INTRASPECIFIC *FLAVOBACTERIUM PSYCHROPHILUM* DIVERSITY AS A FACTOR IN BACTERIAL COLDWATER DISEASE ECOLOGY AND MANAGEMENT

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ABSTRACT

Flavobacterium psychrophilum, causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), causes substantial economic losses worldwide, particularly in salmonid species (Family *Salmonidae*) such as rainbow trout (*Oncorhynchus mykiss*), coho salmon (*O. kisutch*), and Atlantic salmon (*Salmo salar*). Current challenges in managing, preventing, and controlling BCWD outbreaks may partially relate to the considerable intraspecific diversity present within this species, including that revealed via multilocus sequence typing (MLST). Indeed, MLST-based epidemiological studies and others suggest *F. psychrophilum* diversity may influence the ecology and behavior of some variants, affect their detection and diagnosis, and play a role in host specificity, transmission, and environmental persistence. However, controlled studies examining these aspects of BCWD ecology in relation to *F. psychrophilum* genetic diversity are lacking. My dissertation addressed these critical knowledge gaps, with the goal of contributing to enhanced strategies for the management, prevention, and control of BCWD. To improve *F. psychrophilum* recovery and detection, I initially compared colony yields of geographically, temporally, and genetically diverse *F. psychrophilum* isolates on three previously established *F. psychrophilum* culture media. The selected media included the current gold-standard medium, tryptone yeast extract salts (TYES) agar, which yielded the most colonies. With TYES as a foundation, I employed a Plackett-Burman experimental design, culminating in the development of two new culture media (*F. psychrophilum* medium-A and -B). These optimized media significantly improved *F. psychrophilum* recovery in the laboratory and from naturally infected salmonids when compared to TYES, and thus will enhance BCWD research and diagnostic efforts. To assess *F. psychrophilum* host specificity, variants belonging to MLST clonal complexes most associated

with Atlantic salmon, coho salmon, or rainbow trout were cross challenged against each of these salmonid species via immersion. Resultingly, some variants were host specific, as evidenced by only causing disease and mortality in one species, whereas others caused disease and mortality in all three species, although to varying degrees. Variation in molecular serotype and proteolytic activity were also observed among variants. Collectively, findings highlighted the complexities of host-pathogen interactions and may guide the development of BCWD prevent strategies, such as vaccines. In a separate experiment, the shedding dynamics of live and dead Atlantic salmon, coho salmon, and rainbow trout were assessed, marking the first study to evaluate *F. psychrophilum* shedding dynamics in Atlantic salmon and coho salmon. Although both live and dead fish of all species shed *F. psychrophilum*, dead fish shed substantially more bacterial cells and for a longer duration. Furthermore, shedding dynamics varied by *F. psychrophilum* variant and/or host species, a matter that may complicate BCWD management. The persistence of predominating *F. psychrophilum* variants in microcosms composed of sterile well water only, sterile well water with commercial trout feed, and sterile well water with raceway detritus was measured via culture over 13 weeks. All variants remained culturable in each microcosm for at least eight weeks, with bacterial concentrations significantly higher in the presence of raceway detritus. However, significant differences in culturability were observed within and between microcosms, suggesting potential variability in environmental persistence strategies among specific variants. In total, the findings of my dissertation supported my overarching hypothesis that *F. psychrophilum* intraspecific diversity plays an important role in shaping our understanding of BCWD ecology.

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AFS-FHS	American Fisheries Society – Fish Health Section
AK	Alaska
AO	Anacker and Ordal medium
ATCC	American Type Culture Collection
atpA	Adenosine triphosphate synthetase, α subunit
ATS	Atlantic salmon
AUF	Animal Use Form
BC	British Columbia
BCWD	Bacterial Coldwater Disease
BKT	Brook trout
BNT	Brown trout
bp	Base pair
C	Celsius
CA	California
$CaCl_2$	Calcium chloride
CACM	Anacker and Ordal medium with carbohydrates and skimmed milk
CC	Clonal complex
cfu	Colony forming unit
CHS	Chinook salmon
CO	Colorado
COS	Coho salmon
Cq	Quantification cycle

CUT	Cutthroat trout
CV	Coefficient of variation
CZR	Clear zone ratio
DECF	DNA extraction correction factor
df	Degrees of freedom
DNA	Deoxyribose nucleic acid
dnak	Chaperone heat-shock protein 70
EAO	Enriched Anacker and Ordal medium
EAOC	Enriched Anacker and Ordal medium with charcoal
EAOCa	Enriched Anacker and Ordal medium with charcoal and aromatic compounds
EAOS	Enriched Anacker and Ordal medium with horse serum
FBS	Fetal bovine serum
FCS	Fetal calf serum
FLPA	*Flavobacterium psychrophilum* agar
FPM-A	*Flavobacterium psychrophilum* medium-A
FPM-B	*Flavobacterium psychrophilum* medium-B
fumC	Fumarate hydratase class II
g	Gram
g	Gravity
G+C	Guanine plus Cytosine
gDNA	Genomic DNA
gyrb	Topoisomerase II, β subunit
H_2O	Water

