 58°C and 60 s at 72°C. A final extension step at 72°C for 7 min was performed. All amplicons were purified using the QIAquick Purification Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol, and sequenced in-house.

3.3. Housekeeping genes: PCR amplification and gene sequencing

PCR amplification of partial stretches of 3 housekeeping genes, which have been selected on the basis of their presence as a single-copy, protein-encoding, housekeeping gene was conducted using the primers listed in Table 3.2 (*gyrB, murG,* and *tuf*; Fujiwara-Nagata et al. 2013). Each 50 µL reaction included 25 µL of 2x Go-Taq Green master mix (Promega, Madison, Wisconsin), 20 ng of DNA template, 0.25 µM of each primer, and nuclease-free water comprising the remainder. Sterile nuclease free water served as a negative control in all assays, and *F. psychrophilum* ATCC 49418^T was used as a positive control. All genes were amplified using the same touchdown protocol: 94°C for 5 min; 24 cycles at 94°C for 0.5 min, 55°C for 0.5 min (-0.4°C/cycle), and 72°C for 1 min (+2 sec/cycle); 12 cycles at 94°C for 0.5 min, 45°C for 0.5 min, and 72°C for 2 min (+3 sec/cycle); and a final extension step at 72°C for 10 min (Fujiwara-Nagata et al. 2013). Reactions were loaded in a 1.5% agarose gel and electrophoresed at 100 V for 40 min, where the presence of an amplicon of the appropriate size under UV exposure confirmed amplification (Table 3.2). Purification of amplified PCR product was performed using the QIAquick Purification Kit (Qiagen, Inc., Valencia, California) according to the manufacturer's

protocol. Bidirectional sequencing was performed using the corresponding forward and reverse primers.

3.4. Data analysis

For phylogenetic analysis, 7 different alignments were created, 3 corresponding to the alignment of sequences of each gene individually: *gyrB, murG,* and *tuf*, and 3 alignments of two-gene concatenations (i.e., *gyrB* and *murG, gyrB* and *tuf, murG* and *tuf*), and a last alignment of all 3 genes concatenated (*gyrB, murG,* and *tuf*). All sequences were manually verified for confirmation and quality trimmed. All alignments were made using the CLUSTAL W program (Thompson et al. 1997).

Phylogenetic analysis included neighbor-joining trees of DNA and translated amino acid sequences and were conducted using MEGA 5.2. Bootstrap analysis was made with 1000 replicates for all trees. Model testing was conducted in MEGA 5.2. to determine the most appropriate evolutionary model to use. The best model for the majority of datasets in this study was the Tamura three-parameter evolutionary model (Tamura 1992) with a gamma distribution, and this model was used unless otherwise noted. Positions used to denote mutations are based on sequences retrieved from GenBank. The sequenced used for *gyrB* was EU428468.1, and sequence used for *murG* was EU428518.1.

4. Results

4.1. Single gene analyses

4.1.1. *gyrB*

Approximately 1,077 bp-long DNA fragments of the *gyrB* gene were successfully amplified by PCR from all the tested isolates and used to construct a neighbor-joining tree that contained two main clusters (Fig. 3.2). Cluster I was the largest cluster containing 38 isolates, with sequence identity ranging from 99.16-100%. Isolates within cluster I have originated from all sampled locations (i.e., LMRW, SRW, PRW, WLSFH, TSFH, and OSFH), as well as from all three host species (Fig. 3.2). Additionally, both epidemic and non-epidemic isolates were present within this large cluster, however all epidemic isolates grouped together in a sub-cluster (Fig. 3.2). Furthermore, isolate US09 was recovered from an external lesion of a captive rainbow trout, but was not associated with mortality or morbidity, and grouped closely to the epidemic isolate sub-cluster (Fig. 3.2). Cluster I contained both single-species and multiple-species sub-clusters (Fig. 3.2). For example, isolates US04, US25, US41, and US55 were all recovered from Chinook salmon, in addition to branching off another sub-cluster containing only Chinook salmon isolates as well (i.e., US06, US12, US34, US36, US37, and US43; Fig. 3.2). In contrast, isolates US33 and US50 were recovered from Chinook salmon, and grouped with isolate US35 which was recovered from a Coho salmon (Fig. 3.2).

Cluster II was smaller than cluster I, containing only 12 isolates (Fig. 3.2), with sequence identity ranging from 99.53-100%. Only isolates from the Lake Michigan watershed (i.e., locations: LMRW, PRW, and WLSFH) were placed in this cluster. Isolates from all three fish host species and both epidemic and non-epidemic isolates were also within cluster II (Fig. 3.2).

Unlike cluster I, the two epidemic isolates in cluster II were not grouped together within a sub-cluster (Fig. 3.2). All sub-clusters were formed with isolates from the same host species.

The overall sequence similarity ranged from 98.61-100% identity to each other. Isolate US06 recovered from Chinook salmon was the most dissimilar sequence compared to US20, US27, US48, and US52, all of which were recovered from Coho salmon (Table 3.1). Although the neighbor-joining tree based on amino acid sequences provided much less resolution (Fig. 3.3) than the tree based on nucleotide sequences (Fig. 3.2), there was observable amino acid variation. The amino acid sequence similarity of all *F. psychrophilum* isolates in this study ranged from 99.72-100%. Isolate US35 from Coho salmon exhibited the lowest amino acid similarity among all isolates within the data set, and had a single observable amino acid mutation from alanine to valine at position 929 (Fig. 3.3; Table 3.3).

4.1.2. *murG*

Approximately 681 bp-long DNA fragments were successfully amplified via PCR from all tested isolates, and used to construct a neighbor-joining tree (Fig. 3.4). This tree contained two main clusters, with cluster I as the largest containing 42 isolates ranging in sequence identity from 98.97-100% (Fig. 3.4). All 3 sampled host species were represented within cluster I, whereby both single-species and mixed-species sub-clusters were present (Fig. 3.4). A large sub-cluster containing isolates (e.g., US04, US06, US12, US21, US23, US25, US36, US37, US41, US43, and US55) from Chinook salmon was formed, however, another sub-cluster contained both Chinook salmon (e.g., US14, US30, US46, and US47) and rainbow trout (e.g., US18 and US39) isolates (Fig. 3.4). Interestingly, in both the analysis of the *murG* and *gyrB* nucleotide

sequences, Coho salmon isolate US35 was grouped with Chinook salmon isolates. Similarly, rainbow trout isolates US18 and US46 were also consistently grouped with Chinook salmon isolates in both analyses as well. Both epidemic and non-epidemic isolates were present within cluster I, although 11/12 epidemic isolates in cluster I formed an epidemic-only sub-cluster (Fig. 3.4). The remaining epidemic isolate did not group with any other sequences. The same epidemic-only sub-cluster consisting of 11/13 epidemic isolates was present in both the *murG* and *gyrB* nucleotide sequence analyses.

Cluster II was smaller than cluster I, containing only 8 isolates ranging in sequence identity from 99.12-100%. All 3 sampled host species were represented within cluster II, whereby sequences from the same host species most frequently grouped together, however one sub-cluster contained sequences from both rainbow trout (e.g., US28 and US29) and Chinook salmon (e.g., US13 and US31; Fig. 3.4). Cluster II contained both epidemic and non-epidemic isolates, however the single epidemic isolate in cluster II grouped with non-epidemic isolate US09, both of which were recovered from rainbow trout (Fig. 3.4).The overall nucleotide sequence similarity ranged from 98.67-100% identity among all examined Great Lakes basin isolates.

This was the only gene examined that produced significant variation among amino acid sequences. The amino acid sequences ranged from 97.79-100% identity among each other, and there were 57 observable amino acid mutations at 7 sites (Table 3.3). The neighbor-joining tree constructed using the amino acid sequences based on the JTT matrix-based method (Jones et al. 1992) provided less resolution as evidenced by the lower bootstrap values than that based on nucleotide sequences (Fig. 3.5). For example, in the tree constructed on *murG* nucleotide

sequences, a sub-cluster containing 4 Coho salmon isolates were supported with a bootstrap value of 78, whereby this same cluster was formed in the tree based on amino acid sequences, yet the bootstrap value was <50. Nevertheless, trends can still be observed in the tree based on amino acids, and importantly 11/13 epidemic isolates formed the same sub-cluster in the tree based on amino acids as in the tree based on nucleotide sequences (Fig. 3.5). All but one epidemic isolate (US16) exhibited an amino acid mutation at position 662 (based on the *murG* sequence of EU428518.1) from histidine to proline, and 8/12 of them exhibited a mutation at position 688 from valine to isoleucine as well (Table 3.3).

4.1.3. *tuf*

Successful amplification by PCR from all test isolates yielded 795 bp-long quality trimmed DNA fragments of the *tuf* gene and were used to construct a neighbor-joining tree (Fig. 3.6). This tree contained two main clusters with cluster I as the largest (42 isolates; Fig. 3.6). The sequence identity ranged from 98.99-100% among the isolates in cluster I. Sequences in cluster I were recovered from all sampled locations, as well as from all sampled host species (Fig. 3.6). However, both single-location and mixed-location sub-clusters, as well as single-species and mixed-species sub-clusters were present (Fig. 3.6). For example, a sub-cluster containing sequences from Chinook salmon (e.g., US14, US30, US46, and US47), Coho salmon (e.g., US08), and rainbow trout (e.g., US18, and US39) was present in cluster I (Fig. 3.6). Interestingly, rainbow trout isolates US18 and US39 have now been grouped with Chinook salmon isolates in all three individual gene analyses. Conversely, a large sub-cluster containing sequences from only rainbow trout (e.g., US17, US26, US32, US38, US40, US42, US44, US45,

US49, US51, and US53) was also present (Fig. 3.6). Epidemic and non-epidemic isolates were present in cluster I, however the majority of epidemic isolates (i.e., 11/13) grouped together to form a sub-cluster, the remaining epidemic isolates did not cluster together (Fig. 3.6).

Cluster II contained 8 isolates ranged in sequence identity from 99.37-100%. Only sequences from LMRW and PRW were present in cluster II, whereby each sub-cluster contained only sequences from a single location (Fig. 3.6). Similarly, each sub-cluster contained only sequences from a single species, whereby only rainbow trout and Coho salmon isolates were present within cluster II (Fig. 3.6). No epidemic isolates were present in cluster II (Fig. 3.6). Sequence similarity ranged from 98.62-100% among all isolates in the study. The neighbor-joining tree based on amino acid sequences provided no phylogenetic resolution (Fig. 3.7).

4.2. Concatenated sequence analyses

4.2.1. *gyrB* and *murG*

Concatenation of the *gyrB* and *murG* nucleotide sequences yielded 1,758 bp-long sequence fragments that were used to construct a neighbor-joining tree (Fig. 3.8). Two main clusters were present, whereby cluster I contained 37 isolates compared to cluster II containing 13 isolates (Fig. 3.8). Cluster I contained both epidemic and non-epidemic isolates, however all epidemic isolates in cluster I grouped together in an epidemic isolate-only sub-cluster (Fig. 3.8). Single-species sub-clusters consisted of primarily rainbow trout or Chinook salmon isolates (Fig. 3.8), however, few multiple-species groups were also formed (Fig. 3.8). For example, isolate US35 recovered from Coho salmon grouped together with isolates US33 and US50 from Chinook salmon (Fig. 3.8). Similarly, cluster II contained both epidemic and non-epidemic

isolates; however the two epidemic isolates did not group closely together (Fig. 3.8). A strongly supported sub-cluster containing isolates from Coho salmon was formed, although other multiple-species groups were also present (Fig. 3.8).

4.2.2. *gyrB* and *tuf*

Concatenation of the *gyrB* and *tuf* nucleotide sequences produced a sequence fragment 1,872 bp in length. The neighbor-joining tree constructed on the concatenated sequences based on the Tamura-Nei evolutionary model (Tamura and Nei 1993) with a gamma distribution contained two main clusters, both of which had isolates from all three fish host species as well as both epidemic and non-epidemic isolates (Fig. 3.9). Within cluster I, all epidemic isolates grouped together in a strongly supported sub-cluster, whereas the two epidemic isolates within cluster II did not group together (Fig. 3.9). Cluster I contained sub-clusters primarily formed of isolates from a single host species, for example the majority of Chinook salmon isolates grouped together with strongly supported bootstrap values. In cluster II, Coho salmon isolates grouped together with relatively strong values (Fig. 3.9).

4.2.3. *murG* and *tuf*

Using the concatenated *murG* and *tuf* nucleotide sequences (1,476 bp in length), the neighbor-joining tree revealed two main clusters (Fig. 3.10). Cluster I contained 44 isolates recovered from all three fish host species and epidemic and non-epidemic isolates (Fig. 3.10). Highly supported sub-clusters consisting of isolates recovered from a single host species were present (Fig. 3.10). However, few isolates were unable to be placed into single host species

clusters and formed mixed-species sub-clusters (Fig. 3.10). All epidemic isolates were placed in cluster I, however only 11/13 grouped together in a single highly supported sub-cluster (Fig. 3.10). Cluster II contained 6 non-epidemic isolates recovered only from Chinook salmon and Coho salmon. Isolates recovered from the same species grouped together in highly supported sub-clusters (Fig. 3.10).

4.2.4. *gyrB, murG,* and *tuf*

The concatenation of the *gyrB, murG,* and *tuf* nucleotide sequences produced a sequence fragment 2,553 bp in length. The neighbor-joining tree using the concatenated sequences based on the Tamura-Nei evolutionary model (Tamura and Nei 1993) with a gamma distribution yielded two main clusters (Fig. 3.11). Both clusters consisted of isolates recovered from all three fish host species as well as both epidemic and non-epidemic isolates (Fig. 3.11). Cluster I primarily contained sub-clusters consisting of single species isolates, whereby rainbow trout and Chinook salmon isolates most frequently grouped with isolates of the same fish host species. All epidemic isolates in cluster I grouped together in a single sub-cluster (Fig. 3.11). All isolates in cluster II grouped with isolates recovered from the same fish host species with strongly supported bootstrap values (Fig. 3.11). Two epidemic isolates were present within cluster II, however they did not form a sub-cluster together (Fig. 3.11). The concatenation of all three genes provided the most robust phylogenetic tree and was able to group the majority of epidemic isolates together, as well as frequently grouping isolates recovered from the same fish host species (Fig. 3.11).

5. Discussion

The main objective of this study was to reveal the genetic diversity of *F. psychrophilum* strains in the GLB with a goal of uncovering trends between *F. psychrophilum* strains and fish host species and/or association with disease. By identifying strains of clinical significance, the understanding of the epidemiology of this pathogen as it devastates GLB salmonid populations will be clearer. Since the phylogenetic positioning of *F. psychrophilum* isolates is not clearly distinguishable based on their 16S rRNA gene sequences (Izumi et al. 2003; Soule et al. 2005; Hesami et al. 2008; Nicolas et al. 2008), we opted to use alternative loci to better understand the genetic relatedness of *F. psychrophilum* isolates from the GLB.

It is recommended that protein-encoding housekeeping genes be targeted when investigating phylogenetic positioning within a species and between closely related species, as these genes are usually ubiquitous within the population and evolve at a moderate rate (Cooper and Feil 2004; Peeters and Willems 2011). Few loci in this respect have been identified as alternatives for when the 16S rRNA gene does not allow for discrimination between closely related species. In this context, we have demonstrated the usefulness of the *gyrB, murG,* and *tuf* genes individually in terms of phylogenetic positioning of GLB *F. psychrophilum* isolates. The three genes selected are commonly used in the identification of bacteria, including *Flavobacterium* spp. (Nicolas et al. 2008; Peeters and Willems 2011; Skulska 2014). Each gene has only one copy on the *F. psychrophilum* genome, performs a different essential function, and evolves at a moderate rate (Duchaud et al. 2007). The use of *gyrB* seems to generate the most robust phylogenetic tree of the three genes examined, although all genes were able to separate the majority of isolates into groups based on the fish host species they were recovered from

with moderate support. There were, however, a few isolates that could not be placed within species-specific groups. These cases may reflect the broad host range of the isolates themselves rather than on the gene or method used to estimate the phylogeny, as these isolates continuously remain grouped when examining all three genes individually. In the same regard, there has been indication that in another fish pathogenic flavobacterium, *F. columnare*, certain genotypes exhibit strong fish host specificity, while a few others span multiple fish host species (Olivares-Fuster et al. 2007).

I report similar findings using these genes in regards to isolates associated with BCWD outbreaks, which may represent particularly virulent isolates. All genes were able to group 11/13 epidemic isolates together with strong support. It could be argued that the two epidemic isolates in our GLB study that did not group with other epidemic isolates could possibly be less virulent strains and were able to colonize the fish due to increased stress levels of fish in captivity. Conversely, and more likely in this case considering the overt BCWD present at time of isolation, these two isolates could represent other highly virulent genotypes and indicate that multiple sequence variations could be the source of disease outbreaks in Michigan aquaculture facilities.

Considering the aim of this study was to clarify the intraspecific phylogeny of *F. psychrophilum* using different genes, the decision was made to make a joint analysis based on the concatenation of the sequences to improve phylogenetic tree robustness. The congruence between phylogenies based on individual genes of this study indicated that the phylogenetic signals would not be counteracted when the gene sequences were concatenated. Indeed, the phylogenies constructed on the concatenated sequences of this study have improved

robustness over the individual gene trees. The tree based on all three concatenated genes provides the most robust estimation of GLB *F. psychrophilum* isolates. However, the tree based on the concatenated *gyrB* and *tuf* genes also provided a good level of detail with clear delineation and support for groups of epidemic isolates as well as isolates from the same fish host species. The improvement through concatenation could be explained through the use of multiple genes that evolve at different rates and be informative at different levels (Vitorino et al. 2007). For example, the use of the 16S rRNA gene was able to discriminate between the main groups of enterococci but failed in regards to closely related species, in comparison, the *atpA* sequences were able to clearly differentiate all *Enterococcus* spp. examined (Naser et al. 2005). Moreover, it is suggested that the use of several genes dispersed throughout the bacterial genome increases the discriminatory power and robustness of relationships inferred (Stackebrandt et al. 2002), which was seen through results of this study. The use of multiple genes distributed throughout the genome can reduce the effects of location-dependent sequence evolution processes (Cummings et al. 1995), which may have also contributed to the improvement upon the phylogenetic analyses of the initial use of the genes individually.

Although the individual nucleotide sequences of all three genes offered a moderate estimation based on their bootstrap support values of the phylogenetic diversity of GLB *F. psychrophilum*, the amino acid sequences deduced from the *gyrB* and *tuf* nucleotide sequences provided little or no resolution in this regard. This is not entirely surprising considering that nucleotide substitution resulting in amino acid divergence is less frequent than synonymous substitution. However, when considering the phylogeny constructed from the *murG* amino acid sequences, the results of this study indicate a moderate rate of nonsynonymous substitution

within this gene in *F. psychrophilum* isolates from the GLB. Although the tree constructed on the *murG* amino acid sequences is not as robust as that based on nucleotide sequences, a similar topology was formed, whereby clear separation of certain isolates by the fish host species they were recovered from and grouping of epidemic isolates was demonstrated. These results suggest that the rate of nonsynonymous amino acid substitution within the *murG* gene of *F. psychrophilum* is frequent enough to generate a fair estimation of the phylogeny of this species. Each protein can be expected to evolve at its own rate, which influences the rate of amino acid divergence.

When further examining the amino acid divergence among *murG* sequence fragments, it was clear that certain sites were more likely to have a nonsynonymous mutation than others, and that multiple isolates exhibited these mutations at each position. The most common mutation from histine to proline occurred in 20 isolates, whereby no correlation between this mutation and association with disease was found. On the contrary, the second most common mutation from valine to isoleucine occurred in 14 isolates with 9 of those isolates associated with BCWD and high virulence. Although more investigations are needed to fully understand the significance of this correlation, it may hint at a functional mutation among particularly virulent strains of *F. psychrophilum* and could contribute to the efforts of developing better targeted control measures, such treatments or vaccines that target or disrupt the functionality of the potential virulence marker.

We demonstrate herein the ability of the *gyrB, murG,* and *tuf* genes to estimate the intraspecific phylogeny of GLB *F. psychrophilum* and the improvements to these estimates by the concatenation of the genes. In this context, we have recognized the existence of fish host

species-specific *F. psychrophilum* strains in the case of rainbow trout, Chinook salmon, and Coho salmon. Moreover, we have also identified that some strains are less host specific and were found genetically identical to isolates from multiple salmonid species. We have additionally identified strains of clinical significance as genetically identical. As there remains no efficacious approved vaccine for the control of BCWD in North America, elucidation of important epidemiological knowledge is of utmost importance (Gomez et al. 2014). The little information known about *F. psychrophilum* strains regarding their fish host specificity, virulence level, and the mechanisms responsible for these is hypothesized as a primary reason vaccine development against this pathogen has been so difficult (Gomez et al. 2014). Although whole genome and metagenomic approaches are beneficial and have more recently been used in identifying many genes involved in the lifestyle and virulence of *F. psychrophilum,* these methods are often unattainable to many investigators at this current time considering the cost and time. Therefore, the methods described herein present a fast, accurate, and cost and time efficient way of evaluating key intraspecific characteristics of *F. psychrophilum*.

APPENDIX

Table 3.1 Great Lakes basin *Flavobacterium psychrophilum* isolates compared in this study. Isolates with the same superscript letter in the Isolate ID column were recovered from the same fish. All isolates were recovered from locations within Michigan. SRW, Swan River Weir; LMRW, Little Manistee River Weir; PRW, Platte River Weir; OSFH, Oden State Fish Hatchery; WLSFH, Wolf Lake State Fish Hatchery; TSFH, Thompson State Fish Hatchery.

Isolate ID[1]	Location[2]	Watershed	Salmonid Species	Feral/captive	Epidemic/non-epidemic
US04	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US05	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US06	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US07	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US08	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US09	OSFH	Lake Michigan	*O. mykiss*	captive	non-epidemic
US12	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US13	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US14	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US16	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US17	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US18	LMRW	Lake Michigan	*O. mykiss*	feral	non-epidemic
US19	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US20	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US21	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US22	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US23	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US24	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US25	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US26	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US27	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US28	LMRW	Lake Michigan	*O. mykiss*	feral	non-epidemic
US29	LMRW	Lake Michigan	*O. mykiss*	feral	non-epidemic
US30	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US31	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic

Table 3.1 (cont'd)

Isolate ID[1]	Location[2]	Watershed	Salmonid Species	Feral/captive	Epidemic/non-epidemic
US32	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US33	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US34	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US35	PRW	Lake Huron	*O. kisutch*	feral	non-epidemic
US36	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US37	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US38	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US39	LMRW	Lake Michigan	*O. mykiss*	feral	non-epidemic
US40	TSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US41	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US42[a]	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US43	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US44	TSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US45	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US46	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US47	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US48	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US49	TSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US50	LMRW	Lake Michigan	*O. tshawytscha*	feral	non-epidemic
US51	TSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US52	PRW	Lake Michigan	*O. kisutch*	feral	non-epidemic
US53[a]	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US54	WLSFH	Lake Michigan	*O. mykiss*	captive	epidemic
US55	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic
US56	SRW	Lake Huron	*O. tshawytscha*	feral	non-epidemic

Table 3.2 *Flavobacterium psychrophilum*-specific primer sequences used to amplify 3 housekeeping genes and the expected amplicon length for each loci.