HS	Horse serum
ID	Idaho
IPC	Internal positive control
L	Liter
LAT	Lake trout
LMRW	Little Manistee River weir
LOD	Limit of detection
LOQ	Limit of quantification
LWF	Lake whitefish
mAO	Modified Anacker and Ordal medium
MAOG	Modified Anacker and Ordal medium with glucose
MAT	TYES with 1% maltose
MD	Maryland
mg	Milligram
$MgSO_4$	Magnesium sulfate
MI	Michigan
ml	Milliliter
MLST	Multilocus sequence typing
mm	Millimeter
MS-222	Methanesulfonate
MSU	Michigan State University
MT	Montana
mTYES	Modified TYES

murg	Glycosyltransferase murein G
n	Number
N	Number
NA	Nutrient agar
NAC	Nutrient agar with charcoal
NC	North Carolina
NCIMB	National Collection of Industrial Food and Marine Bacteria
ng	Nanogram
NJ	New Jersey
NM	Neutral medium
OD	Optical density
OR	Oregon
OW	Oplinger and Wagner medium
PA	Pennsylvania
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ppm	Parts per million
PRW	Platte River weir
qPCR	Quantitative PCR
R^2	Coefficient of determination
RBT	Rainbow trout
rpm	Revolutions per minute
RTFS	Rainbow Trout Fry Syndrome

SD	Standard deviation
SE	Standard error
SMR	St. Mary's River
SOC	Sockeye salmon
SPL	Splake
SRW	Swan River weir
ST	Sequence type
STT	Steelhead trout
tRNA	Transfer ribonucleic acid
trpB	Tryptophane synthetase β subunit
tuf	Elongation factor Tu
TX	Texas
TYES	Tryptone yeast extract salts agar
U.S.	United States
µl	Microliter
µm	Micrometer
µM	Micromolar
USA	United States of America
UT	Utah
VA	Virginia
w/v	Weight per volume
WA	Washington
WHT	White sturgeon

WI	Wisconsin
WV	West Virginia
WY	Wyoming

INTRODUCTION

Fish currently comprise one of the most important animal protein sources for the ever-expanding human population, much of which is supplied through aquaculture (FAO, 2022). Concurrently, many fish species are the focus of substantial conservation and stock enhancement efforts by fishery resource agencies throughout the world. A significant threat to the productivity and health of hatchery and farm-reared salmon and trout (Family *Salmonidae*) is bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), caused by *Flavobacterium psychrophilum* (Holt, 1987; Loch and Faisal, 2017). This bacterial fish pathogen has been recovered in >20 countries distributed over five continents (e.g., Asia, Australia, Europe, North America, and South America; Starliper, 2011) and continues to devastate both wild and farmed fish species, especially salmonids, by causing up to 90% mortality in affected populations (Barnes and Brown, 2011).

Despite nearly a century of research and various disease management efforts having been undertaken, the efficacy of currently available BCWD prevention and control methods remains inadequate. In this context, recent studies have identified some predominant *F. psychrophilum* genetic variants responsible for BCWD epizootics in the USA and abroad, and made significant advancements towards untangling how such intraspecific diversity relates to bacterial virulence (Sundell et al. 2019; Knupp et al. 2021; Li et al. 2021), mounting antimicrobial resistance (Van Vliet et al. 2017; Li et al. 2021), host species predilections (Knupp et al. 2021), and geographical distribution (Nicolas et al. 2008; Van Vliet et al. 2016; Knupp et al. 2019; Sebastião et al. 2020; Harrison et al. 2021). Notably, these and other studies have highlighted a range of knowledge gaps and raised questions regarding why some of the most damaging USA *F. psychrophilum* variants are so successful through the lens of disease ecology. For example, do some *F. psychrophilum* variants truly have an affinity for a particular host species and if so, what

mechanism(s) may contribute to these associations? Have artificial rearing environments contributed to the persistence of the predominating and seemingly highly successful *F. psychrophilum* variants? Do shedding dynamics (e.g., *F. psychrophilum* shedding loads and duration) vary among *F. psychrophilum* variants and/or salmonid species? Are current *F. psychrophilum* culture media, including the gold-standard medium, tryptone yeast extract salts (TYES), sufficient for BCWD diagnosis and research purposes? At present, these gaps in knowledge contribute to productivity losses for salmonid farms and impede hatchery-based conservation of salmonid populations.

To fill in these knowledge gaps and improve BCWD diagnosis, management, prevention, and control, I reviewed a range of *F. psychrophilum* literature, and then conceptualized, planned, and conducted a series of experiments that collectively comprise my dissertation research.