	Primer sequence (5'-3')		Expected amplicon length (bp)
DNA gyrase, β subunit (*gyrB*)	Forward	CAGGAAACAGCTATGACCGTTGTAATGACTAAAATTGGTG	1077
	Reverse	TGTAAAACGACGGCCAGTCAATATCGGCATCACACAT	
Glycosyltransferase murein G (*murG*)	Forward	CAGGAAACAGCTATGACCTGGCGGTACAGGAGGACATAT	681
	Reverse	TGTAAAACGACGGCCAGTGCATTCTTGGTTTGATGGTCTTC	
Elongation factor Tu (*tuf*)	Forward	CAGGAAACAGCTATGACCGAAGAAAAAGAAAGAGGTATTAC	795
	Reverse	TGTAAAACGACGGCCAGTCACCTTCACGGATAGCGAA	

Table 3.3 Nucleotide divergences and the supposed amino acid changes found in the genes of *Flavobacterium psychrophilum* isolates from the Great Lakes basin. Nucleotide positions are according to those of ATCC 49418^T (*gyrB* EU428468.1; *murG* EU428518.1) Supposed amino acid enclosed in parenthesis. *indicates epidemic isolate.

Gene	Isolate(s)	Nucleotide (amino acid) divergence			
		Position		Divergence	
gyrB	US35	C 929	(Ala)	T	(Val)
	US33, US35, US50	C 308	(Ala)	A	(Glu)
	US22, US54, US56	G 511	(Asp)	A	(Asn)
	US05, US08, US13, US17*, US24, US26*, US28, US29, US31, US32*, US34, US38*, US40*, US42*, US44*, US45*, US49*, US51*, US53*, US54*	A 662	(His)	C	(Pro)
murG	US13, US24, US28, US29, US31, US34	G 671	(Gly)	A	(Asp)
	US09, US13, US16*, US24, US28, US29, US31, US34	G 676	(Glu)	A	(Lys)
	US17, US20, US26*, US27, US32*, US38*, US40*, US42*, US44*, US45*, US48, US49*, US52, US53*	G 688	(Val)	A	(Ile)
	US33, US35, US50	G 727	(Ala)	A	(Thr)

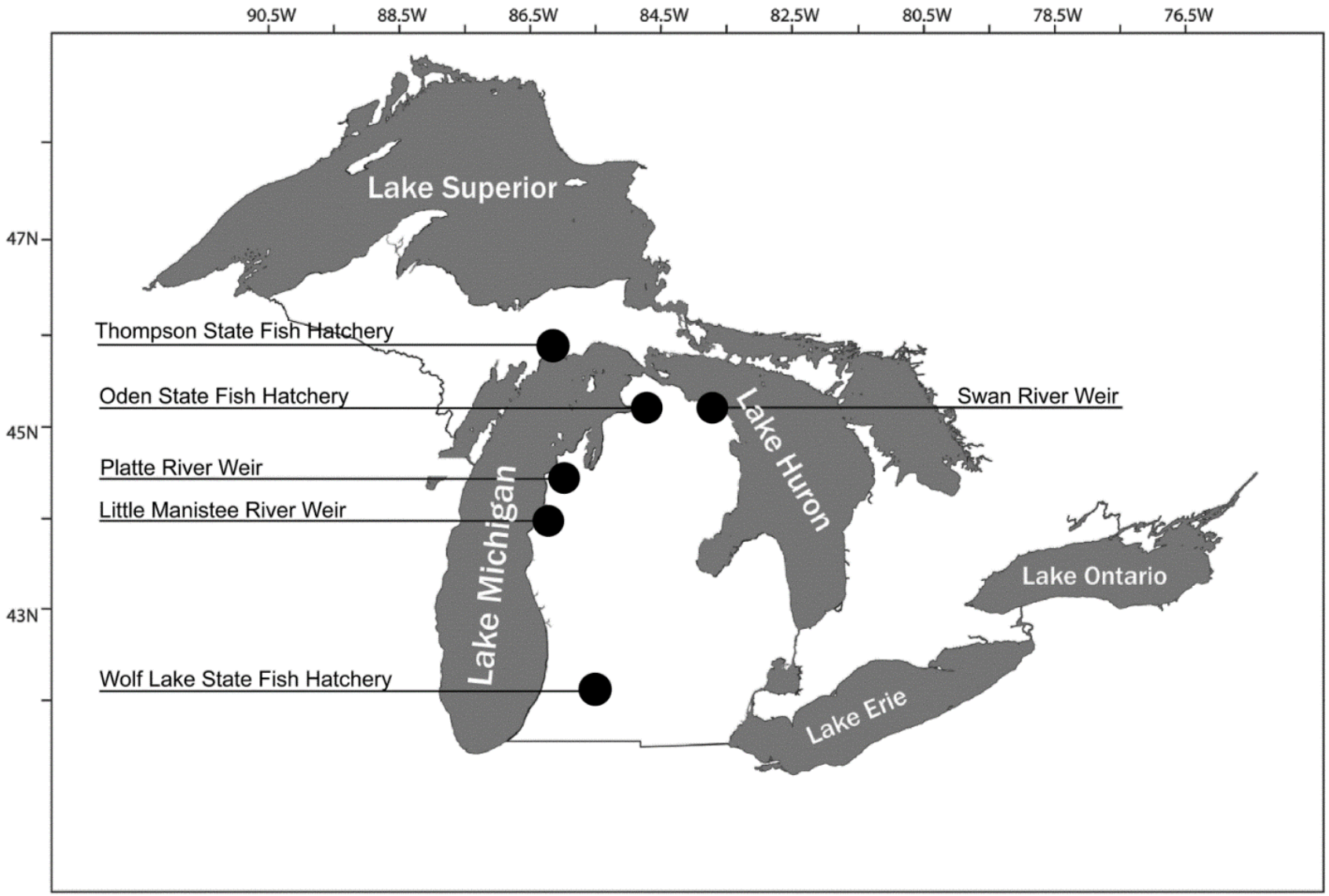

Figure 3.1 Map of locations within the state of Michigan where *Flavobacterium psychrophilum* isolates were recovered.

Figure 3.2 Neighbor-joining tree obtained from *Flavobacterium psychrophilum gyrB* nucleotide sequences 1,077 bp in length based on Tamura three-parameter model distances. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

Figure 3.3 Neighbor-joining tree obtained from *Flavobacterium psychrophilum gyrB* amino acid sequences based on the JTT matrix method. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

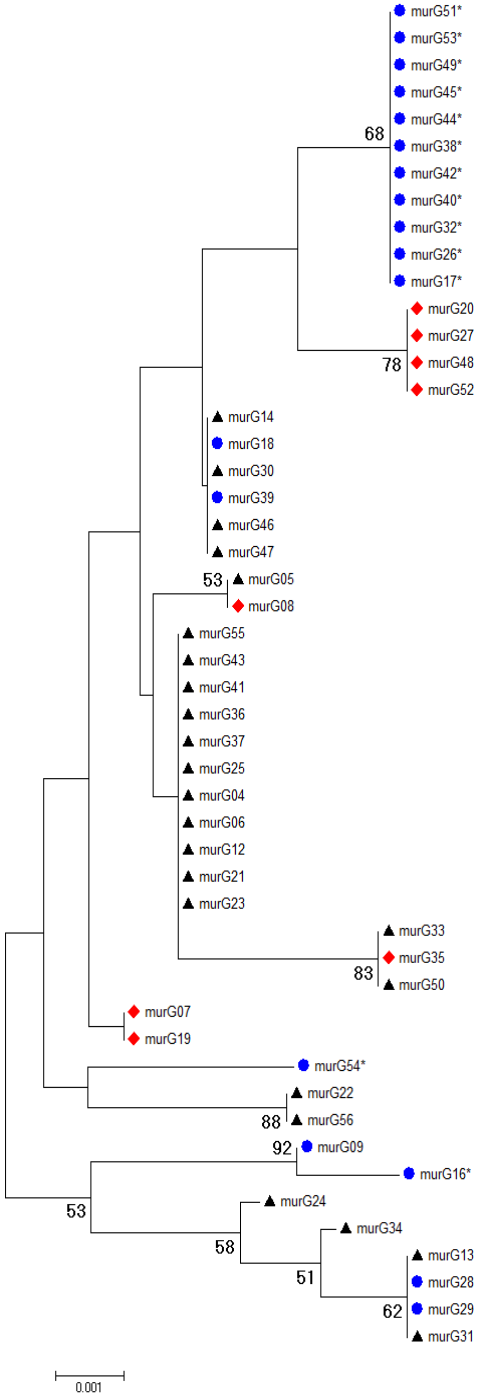

Figure 3.4 Neighbor-joining tree obtained from *Flavobacterium psychrophilum murG* nucleotide sequences 681 bp in length based on Tamura three-parameter model distances. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

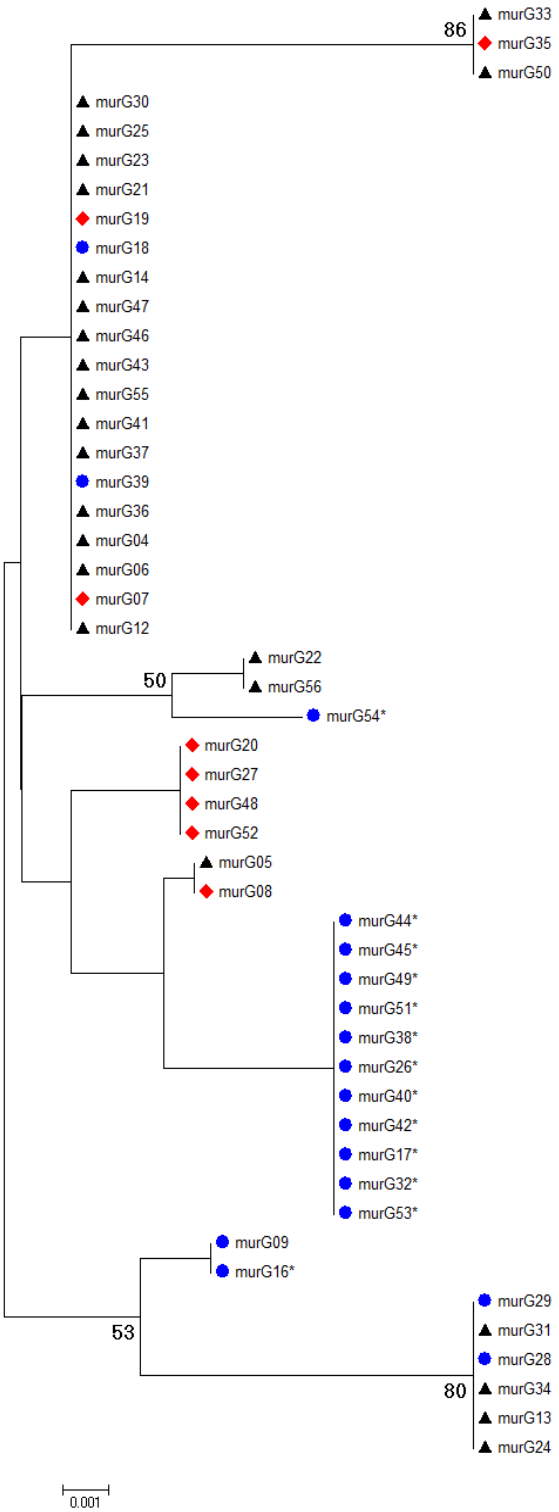

Figure 3.5 Neighbor-joining tree obtained from *Flavobacterium psychrophilum murG* amino acid sequences based on the JTT matrix method. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

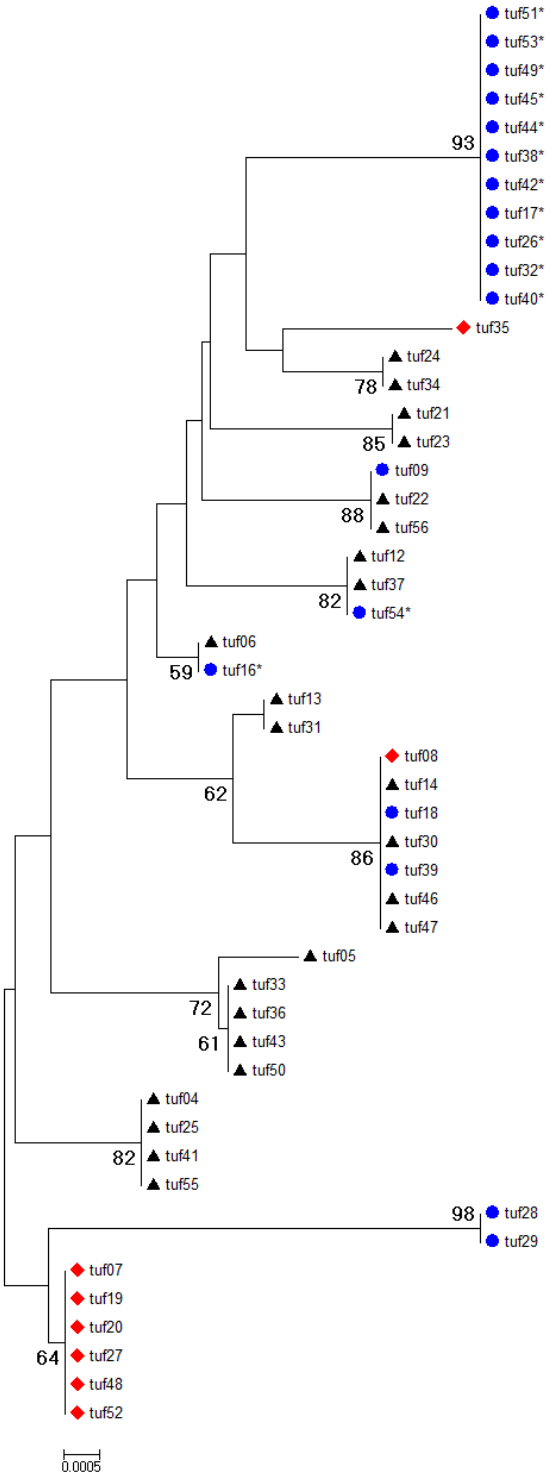

Figure 3.6 Neighbor-joining tree obtained from *Flavobacterium psychrophilum tuf* nucleotide sequences 795 bp in length based on Tamura-Nei model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

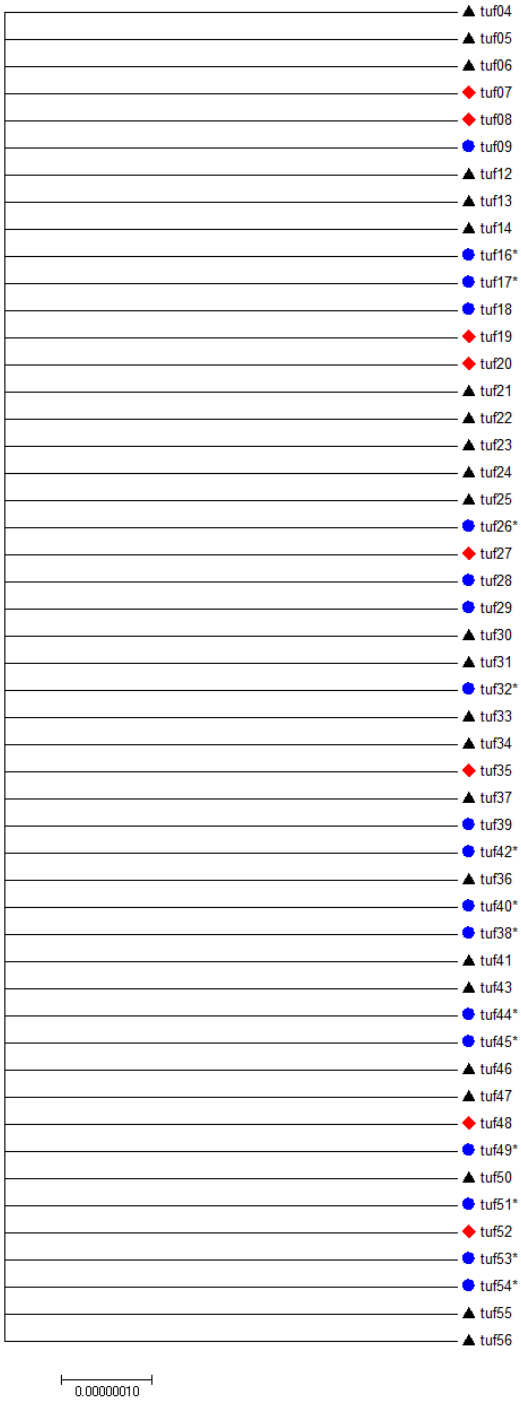

Figure 3.7 Neighbor-joining tree obtained from *Flavobacterium psychrophilum tuf* amino acid sequences based on the JTT matrix method. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

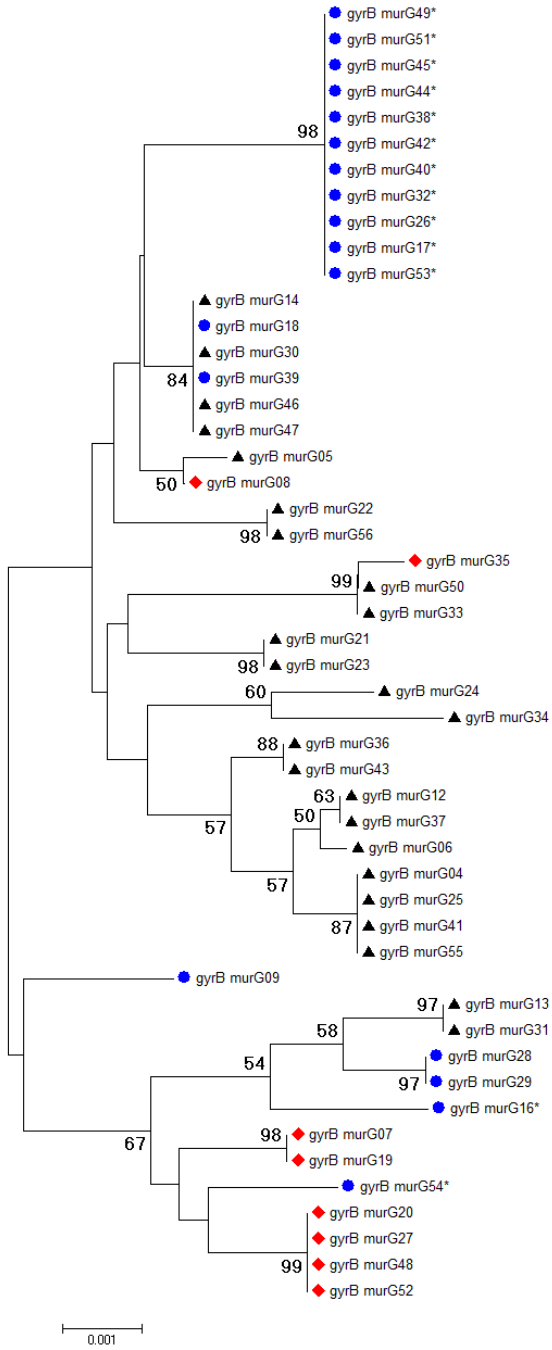

Figure 3.8 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum gyrB* and *murG* nucleotide sequences 1,758 bp in length based on Tamura three-parameter model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

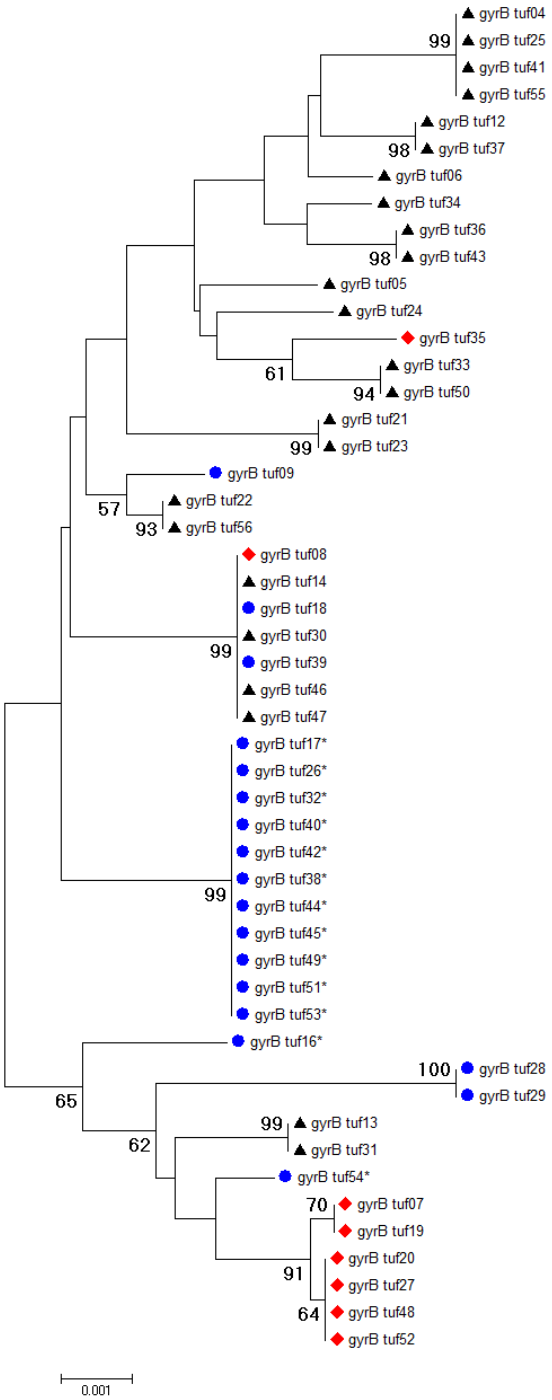

Figure 3.9 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum gyrB* and *tuf* nucleotide sequences 1,872 bp in length based on Tamura-Nei model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