0.1. Study objectives

The overarching goal of my dissertation research is to elucidate how *F. psychrophilum* intraspecific diversity may affect: a) detection in fish tissues and diagnose BCWD, b) BCWD research findings, c) management of BCWD epizootics, and d) development of effective BCWD prevention and control strategies. To accomplish this goal, I reviewed the literature on *F. psychrophilum* and then performed a series of in vitro and in vivo experiments as described below.

In Chapter 1, I reviewed the literature on *F. psychrophilum* and bacterial coldwater disease/RTFS, focusing on: a) the use and development of culture media for *F. psychrophilum* isolation and propagation in vitro; b) *F. psychrophilum*-host associations and contributing mechanisms; c) *F. psychrophilum* shedding dynamics, with a primary focus on horizontal

transmission; and d) the persistence of *F. psychrophilum* outside of its host in various aquatic environments.

In Chapter 2, I compared the recovery of 165 genetically diverse *F. psychrophilum* isolates on three previously published *F. psychrophilum* culture media and selected the medium (e.g., TYES) that achieved the best overall recovery for subsequent comparisons. Next, I used a Plackett-Burman experimental design to quantify the effect 11 medium components had on *F. psychrophilum* recovery to subsequently develop two new culture media (e.g., *F. psychrophilum* medium A and -B; FPM-A and FPM-B). I then compared *F. psychrophilum* recovery on FPM-A and FPM-B to TYES under laboratory conditions, finding both new media significantly improved *F. psychrophilum* recovery by >141%, although FPM-A performed slightly better than FPM-B. Likewise, I compared all three media under field conditions while performing routine health surveillance on wild/feral Michigan salmonid broodstock populations and again found the new media outperformed TYES for *F. psychrophilum* primary isolation.

In Chapter 3, I examined the hypothesis that some *F. psychrophilum* variants had host-associations following close examination of multilocus sequence typing metadata and the completion of a preliminary *F. psychrophilum* challenge study using coho salmon (*Oncorhynchus kisutch*). I tested this hypothesis by exposing Atlantic salmon (*Salmo salar*), coho salmon, and rainbow trout (*O. mykiss*) under controlled laboratory conditions to three distinct *F. psychrophilum* variants, each with putative associations to one of the salmonid species. Study results demonstrated some *F. psychrophilum* variants have strong host associations, whereas others appear to have a wider host range.

In Chapter 4, I assessed whether horizontal transmission strategies differed amongst *F. psychrophilum* variants and/or salmonid species. To do so, I intramuscularly injected rainbow

trout, coho salmon, and Atlantic salmon with one putatively host specific variant per species, and then quantified (via quantitative PCR) *F. psychrophilum* loads that were shed into the water by live and dead fish up to 98 days post-exposure. Findings collectively showed that dead fish were more efficient *F. psychrophilum* shedders than live fish (i.e., *F. psychrophilum* was shed at higher loads for a longer duration), but that other aspects of shedding dynamics may vary depending on *F. psychrophilum* variant and/or affected fish species.

In Chapter 5, I compared the survival of 10 distinct *F. psychrophilum* genetic variants, including some that are most widespread in the USA, in three microcosms that mimic environments common to aquaculture and hatchery facilities, including one with water only, a second with water and trout feed, and the third with water and raceway detritus (i.e., uneaten food, fish byproducts). Overall, all tested isolates persisted in each environment for at least 8 weeks, but survival was best in water with detritus. Notably, some isolates did survive better in water only compared to water with feed, highlighting potential differences in environmental persistence strategies by *F. psychrophilum* variant.

In Chapter 6, I discussed how the new knowledge described herein has not only led to improved detection of *F. psychrophilum* and diagnosis of BCWD across the USA, but also has informed best management practices and the future development of BCWD prevention and control strategies. Ultimately, this research will minimize losses, and reduce the risk of BCWD epizootics in salmonid farms and hatcheries across the USA.

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Chapter 1:

Literature Review

1.1. Bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS)

1.1.1. Losses and economic impact

Flavobacterium psychrophilum is considered one of the most economically devastating flavobacterial fish pathogens, resulting in mortality rates as high as 90% in affected salmonid populations (Cipriano and Holt, 2005; Bernardet and Bowman, 2006; Starliper, 2011; Nilsen et al. 2011a,b). Although many reference the economic impact of *F. psychrophilum*-related losses, an exact monetary value to these losses has not yet been calculated. Considering rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) are the most valuable and farmed salmonid species worldwide and continue to sustain losses on multiple continents (e.g., Asia, Europe, North America, and South America; Van Vliet et al. 2016; Söderlund et al. 2018; Duchaud et al. 2018; Knupp et al. 2019; Avendaño-Herrera et al. 2020; Li et al. 2021), the economic impact of BCWD likely equates to millions of dollars in losses annually (Duchaud et al. 2018).