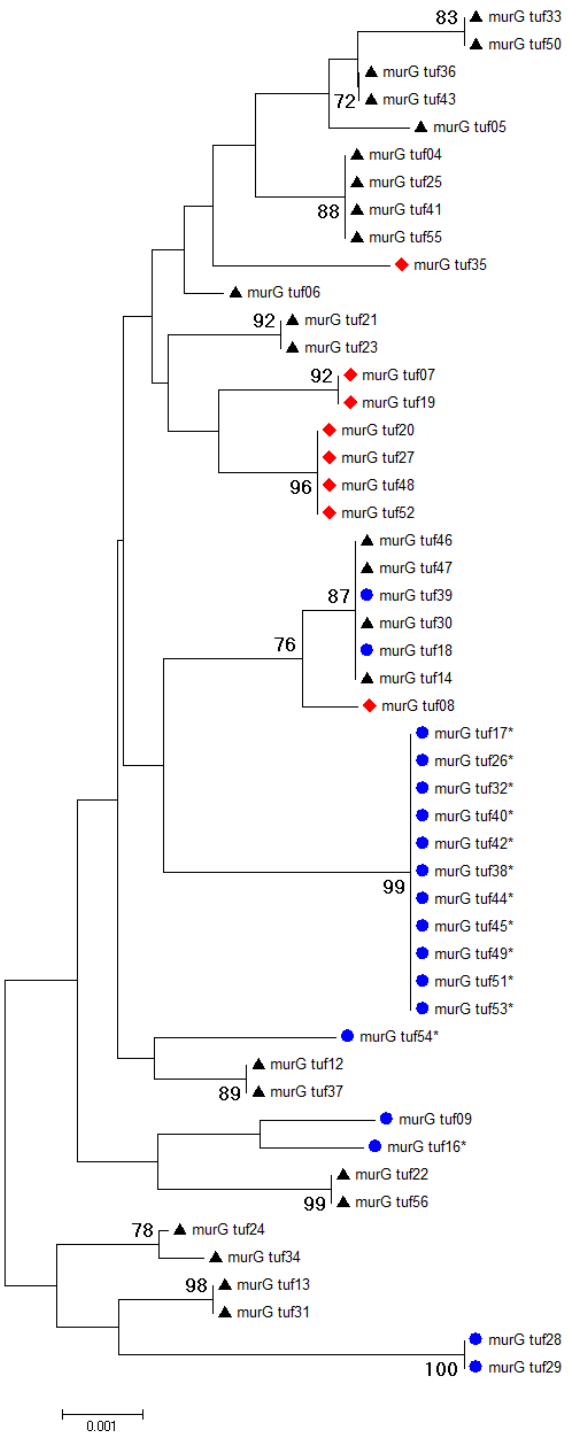

Figure 3.10 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum murG* and *tuf* nucleotide sequences 1,476 bp in length based on Tamura three-parameter model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

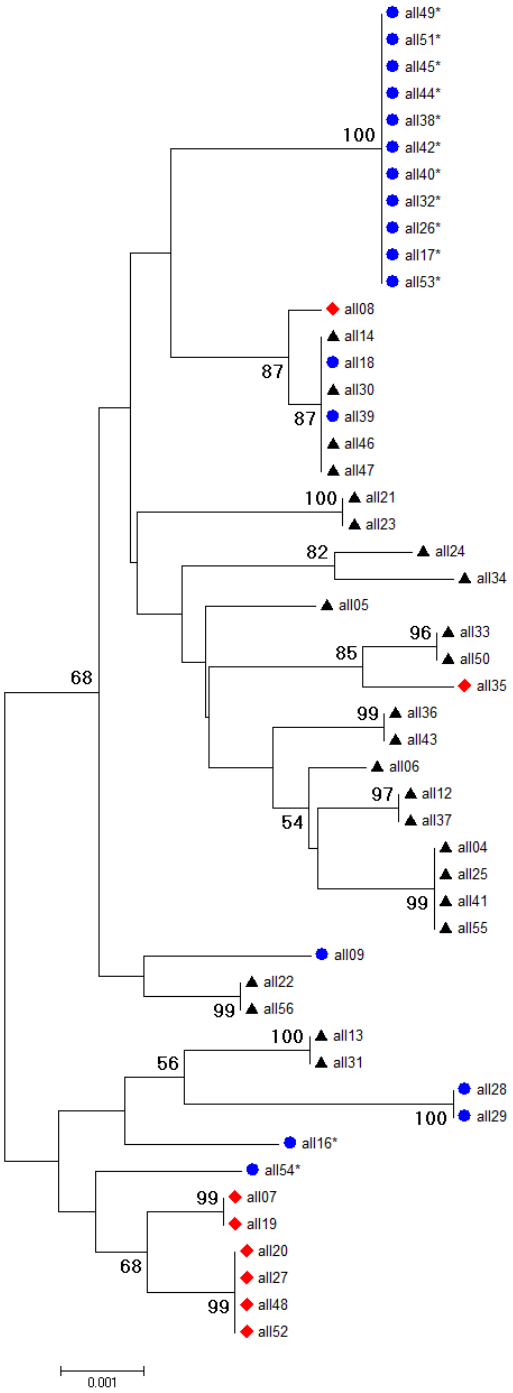

Figure 3.11 Neighbor-joining tree obtained from concatenated *Flavobacterium psychrophilum gyrB, murG,* and *tuf* nucleotide sequences 2,553 bp in length based on Tamura-Nei model distances with a gamma distribution. Tree is rooted at the midpoint. Bootstrap values >50 are displayed on branches (1000 replicates). Diamonds indicate isolates recovered from Coho salmon (*Oncorhynchus kisutch*), triangles indicate isolates from Chinook salmon (*O. tshawytscha*), and circles indicate isolates from rainbow trout (*O. mykiss*). Epidemic isolates are denoted with an asterisk.

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Chapter 4

Genetic diversity of *Flavobacterium psychrophilum* isolated from three *Oncorhynchus* spp. in the U.S.A. revealed by multilocus sequence typing

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1. Abstract

The use of a multilocus sequence typing (MLST) technique has identified the intraspecific genetic diversity of *Flavobacterium psychrophilum* from the United States of America (U.S.A.), an important pathogen of salmonids worldwide. Prior to this analysis, little U.S.A. *F. psychrophilum* genetic information was known, which is of importance when considering targeted control strategies, including vaccine development. Herein, MLST was used to investigate the genetic diversity of 96 *F. psychrophilum* isolates recovered from rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*O. kisutch*), and Chinook salmon (*O. tshawytscha*) that originated from nine U.S.A. states. The isolates fell into 34 distinct sequence types (STs) that clustered in 5 clonal complexes (CCs; n = 63) or were singletons (n = 33). The distribution of STs varied spatially, by host species, and in association with mortality events. Several STs (i.e., ST9, ST10, ST30, and ST78) were found in multiple states, whereas the remaining STs were localized to single states. With the exception of ST256, which was recovered from rainbow trout and Chinook salmon, all STs were found to infect a single host species. Isolates that were collected during bacterial coldwater disease outbreaks most frequently belonged to CC-ST10 (e.g., ST10 and ST78). Collectively, the results of this study clearly demonstrate the genetic diversity of *F. psychrophilum* within the U.S.A., and identify STs of clinical significance. Although the majority of STs described herein were novel, some were previously recovered on other continents (e.g., ST9, ST10, ST13, ST30 and ST31), which demonstrates the transcontinental distribution of *F. psychrophilum* STs.

2. Introduction

The causative agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS), *Flavobacterium psychrophilum*, is an important bacterial pathogen of wild and farmed salmonids worldwide (Starliper 2011). In addition to horizontal transmission, *F. psychrophilum* is suspected to be vertically transmitted (Brown et al. 1997; Taylor 2004; Cipriano 2005; Kumagai and Nawata 2010a) and appears to resist standard povidone-iodine treatment during egg disinfection (Taylor 2004; Kumagai and Nawata 2010b; Sundell and Wiklund 2015), which make efforts to control this bacterium particularly problematic. Since its initial isolation in North America (Borg 1948), *F. psychrophilum* infections have been reported in Europe, South America, Asia, and Australia (Bernardet and Kerouault 1989; Lee and Heo 1998; Nematollahi et al. 2003), as well as from all of the major areas of intensive salmonid aquaculture that have been studied (Bernardet and Bowman 2006).

Despite the fact that the trade of live fish and their eggs has been hypothesized as a major factor that drove the transcontinental spread of *F. psychrophilum* (Wakabayashi et al. 1994; Kumagai and Takahashi 1997), the epidemiological details to support this have not been fully elucidated. A number of molecular-based assays have been employed to study the genetic diversity of *F. psychrophilum* in an attempt to define host specificity, geographical associations, and virulence (Chakroun et al. 1998; Soule et al. 2005; Ramsrud et al. 2007; Hesami et al. 2008). However, despite these concerted efforts, the lack of standardized, reproducible, and comparable assays (Izumi et al. 2003; Hesami et al. 2008; Nicolas et al. 2008) left the intraspecific heterogeneity of *F. psychrophilum* as it relates to distribution and transmission routes incompletely understood.

Multilocus sequence typing (MLST) is a robust, reproducible, and established technique to identify and characterize strain diversity of human and animal bacterial pathogens (Cooper and Feil 2004; Maiden 2006), including those affecting fish (Martino et al. 2001; Bastardo et al. 2012; Yang et al. 2013; Habib et al. 2014; Zhang et al. 2014). MLST is based on the sequencing of typically 7 housekeeping gene loci, whereby an isolate is characterized by the allele types (ATs) found at the loci. Each specific combination of ATs is referred to as a sequence type (ST), which can be further grouped into clonal complexes (CCs) based upon their suspected evolutionary relatedness. A MLST scheme for *F. psychrophilum* was developed (Nicolas et al. 2008) and optimized (Siekoula-Nguedia et al. 2012; Fujiwara-Nagata et al. 2013), and has since linked some *F. psychrophilum* STs/CCs recovered from infected salmonids in Europe (Siekoula-Nguedia et al. 2012; Apablaza et al. 2013; Strepparava et al. 2013; Nilsen et al. 2014), Chile (Apablaza et al. 2013; Avendaño-Herrera et al. 2014), and Japan (Fujiwara-Nagata et al. 2013) with enhanced virulence (Siekoula-Nguedia et al. 2012; Strepparava et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014) and host species predilections (Nicolas et al. 2008; Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014).

However, the genetic diversity of *F. psychrophilum* in the United States has not yet been adequately addressed, a matter of importance considering the potential of this knowledge to contribute to targeted control strategies, including vaccine development. Particular consideration should be focused on deciphering host specificity and virulence levels of certain *F. psychrophilum* STs found in the U.S.A., where *F. psychrophilum* – susceptible salmonids have been artificially propagated since the end of the nineteenth century (Parker 1989). Similarly, captive and feral salmonid populations that were intentionally introduced into the Great Lakes

basin (GLB) over the last century (Kocik and Jones 1999) continue to suffer from *F. psychrophilum* infections (Van Vliet et al. 2015), yet little is known about the pathogen population structure there. Herein, we investigated the population structure of *F. psychrophilum* within the U.S.A. utilizing a comprehensive MLST approach, with the goal of characterizing the distribution of clonal complexes and their association with local BCWD outbreaks in feral and farmed *Oncorhynchus* spp. stocks.

3. Materials and Methods

3.1. Fish collection and isolation of *F. psychrophilum*

This study analyzed 96 *F. psychrophilum* isolates originating from 9 states within the U.S.A. (Fig. 4.1). Among these, 50 isolates originated from Michigan (MI) and accounted for two watersheds (i.e., lakes Michigan and Huron) of the GLB. The remainder originated from Idaho (ID; n = 18), Washington (WA; n = 8), Utah (UT; n = 6), North Carolina (NC; n = 5), West Virginia (WV; n = 3), Colorado (CO; n = 2), New Mexico (NM; n = 2), and Oregon (OR; n = 2). Isolates were recovered from rainbow trout (*O. mykiss*; n = 54), Chinook salmon (*O. tshawytscha*; n = 26), and Coho salmon (*O. kisutch*; n = 16) sampled between 1981 and 2013 (2009-2013 for the GLB isolates; Table 4.1a). Samples were collected from kidneys, spleens, brains, ovarian fluids, and/or external lesions from multiple host life-stages (i.e., eggs, fry, juveniles, and sexually mature adults; Table 4.1a). The majority of *F. psychrophilum* isolates were recovered from fish exhibiting gross disease signs commonly associated with BCWD (e.g., muscle ulceration, fin erosion, exophthalmia, and swollen internal organs), whereas others were occasionally recovered from apparently healthy fish.

Bacterial isolation was performed by a number of fish health professionals either on-site or upon receiving the fish at the laboratory. Tissues were inoculated onto flavobacterial-selective media, such as cytophaga agar (CA) with 11% agar concentration (Anacker and Ordal 1959) supplemented with neomycin sulfate at 4 mg l^{-1} and tryptone yeast extract salts (TYES) medium with 15% agar concentration (Holt 1987). In several rainbow trout outbreaks, multiple isolates were saved from single fish to examine the presence of co-infection. Otherwise, one colony-forming unit from each fish was sub-cultured for further analyses. Isolates were then cryopreserved in CA or TYES broth supplemented with glycerol (20% v/v) and then immediately frozen at -80°C for future analyses. Also included in this analysis was strain CSF259-93, originating from rainbow trout in ID (Table 4.1a; Table 4.1b), whose ST (ST10) was already available (Nicolas et al. 2008).

DNA was extracted from each suspected *F. psychrophilum* isolate using the DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, California), according to the manufacturers protocol for Gram-negative bacteria. For a portion of the isolates, the *F. psychrophilum*-specific primers of Toyama et al. (1994) were used to PCR-amplify a partial stretch of the 16S rRNA gene. Each reaction included 10 µL of 2x Go-Taq Green master mix (Promega, Madison, WI), 10 ng of DNA template, 5 ng of each primer, and nuclease-free water for a total reaction volume of 20 µL for each sample. The PCR parameters included an initial denaturation step at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec. A final elongation step was performed at 72°C for 7 min. Gel electrophoresis was conducted using 1.5% agarose get at 100 V for 45 min. Amplicons were then visualized under UV exposure, with an amplicon present at approximately 1100 bp considered confirmatory for

F. psychrophilum (Toyama et al. 1994). Isolates confirmed as *F. psychrophilum* by Sanger sequencing were prepared and described by Loch et al. (2013). Briefly, a partial 16S rRNA gene stretch was amplified using the universal primers 27F (5'-AGAGTTTGATCMTGGCTGAG-3') and 1387R (5'-GGGCGCWGTGTACAAGGC-3'; Marchesi et al. 1998). Amplification was performed with the following PCR parameters: initial denaturation at 95°C for 5 min, followed by 32 cycles of 30 s at 95°C, 30 s at 58°C and 60 s at 72°C. A final extension step at 72°C for 7 min was performed. All amplicons were purified using the QIAquick Purification Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol, and sequenced in-house.

3.2. MLST

PCR amplification of partial sequences of 7 housekeeping genes (*atpA, dnaK, fumC, gyrB, murG, trpB*, and *tuf;* Table 4.3) as originally described by Nicolas et al. (2008) and modified by Siekoula-Nguedia et al. (2012) was performed using primer sequences previously described by Fujiwara-Nagata et al. (2013) in a 50 µL reaction volume. Each reaction included 25 µL of 2x Go-Taq Green master mix (Promega, Madison, Wisconsin), 20 ng of DNA template, 0.25 µM of each primer, and nuclease-free water comprising the remainder. Sterile nuclease free water served as a negative control in all assays, and *F. psychrophilum* ATCC 49418^T was used as a positive control. All genes were amplified using the same touchdown protocol: 94°C for 5 min; 24 cycles at 94°C for 0.5 min, 55°C for 0.5 min (-0.4°C/cycle), and 72°C for 1 min (+2 sec/cycle); 12 cycles at 94°C for 0.5 min, 45°C for 0.5 min, and 72°C for 2 min (+3 sec/cycle); and a final extension step at 72°C for 10 min, as detailed in Fujiwara-Nagata et al. (2013). Reactions were run on a 1.5% agarose gel at 100 V for 40 min, where the presence of an amplicon of the

appropriate size under UV exposure confirmed amplification (Fujiwara-Nagata et al. 2013; Table 4.3). Purification of amplified PCR product was performed using the QIAquick Purification Kit (Qiagen, Inc., Valencia, California) according to the manufacturer's protocol. Bidirectional sequencing was performed using the corresponding forward and reverse primers (Fujiwara-Nagata et al. 2013), or *F. psychrophilum*-specific MLST sequencing primers (M13a: 5'-CAGGAAACAGCTATGACC-3'; M13b: 5'-TGTAAAACGACGGCCAGT-3'; 29). All sequences have been deposited in GenBank (accession numbers KT809511-KT810182).

3.3. MLST data analysis

All chromatograms were manually verified before assigning ATs and STs using an in-house script (P. Nicolas, INRA). ATs profiles were analyzed to delineate CCs and other relationships using eBURST v3 (eburst.mlst.net; Feil et al. 2004; Spratt et al. 2004) on the basis of Single Locus Variants (SLVs). STs not belonging to any CC were referred to as singletons. The predicted founder ST of a CC is defined as the ST with the highest number of single-locus variants. The CC is named after the predicted founder ST (e.g., CC-ST10), in cases where the founding ST could not be predicted (e.g., when only two STs in the complex) the CC was named after the most abundant ST. On the occasion that both STs were equally represented, the CC was named after the earliest identified ST. In the event that both STs were equally represented and found during the same year, the CC was named after both STs (e.g., CC-ST8/18). The entire publically available *F. psychrophilum* MLST database at the time of examination (n = 995; http://pubmlst.org/fpsychrophilum/) was used in the eBURST analysis. Average pairwise diversity at gene (i.e., locus) and nucleotide levels were computed as the mean number of

differences between pairs of STs. The sequence data collected in this study is available through the *F. psychrophilum* MLST database (http://pubmlst.org/fpsychrophilum/; Jolley et al. 2010). Statistical associations between STs and variables of interest (host species, geographical origin) were investigated using the Fisher's exact test. The Fisher's exact test was also used to investigate the associations for a specific ST by analyzing condensed contingency tables. All statistical analyses were conducted using SAS software (SAS statistical software – version 9.1; SAS Institute Inc.).

4. Results

4.1. Genetic diversity of *F. psychrophilum* in the U.S.A.

MLST analysis on the 96 U.S.A. *F. psychrophilum* isolates revealed 34 distinct STs (Table 4.1b). Among these, 28 STs (n = 60 isolates) were novel, whereas the remaining 6 (n = 36 isolates) have previously been reported from other locations worldwide (Table 4.2). The most abundant ST was ST10, which accounted for 23/96 of our isolates. The second most abundant ST (n = 15/96) was novel ST78. The remaining 34 STs were each identified less than 6 times in our collection of isolates (Table 4.1b).

The mean gene diversity of the U.S.A. *F. psychrophilum* isolates was 0.75±0.03, and the mean nucleotide diversity amounted to 4.4 Kbp^{-1}. The diversity indices were also computed between the 34 STs, whereby the mean gene diversity was 0.925± 0.02, and the mean nucleotide diversity was 5.4 Kbp^{-1}. Gene diversity indices varied between isolates retrieved from different fish host species. The highest gene diversity was from Chinook salmon isolates

(0.89 ± 0.02), followed by Coho salmon isolates (0.79 ± 0.05). The lowest gene diversity of 0.32 ± 0.07 was computed from isolates collected from rainbow trout.

4.2. Identification of clonal complexes

The eBURST analysis identified a few clonal complexes with the SLV-link criterion (Fig. 4.2). The largest CC in this analysis was CC-ST10, which contains 6 STs (Fig. 4.2) including the two most abundant: ST10 and ST78. Besides CC-ST10 which accounted for nearly half of the isolates in the U.S.A. dataset (n = 46), two other smaller complexes, CC-ST256 (n = 7 isolates, n = 2 STs) and CC-ST9 (n = 5 isolates, n = 2 STs), were detected (Fig. 4.2).

An eBURST analysis was also conducted to depict the connection between the 96 isolates and the 995 isolates currently in the *F. psychrophilum* MLST database as of June 2015 (Fig. 4.3). This revealed additional SLV-links between the STs of this study and those identified in other studies. Namely, ST31, ST262, and ST267 could be identified as part of small clonal complexes (designated CC-ST31, CC-ST262, and CC-ST191, respectively). In particular, ST267 discovered in Michigan clusters with CC-ST191, and is the first ST from North America in this CC. Nevertheless, most of the STs identified in this study (21/34) were singletons (Fig. 4.2). Out of the 21 singleton STs, most (n = 13) were represented by a unique isolate. When several isolates were identified as the same novel singleton ST, all isolates were recovered from the same geographic location (i.e., U.S.A. state) during a short time period (i.e., 1-3 years).

4.3. Association between STs and fish host species

When investigating the association between ST and fish host species, the Fisher's exact test revealed an overall association between these two variables ($P < 0.001$). Indeed, all but one of the 34 STs of this study were retrieved from a single host fish species (Table 4.4).

For rainbow trout, which was the best represented fish host (n = 54 isolates, Table 4.1a), 23 isolates belonged to ST10 and 15 to ST78 (Table 4.4). Both ST10 and ST78 were found statistically significantly associated with rainbow trout ($P < 0.001$). Association between genotype and host fish species extended beyond the ST-level, since all CC-ST10 isolates were retrieved exclusively from rainbow trout. Overall the 6 STs in CC-ST10 accounted for 85% (n = 46, Table 4.1a; Table 4.1b) of the isolates from this fish host. Of note, all isolates that belonged to CC-ST10 were retrieved from captive fish and the vast majority (n = 44/46 isolates) were recovered during high mortality and/or morbidity events (Table 4.1a; Table 4.1b). Furthermore, on five occasions multiple isolates from the same rainbow trout host were retrieved and analyzed. In three of those cases, analysis of both external lesions and internal tissues (kidney or spleen) lead to the identification of the same ST (i.e., ST78 or ST82; Table 1). However, in two instances, multiple STs were found to be co-infecting the same individual fish (i.e., ST10 with either ST82 or ST84; Table 4.1a; Table 4.1b).

The 26 Chinook salmon isolates fell into 14 STs (Table 4.4), among which the two most abundant STs were ST29 and ST256 (represented by 4 isolates each). ST29 was isolated exclusively from Chinook salmon, while ST256 was isolated from rainbow trout as well (Table 4.4). The 16 isolates recovered from Coho salmon belonged to 9 different STs, among which the

most abundant ST was ST258 (n = 4, Table 4.4). It was noticeable that all isolates in CC-ST9 (i.e., ST9 and ST13) originated from Coho salmon (Table 4.1a; Table 4.1b).

The only detected exception to the strict association between STs and host fish was for ST256 which was isolated both from rainbow trout (n = 2) and Chinook salmon (n = 4, Table 4.4). Interestingly, another ST belonging to the same CC-ST256 was also identified in Coho salmon (n = 1, Table 4.1a; Table 4.1b). All these hosts were feral fish from the GLB (Table 4.1a).

4.4. Geographical origin of the STs

The strong association between ST and host fish species, combined with the limited number of locations sampled did not allow for any statistical associations between ST and geographical origin to be assessed. Nevertheless, a particularly striking association between ST and geographical origin of the two most abundant STs (i.e., ST10 n = 23, and ST78 n = 15) in this study was observed. ST10 and ST78 isolates were recovered exclusively from captive rainbow trout, and differed clearly by their geographical distributions (Table 4.1a; Table 4.1b). ST10 isolates originated from Idaho (n = 13), Utah (n = 5), North Carolina (n = 2), New Mexico (n = 2), and West Virginia (n = 1). ST78 was identified primarily in Michigan (n = 11), and secondarily in Colorado (n = 2), and West Virginia (n = 2).

The state of Michigan accounted for 50 of our isolates, which could be divided in two sub-groups reflecting two of the watersheds within the GLB (e.g., Lake Michigan and Lake Huron). This provided the opportunity to further examine a potential link between ST and the geographical origin within a particular geographical area. Feral fish in the Lake Michigan watershed accounted for the highest number of isolates (n = 24) and number of distinct STs (n =

12), 9 of which are newly described herein (Table 4.1a; Table 4.1b). The most abundant ST in the Lake Michigan watershed was ST256 (n = 5 isolates). From the Lake Huron watershed, 12 isolates recovered from feral fish resulted in identification of 8 STs, 7 of which were also newly described herein (Table 4.1a; Table 4.1b). Captive hosts in the GLB were represented by 14 isolates from 3 Michigan state fish hatcheries, and resulted in 4 novel STs (i.e., ST78, ST253, ST257, and ST267; Table 4.1a; Table 4.1b). The most abundant ST observed in Michigan state fish hatcheries was ST78 (n = 11 isolates; 22%; CC-ST10). A Fisher's exact test (based on STs) revealed a significant association between all 6 locations within the state of Michigan and ST ($P < 0.001$). Most STs were unique to single locations within the GLB (e.g., ST259 and ST260 were only recovered from the Lake Michigan watershed), but others were widespread throughout the state (e.g., ST256 was recovered from both Lake Michigan and Lake Huron watersheds; Table 4.1a; Table 4.1b).

5. Discussion

Previous MLST studies have demonstrated the genetic heterogeneity of *F. psychrophilum* populations in other parts of the world. Despite the fact that the 96 isolates of this study may not fully represent the entire diversity of the U.S.A. *F. psychrophilum* population, the observed 34 distinct STs, of which 28 were novel, clearly depict a similar genetic heterogeneity within the U.S.A. Upon comparison to the global MLST database, it also became clear that some U.S.A. *F. psychrophilum* STs and/or CCs have transcontinental distributions, whereas others seem to be more geographically limited. The results of this study shed light on

the population structure of *F. psychrophilum* in the U.S.A. and highlight the similarities and differences between this population and *F. psychrophilum* populations elsewhere.

Average pairwise diversity measures allow for a direct comparison of datasets from distinct regions of the world. The average gene diversity of the 96 U.S.A. isolates (i.e., 0.75 ± 0.03) was higher than any previously reported values from other regions of the world, where it varied from 0.43 in France (Siekoula-Nguedia et al. 2012), 0.48 in Chile (Avendaño-Herrera et al. 2014), 0.61 in Nordic countries (Nilsen et al. 2014), and 0.68 in Japan (Fujiwara-Nagata et al. 2013). However, the observed diversity may depend on the sampling scheme and the epidemiological characteristics of each *F. psychrophilum* population under investigation. In particular, the number of fish species in these previous studies varied from 1 to 15 (Siekoula-Nguedia et al. 2012; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014), whereby this study focused on 3 *Oncorhynchus* spp. Interestingly, the average pairwise nucleotide diversity of the 34 STs in the current study (i.e., 5.4Kbp^{-1}) is strikingly similar to that reported in Japan (i.e., 5.4Kbp^{-1}, based on 35 STs; Fujiwara-Nagata et al. 2013), which may suggest that the genetic diversity of *F. psychrophilum* may be roughly comparable between the temperate regions of the Northern hemisphere.

Studies on other fish-pathogenic flavobacteria have linked genetic differences to virulence and host specificity. For example, Olivares-Fuster et al. (2007) found that genomovar I of the fish-pathogenic *F. columnare* is predominantly associated with the threadfin shad (*Dorosoma pretenense*), while Shoemaker et al. (2008) linked genomovar II of the same bacterium to channel catfish (*Ictalurus punctatus*). Host-specific trends for different *F. psychrophilum* STs are also becoming evident (Nicolas et al. 2008; Fujiwara-Nagata et al. 2013;

Nilsen et al. 2014), and the findings from this study suggest that the same may be true for some STs found in the U.S.A. (Table 4.2). For example, isolates belonging to CC-ST10 were recovered exclusively from rainbow trout, thus supporting the hypothesis that CC-ST10 strains are particularly adapted to this species, which is highly susceptible to BCWD, or its rearing conditions (Nilsen et al. 2014). A statistical association between ST10 and ST78 (both CC-ST10) and rainbow trout hosts has been demonstrated, however, statistically significant associations between rainbow trout and the other STs in CC-ST10 may become evident as more isolates are analyzed. Likewise, Avendaño-Herrera et al. (2014) indicated a probable association between CC-ST9 strains and Coho salmon, which coincides with the findings of this study, as all CC-ST9 isolates recovered in the U.S.A. were isolated from Coho salmon. Further investigation into the mechanisms responsible for the apparent association between STs and host species is greatly needed.

However, other factors may also contribute to these observed associations. STs belonging to CC-ST10 have been found to circulate in more than one host species in different regions of the world: rainbow trout and Atlantic salmon in Chile (Avendaño-Herrera et al. 2014), and rainbow trout and brown trout in Switzerland (Strepparava et al. 2013). These patterns might be explained by the interconnection between the rearing systems of the different fish species. In this study, the absence of CC-ST10 in feral rainbow trout raises the suspicion that the observed association between CC-ST10 and this host species may result from a combination of some degree of host specificity and intensive culture conditions. It is also interesting to note that for the feral fish in the GLB and in particular for Chinook salmon (which harbor the highest *F. psychrophilum* infection prevalence among three *Oncorhynchus* spp. that

were recently examined; Van Vliet et al. 2015), data from this study does not seem to indicate an epidemic population structure as demonstrated by the lack of dominant STs or CCs. It is also only among these fish that we observed infections by the same ST (e.g., ST256) or CC (e.g., CC-ST256) in different fish host species. Collectively, these observations give additional support to the idea that fish life conditions are also an important determinant of the *F. psychrophilum* genetic population structure.

Similar to the virulence trends identified in *F. columnare* (Shoemaker et al. 2008), previous *F. psychrophilum* MLST studies suggest that some STs may be associated with higher virulence. For example, the founding and sub-founding STs of CC-ST10 (i.e., ST2 and ST10) cause severe disease outbreaks in Europe and Chile and are proposed to have given rise to multiple highly pathogenic sub-lineages (Siekoula-Nguedia et al. 2012; Strepparava et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014). Findings of this study support this hypothesis, as all but 2 CC-ST10 isolates recovered in the U.S.A. were isolated from rainbow trout undergoing clinical BCWD. Within this complex, ST10, and its SLV ST78, seem to be dominant in North America. While ST10 has a worldwide distribution (Strepparava et al. 2013; Nilsen et al. 2014), ST78 has emerged as a ST of clinical significance, at least in Michigan. Indeed, only 2 isolates recovered during clinical disease epizootics in Michigan state fish hatcheries did not belong to this ST. Similarly, CC-ST191 contains isolates from disease outbreaks in farmed rainbow trout (Nilsen et al. 2014), including ST267, which was isolated during a disease outbreak at a Michigan state fish hatchery. The reason(s) for the apparent increase in virulence that certain STs display remains to be determined. However, a recent study demonstrated that members of CC-ST10 exhibit enhanced adherence to fish mucus, thereby facilitating their

colonization, and are also resistant to antibiotics commonly used in aquaculture (Sundell and Wiklund 2015). Both the adherence and antimicrobial resistance properties, in conjunction with the high susceptibility of rainbow trout to *F. psychrophilum* in general, may be contributing to dominance of these STs in aquaculture facilities.

These results bring new data on the history of CC-ST10, which is the largest established and most widespread CC, based on all available *F. psychrophilum* MLST data. CC-ST10 is composed of a total of 34 STs recovered in North America, Europe, Asia, and South America (Nicolas et al. 2008; Siekoula-Nguedia et al. 2012; Strepparava et al. 2013; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014). Likewise, this study demonstrated that CC-ST10 is widespread in U.S.A. rainbow trout farms. This finding, along with the history of dissemination of rainbow trout/eggs from the U.S.A. to other countries, suggests that CC-ST10 originated from North America. Inclusion of U.S.A. isolates into the global *F. psychrophilum* MLST database and eBURST analyses suggest that ST10 is the likely founder of CC-ST10, rather than ST2 (Nicolas et al. 2008; Siekoula-Nguedia et al. 2012; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014), as the novel U.S.A. STs are SLVs of ST10 rather than ST2. Furthermore, it is notable that neither ST2 nor its SLVs (aside of ST10) have yet to be observed in the U.S.A., suggesting that evolution and diversification of the ST2 lineage within CC-ST10 may have occurred outside of this country.

In addition to globally distributed STs in the U.S.A., this study revealed the presence of *F. psychrophilum* populations with lower prevalence and probably more limited geographical distribution. This was exemplified by the relatively high number of novel singletons in the salmonid populations of the GLB, which aside from two occasions (i.e., ST257 and ST267), were

not associated with morbidity or mortality. Furthermore, the majority (e.g., 6/8) of the singletons from outside of the GLB were also not associated with morbidity or mortality which coincides with the suggestion that many of these singletons may correspond to less virulent STs (Siekoula-Nguedia et al. 2012; Nilsen et al. 2014; Sundell and Wiklund 2015). These may be representatives of endemic *F. psychrophilum* populations whose characteristics with respect to pathogenicity, fish host, and geographical distribution remains to be clarified.

Although the trade of live fish and/or fish eggs may be a major source of pathogen transmission, natural dissemination (e.g., animal migration and transmission within ballast water) may also contribute to the broad distribution of some STs. The anadromous lifestyle of many salmonid species allows for mixing and encountering of various salmonid populations from wide geographic locations (Healy 1991). As proposed by Fujiwara-Nagata et al. (2013), neutral population genetics models predict that these long-distance dissemination events do not need to be frequent in order to have a dramatic homogenizing effect on the population genetics of *F. psychrophilum*.

In conclusion, the MLST investigation of *F. psychrophilum* isolates recovered from feral and hatchery-reared salmonids in the United States revealed marked genetic diversity. Several of the U.S.A. *F. psychrophilum* STs are found worldwide, whereas others seem specific to the continental U.S.A. These results shed light on the historical links between different global *F. psychrophilum* populations. Furthermore, we demonstrated the association between particular STs and host species, as well as high virulence, in the U.S.A. This information can be used to more appropriately investigate preventative control measures to reduce the spread and severity of BCWD.

APPENDIX

Table 4.1a Characteristics of the 96 United States *Flavobacterium psychrophilum* isolates investigated using multilocus sequence typing. Isolates designated WLSFH, TSFH, LMRW, PRW, and OSFH recovered from Lake Michigan watershed. Isolates designated SRW recovered from Lake Huron watershed. [1]Isolates originating from the same fish denoted by the same superscript letter.[2]United States abbreviations: ID, Idaho; NC, North Carolina; NM, New Mexico; UT, Utah; WV, West Virginia; CO, Colorado; MI, Michigan; OR, Oregon; WA, Washington. [*]Isolates recovered during high levels of morbidity and mortality.

Isolate ID[1]	Isolate Designation	Year	U.S.A. State[2]	Salmonid Species	Isolation Tissue	Feral/ captive	Life-stage
526	CSF201-91	1991	ID	*O. mykiss*	spleen	captive	juvenile*
527	CSF408-92	1992	ID	*O. mykiss*	spleen	captive	juvenile*
50	CSF259-93	1993	ID	*O. mykiss*	spleen	captive	juvenile*
529	CSF060-99	1999	ID	*O. mykiss*	spleen	captive	juvenile*
530	CSF016-00	2000	ID	*O. mykiss*	spleen	captive	juvenile*
531	CSF054-01	2001	ID	*O. mykiss*	spleen	captive	juvenile*
533	CSF088-03	2003	ID	*O. mykiss*	spleen	captive	juvenile*
534	CSF352-04	2004	ID	*O. mykiss*	spleen	captive	juvenile*
535	CSF226-05	2005	ID	*O. mykiss*	spleen	captive	juvenile*
536	CSF009-06	2006	ID	*O. mykiss*	spleen	captive	juvenile*
537[a]	ARS-01S-08	2008	ID	*O. mykiss*	spleen	captive	juvenile*
539[a]	ARS-01L-08	2008	ID	*O. mykiss*	lesion	captive	juvenile*
542	ARS-03S-08	2008	ID	*O. mykiss*	spleen	captive	juvenile*
545[b]	ARS-05K1-09	2009	NC	*O. mykiss*	kidney	captive	juvenile*
548[b]	ARS-05S2-09	2009	NC	*O. mykiss*	spleen	captive	juvenile*
519	F12 K1 17	2002	NM	*O. mykiss*	kidney	captive	fry*

Table 4.1a (cont'd)

Isolate ID[1]	Isolate Designation	Year	U.S.A. State[2]	Salmonid Species	Isolation Tissue	Feral/ captive	Life-stage
520	F12 6 17	2002	NM	*O. mykiss*	kidney	captive	fry*
523	03-009	2003	UT	*O. mykiss*	unknown	captive	juvenile*
556	08-114	2008	UT	*O. mykiss*	spleen	captive	juvenile*
553	09-041	2009	UT	*O. mykiss*	spleen	captive	juvenile*
554	09-080	2009	UT	*O. mykiss*	spleen	captive	juvenile*
557	09-083	2009	UT	*O. mykiss*	spleen	captive	juvenile*
552	ARS-002-07	2007	WV	*O. mykiss*	kidney/brain	captive	juvenile*
515[c]	463-96 Fsp RBT5	1996	CO	*O. mykiss*	kidney/lesion	captive	unknown*
521[c]	464-96 Fsp RBT1B	1996	CO	*O. mykiss*	kidney/lesion	captive	unknown*
US17	WLSFH	2010	MI	*O. mykiss*	brain	captive	juvenile*
US26	WLSFH	2010	MI	*O. mykiss*	brain	captive	juvenile*
US32	WLSFH	2010	MI	*O. mykiss*	brain	captive	juvenile*
US45	WLSFH	2010	MI	*O. mykiss*	kidney	captive	juvenile*
US38	WLSFH	2011	MI	*O. mykiss*	kidney	captive	juvenile*
US40	TSFH	2011	MI	*O. mykiss*	kidney	captive	juvenile*
US42[d]	WLSFH	2011	MI	*O. mykiss*	kidney	captive	juvenile*
US53[d]	WLSFH	2011	MI	*O. mykiss*	ext. lesion	captive	juvenile*
US44	TSFH	2011	MI	*O. mykiss*	kidney	captive	juvenile*
US49	TSFH	2011	MI	*O. mykiss*	kidney	captive	juvenile*
US51	TSFH	2011	MI	*O. mykiss*	kidney	captive	juvenile*
549	ARS-001-06	2006	WV	*O. mykiss*	fin	captive	adult
550	ARS-002-06	2006	WV	*O. mykiss*	fin	captive	adult
538[a]	ARS-01K-08	2008	ID	*O. mykiss*	kidney	captive	juvenile*
540[e]	ARS-02S-08	2008	ID	*O. mykiss*	spleen	captive	juvenile*

Table 4.1a (cont'd)

Isolate ID[1]	Isolate Designation	Year	U.S.A. State[2]	Salmonid Species	Isolation Tissue	Feral/ captive	Life-stage
541[e]	ARS-02L-08	2008	ID	*O. mykiss*	lesion	captive	juvenile*
543	ARS-07S-08	2008	ID	*O. mykiss*	spleen	captive	juvenile*
546[b]	ARS-05K2-09	2009	NC	*O. mykiss*	kidney	captive	juvenile*
547[b]	ARS-05S1-09	2009	NC	*O. mykiss*	spleen	captive	juvenile*
532	CSF067-02	2002	ID	*O. mykiss*	spleen	captive	juvenile*
555	09-032	2009	UT	*O. mykiss*	spleen	captive	juvenile*
US18	LMRW	2013	MI	*O. mykiss*	kidney	feral	adult
US39	LMRW	2013	MI	*O. mykiss*	kidney	feral	adult
US46	LMRW	2011	MI	*O. tshawytscha*	kidney	feral	adult
US47	SRW	2011	MI	*O. tshawytscha*	egg	feral	egg
US14	LMRW	2013	MI	*O. tshawytscha*	kidney	feral	adult
US30	LMRW	2013	MI	*O. tshawytscha*	kidney	feral	adult
US08	PRW	2011	MI	*O. kisutch*	kidney	feral	adult
503	SH3-81	1981	OR	*O. kisutch*	kidney	captive	juvenile*
502	Quilcene C7	2000	WA	*O. kisutch*	OF	feral	adult
504	Quilcene C5	2000	WA	*O. kisutch*	OF	feral	adult
US07	PRW	2010	MI	*O. kisutch*	kidney	feral	adult
US19	PRW	2010	MI	*O. kisutch*	kidney	feral	adult
US28	LMRW	2010	MI	*O. mykiss*	kidney	feral	adult
US29	LMRW	2010	MI	*O. mykiss*	kidney	feral	adult
US33	LMRW	2010	MI	*O. tshawytscha*	kidney	feral	adult
US50	LMRW	2010	MI	*O. tshawytscha*	kidney	feral	adult
US54	WLSFH	2013	MI	*O. mykiss*	kidney	captive	juvenile*
US04	SRW	2011	MI	*O. tshawytscha*	egg	feral	egg
US25	SRW	2011	MI	*O. tshawytscha*	kidney	feral	adult

Table 4.1a (cont'd)

Isolate ID[1]	Isolate Designation	Year	U.S.A. State[2]	Salmonid Species	Isolation Tissue	Feral/ captive	Life-stage
US41	LMRW	2011	MI	*O. tshawytscha*	kidney	feral	adult
US55	SRW	2011	MI	*O. tshawytscha*	kidney	feral	adult
US48	PRW	2012	MI	*O. kisutch*	kidney	feral	adult
US52	PRW	2012	MI	*O. kisutch*	kidney	feral	adult
US20	PRW	2013	MI	*O. kisutch*	kidney	feral	adult
US27	PRW	2013	MI	*O. kisutch*	kidney	feral	adult
505	W98-317-16K	1998	OR	*O. kisutch*	kidney	captive	unknown
506	EC98-305-5402K	1998	WA	*O. kisutch*	kidney	captive	unknown
511	AFTC P-3	2000	WA	*O. tshawytscha*	OF	feral	adult
512	AFTC C2	2000	WA	*O. tshawytscha*	OF	feral	adult
US13	LMRW	2012	MI	*O. tshawytscha*	kidney	feral	adult
US31	LMRW	2012	MI	*O. tshawytscha*	kidney	feral	adult
US21	SRW	2009	MI	*O. tshawytscha*	kidney	feral	adult
US23	SRW	2013	MI	*O. tshawytscha*	kidney	feral	adult
US22	SRW	2010	MI	*O. tshawytscha*	kidney	feral	adult
US56	SRW	2010	MI	*O. tshawytscha*	kidney	feral	adult
US36	LMRW	2008	MI	*O. tshawytscha*	kidney	feral	adult
US43	LMRW	2008	MI	*O. tshawytscha*	kidney	feral	adult
508	03-398-1	2003	WA	*O. kisutch*	kidney	captive	adult
510	03-449-5	2003	WA	*O. kisutch*	kidney	captive	adult
513	03-169	2003	WA	*O. kisutch*	kidney	captive	juvenile*
544	ARS-03B-09	2009	NC	*O. mykiss*	brain	captive	juvenile*
US05	LMRW	2011	MI	*O. tshawytscha*	egg	feral	egg
US06	LMRW	2011	MI	*O. tshawytscha*	egg	feral	egg

Table 4.1a (cont'd)

Isolate ID[1]	Isolate Designation	Year	U.S.A. State[2]	Salmonid Species	Isolation Tissue	Feral/ captive	Life-stage
US09	OSFH	2013	MI	*O. mykiss*	ext. lesion	captive	adult
US12	SRW	2013	MI	*O. tshawytscha*	kidney	feral	adult
US16	WLSFH	2013	MI	*O. mykiss*	kidney	captive	juvenile*
US24	SRW	2009	MI	*O. tshawytscha*	kidney	feral	adult
US34	SRW	2012	MI	*O. tshawytscha*	kidney	feral	adult
US35	PRW	2011	MI	*O. kisutch*	kidney	feral	adult
US37	SRW	2012	MI	*O. tshawytscha*	kidney	feral	adult

Table 4.1b Multilocus sequence typing allele and sequence types (STs) and association with clonal complexes (CC) of 96 United States *F. psychrophilum* isolates. Underlined text in Allelic profile and ST column denote novel allele types and STs.