1.1.2. Host range

Flavobacterium psychrophilum primarily affects fish within the family *Salmonidae*. Although coho salmon (*O. kisutch*) and rainbow trout are considered most susceptible (Holt, 1987), reports of BCWD epizootics in Atlantic salmon are also frequent (Nilsen et al. 2011b; Avendaño-Herrera et al. 2020; Macchia et al. 2022). *F. psychrophilum* has also been recovered from many other salmonids, including amago (*O. rhodurus*), Apache trout (*O. apache*), Arctic char (*S. alpinus*), Arctic grayling (*Thymallus arcticus*), brown trout (*S. trutta*), brook trout (*Salvelinus fontinalis*), Chinook salmon (*O. tshawytscha*), chum salmon (*O. keta*), coruh trout (*S. coruhensis*), cutthroat trout (*O. clarkii*), European grayling (*T. thymallus*), lake trout (*S. namaycush*), lake whitefish (*Coregonus clupeaformis*), masou salmon (*O. masou*),

pink salmon (*O. gorbuscha*), sockeye salmon (*O. nerka*), splake (*S. namaycush* x *S. fontinalis*), and white spotted char (*S. leucomaenis*; Davis 1946, Rucker et al. 1953, Borg 1948, Schachte 1983, Holt 1987, Iida and Mizokami 1996, Ekman et al. 1999, Madetoja et al. 2001, Cipriano and Holt 2005, Fujiwara-Nagata et al. 2013; Saticioglu et al. 2018; Knupp et al. 2019; Harrison et al. 2022; Loch and Knupp, unpublished). *F. psychrophilum* has also been recovered from non-salmonid species, including ayu (*Plecoglossus altivelis*), common carp (*Cyprinnus carpio*), crucian carp (*Carassius carassius*), European eel (*Anguilla Anguilla*), European flounder (*Platichthys flesus*), far eastern brook lamprey (*Lethenteron reissneri*), forktongue goby (*Chaenogobius urotaenia*), goldfish (*C. auratus*), Indian catfish (*Clarias batrachus*), Japanese eel (*A. japonica*), Japanese crucian carp funbuna (*C. auratus langsdorfii*), Japanese dace (*Trybolodon hakonensis*), Japanese smelt (*Hypomesus nipponensis*), lake goby (*Rhinogobius brunneus*), numachichibu (*Tridentiger brevispinis*), pale chub (*Zacco platypus*), perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), sea lamprey (*Petromyzon marinus* L.), takahaya (*Phoxinus jouyi*), tench (*Tinca tinca*), three-spined stickleback (*Gasteroosteus aculeatus*), and white sturgeon (*Acipenser transmontanus*; Lehmann et al. 1991; Wakabayashi et al. 1994; Amita et al. 2000; Iida and Mizokami 1996; Madetoja et al. 2002; Starliper 2011; Fujiwara-Nagata et al. 2013; Harrison et al. 2021). *Flavobacterium psychrophilum* has also been recovered from other aquatic/semi-aquatic hosts, such as freshwater leeches (*Myzobdella lugubris;* Schulz and Faisal, 2010), benthic diatoms (Izumi et al. 2005); newts (Brown et al. 1997), aquatic plants (e.g., algae; Amita et al. 2000) and insects (e.g., caddisfly; Fujiwara-Nagata et al. 2013).

1.1.3. Geographic distribution

Flavobacterium psychrophilum is geographically widespread, having been recovered from fishes inhabiting at least 24 countries on five continents (e.g., Asia, Australia, Europe,

North America, and South America). In Asia, *F. psychrophilum* has been reported in the Korean Peninsula (Lee and Heo, 1998), Japan (Fujiwara-Nagata et al. 2013), Turkey (Kum et al. 2008; Saticioglu et al. 2018), China (Li et al. 2021), and Russia (Sundell et al. 2019). In Europe, *F. psychrophilum* has been reported in Belgium (Nematollahi et al. 2003), Denmark (Lorenzen et al. 1991; Nilsen et al. 2014), Estonia (Madetoja et al. 2001), Finland (Dalsgaard and Madsen, 2000; Madetoja et al. 2001), France (Siekoula-Nguedia et al. 2012), Germany (Nilz et al. 2009), Italy (Sarti et al. 1992), Ireland (Lorenzen et al. 1997), Norway (Nilsen et al. 2014), Scotland (Starliper, 2011), Spain (Toranzo and Barja, 1993), Sweden (Ekman et al. 1999), Switzerland (Strepparava et al. 2013), and the United Kingdom (Austin and Stobie, 1991). In South America, *F. psychrophilum* has been reported in Chile (Avendaño-Herrera et al. 2014; Avendaño-Herrera et al. 2020) and Peru (Leon et al. 2009). *Flavobacterium psychrophilum* has been reported in Australia (Schmidtke and Carson, 1995). In North America, *F. psychrophilum* has been reported in multiple states of the USA, including Alaska, California, Colorado, Idaho, Indiana, Maryland, Michigan, Minnesota, Missouri, Montana, North Carolina, New Jersey, New Mexico, New York, Oregon, Pennsylvania, South Dakota, Utah, Vermont, Virginia, Washington, Wisconsin, West Virginia, Wyoming (Holt, 1987; Van Vliet et al. 2016; Knupp et al. 2019; Loch and Knupp, unpublished, https://pubmlst.org/fpsychrophilum), as well as in the Canadian provinces of British Columbia and Ontario (Hesami et al. 2008; Knupp et al. 2019).