Isolate ID[1]	Allelic profile							ST	CC
	trpB	*gyrB*	*dnaK*	*fumC*	*murG*	*tuf*	*atpA*		
526	2	8	2	2	2	2	2	ST10	CC-ST10
527	2	8	2	2	2	2	2	ST10	CC-ST10
50	2	8	2	2	2	2	2	ST10	CC-ST10
529	2	8	2	2	2	2	2	ST10	CC-ST10
530	2	8	2	2	2	2	2	ST10	CC-ST10
531	2	8	2	2	2	2	2	ST10	CC-ST10
533	2	8	2	2	2	2	2	ST10	CC-ST10
534	2	8	2	2	2	2	2	ST10	CC-ST10
535	2	8	2	2	2	2	2	ST10	CC-ST10
536	2	8	2	2	2	2	2	ST10	CC-ST10
537[a]	2	8	2	2	2	2	2	ST10	CC-ST10
539[a]	2	8	2	2	2	2	2	ST10	CC-ST10
542	2	8	2	2	2	2	2	ST10	CC-ST10
545[b]	2	8	2	2	2	2	2	ST10	CC-ST10
548[b]	2	8	2	2	2	2	2	ST10	CC-ST10
519	2	8	2	2	2	2	2	ST10	CC-ST10
520	2	8	2	2	2	2	2	ST10	CC-ST10
523	2	8	2	2	2	2	2	ST10	CC-ST10
556	2	8	2	2	2	2	2	ST10	CC-ST10
553	2	8	2	2	2	2	2	ST10	CC-ST10
554	2	8	2	2	2	2	2	ST10	CC-ST10
557	2	8	2	2	2	2	2	ST10	CC-ST10
552	2	8	2	2	2	2	2	ST10	CC-ST10
515[c]	2	8	2	2	2	41	2	ST78	CC-ST10
521[c]	2	8	2	2	2	41	2	ST78	CC-ST10
US17	2	8	2	2	2	41	2	ST78	CC-ST10
US26	2	8	2	2	2	41	2	ST78	CC-ST10
US32	2	8	2	2	2	41	2	ST78	CC-ST10
US45	2	8	2	2	2	41	2	ST78	CC-ST10
US38	2	8	2	2	2	41	2	ST78	CC-ST10
US40	2	8	2	2	2	41	2	ST78	CC-ST10
US42[d]	2	8	2	2	2	41	2	ST78	CC-ST10
US53[d]	2	8	2	2	2	41	2	ST78	CC-ST10

Table 4.1b (cont'd)

Isolate ID[1]	Allelic profile							ST	CC
	trpB	*gyrB*	*dnaK*	*fumC*	*murG*	*tuf*	*atpA*		
US44	2	8	2	2	2	41	2	ST78	CC-ST10
US49	2	8	2	2	2	41	2	ST78	CC-ST10
US51	2	8	2	2	2	41	2	ST78	CC-ST10
549	2	8	2	2	2	41	2	ST78	CC-ST10
550	2	8	2	2	2	41	2	ST78	CC-ST10
538[a]	2	8	2	2	2	44	2	ST82	CC-ST10
540[e]	2	8	2	2	2	44	2	ST82	CC-ST10
541[e]	2	8	2	2	2	44	2	ST82	CC-ST10
543	2	8	2	2	2	44	2	ST82	CC-ST10
546[b]	2	41	2	2	2	41	2	ST84	CC-ST10
547[b]	2	41	2	2	2	41	2	ST84	CC-ST10
532	2	8	2	2	2	43	2	ST81	CC-ST10
555	2	8	2	3	2	2	2	ST86	CC-ST10
US18	1	28	4	2	25	25	59	ST256	CC-ST256
US39	1	28	4	2	25	25	59	ST256	CC-ST256
US46	1	28	4	2	25	25	59	ST256	CC-ST256
US47	1	28	4	2	25	25	59	ST256	CC-ST256
US14	1	28	4	2	25	25	59	ST256	CC-ST256
US30	1	28	4	2	25	25	59	ST256	CC-ST256
US08	1	28	4	2	16	25	59	ST252	CC-ST256
503	4	7	6	5	6	5	4	ST9	CC-ST9
502	4	7	6	5	6	5	4	ST9	CC-ST9
504	4	7	6	5	6	5	4	ST9	CC-ST9
US07	4	7	6	5	6	8	4	ST13	CC-ST9
US19	4	7	6	5	6	8	4	ST13	CC-ST9
US28	3	19	13	9	12	16	15	ST31	CC-ST31
US29	3	19	13	9	12	16	15	ST31	CC-ST31
US33	6	9	7	3	49	5	7	ST262	CC-ST262
US50	6	9	7	3	49	5	7	ST262	CC-ST262
US54	4	73	22	3	3	3	3	ST267	CC-ST191
US04	8	17	14	9	13	17	16	ST29	
US25	8	17	14	9	13	17	16	ST29	
US41	8	17	14	9	13	17	16	ST29	
US55	8	17	14	9	13	17	16	ST29	
US48	42	18	26	5	47	8	14	ST258	
US52	42	18	26	5	47	8	14	ST258	
US20	42	18	26	5	47	8	14	ST258	
US27	42	18	26	5	47	8	14	ST258	
505	11	18	7	5	14	18	17	ST30	

Isolate ID[1]	Allelic profile							ST	CC
	trpB	*gyrB*	*dnaK*	*fumC*	*murG*	*tuf*	*atpA*		
506	11	18	7	5	14	18	17	ST30	
511	8	19	7	1	29	40	39	ST76	
512	8	19	7	1	29	40	39	ST76	
US13	41	68	10	9	12	18	14	ST255	
US31	41	68	10	9	12	18	14	ST255	
US21	27	69	15	5	13	36	26	ST259	
US23	27	69	15	5	13	36	26	ST259	
US22	11	23	28	1	48	1	8	ST260	
US56	11	23	28	1	48	1	8	ST260	
US36	18	72	9	3	13	5	11	ST266	
US43	18	72	9	3	13	5	11	ST266	
508	8	38	8	2	25	38	37	ST74	
510	27	22	15	7	12	39	38	ST75	
513	28	39	4	2	30	12	4	ST77	
544	1	40	16	2	31	45	2	ST83	
US05	40	66	4	5	16	47	57	ST250	
US06	4	67	15	10	13	12	58	ST251	
US09	1	13	8	1	1	1	1	ST253	
US12	4	47	15	3	13	3	11	ST254	
US16	1	2	2	3	46	12	2	ST257	
US24	21	14	32	3	34	13	60	ST261	
US34	11	70	33	10	50	13	61	ST263	
US35	6	71	7	3	49	60	7	ST264	
US37	18	47	8	3	13	3	62	ST265	

Table 4.2 Sequence types (STs) identified in this study and have been found in other locations in the world.

ST	Current analysis		Prior analyses	
	Host	**Location(s)**	**Host**	**Location(s)**
9	*O. kisutch*	OR; WA	*O. kisutch*	Oregon (Nicolas et al. 2008) British Columbia (Nicolas et al. 2008) Chile (Nicolas et al. 2008; Avendaño-Herrera et al, 2014) Japan (Fujiwara-Nagata et al. 2013)
10	*O. mykiss*	ID; NC; NM; UT; WV	*O. mykiss*; *Salvelinus* sp.; tank water	Idaho (Nicolas et al. 2008) Oregon (Nicolas et al. 2008) Chile (Avendaño-Herrera et al. 2014) Denmark (Inger Dalsgaard http://pubmlst.org/fpsychrophilum/; Nilsen et al. 2014) Finland (Tom Wiklund http://pubmlst.org/fpsychrophilum/) Scotland (Nicolas et al. 2008) Spain (Nicolas et al. 2008) Switzerland (Strepparava et al. 2013) Sweden (Nilsen et al. 2014) Japan (Nicolas et al. 2013)
13	*O. kisutch*	MI	*O. kisutch*; *Salmo trutta*	Washington (Nicolas et al. 2008) Finland (Tom Wiklund http://pubmlst.org/fpsychrophilum/) Japan (Nicolas et al. 2008)
29	*O. tshawytscha*	MI	*O. tshawytscha*	Oregon (Nicolas et al. 2008)

Table 4.2 (cont'd)

ST	Current analysis		Prior analyses	
	Host	Location(s)	Host	Location(s)
30	*O. kisutch*	OR; WA	*O. kisutch*	Japan (Nicolas et al. 2008; Fujiwara-Nagata et al. 2013)
31	*O. mykiss*	MI	*O. mykiss*	Denmark (Inger Dalsgaard http://pubmlst.org/fpsychrophilum/) Switzerland (Nicolas et al. 2008; Strepparava et al. 2013)

Table 4.3 *Flavobacterium psychrophilum*-specific primer sequences used to amplify 7 loci for multilocus sequence typing analysis.

	Primer sequence (5'-3')		Expected amplicon length (bp)
Tryptophane synthetase, β subunit (*trpB*)	Forward	CAGGAAACAGCTATGACCAAGATTATGTAGGCCGCCC	789
	Reverse	TGTAAAACGACGGCCAGTTGATAGATTGATGACTACAATATC	
DNA gyrase, β subunit (*gyrB*)	Forward	CAGGAAACAGCTATGACCGTTGTAATGACTAAAATTGGTG	1077
	Reverse	TGTAAAACGACGGCCAGTCAATATCGGCATCACACAT	
Chaperone heat shock protein 70 (*dnaK*)	Forward	CAGGAAACAGCTATGACCAAGGTGGAGAAATTAAAGTAGG	873
	Reverse	TGTAAAACGACGGCCAGTCCACCCATAGTTTCGATACC	
Fumarate hydratase class II (*fumC*)	Forward	CAGGAAACAGCTATGACCCCAGCAAACAAATACTGGGG	750
	Reverse	TGTAAAACGACGGCCAGTGGTTTACTTTTCCTGGCATGAT	
Glycosyltransferase murein G (*murG*)	Forward	CAGGAAACAGCTATGACCTGGCGGTACAGGAGGACATAT	681
	Reverse	TGTAAAACGACGGCCAGTGCATTCTTGGTTTGATGGTCTTC	
Elongation factor Tu (*tuf*)	Forward	CAGGAAACAGCTATGACCGAAGAAAAAGAAAGAGGTATTAC	795
	Reverse	TGTAAAACGACGGCCAGTCACCTTCACGGATAGCGAA	
ATP synthetase, α subunit (*atpA*)	Forward	CAGGAAACAGCTATGACCCTTGAAGAAGATAATGTGGG	834
	Reverse	TGTAAAACGACGGCCAGTTGTTCCAGCTACTTTTTTCAT	

Table 4.4 MLST sequence types (STs) in this study summarized by fish host species. Numbers in parentheses indicate the number of isolates identified as that ST in this study.

Salmonid host species	STs unique to fish host species	STs found in more than 1 fish host species
O. mykiss	ST10 (23); ST78 (15); ST82 (4); ST31 (2); ST84 (2); ST81 (1); ST83 (1); ST86 (1); ST253 (1); ST257 (1); ST267 (1)	ST256 (2)
O. tshawytscha	ST29 (4); ST76 (2); ST255 (2); ST259 (2); ST260 (2); ST262 (2); ST266 (2); ST250 (1); ST251 (1); ST254 (1); ST261 (1); ST263 (1); ST265 (1)	ST256 (4)
O. kisutch	ST258 (4); ST9 (3); ST13 (2); ST30 (2); ST252 (1); ST74 (1); ST75 (1); ST77 (1); ST264 (1)	

Figure 4.1 The number of *F. psychrophilum* isolates from each location where samples were collected in the United States. Isolates were recovered from three *Oncorhynchus* spp. *O. mykiss* (n = 54), *O. tshawytscha* (n = 26), and *O. kisutch* (n = 16).

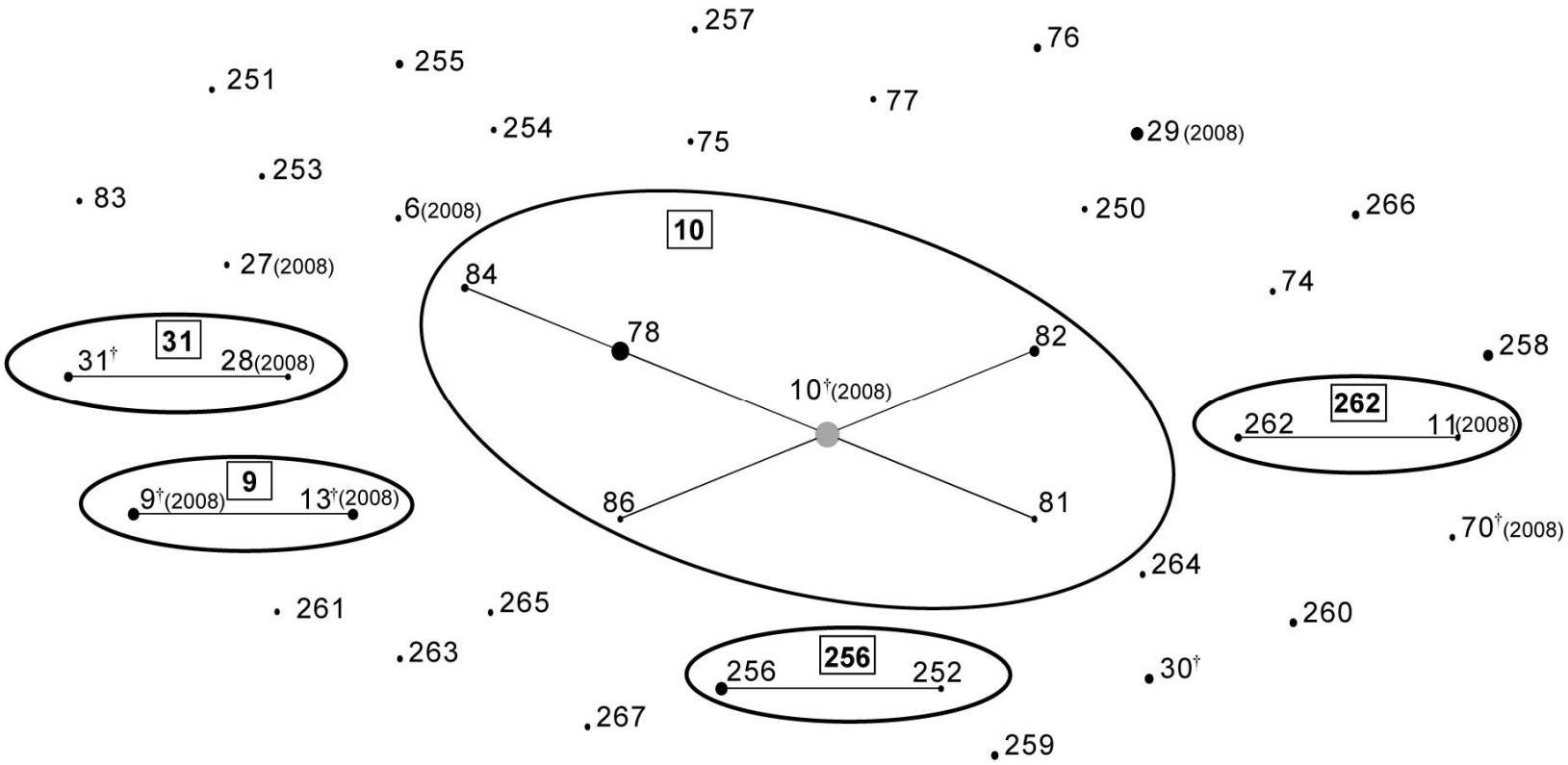

Figure 4.2 eBURST diagram depicting the relationships of the 96 U.S.A. *F. psychrophilum* isolates of this study and the 10 previously typed U.S.A. isolates (Nicolas et al. 2008). Sequence types (STs) followed by (2008) denote isolates previously typed by Nicolas et al. (2008). STs 9, 10, 13, and 29 were detected in the U.S.A. in both the current U.S.A. study as well as in Nicolas et al. (2008). †: STs found in both the U.S.A. and abroad. All other isolates are unique to the U.S.A. Light gray denotes the predicted founder ST. Clonal complexes (CC; numbers within rectangles) are named after the predicted founding ST. In CCs composed of two STs, the CC is named after the most abundant ST; if both STs are equally represented, then the CC is named for the earliest found ST.

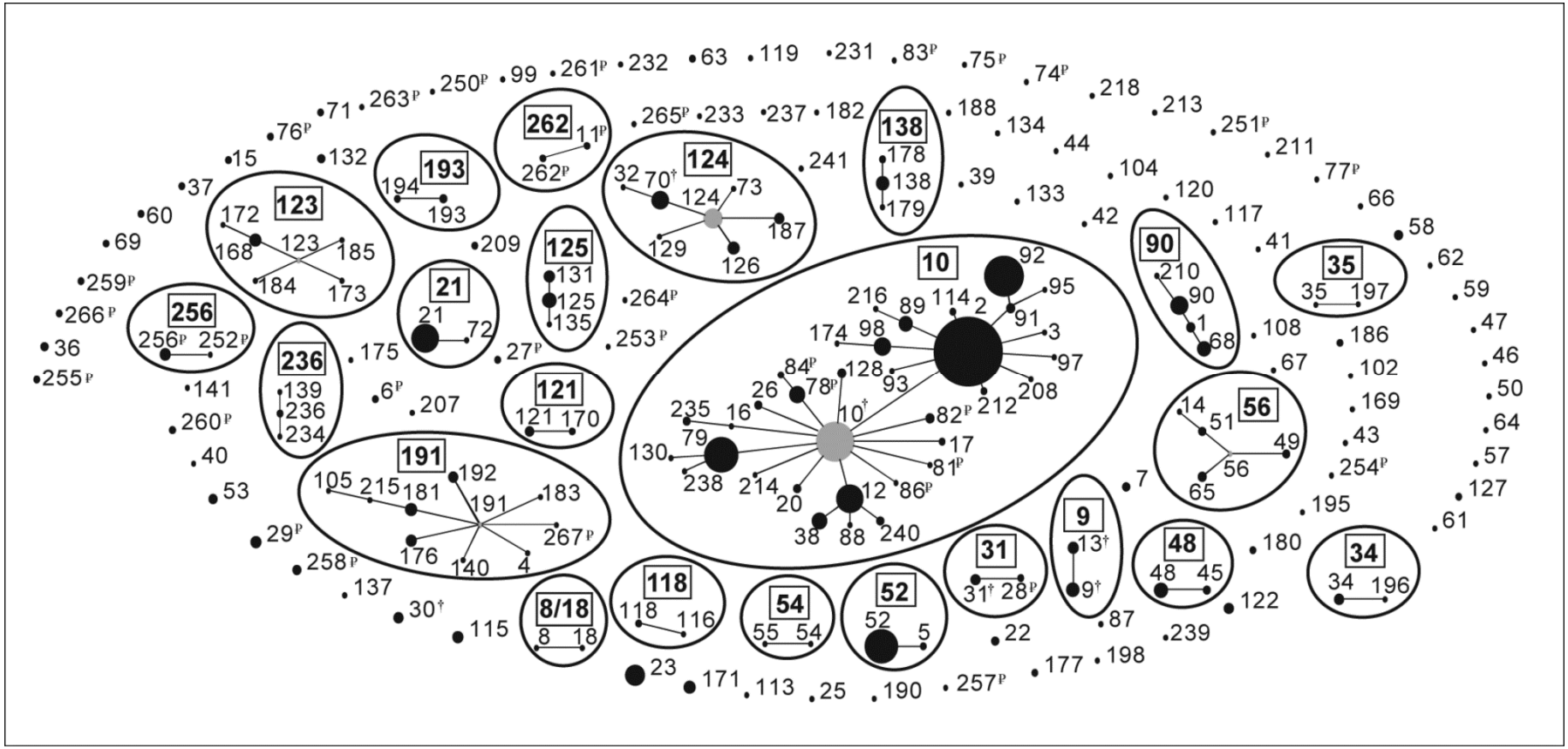

Figure 4.3 eBURST diagram depicting the relationships among global *F. psychrophilum* isolates, including the isolates of this North American study (n = 1091). †: Sequence types (STs) found in both North America and abroad. Ᵽ: STs currently present only in North America. Light gray denotes predicted founder ST. Clonal complexes (CC; numbers within rectangles) are named after the predicted founding ST. In CCs composed of two STs, the CC is named after the most abundant ST; if both STs are equally represented, then the CC is named for the earliest found ST.

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Chapter 5

Antimicrobial susceptibilities of *Flavobacterium psychrophilum* isolates from the Great Lakes basin, Michigan, U.S.A.

1. Abstract

Flavobacterium psychrophilum devastates salmonids worldwide, a matter that is compounded by the lack of an approved vaccine. As a result, biosecurity measures and antimicrobial agents remain the only available methods to control diseases caused by *F. psychrophilum.* Continuous antimicrobial use may have led to the emergence of resistant *F. psychrophilum* strains. Therefore, the primary objective of this study was to determine the antimicrobial susceptibility profiles of 50 *F. psychrophilum* isolates from Michigan in response to 10 antimicrobial compounds that are being used in the treatment of bacterial infections. As advocated by the World Organization of Animal Health (OIE), a standardized microdilution broth assay has been employed to determine the minimum inhibitory concentrations (MICs) for ampicillin, gentamicin, enrofloxacin, oxolinic acid, flumequine, trimethoprim-sulphamethoxazole, ormetoprim-sulphadimethoxine, erythromycin, florfenicol, and oxytetracycline. Epidemiological cut-off values were calculated using both the normalized resistance interpretation (CO_{NRI}) and the ECOFFinder analysis method (CO_{ECOFF}), which were in agreement 50% of the time. The MIC distributions in response to oxytetracycline exhibited bimodality, indicating both susceptible and resistant isolates present in Michigan. The oxytetracycline epidemiological cut-off values (CO_{ECOFF} <0.06 μg mL^{-1}; CO_{NRI} <0.12 μg mL^{-1}) demonstrated that 24% of Michigan isolates are resistant to this commonly used drug. No other antimicrobial exhibited a bimodal distribution of MICs. The data generated in this study represents the first report of antimicrobial susceptibility assessment of United States *F. psychrophilum* strains, a matter that will contribute to the worldwide validation efforts to determine universal epidemiological cut-off values of this deadly fish pathogen.

2. Introduction

The Gram-negative bacterium *Flavobacterium psychrophilum* is the causative agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS), both of which devastate farmed and free-ranging salmonid populations worldwide (Starliper 2011). Despite the efforts to develop a vaccination strategy to prevent *F. psychrophilum* infections in North America, an effective, licensed vaccine is still lacking (Gomez et al. 2014). Although other preventative measures, such as broodstock culling (Long et al. 2014), and the development of a genetically resistant rainbow trout line have been under investigation (Silverstein et al. 2009; Leeds et al. 2010; Wiens et al. 2013), antimicrobial treatments remain the only choice currently available for controlling these infections and minimizing economic losses. In the United States (U.S.), it's estimated that 150 pounds of antimicrobials are used per acre of salmon harvested (FAO 2005), and only two antibiotics (e.g., oxytetracycline and florfenicol) are approved by the U.S. Food and Drug Administration (US-FDA) to treat *F. psychrophilum* infections in food fish in the U.S. (USFWS-AADAP).

The intense use of antimicrobial agents in the ever-growing aquaculture industry is believed to play a key role in the emergence of resistant strains of fish-pathogenic bacteria (FAO 2005; Cabello 2006; Sekkin and Kum 2011). Over the past decade, there have been reports of antimicrobial resistance associated with *F. psychrophilum* strains from Europe, Chile, and Canada (Bruun et al. 2000; Dalsgaard and Madsen 2000; Kum et al. 2008; Hesami et al. 2010; Henriquez-Nunez et al. 2012), however, there is no information currently available on antimicrobial susceptibility of *F. psychrophilum* in the United States in general and the Great Lakes basin in particular. This constitutes a void in our knowledge since salmonid populations in

Michigan are known to be widely and heavily infected with *F. psychrophilum,* and Michigan aquaculture facilities frequently rely on antimicrobial treatments to control these infections (Van Vliet et al. 2015). Therefore, the first objective of this study was to investigate the antimicrobial susceptibility profiles of *F. psychrophilum* isolates from the section of the Great Lakes basin within Michigan.

The variation in assessing antimicrobial susceptibility of bacterial pathogens urged the World Organization for Animal Health (OIE) to advocate for the development of an accurate and standardized antimicrobial susceptibility testing protocol that can be used worldwide (OIE Aquatic Animal Health Code). In fulfillment of the goals set by OIE, the Clinical and Laboratory Standard Institute published guidelines to ensure that antimicrobial susceptibility profiles of aquatic bacterial pathogens, including *F. psychrophilum,* are being performed in a standardized manner (Gieseker et al. 2012; CLSI 2014a; CLSI 2014b). This optimized microdilution broth assay generates data necessary to establish epidemiological cut-off values that could be used to interpret bacterial susceptibility and identify the potential risk of antimicrobial resistance. A major reason for the lack of universally approved *F. psychrophilum*-specific epidemiological cut-off values is the limited data available for accurate calculations (CLSI 2014a; Smith et al. 2016).

To this end, we build on the studies of Smith et al. (2016) performed by two European laboratories following CLSI guidelines (CLSI 2014a; CLSI 2014b) to calculate epidemiological cut-off values from Michigan *F. psychrophilum* minimum inhibitory concentration (MIC) data and compare these results to the currently proposed values. The generated data of this study are important in the validation of epidemiological cut-off values that can be internationally adopted.

3. Materials and Methods

3.1. *F. psychrophilum* collection and identification

The antibiotic susceptibility profiles of 50 *F. psychrophilum* isolates originating from both feral (n = 36) and captive (n = 14) salmonid populations from six Great Lakes basin locations were analyzed in this study (Table 5.1; Fig. 5.1). Isolates from adult feral Chinook salmon (*O. tshawytscha*) or their eggs were recovered from the Swan River weir (SRW; Presque Isle County; Lake Huron watershed; n = 12), and the Little Manistee River weir (LMRW; Manistee County; Lake Michigan watershed; n = 12). Isolates originating from adult feral steelhead (*O. mykiss*; n = 4) were also collected from LMRW. Additionally, isolates from adult feral Coho salmon (*O. kisutch*) or their eggs were recovered from Platte River weir (PRW; Benzie County; Lake Michigan watershed; n = 8). Isolates from captive *O. mykiss* populations were recovered from Wolf Lake State Fish Hatchery (WLSFH; Van Buren County; Lake Michigan watershed; n = 9), Thompson State Fish Hatchery (TSFH, Schoolcraft County; Lake Michigan watershed; n = 4), and Oden State Fish Hatchery (OSFH, Emmett County; Lake Michigan watershed, n = 1). Isolates were recovered from multiple tissues (e.g., kidneys, external lesions, brains, gametes/eggs) from the years 2008-2013. The majority of fish examined exhibited gross disease signs commonly associated with BCWD (e.g., muscle ulceration, fin erosion, exophthalmia, and/or swollen internal organs); however some isolates were occasionally recovered from apparently healthy fish. All tissues were collected aseptically and cultured immediately onto cytophaga agar (CA; Anacker and Ordal 1959) supplemented with neomycin sulfate at 4 mg l^{-1}, and incubated at 15°C for 72-144 hours, as per the guidelines of the

American Fisheries Society – Fish Health Section (AFS-FHS 2012). Suspect *F. psychrophilum* isolates underwent confirmatory testing using an *F. psychrophilum* specific PCR assay (Toyama et al. 1994) or a PCR assay using universal degenerate primers (Marchesi et al. 1998) and subsequent Sanger sequencing, as previously described (Loch et al. 2013; Van Vliet et al. 2015). Isolates were cryogenically frozen in CA broth supplemented with 20% glycerol at -80°C for future analyses. MLST analysis has been previously conducted on these 50 isolates, and their STs are known (Van Vliet et al. in press). Epidemic isolates/STs refer to isolates that were collected during periods of morbidity and/or mortality characteristic of BCWD.

3.2. Antimicrobial susceptibility testing

3.2.1. Antimicrobial agents

The MICs of 10 drugs were assessed using custom-designed Sensititre susceptibility plates (Trek Diagnostic Systems, Oakwood Village, OH) prepared with dilute cation adjusted Mueller Hinton broth (DCAMHB; 4 g L^{-1}). The 96-well plates contained twofold serial dilutions of antimicrobials from multiple drug classes. Tetracyclines were represented by 10 concentrations of oxytetracycline (OXY; 0.015-8 µg mL^{-1}), phenicols were represented by 10 concentrations of florfenicol (FFN; 0.03-16 µg mL^{-1}), sulphonamides/potentiated sulphonamides were represented by 10 concentrations of ormetoprim-sulphadimethoxine (PRI; 0.008/0.15-4/76 µg $mL^{-}1$) and 7 concentrations of trimethoprim-sulphamethoxazole (SXT; 0.015/0.3-1/19 µg mL^{-1}). All of the aforementioned drugs are approved for aquaculture use in North America (USFWS AADAP; Canadian Food Inspection Agency, CFIA). Due to the common practice of extra-label use of erythromycin under the direction of a veterinarian in aquaculture, this antimicrobial

compound was used to represent the macrolide drug class (ERY; 0.25-128 µg mL^{-1}). Quinolones/fluoroquinolones were represented by 10 concentrations each of oxolinic acid (OXO; 0.004-2 µg mL^{-1}), flumequine (FLUQ; 0.008-4 µg mL^{-1}), and enrofloxacin (ENRO; 0.002-1 µg mL^{-1}). Aminoglycosides were represented by gentamicin (GEN; 7 concentrations; 0.06-4 µg mL^{-1}), aminopenicillins were represented by ampicillin (AMP; 10 concentrations; 0.03-164 µg mL^{-1}).

3.2.2. Broth dilution preparation

All broth dilution preparations were performed according to CLSI (2014a) in a sterile hood. Briefly, *F. psychrophilum* isolates were revived from cryogenically frozen stock on fresh tryptone yeast extract salts (TYES; Holt 1987) plates and incubated at 18°C for 72-96 hrs. Isolates were then subcultured in 5 mL of fresh TYES broth and grown statically at 18°C for 72 hrs. The suspensions were then adjusted to a 0.5 McFarland standard turbidity-equivalent with the addition of sterile 0.85% saline solution. An aliquot of the suspension (220 µL) was then transferred to 11 mL of sterile DCAHMB (Becton, Dickson Company, Franklin Lakes, NJ), vortexed, and 50 µL inoculated into 96-well plates within 15 minutes of preparation. Control wells contained no antimicrobial compounds. The negative control well received 50 µL of sterile DCAMHB, and the positive growth control well received 50 µL of inoculum. Each plate was then covered with an adhesive seal and placed in an 18°C incubator stacked no more than 4 plates high to allow for proper ventilation and airflow. To ensure the appropriate inocula concentration (e.g., 5.0 X 10^5 cfu mL^{-1}, as recommended by the manufacturer), colony counts were performed from each plate via serial dilution. After 96 hrs of incubation, Sensititre plates

were visually examined for the presence of bacterial growth in each well. The MICs were recorded as the lowest concentration of drug that inhibited detectable growth of bacteria (CLSI 2014a). As noted by CLSI, antagonists in the medium with SXT and PRI may allow some slight bacterial growth, because of this the MIC recorded was the concentration that inhibited ~50% of growth in relation to the positive control well (CLSI 2014a). Quality control strains ATCC 25922^T of *Escherichia coli* and ATCC 33658^T of *Aeromonas salmonicida* subsp. *salmonicida* were prepared alongside each group of *F. psychrophilum* isolates in a similar manner with minor modifications. The growth media used to culture quality control strains prior to inoculation in DCAMHB was tryptone soya agar with 5% sheep's blood. Furthermore, to adjust the suspensions of the quality control strains to a turbidity equivalent to a 0.5 McFarland standard, isolated colonies were removed from an agar plate and added to 5 mL sterile 0.85% saline solution until the desired concentration was reached, upon which 110 µL was then added to 11 mL DCAMHB.

3.2.3. Epidemiological cut-off values

As suggested by Smith et al. (2016), the acronym ECV should be reserved for cut-off values set officially by CLSI and the acronym ECOFF should be reserved for cut-off values set officially by The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Because the calculated epidemiological cut-off values generated in this study have not yet been accepted by CLSI or EUCAST, the acronym CO_{WT} (cut-off wild type) will be used to denote the epidemiological cut-off value developed in this study. Specifically, CO_{WT} values that were

calculated by the NRI method will be denoted CO_{NRI}, and CO_{WT} values that were calculated by the ECOFFinder analysis will be denoted CO_{ECOFF}.

Epidemiological cut-off values (CO_{WT}) were calculated by the normalized resistance interpretation (NRI) method (Kronvall 2010; CO_{NRI}). The settings used included 97.7% of the putative wild type isolates. The NRI values are continuous variables; however they are expressed as ordinal categories with values relating to the concentrations used in the dilutions of the test protocol. The experimentally determined cut-off values were rounded up to the next highest dilution category. The NRI method was used with permission from the patent holder, Bioscand AB, TÄBY, Sweden (European patent No 1383913, US Patent No. 7,465,559).

CO_{WT} values were also calculated using the ECOFFinder analysis as based on the methods of Turnidge et al. (2006; CO_{ECOFF}), and provided by CLSI (http://clsi.org/standards/micro/ecoffinder/). The settings used for this method included 99% of the putative wild type isolates.

3.3. PCR of tetracycline genes

All isolates were further investigated on a genetic basis to identify antibiotic resistance genes (ARGs) associated with oxytetracycline resistant phenotypes. For assessing the presence of ARGs, DNA extraction was performed on all isolates as previously described. The identification of tetracycline resistance genes was performed using PCR amplification with specific primers (Table 5.2) that have been previously described (Aareustrup et al. 2000; Schmidt et al. 2001; Macauley et al. 2007). The genes investigated in this study were *tetA, tetE, tetH,* and *tetM.* All genes have been previously recognized as tetracycline resistant genes and

have been associated with resistant phenotypes in other *Flavobacteria* (Akinbowale et al. 2007; MaCauley et al. 2007). All PCR reactions were performed in a Mastercyler® Gradient Thermalcycler (Eppendorf, Hauppauge, New York) with a total reaction volume of 25 or 50 µL for each sample. For *tetA* and *tetM*, each 25 µL reaction consisted of 12.5 µL of 2x Go-Taq Green master mix (Promega, Madision WI), 20 ng of DNA template, 20 ng of each primer, and nuclease-free water for the remainder of the reaction. For *tetE*, each 50 µL reaction consisted of 25 µL of 2x Go-Taq Green master mix (Promega, Madision WI), 40 ng of DNA template, 40 ng of each primer, and nuclease-free water for the remainder of the reaction. For *tetH*, each 25 µL reaction consisted of 12.5 µL of 2x Go-Taq Green master mix (Promega, Madision WI), 20 ng of DNA template, 30 ng of each primer, and nuclease-free water for the remainder of the reaction. Amplification of *tetA* and *tetE* was performed with the following PCR parameters: initial denaturation at 95°C for 5 min, followed by 23 cycles of 30 s at 95°C, 30 s at 62°C and 45 s at 72°C. A final elongation step at 72°C for 7 min was performed. Amplification of *tetH* was performed with the following PCR parameters: initial denaturation at 94°C for 5 min, followed by 25 cycles of 5 s at 94°C, and 30 s at 61°C, with a final elongation step at 61°C for 7 min. Amplification of *tetM* was performed with the following PCR parameters: initial denaturation at 95°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min 30 s at 72°C. A final elongation step at 72°C for 7 min was performed. All products were combined with SYBR® Green gel stain (Cambrex Bio Science Rockland, Inc., Rockland, Maine) and run on 1.5% agarose gel and electrophoresed for 35 min at 100 V. A 1-kb Plus DNA ladder (Life Technologies, Grand Island, New York) was used as a molecular marker. Amplicons were then visualized under UV exposure (UVP, LCC, Upland, California). The *E. coli* strain R08 was used as a positive control for

tetA (provided by Dr. Shannon Manning, Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI).

4. Results

4.1. Antimicrobial susceptibility profiles and epidemiological cut-off values.

The MIC values produced by the two quality control strains grown at 18°C were within accepted CLSI ranges (CLSI 2014b). The MIC range, MIC_{50}, MIC_{90}, and CO_{WT} values for each antimicrobial tested are listed in Table 5.3. The MIC for each antimicrobial agent varied between isolates and their classified STs (Table 5.4).

4.1.1. Oxytetracycline

For oxytetracycline the CO_{NRI} value was 0.125 μg mL^{-1}, and the CO_{ECOFF} value was 0.06 μg/mL μg mL^{-1} (Table 5.3). A bimodal MIC distribution in response to oxytetracycline was observed (Fig. 5.2). The first group (group 1) consisted of 38 isolates (76%) inhibited by concentrations of oxytetracycline ranging from 0.015-0.06 μg mL^{-1}, and the second (group 2) contained 11 isolates (22%) inhibited by concentrations of oxytetracycline $\geq$4 μg mL^{-1} (Fig. 5.2). In fact, the MIC for a single epidemic isolate (US32, ST78; Table 5.4) exceeded the highest employed oxytetracycline concentration. The majority of isolates requiring higher concentrations of oxytetracycline were closely related epidemic STs (Table 5.4); however three non-epidemic isolates also had high oxytetracycline MICs (e.g., US09, ST253; US28, ST31; and US29, ST31).

To determine if MIC values for oxytetracycline were associated with MIC values for other antimicrobials, scatter plots were constructed (Fig. 5.3). However, no associations were observed.

4.1.2. Florfenicol

The CO_{WT} value calculated from the florfenicol MIC data by both methods was 2 μg mL^{-1} (Table 5.3). Isolates exhibited an MIC range of 0.25-1 μg mL^{-1} in response to florfenicol (Table 5.3; Fig. 5.2). The most frequently observed MIC value was 0.5 μg mL^{-1} (n = 25, 50%), however the next highest concentration of 1 μg mL^{-1} was also highly represented (n = 24, 48%). Both concentrations contained epidemic and non-epidemic isolates (Fig. 5.2).

4.1.3. Ormetoprim-sulphadimethoxine

The CO_{WT} values calculated from the ormetoprim-sulphadimethoxine MIC data were 2/38 μg mL^{-1} (CO_{NRI}) and 1/19 μg mL^{-1} (CO_{ECOFF}; Table 5.3). A broad range of ormetoprim-sulphadimethoxine concentrations (0.015/0.3-1/19 μg mL^{-1}) was required for isolate inhibition (Table 5.3). The most frequently observed MIC value was 0.12/2.38 μg mL^{-1}, and consisted of both epidemic and non-epidemic isolates (Fig. 5.2). All isolates had MICs below the CO_{WT} values calculated by both methods (Fig. 5.2), however, a single isolate (US17, ST78) was approaching the CO_{NRI} with an MIC of 1/19 μg mL^{-1} (Table 5.4). Furthermore, the majority of isolates with lower MIC values consisted of non-epidemic isolates, and as ormetoprim-sulphadimethoxine concentrations increased, the proportion of epidemic isolates increased as well (Fig. 5.2).

4.1.4. Erythromycin

The CO_{NRI} and CO_{ECOFF} values were both 8 µg mL^{-1} when calculated from erythromycin MIC data (Table 5.3), and all isolates fell below this threshold (Fig. 5.2). The erythromycin MICs ranged from 0.5-4 µg mL^{-1} with the most frequently observed MIC of 2 µg mL^{-1} (n = 21, 42%; Table 5.3; Fig. 5.2). Both epidemic and non-epidemic isolates had broad MIC values in response to erythromycin (Fig. 5.2).

4.1.5. Oxolinic acid

Both the CO_{NRI} and CO_{ECOFF} value calculated from the oxolinic acid data was 0.25 µg mL^{-1} (Table 5.3). All isolates fell below the CO_{WT} as the range of MICs was 0.06-0.25 µg mL^{-1} (Table 5.3; Fig. 5.4). The majority of isolates had an MIC of 0.12 µg mL^{-1} (n = 31, 62%; Fig. 5.4). No association between MIC and whether the isolate was epidemic or not was observed, as both epidemic and non-epidemic isolates were distributed among all MIC values produced (Fig. 5.4).

4.1.6. Flumequine

The CO_{WT} value calculated from the flumequine MIC data was 0.12 µg mL^{-1} (CO_{NRI} and CO_{ECOFF}; Table 5.3). Flumequine MICs were unimodal (0.03-0.12 µg mL^{-1}) and all fell below the CO_{WT} value (Table 5.3; Fig. 5.4). The majority of isolates (epidemic and non-epidemic) had an MIC value of 0.06 µg mL^{-1} (n = 31, 62%; Fig. 5.4). Two non-epidemic isolates had the highest MIC value (0.12 µg mL^{-1}; US22, ST260; US34, ST263; Table 5.4).

4.1.7. Trimethoprim-sulphamethoxazole

Although the CO_{WT} values were different based on the method used (CO_{NRI} 0.5/9.5 µg mL^{-1}; CO_{ECOFF} 0.25/9.5 µg mL^{-1}; Table 5.3), all isolates were below both thresholds as the MIC values ranged 0.015/0.3-0.25/4.75 µg mL^{-1} (Table 5.3). The most frequently observed MIC was 0.06/1.19 µg mL^{-1} (n = 18, 36%) and encompassed both epidemic and non-epidemic isolates (Fig. 5.4). However, the lowest MIC value (0.015/0.3 µg mL^{-1}) did not contain any epidemic isolates, and the highest MIC value (0.25/4.75 µg mL^{-1}) did not contain any non-epidemic isolates (Fig. 5.4).

4.1.8. Enrofloxacin

The CO_{NRI} for enrofloxacin was 0.03 µg mL^{-1} and the CO_{ECOFF} was 0.015 µg mL^{-1} (Table 5.3). Isolates ranged in MICs from <0.002-0.015 µg mL^{-1} (Table 5.3). All but one isolate (non-epidemic US37, ST265; Table 5.4) produced an MIC value below the CO_{ECOFF} value (0.015 µg mL^{-1}), and all isolates were below the CO_{NRI} value (0.03 µg mL^{-1}; Fig. 5.4). The most frequently observed MIC was 0.008 µg mL^{-1} (n = 25, 50%; Fig. 5.4). Enrofloxacin MICs of 10% of the isolates (n = 5), which all belonged to ST256, could not be determined (Table 5.4).

4.1.9. Gentamicin

The large percentage of putative wild type isolates did not allow for the calculation of statistically valid CO_{WT} values (NRI or ECOFF; Fig. 5.4). The MICs for gentamicin could not be determined for 24% (n = 12) of the samples, which were composed of both epidemic and non-

epidemic isolates (Table 5.4). The most frequently observed MIC was 0.12 μg mL^{-1} (n = 17, 34%) and encompassed both epidemic and non-epidemic isolates.

4.1.10. Ampicillin

The ampicillin MICs of 92% (n = 46) of the isolates could not be determined with the drug concentrations used in the Sensititre plates (i.e., lower drug concentrations required). The remaining 4 isolates, which were non-epidemic, were inhibited by ampicillin values of 0.06 μg $mL^{-1}$and belonged to ST13 (n = 2) and ST260 (n =2; Fig. 5.4; Table 5.4). Because of the large percentage of putative wild type isolates, statistically valid CO_{WT} values could not be calculated using either method (NRI or ECOFF; Table 5.3).

4.1.11. Comparison between CO_{NRI} and CO_{ECOFF} values

Epidemiological cut-off values were calculated for 8/10 antimicrobial MIC distributions. Among these, the CO_{NRI} and CO_{ECOFF} values were in agreement 50% of the time (n = 4), producing identical CO_{WT} values for oxolinic acid (2 μg mL^{-1}), flumequine (0.12 μg mL^{-1}), erythromycin (8 μg mL^{-1}), and florfenicol (2 μg mL^{-1}; Table 5.3). For enrofloxacin, trimethoprim-sulphamethoxazole (SXT), ormetoprim-sulphadimethoxine (PRI), and oxytetracycline, the CO_{NRI} and CO_{ECOFF} values were in disagreement (Table 5.3). For the cases in which CO_{NRI} and CO_{ECOFF} values were different, the CO_{ECOFF} value was consistently lower by 1 dilution than the CO_{NRI} value.

4.2. *tet* gene detection

No detection of *tetA, tetE, tetH,* or *tetM* in any of the tested *F. psychrophilum* isolates occurred.

5. Discussion

Of all antimicrobial agents examined in this study, oxytetracycline is the only drug in which *F. psychrophilum* isolates from Michigan have shown resistance to at this time. Oxytetracycline has long been widely used to treat *F. psychrophilum* infections worldwide (Bruun et al. 2000; Cipriano and Holt 2005; Hesami et al. 2010), and is in fact the most used antibiotic in North American aquaculture (Shao 2001; Soule et al. 2005). This study identifies the first report of oxytetracycline resistance in *F.psychrophilum* isolates retrieved from both free-ranging and aquacultured Michigan salmonid populations. However, the majority of the Michigan *F. psychrophilum* isolates resistant to oxytetracycline were recovered from captive salmonid populations. This may indicate that the resistance risk stems from the use of this compound in aquaculture settings. Treatment with oxytetracycline in Michigan aquaculture facilities occurs frequently, which has provided many opportunities for repeated exposure to these isolates. Although hatcheries are disinfected between rearing seasons, *F. psychrophilum* may linger in areas of hatchery infrastructure that are rarely completely disinfected, increasing the risk that these isolates will re-emerge and perpetuate in the hatchery system.

Because of the MLST work previously done on these GLB isolates, we are able to identify that the majority of the isolates resistant to oxytetracycline are genetically identical and are and represent one ST (i.e., ST78; Van Vliet et al. in press). These ST78 isolates (and their closely

related single-locus variants) are also frequently associated with BCWD mortality events, and are considered highly virulent to rainbow trout around the world (Strepparava et al. 2013; Nilsen et al. 2014; Van Vliet et al. in press). In general, our data suggest a correlation between highly virulent isolates and their resistance to oxytetracycline. Sundell and Wiklund (2015) reached a similar conclusion using European isolates. Oxytetracycline resistance, however, has also been found in isolates retrieved from apparently healthy fish (i.e., US09, US28, and US29). Additionally, some epidemic-associated isolates have demonstrated wild type susceptibility to oxytetracycline, a matter that cannot be explained solely by data generated in this study.

Fortunately, the majority of Michigan *F. psychrophilum* isolates are not showing resistance to the other antimicrobials examined in this study. However, one isolate in the case of enrofloxacin showed resistance as revealed by their CO_{ECOFF} values of 0.015 µg mL^{-1}. Similarly, by way of a comparable broth dilution assay, Hesami et al. (2010) demonstrated that >80% of *F. psychrophilum* isolates recovered in Ontario showed reduced susceptibility to oxolinic acid. None of these antimicrobials are commonly used in North American salmonid aquaculture, and these perplexing results warrant further investigation.

Although a similar study has been conducted in Canada (Hesami et al. 2010), the contrasting results from this study indicate the importance of continued investigation of resistance patterns of *F. psychrophilum* isolates from different geographic regions. Canadian isolates are showing resistance to both flumequine and ampicillin, however no GLB isolates from the sampling sites in this study were found to have similar results. In fact, the majority of Michigan isolates required ampicillin concentrations lower than what were used during analyses to determine the MICs. While the mechanism for this may remain unknown, these

findings demonstrate the variability of resistance patterns of *F. psychrophilum* isolates, even from adjacent geographical regions.

Antimicrobial resistance can occur through various mechanisms, with genetics playing an important role. As previously discussed, ST78 isolates are commonly associated with BCWD outbreaks and are frequently resistant to oxytetracycline, however the exact mechanism(s) linking the apparent oxytetracycline resistance to virulence is sorely needed and could help guide future control efforts of this pathogen. On the contrast, ST256 isolates were shown to have increased susceptibility to enrofloxacin, whereby the concentrations used in this study were not low enough to determine MICs for 5/6 ST256 isolates. Enrofloxacin is a bactericidal agent that works by inhibiting DNA gyrase and topoisomerase (Sekkin and Kum 2011). One of the housekeeping genes used in the MLST scheme is *gyrB* (Van Vliet et al. in press) and ST256 isolates possess an allele type (AT) for this locus that is relatively rare in the *F. psychrophilum* population for which MLST data is available (http://pubmlst.org/fpsychrophilum/). This particular AT could be linked to sensitivity to enrofloxacin. Furthermore, antibiotic resistance can occur by possession of antibiotic resistance genes. Although *tetA, tetE, tetH,* and *tetM* are some of the most widespread tetracycline resistance genes (Roberts 2005), and have been shown to be prevalent in bacterial strains isolated from aquaculture facilities, including present in *Flavobacterium* sp. (Akinbowale et al. 2007; MaCauley et al. 2007), the results of this study indicate that these particular *tet* genes do not currently circulate in the *F. psychrophilum* population of the GLB.