1.1.4. Modes of transmission

1.1.4.1. Vertical

Flavobacterium psychrophilum is known to be present in the reproductive fluids (e.g., ovarian fluid and milt) and egg surfaces of spawning-aged salmonids (Rangdale et al. 1996; Brown et al. 1997; Taylor 2004; Madsen et al. 2005; Cipriano, 2005; Van Vliet et al. 2016). In

addition, there is strong evidence that *F. psychrophilum* is also present intra-ova, occupying the perivitelline space, which may allow it to circumvent the current and widely utilized egg-surface disinfectant, iodophor (Brown et al. 1997; Cipriano, 2005). Importantly, vertical transmission appears important for the infiltration of fish hatcheries by some *F. psychrophilum* multilocus sequence typing variants (Knupp et al. 2019) and may account for this bacterium's intercontinental dispersion via the trade of infected eggs (Nicolas et al. 2008; Knupp et al. 2019; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Li et al. 2021).

1.1.4.2. Horizontal

Flavobacterium psychrophilum has been recovered from and detected in multiple freshwater sources (Wiklund et al. 2000; Strepparava et al. 2013; Nilsen et al. 2014; Nguyen et al. 2018). In vivo cohabitation/contact studies involving infected and naive fish (Madsen and Dalsgaard, 1999; Madetoja et al. 2000) have provided evidence for this bacterium's horizontal transmission. Seemingly key to *F. psychrophilum* transmission is shedding from both live and dead infected fish (Madetoja et al. 2000; Madetoja et al. 2002). Indeed, Madetoja et al. (2000) found live infected rainbow trout shed ~10^3 – 10^6 cells/fish/hour and for 10 – 21 days, depending on water temperature. Notably, the same authors also found dead rainbow trout shed ~10^4 – 10^8 cells/fish/hour and for at least 80 days. Brenden et al. (2023) used this data to develop a model predicting the transmission risk of *F. psychrophilum* in rainbow trout, and highlighted the importance of removing dead fish from rearing units. However, the applicability of these findings to other *F. psychrophilum* variants and other BCWD-susceptible salmonid species remains an open question, as these studies were conducted solely in rainbow trout and with one *F. psychrophilum* variant.

1.1.5. Prevention and control

Various methods for preventing and controlling BCWD have been thoroughly reviewed elsewhere (Nematollahi et al. 2003; Starliper, 2011; Barnes and Brown, 2011; Loch and Faisal 2017). In brief, BCWD prevention largely depends on comprehensive management strategies. These not only aim to reduce the likelihood of pathogen exposure (e.g., stringent biosecurity measures, disinfection of eggs with iodophor, health monitoring of fish, and the use of water sources free from *F. psychrophilum*; Starliper, 2011; Van Vliet et al. 2015; Loch and Faisal 2017), but also strive to minimize stress on the fish (Starliper, 2011). Additionally, maintaining optimal fish nutrition, water quality, and rearing conditions is critical (Starliper, 2011). These comprehensive best management practices are essential, particularly given that effective, licensed vaccines for BCWD remain unavailable in the USA at this time (Gomez et al. 2014). Alternative prevention methods are also being explored, including the use of genetically resistant fish (Wiens et al. 2013; Lee et al. 2023), probiotics (Burbank et al. 2012), and phage therapy (Christiansen et al. 2014). Although these strategies have shown promise in reducing the risk of BCWD and minimizing related losses, BCWD outbreaks continue to cause substantial losses in salmonid farming across the globe.

Control of BCWD epizootics relies heavily upon antibiotic and chemotherapeutic treatments (e.g., Chloramine-T and hydrogen peroxide). In the USA, Terramycin® (e.g., oxytetracycline dihydrate) and Aquaflor® (florfenicol) are the only two Food and Drug Administration-approved antibiotics for use in food fish. Concerningly, reports of mounting resistance to oxytetracycline have been reported in salmonid farms worldwide, including in the USA (Bruun et al. 2000; Schmidt et al. 2000; Van Vliet et al. 2017; Ngo et al. 2018).

1.2. *Flavobacterium psychrophilum,* causative agent of BCWD and RTFS

1.2.1. Taxonomy

Flavobacterium psychrophilum was originally placed within the genus *Cytophaga* by Borg (1948), and because of this species' affinity for low temperatures, it was given the name *Cytophaga psychrophila*. Because *F. psychrophilum* does not produce fruiting bodies or degrade polysaccharides, *C. psychrophila* was re-classified as *Flexibacter psychrophilus* (Bernardet and Grimont 1989), but subsequent analysis of DNA G+C content indicated that *F. psychrophilus* was most like bacteria within the genus *Flavobacterium*. In 1996, Bernardet et al. (1996) emended the description of the genus *Flavobacterium* and renamed *Flexibacter psychrophilus* as *Flavobacterium psychrophilum* (Phylum Bacteroidota; Class Flavobacteriia; Order Flavobacteriales; Family *Flavobacteriaceae*).

1.2.2. Phenotypic characteristics

Flavobacterium psychrophilum is a Gram-negative, rod-shaped (0.5-µm wide × 1 – 5-µm long), and weakly refractile bacterium (Pacha 1968; Bernardet and Kerouault 1989). In addition, *F. psychrophilum* is strictly aerobic and produces yellow-pigmented (due to the presence of a flexirubin-type pigment) colonies with a raised center and a smooth or thinly spreading edge (Bernardet and Kerouault 1989, Holt et al. 1987), although colony morphology varies (Hogfors-Ronnholm and Wiklund, 2010). This bacterium grows in the presence of up to 1.0% NaCl and at a pH range of 4.0 - 8.0 (Bernardet and Kerouault 1989). The bacterium degrades multiple substrates (e.g., tributyrin, tyrosine, lecithin, tween 20, and tween 80; Bernardet and Kerouault 1989) and proteolyzes many host tissue constituents (e.g., gelatin, casein, elastin, collagen, fibrinogen, chondroitin sulphate, and fish muscle extract; Holt, 1987; Lorenzen et al. 1997; Soule et al. 2005). In contrast, *F. psychrophilum* cannot hydrolyze

xanthine, chitin, starch, agar, carboxymethylcellulose, or esculin. Some *F. psychrophilum* variants reportedly use simple or complex carbohydrates, but this trait appears uncommon (Bernardet and Kerouault 1989; Cepeda et al. 2004; Cipriano and Holt 2005). This bacterium is weakly positive for both cytochrome oxidase and catalase, does not produce hydrogen sulfide, indole, arginine dihydrolase, lysine decarboxylase, or ornithine decarboxylase (Bernardet and Kerouault 1989; Cipriano and Holt 2005). *Flavobacterium psychrophilum* cannot absorb congo red nor reduce nitrate to nitrite (Bernardet and Kerouault 1989).

1.2.3. Proteolytic activity

Although *F. psychrophilum* reportedly proteolyzes multiple protein substrates, including albumin, casein, chondroitin sulfate, collagen, elastin, fibrinogen, and fish muscle extract (Pacha, 1968; Otis, 1984; Holt, 1987; Bertolini et al. 1994; Dalsgaard and Madsen, 2000; Madetoja et al. 2001; Madetoja et al. 2002; Soule et al. 2005; Sundell and Wiklund, 2015; Rochat et al. 2019; Sundell et al. 2019; Knupp et al. 2021), most studies assessing this bacterium's proteolytic activity have focused on the degradation of casein, elastin, and gelatin (Pacha, 1968; Otis, 1984; Holt, 1987; Bertolini et al. 1994; Dalsgaard and Madsen, 2000; Madetoja et al. 2001; Madetoja et al. 2002; Soule et al. 2005; Sundell and Wiklund, 2015; Rochat et al. 2019; Sundell et al. 2019; Knupp et al. 2021). In this context, the casein, elastin, and gelatin-degrading activity of >260 *F. psychrophilum* isolates recovered from numerous host species (e.g., Arctic char, Atlantic salmon, ayu, brook trout, brown trout, chinook salmon, coho salmon, flounder, perch, rainbow trout, sea trout, tench, and white sturgeon) in Asia, Europe, North America, and South America has been evaluated, although most isolates were recovered from rainbow trout. Nevertheless, of these *F. psychrophilum* isolates, only two did not degrade casein and/or gelatin (e.g., WB-1 recovered from ayu and FPS-S11B from rainbow trout; Nakayama et al. 2016; Sundell et al.

2019) and interestingly, were avirulent to their respective host species, possibly suggesting at least some proteolytic activity is required for virulence. In contrast to the nearly universal observation that *F. psychrophilum* degrades casein and gelatin, the ability of *F. psychrophilum* to degrade elastin is far more variable and may relate to the possession of a recently described elastase gene, *FP0506* (Rochat et al. 2019). However, elastase activity does not dictate the capacity to cause mortality in rainbow trout, as many elastase negative *F. psychrophilum* isolates have been recovered from diseased trout suffering mortality events (Dalsgaard and Madsen, 2000; Madetoja et al. 2002; Soule et al. 2005; Sundell and Wiklund, 2015; Rochat et al. 2019; Sundell et al. 2019). Likewise, multiple *F. psychrophilum* elastase negative isolates were virulent to rainbow trout following in vivo challenge, though fish were challenged via injection (Sundell et al. 2019), a route that bypasses some immune defenses (Fast et al. 2002). Besides virulence, elastase activity may also be associated with genetic lineage (Soule et al. 2005) and/or MLST variant, considering most *F. psychrophilum* isolates belonging to CC-ST10 degrade elastin (Sundell et al. 2019; Rochat et al. 2019; Knupp et al. 2021).