Epidemiological cut-off values were calculated using two statistical approaches (NRI and ECOFFinder; Turnidge et al. 2006; Kronvall 2010; Kronvall et al. 2011). In some cases (i.e.,

oxolinic acid, flumequine, erythromycin, and florfenicol), both the CO_{NRI} and CO_{ECOFF} methods were in agreement and produced the same CO_{WT} value. For the antimicrobials in which the MIC distributions produced variable CO_{WT} values dependent on the method used, the CO_{ECOFF} method consistently produced values lower by 1 dilution than that of the CO_{NRI} method. Because of this disagreement between the two CO_{WT} calculation methods in regards to enrofloxacin, the MIC distributions included isolates that varied in their wild type/resistance status depending on the CO_{WT} calculation methods. Conversely, even though the CO_{NRI} and CO_{ECOFF} values were different for the trimethoprim-sulphamethoxazole, ormetoprim-sulphadimethoxine, and oxytetracycline distributions, all isolates produced MICs below both values. Since both methods are relatively recent, and are improvements upon the older subjective visual method (Kronvall et al. 2011), they should both continue to be used when calculating epidemiological cut-off values for a variety of bacterial species until a gold standard can be accepted.

As no epidemiological cut-off values are currently established for *F. psychrophilum* the interpretation and comparison of MIC data among studies is difficult. As the first attempt to generate these values specific for *F. psychrophilum*, Smith et al. (2016) examined 61 European isolates to produce preliminary values based on the NRI method. In comparison among preliminary values of Smith et al. (2016) and the CO_{NRI} values calculated solely on the Michigan data, agreement among florfenicol (2 µg mL^{-1}), oxytetracycline (0.125 µg mL^{-1}), and oxolinic acid (0.25 µg mL^{-1}) was found. The CO_{NRI} values for erythromycin and trimethoprim-sulphamethoxazole from Michigan isolates were one dilution lower than that proposed by Smith et al. (2016). Overall, isolates from both studies are seemingly responding in similar

manners to the antimicrobials examined. As more studies are conducted using the standardized test protocol described herein, the larger the dataset for which epidemiological cut-off values can be calculated from which will help to validate these important and greatly needed values. Upon acceptance of epidemiological cut-off values for *F. psychrophilum,* a full standard protocol will have been developed and allow for generation of comparable data from different geographical regions and time periods to assess the consequences of antimicrobial use in regards to *F. psychrophilum* (Smith et al. 2016).

The evidence of isolates with reduced susceptibility to commonly used drugs, particularly oxytetracycline, along with the lack of an approved vaccine (Gomez et al. 2014), increases the importance of other control methods. Strengthened biosecurity measures have been shown to help reduce the effects of *F. psychrophilum* infections (Oplinger and Wagner 2013; Van Vliet et al. 2015). Increased awareness of hatchery staff, and diligent caretaking can also help in identifying *F. psychrophilum* infections early enough to ward off massive mortalities. Because *F. psychrophilum* can be vertically transmitted from infected parent to progeny (Brown et al. 1997; Taylor 2004; Cipriano 2005), culling of heavily infected broodstock has seemingly shown success in reducing the vertical transmission component (Long et al. 2014). Furthermore, the development of a *F. psychrophilum* resistant strain of rainbow trout is also showing promise (Wiens et al. 2013).

For the first time, data generated on the antimicrobial susceptibility profiles of U.S. *F. psychrophilum* isolates using a standardized antimicrobial susceptibility testing protocol identifies the risk of oxytetracycline resistance in this region. The epidemiological cut-off value data produced in this study will contribute to the validation of these values worldwide and

provide a better means of interpretation and comparison across studies. Identification of resistance trends early can help mitigate the risk of further emergence, and thus it is recommended that antibiotic susceptibility profiling continue with *F. psychrophilum* isolates from many other regions using the standardized protocol employed in this study.

APPENDIX

Table 5.1 Characteristics of *Flavobacterium psychrophilum* isolates analyzed in this study. ST, sequence type; CC, clonal complex. [1]Isolate IDs with an asterisk indicate *F. psychrophilum* recovery during times of morbidity/mortality characteristic of bacterial coldwater disease [2]Isolates recovered from the same fish marked with the same superscript letter.

Isolate ID[1,2]	Year	Salmonid Species	GLB Location	ST
US07	2010	*O. kisutch*	PRW	ST13
US19	2010	*O. kisutch*	PRW	ST13
US41	2011	*O. tshawytscha*	LMRW	ST29
US04	2011	*O. tshawytscha*	SRW	ST29
US25	2011	*O. tshawytscha*	SRW	ST29
US55	2011	*O. tshawytscha*	SRW	ST29
US28	2010	*O. mykiss*	LMRW	ST31
US29	2010	*O. mykiss*	LMRW	ST31
US17*	2010	*O. mykiss*	WLSFH	ST78
US26*	2010	*O. mykiss*	WLSFH	ST78
US32*	2010	*O. mykiss*	WLSFH	ST78
US45*	2010	*O. mykiss*	WLSFH	ST78
US40*	2011	*O. mykiss*	TSFH	ST78
US44*	2011	*O. mykiss*	TSFH	ST78
US49*	2011	*O. mykiss*	TSFH	ST78
US51*	2011	*O. mykiss*	TSFH	ST78
US38*	2011	*O. mykiss*	WLSFH	ST78
US42*[a]	2011	*O. mykiss*	WLSFH	ST78
US53*[a]	2011	*O. mykiss*	WLSFH	ST78
US05	2011	*O. tshawytscha*	LMRW	ST250
US06	2011	*O. tshawytscha*	LMRW	ST251
US08	2011	*O. kisutch*	PRW	ST252
US09	2013	*O. mykiss*	OSFH	ST253
US12	2013	*O. tshawytscha*	SRW	ST254
US13	2012	*O. tshawytscha*	LMRW	ST255
US31	2012	*O. tshawytscha*	LMRW	ST255
US46	2011	*O. tshawytscha*	LMRW	ST256
US47	2011	*O. tshawytscha*	SRW	ST256
US14	2013	*O. tshawytscha*	LMRW	ST256
US18	2013	*O. mykiss*	LMRW	ST256
US30	2013	*O. tshawytscha*	LMRW	ST256
US39	2013	*O. mykiss*	LMRW	ST256
US16*	2013	*O. mykiss*	WLSFH	ST257
US48	2012	*O. kisutch*	PRW	ST258
US52	2012	*O. kisutch*	PRW	ST258

Table 5.1 (cont'd)

Isolate ID[1,2]	Year	Salmonid Species	GLB Location	ST
US20	2013	*O. kisutch*	PRW	ST258
US27	2013	*O. kisutch*	PRW	ST258
US21	2009	*O. tshawytscha*	SRW	ST259
US23	2013	*O. tshawytscha*	SRW	ST259
US22	2010	*O. tshawytscha*	SRW	ST260
US56	2010	*O. tshawytscha*	SRW	ST260
US24	2009	*O. tshawytscha*	SRW	ST261
US33	2010	*O. tshawytscha*	LMRW	ST262
US50	2010	*O. tshawytscha*	LMRW	ST262
US34	2012	*O. tshawytscha*	SRW	ST263
US35	2011	*O. kisutch*	PRW	ST264
US37	2012	*O. tshawytscha*	SRW	ST265
US36	2008	*O. tshawytscha*	LMRW	ST266
US43	2008	*O. tshawytscha*	LMRW	ST266
US54*	2013	*O. mykiss*	WLSFH	ST267

Table 5.2 Primer sequences used to detect tetracycline resistance genes.

Gene	Primer sequence (5' to 3')		Reference
tetA	Forward	GTAATTCTGAGCACTGTCGC	Schmidt et al. 2001
	Reverse	CTGCCTGGACAACATTGCTT	
tetE	Forward	GTGATGATGGCACTGGTCAT	Schmidt et al. 2001
	Reverse	CTCTGCTGTACATCGCTCTT	
tetH	Forward	CAGTGAAAATTCACTGGCAAC	Macauley et al. 2007
	Reverse	ATCCAAAGTGTGGTTGAGAAT	
tetM	Forward	GTTAAATAGTGTTCTTGGAG	Aarestrup et al. 2000
	Reverse	CTAAGATATGGCTCTAACAA	

Table 5.3 Summary of *Flavobacterium psychrophilum* antimicrobial susceptibility testing data by broth microdilution. Epidemiological cut-off values (CO_{WT}) were calculated using both the ECOFFinder analysis (CO_{ECOFF}) and normalized resistance interpretation (CO_{NRI}) method. MIC value reported for trimethoprim-sulphamethoxazole and ormetoprim-sulphadimethoxine is drug concentration that produced ~50% bacterial growth compared to positive control. CO_{WT} could not be calculated for gentamicin or ampillicin data.

Drug	MIC (µg mL^{-1})			Epidemiological cut-off values (CO_{WT}; µg mL^{-1})			
	Range	MIC_{50}	MIC_{90}	CO_{ECOFF}	% wild type	CO_{NRI}	% wild type
Oxytetracycline	0.03 to >8	0.06	8	0.06	76	0.12	76
Florfenicol	0.25 to 1	0.5	1	2	100	2	100
Ormetoprim-sulphadimethoxine	0.015/0.3 to 1/19	0.12/2.38	0.25/4.75	1/19	100	2/38	100
Erythromycin	0.5 to 4	2	4	8	100	8	100
Oxolinic acid	0.06 to 0.25	0.12	0.25	0.25	100	0.25	100
Flumequine	0.03 to 0.12	0.06	0.12	0.12	100	0.12	100
Trimethoprim-sulphamethoxazole	0.015/0.3 to 0.25/4.75	0.06/1.19	0.12/4.75	0.25/9.5	100	0.5/9.5	100
Enrofloxacin	<0.002 to 0.03	0.008	0.015	0.015	98	0.03	100
Gentamicin	<0.06 to 2	0.12	1	-	-	-	-
Ampicillin	<0.03 to 0.06	>0.03	0.06	-	-	-	-

Table 5.4. Characteristics of *Flavobacterium psychrophilum* isolates analyzed in this study. ST, sequence type; MIC, minimum inhibitory concentration; AMP, ampicillin; GEN, gentamicin; ENRO, enrofloxacin; OXO, oxolinic acid; FLUQ, flumequine; SXT, trimethoprim-sulphamethoxazole; PRI, ormetoprim-sulphadimethoxine; ERY, erythromycin; FFN, florfenicol; OXY, oxytetracycline. [1]Isolate IDs with an asterisk indicate *F. psychrophilum* recovery during times of morbidity/mortality characteristic of bacterial coldwater disease. [2]Isolates recovered from the same fish marked with the same superscript letter. [3]OS, off scale = lower drug concentrations required to determine MIC. Trailing = higher drug concentrations required to determine MIC.[4]MIC value reported is drug concentration that produced ~50% bacterial growth compared to positive control.

Isolate ID[1,2]	ST	MIC (μg mL^{-1})[3]									
		OXY	FFN	PRI[4]	ERY	OXO	FLUQ	SXT[4]	ENRO	GEN	AMP
US07	ST13	0.03	1	0.06/1.19	4	0.06	0.03	0.03/0.59	0.004	0.12	0.06
US19	ST13	0.03	0.5	0.12/2.38	4	0.12	0.06	0.12/2.38	0.008	1	0.06
US41	ST29	0.06	0.5	0.12/2.38	2	0.06	0.06	0.06/1.19	0.008	0.25	OS
US04	ST29	0.06	0.5	0.12/2.38	1	0.06	0.03	0.06/1.19	0.008	0.12	OS
US25	ST29	0.06	0.5	0.25/4.75	2	0.06	0.06	0.12/2.38	0.008	1	OS
US55	ST29	0.06	0.5	0.12/2.38	0.5	0.12	0.06	0.06/1.16	0.004	OS	OS
US28	ST31	4	0.5	0.25/4.75	2	0.12	0.06	0.12/4.75	0.008	OS	OS
US29	ST31	4	0.5	0.25/4.75	2	0.12	0.06	0.12/2.38	0.008	0.12	OS
US17*	ST78	0.06	1	1/19	2	0.25	0.06	0.25/4.75	0.015	0.12	OS
US26*	ST78	8	1	0.25/4.75	2	0.12	0.06	0.06/1.19	0.008	OS	OS
US32*	ST78	trailing	1	0.5/9.5	2	0.12	0.06	0.12/2.38	0.008	0.12	OS
US45*	ST78	8	0.5	0.25/4.75	2	0.12	0.06	0.06/1.19	0.008	OS	OS
US40*	ST78	4	0.5	0.25/4.75	4	0.12	0.03	0.12/2.38	0.008	0.12	OS
US44*	ST78	8	0.5	0.5/9.5	2	0.12	0.06	0.12/2.38	0.008	0.25	OS
US49*	ST78	8	1	0.12/2.38	1	0.12	0.06	0.03/0.59	0.008	0.12	OS
US51*	ST78	8	0.5	0.25/4.75	1	0.12	0.06	0.06/1.19	0.004	OS	OS
US38*	ST78	0.06	0.5	0.12/2.38	1	0.12	0.06	0.06/1.19	0.008	0.12	OS
US42*[a]	ST78	0.06	1	0.25/4.75	2	0.12	0.06	0.06/1.19	0.004	0.12	OS
US53*[a]	ST78	0.03	0.5	0.12/2.38	1	0.06	0.03	0.06/1.19	0.004	OS	OS
US05	ST250	0.06	1	0.06/1.19	2	0.06	0.03	0.03/0.59	0.008	0.25	OS
US06	ST251	0.06	1	0.12/2.38	2	0.12	0.03	0.03/0.59	0.008	0.25	OS

Table 5.4 (cont'd)

Isolate ID[1,2]	ST	MIC (μg mL^{-1})[3]									
		OXY	FFN	PRI[4]	ERY	OXO	FLUQ	SXT[4]	ENRO	GEN	AMP
US08	ST252	0.06	1	0.12/2.38	4	0.06	0.06	0.06/1.19	0.004	0.5	OS
US09	ST253	4	1	0.12/2.38	2	0.06	0.06	0.06/1.19	0.008	0.5	OS
US12	ST254	0.06	1	0.25/4.75	4	0.12	0.06	0.06/1.19	0.015	1	OS
US13	ST255	0.06	1	0.12/2.38	4	0.06	0.03	0.06/1.19	0.008	1	OS
US31	ST255	0.06	1	0.12/2.38	2	0.06	0.03	0.015/0.3	0.004	0.5	OS
US46	ST256	0.03	0.5	0.015/0.3	1	0.12	0.03	0.015/0.3	OS	0.12	OS
US47	ST256	0.03	0.5	0.03/0.59	1	0.06	0.03	0.015/0.3	OS	OS	OS
US14	ST256	0.06	0.5	0.12/2.38	2	0.06	0.03	0.03/0.59	OS	0.12	OS
US18	ST256	0.06	1	0.06/1.19	4	0.06	0.03	0.03/0.59	OS	1	OS
US30	ST256	0.06	0.5	0.12/2.38	1	0.12	0.06	0.015/0.3	OS	OS	OS
US39	ST256	0.06	1	0.25/4.75	2	0.12	0.03	0.06/1.19	0.004	0.5	OS
US16*	ST257	4	1	0.12/2.38	4	0.06	0.03	0.12/2.38	0.008	1	OS
US48	ST258	0.06	1	0.12/2.38	1	0.12	0.06	0.015/0.3	0.004	0.12	OS
US52	ST258	0.03	0.5	0.03/0.59	1	0.06	0.03	0.015/0.3	0.004	0.25	OS
US20	ST258	0.03	0.5	0.03/0.59	2	0.12	0.03	0.015/0.3	0.015	0.12	OS
US27	ST258	0.03	0.5	0.03/0.59	1	0.12	0.06	0.015/0.3	0.008	OS	OS
US21	ST259	0.06	1	0.03/0.59	4	0.06	0.03	0.015/0.3	0.008	0.25	OS
US23	ST259	0.06	1	0.06/1.19	2	0.12	0.06	0.03/0.59	0.008	0.25	OS
US22	ST260	0.06	1	0.12/2.38	4	0.25	0.12	0.06/1.19	0.008	2	0.06
US56	ST260	0.06	0.5	0.03/0.59	1	0.12	0.06	0.015/0.3	0.004	OS	0.06
US24	ST261	0.06	1	0.12/2.38	1	0.12	0.06	0.015/0.3	0.008	OS	OS
US33	ST262	0.06	0.5	0.06/1.19	1	0.12	0.06	0.03/0.59	0.008	0.25	OS
US50	ST262	0.06	0.5	0.06/1.19	1	0.12	0.06	0.03/0.59	0.004	0.12	OS
US34	ST263	0.06	1	0.25/4.75	4	0.12	0.12	0.12/2.38	0.015	2	OS
US35	ST264	0.03	0.5	0.03/0.59	2	0.06	0.06	0.015/0.3	0.008	OS	OS
US37	ST265	0.06	1	0.12/2.38	2	0.12	0.06	0.06/1.19	0.03	0.12	OS

Table 5.4 (cont'd)

Isolate ID[1,2]	ST	MIC (µg mL^{-1})[3]									
		OXY	FFN	PRI[4]	ERY	OXO	FLUQ	SXT[4]	ENRO	GEN	AMP
US36	ST266	0.06	1	0.12/2.38	4	0.12	0.06	0.06/1.19	0.015	0.5	OS
US43	ST266	0.06	0.5	0.03/0.59	1	0.12	0.06	0.06/1.19	0.015	0.12	OS
US54*	ST267	4	0.25	0.06/1.19	2	0.12	0.06	0.03/0.59	0.004	0.12	OS

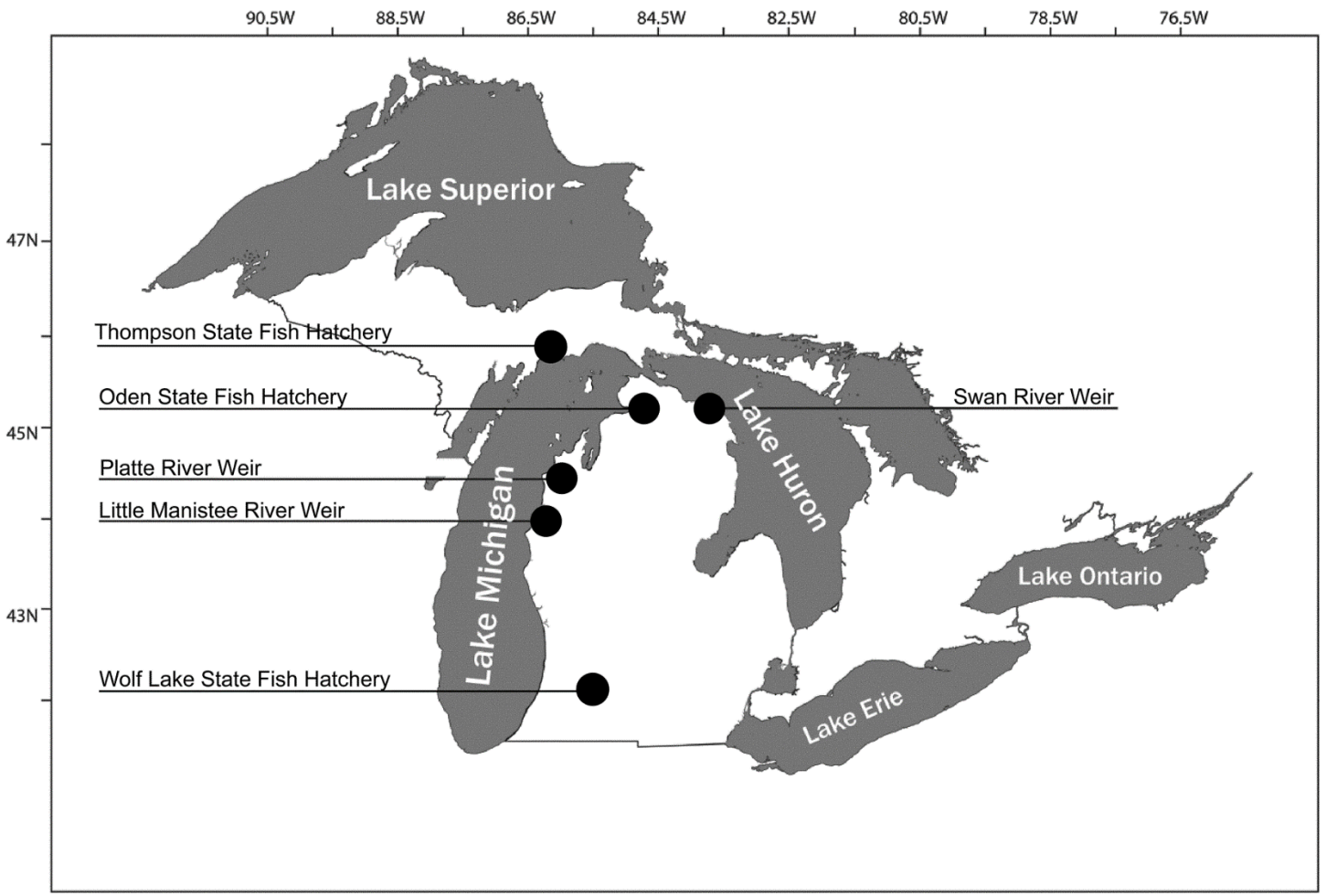

Figure 5.1 Map of locations where *Flavobacterium psychrophium* isolates were collected.