1.2.4. Genetic diversity and epidemiology

The diversity and epidemiology of *F. psychrophilum* has been studied for decades using multiple approaches, including pulsed field gel electrophoresis (Arai et al. 2007; del Cerro et al. 2010), plasmid profiling (Holt, 1987; Lorenzen et al. 1997; Chakroun et al. 1998; Izumi 2004; Madsen and Dalsgaard, 2000), randomly amplified polymorphic DNA analysis (Chakroun et al. 1997, Valdebenito and Avendaño-Herrera 2009), restriction fragment length polymorphism analysis (Izumi et al. 2003; Soule et al. 2005; Hesami et al. 2008), and ribotyping (Cipriano et al. 1996; Chakroun et al. 1998; Madsen and Dalsgaard, 2000). Currently, the most widespread method for characterizing *F. psychrophilum* intraspecific diversity is multilocus sequence typing

(Nicolas et al. 2008), which has been applied to >1500 *F. psychrophilum* isolates recovered across Asia, Australia, Europe, North America, and South America, revealing the existence of >260 different sequence types (STs; i.e., genetic variants; https://pubmlst.org/fpsychrophilum). Notably, MLST-based findings are largely consistent with whole genome-MLST-based findings (Duchaud et al. 2018). Most *F. psychrophilum* genetic variants in North America differ from those found in other continents (Nicolas et al. 2008; Siekoula-Nguedia et al. 2012; Fujiwara-Nagata et al. 2013; Strepparava et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019; Li et al. 2021) and some variants also appear to differ in host species association (Nicolas et al. 2008; Knupp et al. 2019; Knupp et al. 2021) and recovery environment (Van Vliet et al. 2016; Sebastião et al. 2020; Knupp et al. 2019). However, whether some *F. psychrophilum* variants are host specific, better suited to the artificial rearing environment, differ in transmission strategies, and/or are circumventing current gold-standard detection methods (e.g., culture), remains to be determined.

1.2.4.1. Multilocus sequence typing

Multilocus sequence typing (MLST) is a highly discriminatory and reproducible technique for characterizing the intraspecific diversity of bacteria. Originally developed for *Neisseria meningitidis*, a cause of meningitis in humans (Maiden et al. 1998), MLST has since been applied to multiple bacterial pathogens affecting multiple taxa, including fish (Nicolas et al. 2008). Nicolas et al. (2008) developed a MLST scheme for *F. psychrophilum* in 2008, relying upon partial sequences of seven conserved housekeeping genes, including *trpB, gyrB, dnaK, tuf, fumC, murG,* and *atpA*. In that pioneering study, 50 *F. psychrophilum* isolates recovered from 10 fish species in Asia, Europe, North America, and South America were genotyped, revealing the existence of 30 distinct sequence types (STs; i.e., genetic variants), some of which (e.g. ST2,

ST10, and ST13) were seemingly associated with a specific host species (e.g., rainbow trout, *Oncorhynchus mykiss* or coho salmon, *O. kisutch*; Nicolas et al. 2008). Later, Siekoula-Nguedia et al. (2012) examined 66 *F. psychrophilum* isolates from farmed rainbow trout in France, uncovering previously identified ST2, as well as 14 new genetic variants, most of which belonged to clonal complex (CC; i.e., a group of closely related variants)-ST2. Fujiwara-Nagata et al. (2013) studied 114 *F. psychrophilum* isolates recovered from 15 different fish species in Japan, finding 32 new genetic variants, some of which (e.g., ST40, ST45, ST54, and ST55) appeared associated with ayu (*Plecoglossus altivelis*) or masou salmon (*O. masou*). Moreover, some previously detected genetic variants (e.g., ST10, in CC-ST2 and ST13, in CC-ST9) were discovered, and again infecting either rainbow trout or coho salmon, continuing to provide observational evidence these STs/CCs may have host associations. In the same year, Strepparava et al. (2013) genotyped 112 *F. psychrophilum* isolates from Switzerland, finding CC-ST2 variants to be responsible for BCWD in their rainbow trout farms. Nilsen et al. (2014) analyzed 560 *F. psychrophilum* isolates recovered from 10 fish species in Denmark, Finland, Norway, and Sweden, revealing 81 different *F. psychrophilum* genetic variants. At this time, CC-ST2 was renamed CC-ST10 as ST10 had the most single locus variants. Moreover, the variants belonging to CC-ST10 and described in this study were almost exclusively recovered from rainbow trout. In the same year, Avendaño-Herrera et al. (2014) examined 91 *F. psychrophilum* isolates recovered from Atlantic salmon (*Salmo salar*), coho salmon and rainbow trout in Chile, and found most CC-ST10 variants were recovered from rainbow trout, whereas those belonging to CC-ST9 and CC-ST21 were mostly recovered from coho salmon and Atlantic salmon, respectively.