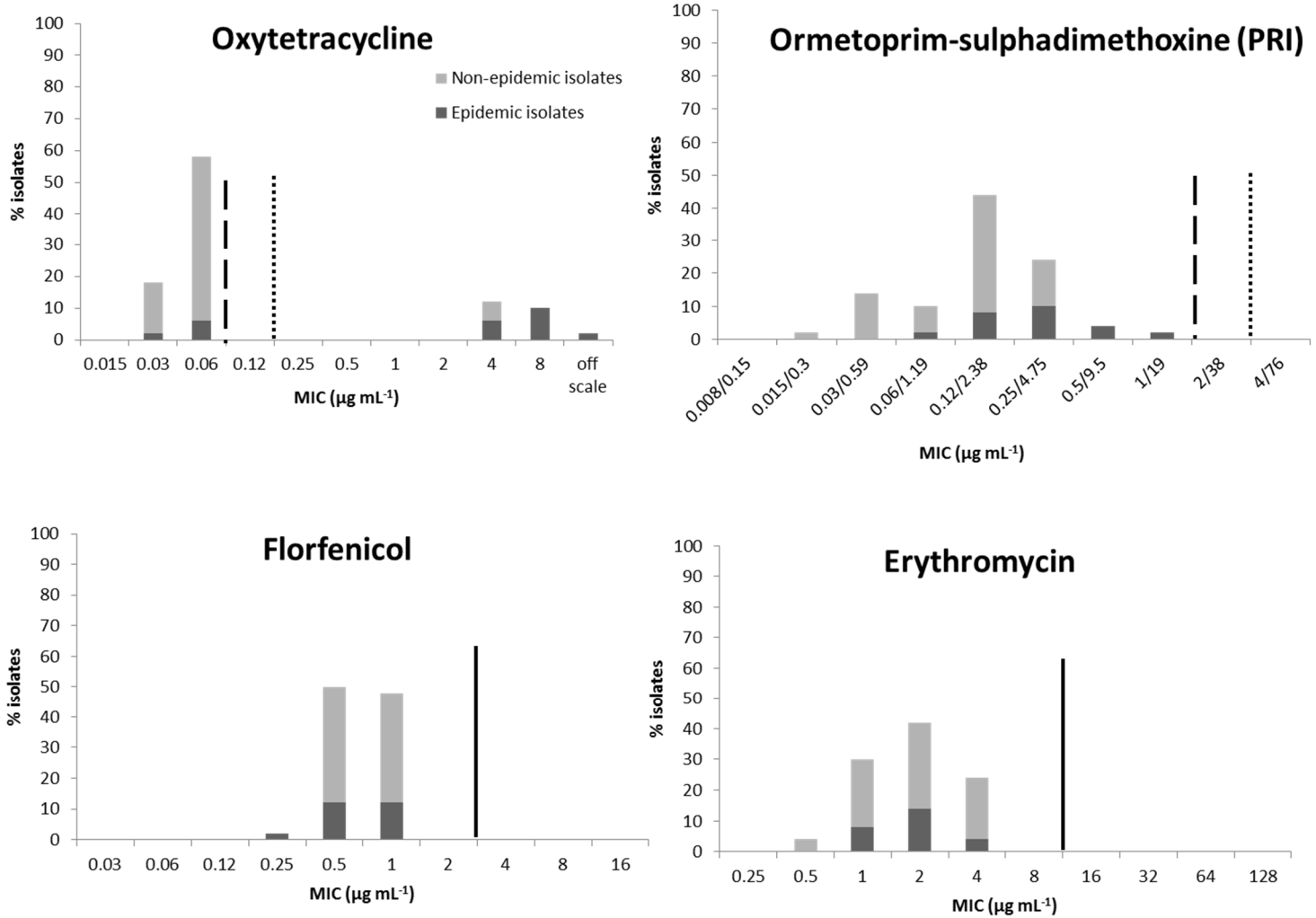

Figure 5.2 The distrubution of *Flavobacterium psychrophilum* isolates (%) according to determined minimum inhibitory concentrations (MICs) for 4 antimicrobial compounds frequently used in Great Lakes basin aquaculture. Dotted lines represent epidemiological cut-off values calculated by the national resistance interpretation method (CO_{NRI}), dashed lines represent epidemiological cut-off values calculated by the ECOFFinder analysis (CO_{ECOFF}), and solid lines represent epidemiological cut-off values that were of the same result from both methods.

A

Flofenicol

Oxytetracycline	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
0.015										
0.03					8	1				
0.06					11	18				
0.12										
0.25										
0.5										
1										
2										
4				1	3	2				
8					3	2				
>8						1				

Figure 5.3 Scatter plot comparision of oxytetracycline (y-axis) and A) florfenicol (x-axis), B) ormetoprim-sulphadimethoxine (x-axis), C) erythromycin (x-axis), D) oxolinic acid (x-axis), E) flumequine (x-axis), F) trimethoprim-sulphamethoxazole (x-axis), G) enrofloxacin (x-axis), H) gentamicin (x-axis), and I) amplicillin (x-axis) MIC values for *Flavobacterium psychrophilum.* The number of isolates with the given combination of MIC values is indicated in each square. Dashed lines indicate the epidemiolocigal cut-off (CO_{WT}) values calculated by the normalized resistance interpretation (CO_{NRI}) method, and dotted lines indicate the CO_{WT} values calculated by the ECOFFinder (CO_{ECOFF}) analysis. The bolded solid lines indicate the CO_{WT} values that were of the same result from both methods. No CO_{WT} values could be calculated for the gentamicin or ampicillin MIC data.

Figure 5.3 (cont'd)

B

Ormetoprim-sulphadimethoxine

Oxytetracycline	0.008/0.15	0.015/0.3	0.03/0.59	0.06/1.19	0.12/2.38	0.25/4.75	0.5/9.5	1/19	2/38	4/76
0.015										
0.03		1	5	1	2					
0.06			3	5	15	5		1		
0.12										
0.25										
0.5										
1										
2										
4				1	2	3				
8					1	3	1			
>8							1			

Figure 5.3 (cont'd)

C

Erythromycin

Oxytetracycline	0.25	0.5	1	2	4	8	16	32	64	128
0.015										
0.03			5	2	2					
0.06		1	9	11	8					
0.12										
0.25										
0.5										
1										
2										
4				4	2					
8			2	3						
>8				1						

D

Oxolinic acid

Oxytetracycline	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2
0.015										
0.03					5	4				
0.06					10	17	2			
0.12										
0.25										
0.5										
1										
2										
4					2	4				
8						5				
>8						1				

Figure 5.3 (cont'd)

E

Flumequine

Oxytetracycline	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4
0.015										
0.03			6	3						
0.06			9	18	2					
0.12										
0.25										
0.5										
1										
2										
4			2	4						
8				5						
>8				1						

F

Trimethoprim-sulphamethoxazole

Oxytetracycline	0.015/0.3	0.03/0.59	0.06/1.19	0.12/2.38	0.25/4.75	0.5/9.5	1/19
0.015							
0.03	6	1	1	1			
0.06	6	7	13	2	1		
0.12							
0.25							
0.5							
1							
2							
4		1	1	4			
8		1	3	1			
>8				1			

Figure 5.3 (cont'd)

G

Enrofloxacin

Oxytetracycline	<0.002	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1
0.015											
0.03	2		3	3	1						
0.06	3		8	12	5	1					
0.12											
0.25											
0.5											
1											
2											
4			1	5							
8			1	4							
>8				1							

H

Gentamicin

Oxytetracycline	<0.06	0.06	0.12	0.25	0.5	1	2	4
0.015								
0.03	4		3			1		
0.06	4		9	6	4	4	2	
0.12								
0.25								
0.5								
1								
2								
4	2		3		1	1		
8	3		1	1				
>8			1					

Figure 5.3 (cont'd)

I

Ampicillin

Oxytetracycline	<0.03	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
0.015											
0.03	7		2								
0.06	27		2								
0.12											
0.25											
0.5											
1											
2											
4	6										
8	5										
>8	1										

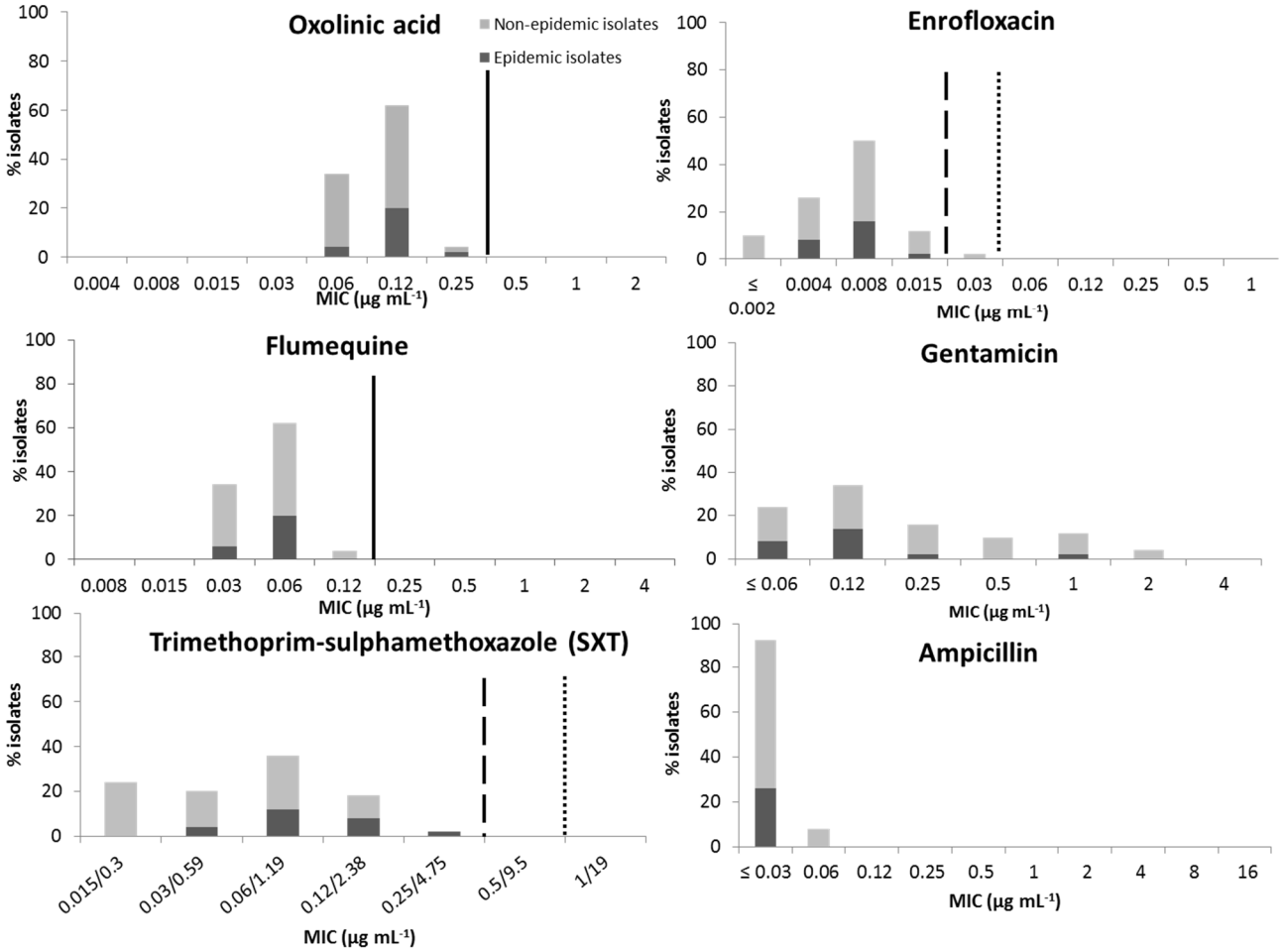

Figure 5.4 The distrubution of *Flavobacterium psychrophilum* isolates (%) according to determined minimum inhibitory concentrations (MICs) for 6 antimicrobial compounds that are not approved for use in United States aquaculture. Dotted lines represent epidemiological cut-off values calculated by the national resistance interpretation method (CO_{NRI}), dashed lines represent epidemiological cut-off values calculated by the ECOFFinder analysis (CO_{ECOFF}), and solid lines represent epidemiological cut-off values that were of the same result from both methods. Epidemiological cut-off values could not be calculated for genetamicin or ampicillin data.

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Chapter 6

Conclusions and Future Research

1. Conclusions

Although *Flavobacterium psychrophilum* has been a known pathogen of salmonid fishes for over 60 years, details of its epidemiology including the distribution and role of vertical transmission, genetic diversity as it pertains to identification of fish host species specificity and highly virulent strains, as well as the risk of antimicrobial resistance have not fully been elucidated in the Great Lakes basin (GLB) of Michigan. These large knowledge gaps may be hindering the lack of appropriate control strategies, such as vaccine development based on particular fish host species specific or highly virulent *F. psychrophilum* strains, as well as revised chemotherapeutic treatments. Furthermore, little is known regarding the pathogen dissemination dynamics between salmonid populations from different geographic regions or between the broodstock population and their resultant progeny. Considerable research has now shown the prevalence and incidence of *F. psychrophilum* infections among valuable GLB salmonid stocks. The results of this dissertation recognize that these infections are caused by a genetically diverse population of *F. psychrophilum.* As antimicrobial therapy currently remains the most commonly used method to control *F. psychrophilum*, it is vital to monitor and continue surveillance of antimicrobial susceptibility profiles. In this context, supporting research has identified a risk of reduced susceptibility to oxytetracycline in the GLB. Collectively, the research presented in this dissertation reveals the heterogeneity of *F. psychrophilum* infections and strains and how it may relate to our understanding of the global dissemination, pathogenesis, and vaccination development against this deadly pathogen.

Chapter 1 identified multiple gaps of knowledge that we need to bridge in order to better understand the pathogenesis and epidemiology of this deadly bacterium. Three of the knowledge gaps were addressed in the following four chapters. For example, the prevalence and distribution of *F. psychrophilum* was not known in seven salmonid broodstocks that form the backbone of recreational fisheries in the GLB. Therefore, in Chapter 2, I analyzed the prevalence of *F. psychrophilum* infections among multiple GLB salmonid stocks, as health history of federal and state hatcheries in the GLB report the association between this bacterium and devastating mortalities. Apart from demonstrating that *F. psychrophilum* is widespread in multiple valuable salmonid broodstocks, it was unraveled, for the first time, that a salmonid like Chinook salmon (*Oncorhynchus tshawytscha*) was able to protect its offspring from an infection that exceeded 85% in the spawning broodstock. Analyses of steelhead (*O. mykiss*) data showed the contrary. One can argue that differential host susceptibility to certain pathogens has been shown and that particularly virulent bacterial strains may have a tendency to colonize certain fish host species. Nevertheless, the mechanism by which Chinook salmon protected its progeny should be further investigated, along with the role the immune system can play in preventing the pathogen from accessing its eggs. Unfortunately, the lack of commercially available biological material hindered me from continuing this line of research, this I opted to look for other factors from the pathogen side such as if certain *F. psychrophilum* strains infect a specific host in a certain locale, or if a specific strain is always associated with morbidity and mortality and vice versa. Identifying such “strains of concern” and being able to detect them by molecular biomarkers was a goal that spurred from and was guided by the initial epidemiological study depicted in Chapter 3.

With widespread and devastating *F. psychrophilum* infections throughout the GLB, Chapter 3 focused on elucidating the genetic diversity of these strains with a goal of understanding the role fish host specific and/or highly virulent *F. psychrophilum* strains play in the epidemiology of this pathogen in the GLB. The use of appropriate loci was integral in revealing the molecular epidemiology among these strains, as prior studies have provided variable results based on the gene target used. Careful consideration was taken to choose single-copy, protein-encoding alternative loci, including *gyrB, murG,* and *tuf*, that were all successful when used individually in revealing a robust phylogeny of GLB *F. psychrophilum.* The results based on the use of these genes individually revealed for the first time among GLB *F. psychrophilum* isolates a high frequency of fish host species specific strains, as well as strains of concern that are seemingly dominant among BCWD outbreaks in Michigan aquaculture facilities. This work has also demonstrated the low likelihood of specific *F. psychrophilum* strain transmission between broodstocks and progeny populations, as no specific strains recovered from broodstock were identified in progeny, which highlights the need for better understanding of the pathogen trafficking dynamics between these populations, as well as what the source of infection really is. Further analyses based on the concatenation of these genes improved the phylogenetic robustness and guided the continued molecular epidemiological investigation of Chapter 4.

The problems associated with BCWD extend far beyond the GLB, and in fact are of major concern in both captive and feral salmonid stocks across the nation. In Chapter 4, I expanded upon the molecular epidemiological investigation of the GLB *F. psychrophilum* to include isolates from a total of 9 U.S. states. Additionally, guided by the improvements through

concatenation of the individual genes used in Chapter 3, the use of 7 single-copy, protein encoding housekeeping genes were used in a multilocus sequence typing (MLST) approach. This work has demonstrated the remarkable genetic diversity of this pathogen in the U.S., and has identified 28 novel sequence types (STs) within the known *F. psychrophilum* population. Indeed, the initial isolation of *F. psychrophilum* occurred in the Pacific Northwest of North America, however the exact route of national and global dissemination of this pathogen still needs investigation. The novel STs described in this work have given strength to the idea that at least a particularly dominant and transcontinental clonal complex (CC; CC-ST10) did in fact arise in the United States. Analyses have also identified STs of clinical significance that should be the target of further vaccination efforts as they are consistently associated with severe morbidity and mortality among rainbow trout (*O. mykiss*) populations. Particularly in the GLB, ST78 should be the primary focus of eradication efforts in regards to rainbow trout aquaculture, and a cost and time efficient method to identify this ST would greatly help improve the detection and control of this strain.

After genetically characterizing GLB *F. psychrophilum* strains, the phenotypic diversity was investigated in Chapter 5 in regards to antimicrobial susceptibilities. Apart from establishing the use of a standardized and approved assay to examine the antimicrobial susceptibility profiles of GLB *F. psychrophilum*, this work demonstrated for the first time resistance to oxytetracycline in approximately 20% of the isolates tested. This is alarming considering oxytetracycline is one of only two antimicrobial agents approved to treat *F. psychrophilum* infections in the GLB. Fortunately, no resistance to the other approved drug, florfenicol, was detected at this point however the distribution of minimum inhibitory

concentrations (MICs) are approaching the epidemiological cut-off value for this drug. These results point to the indication that changes should be made soon to the current chemotherapeutic strategies to ensure an efficacious antimicrobial treatment remains.

Collectively, this dissertation underscores the significance of *F. psychrophilum* as a pathogen of GLB salmonid stocks and demonstrates the dire need for improvement upon its treatment, prevention, and control methods. These epidemiological results offer a basis for which the improvements can be made, including focus on fish stocks identified most at risk for infection, vaccination approaches using fish host species specific *F. psychrophilum* strains and/or strains shown to be dominant and highly virulent, and revised chemotherapeutic plans. Furthermore, this work highlights the need for continued investigation among multiple aspects of *F. psychrophilum* epidemiology, including pathogen tracking between salmonid populations, dissemination routes, and the use of biomarkers to quickly identify strains of clinical significance.

2. Future research

The results presented in this dissertation have contributed substantially to further our knowledge of *F. psychrophilum* epidemiology; particularly how it relates to Great Lakes basin salmonid populations. Moreover, it has demonstrated the genetic diversity and has identified important STs (i.e., strains) circulating in the GLB and other areas of the U.S., as well as isolates exhibiting reduced susceptibility to commonly used and relied upon antimicrobials. However, much more work is needed to draw large scale conclusions regarding the prevalence, persistence, and diversity of *F. psychrophilum* in GLB salmonid populations, and aims at

establishing improved control methods including vaccinations based on fish host species specific and/or highly virulent *F. psychrophilum* strains, revised chemotherapeutic strategies, and a better understanding of vertical transmission components should be a target of future research.

In particular, the mechanism of, and what may influence, the likely vertical transmission is not completely understood. For example, perplexing results from Chapter 2 show high infection prevalence among Chinook salmon broodstock yet low incidence of BCWD in their progeny with a reverse trend in the case of steelhead. Although vertical transmission may indeed play a role in these results, other factors including facility source water and microclimate may influence these results. In this context, a controlled study investigating the link between the prevalence and intensity of broodstock infections and the disease among the associated progeny should be thoroughly conducted. Through this future research, it is hoped that an understanding of the clinical relevance and risk of vertical transmission of this pathogen will be revealed.

Following the analysis of the clinical relevance of vertical transmission, the identification of an efficacious chemotherapeutic agent that can penetrate within the egg and kill the bacterium is sorely needed. At present, the biosecurity measures employed by the Michigan Department of Natural Resources are sufficient at surface disinfecting the eggs using an iodophor solution; however this solution does not migrate inside the egg. The practice of water hardening fish eggs in an erythromycin solution is generally accepted to reduce the risk of vertical transmission of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), and is thought to work by invasion through the egg's micropyle before it seals.

As results from Chapter 5 of this dissertation show, some *F. psychrophilum* strains are indeed susceptible to erythromycin, yet this strategy has not been fully examined in regards to *F. psychrophilum* control and this drug is not currently approved for this use. In a similar manner, the efficacy and invasion capabilities of other chemotherapeutic agents should be thoroughly investigated as a source of vertical transmission reduction.

Along these lines, the investigation of the exact mechanisms of pathogen load and transmission through gametes is required to better understand the role of infected broodfish. Another strategy that has shown promise in reduction of the vertical transmission of *R. salmoninarum* includes culling of infected broodstock. Although recent work has suggested the use of this method pertaining to control of *F. psychrophilum*, better diagnostic assays are needed to identify infection status of the broodstock. There is a lack of knowledge regarding what the influence of the pathogen load among certain tissue types (i.e., kidney, spleen, gametes) and the link between the risks of vertical transmission is. For example, it is unknown if there is an association between the *F. psychrophilum* load in the kidneys and the risk of vertical transmission, or if the risk stems directly from the pathogen load in the gametes (ovarian fluids, eggs, milt). This could be problematic if the latter is true, because enzyme-linked immunosorbent assays (ELISA) exist for the detection and semi-quantification of *F. psychrophilum*; however the use of these assays with reproductive fluids has been problematic and requires much further optimization. Along these same lines, the role of carrier fish and the improvement of diagnostic assays to detect subclinical infections should be further investigated as the role of these individuals and their contribution to vertical transmission of *F. psychrophilum* is unclear.

While vertical transmission may indeed be a source of infection among salmonid stocks in the GLB, a thorough investigation of other potential sources is required to fully understand the risk of BCWD. The continued used of molecular epidemiological techniques will help in identifying the local pathogen trafficking. For example, if STs are consistently found among feral broodstock populations as well as among their associated hatchery-reared progeny, the risk of vertical transmission may be furthered confirmed. Similarly, if particular STs are consistently recovered from hatchery infrastructure and from disease outbreaks, it can be hypothesized that those particular STs are being maintained within the hatchery facility itself. Studies such as these can help managers focus control efforts to the areas presenting the most risk as sources of infection. Biosecurity measures should continue to be employed throughout the hatchery facility and gamete collection locations; however targeted control methods could be developed in response to the information regarding infection sources.

Along the same lines as local pathogen trafficking, the global dissemination of *F. psychrophilum* can be more confidently understood through the continued used of molecular epidemiological techniques. It has been hypothesized that the trade of live fish and their eggs have contributed to the transcontinental distribution of this deadly pathogen, yet the details to confirm this have not been fully elucidated.

A major finding through the molecular epidemiological work of this dissertation was the identification of strains of concern, including fish host species specific and/or highly virulent strains. The mechanisms that have allowed these strains to possess seemingly specific characteristics need further investigation. Little work has been done in identification of components that make *F. psychrophilum* strains particularly virulent, now with strains known to

be clinically significant and associated with devastating morbidity and mortality, these strains could form the basis for which the virulence of *F. psychrophilum* strains can be explored. The identification of biomarkers that can easily discriminate between strains of concern would also be beneficial in helping to prepare for and control BCWD.

Moreover, these strains of clinical significance were shown to predominantly be resistant to oxytetracycline. This is alarming considering these are the strains causing the most problems in Michigan aquaculture facilities, yet the risk of resistance to one of only two approved drugs is very high. First, the genetic mechanisms relating to the resistant phenotypes should be considered. Preliminary work conducted in this dissertation did not confirm the presence of 4 of the major genes associated with tetracycline resistance, yet over 30 of these genes are known to circulate through bacterial populations. Thorough investigation of the presence of these genes, along with the role of plasmid-mediated antimicrobial resistance should be a research focus. In terms of relating the *in vitro* antimicrobial susceptibility patterns to clinically relevant treatment options, the establishment of epidemiological cut-off value specific to *F. psychrophilum* needs to first occur. These values require the support of a large dataset, and concurrent work has been focused on the approval of these values.

Considering the significance of this pathogen to global salmonid populations, and the increasing growth and importance of aquaculture the research recommendations provided herein will help to understand and mitigate the risks BCWD poses to valuable fish stocks worldwide.