Despite the extensive MLST-based analyses conducted to this point in Europe, Asia, and South America, only 10 *F. psychrophilum* isolates originating from North America had been typed using the same method (Nicolas et al. 2008). Then, Van Vliet et al. (2016) studied 96 *F. psychrophilum* isolates recovered from Chinook salmon (*O. tshawytscha*), coho salmon, and rainbow trout in nine different USA states, and identified 34 STs. Like previous studies, most BCWD epizootics in rainbow trout were caused by CC-ST10 variants, whereas CC-ST9 was most prevalent among isolates recovered from coho salmon. Next, Knupp et al. (2019) genotyped 314 *F. psychrophilum* isolates recovered from 10 fish species in 20 states in the USA, as well as one Canadian province, and found 66 genetic variants, 47 of which were newly described. Most isolates belonging to a CC-ST10 variant were recovered from rainbow trout. Similarly, CC-ST9 variants were recovered from coho salmon. Additional CCs with apparent host associations for Atlantic salmon (e.g., CC-ST232) and rainbow trout (e.g., CC-ST191, CC-ST281, and CC-ST310) were also described, as was at least one generalist CC (e.g., CC-ST256) that was isolated from naturally infected rainbow trout and chinook salmon. Moreover, it was proposed that some genetic variants (e.g., ST253) may be better suited to hatchery environments and fish farms, as evidenced by their repeated recovery from the same facility over multiple years. This contrasted with other *F. psychrophilum* variants, such as ST256 and ST257, that were only recovered from wild/feral fish. Sebastião et al. (2020) genotyped 49 *F. psychrophilum* isolates recovered from Chinook salmon and rainbow trout in California, USA and found most isolates recovered from rainbow trout belonged to CC-ST10. Li et al. (2021) used MLST to assess genetic diversity among 31 *F. psychrophilum* isolates recovered from brook trout (*Salvelinus fontinalis*), rainbow trout, and masou salmon in China, and described five genetic variants, including two that belonged to CC-ST10, both of which were recovered from rainbow

trout. Calvez et al. (2021) genotyped 31 *F. psychrophilum* isolates recovered from rainbow trout in France, and most belonged to CC-ST10.

Flavobacterium psychrophilum genetic diversity according to MLST has been studied for nearly two decades and observations suggest some variants may be host specific or generalists. Although a few studies have directly or indirectly investigated such associations under in vivo laboratory conditions (Holt, 1987; Ekman and Norrgren, 2003; Fredriksen et al. 2016), most have used a less natural exposure route (e.g., injection) that bypasses important immune defenses (Fast et al. 2002; Dash et al. 2018). In contrast, at least one study reported the virulence of two host-associated *F. psychrophilum* variants (e.g., US19-coho salmon in ST9 and US53-rainbow trout in ST78) in coho salmon following laboratory immersion exposure (Knupp et al. 2021), and found US19-COS caused disease and mortality in coho salmon but US87-RBT did not. Although this study provided evidence some *F. psychrophilum* variants are host specific, a study has yet to simultaneously cross-challenge multiple salmonid species of a similar age with multiple putatively host specific *F. psychrophilum* variants. Besides host specificity, some *F. psychrophilum* variants appear to be associated with the fish farming environment; however, the long-term survival of different *F. psychrophilum* variants within microenvironments simulating fish farm/hatchery conditions has not been attempted.

1.2.5. Serotypic diversity

The serotypic diversity of *F. psychrophilum* has also been studied for decades (Pacha, 1968; Cipriano and Holt, 2005), although varying methodologies (e.g., slide agglutination and enzyme-linked immunosorbent assay; Wakabayashi et al. 1994; Lorenzen and Olesen, 1997; Izumi et al. 2003; Mata et al. 2002) and reagents have precluded many interlaboratory comparisons. Most recently, however, a reproducible PCR-based serotyping assay, which is

based on a widely accepted serotyping scheme (e.g., Fp^T, Fd, and Th) of Lorenzen and Olesen (1997) and targets putative *O*-polysaccharide genes, was developed by Rochat et al. (2017) to detect four serotypes (e.g., Type-0 – Type-3). Notably, Type-0 has a less conserved genomic structure and thus encompasses isolates not belonging to Types 1 – 3 (i.e., could contain several to many yet to be defined molecular serotypes). In this pioneering study, which examined 244 *F. psychrophilum* isolates from Canada, Chile, Denmark, Finland, France, Germany, Israel, Italy, Japan, Norway, Oregon, Scotland, Spain, Switzerland, Tasmania, and the USA, it was apparent that some serotypes were associated with specific host species. For example, most (*n* = 22/23 = 95.6%) isolates recovered from coho salmon belonged to Type-0, most isolates recovered from rainbow trout belonged to Type-1 (*n* = 59/151, 39.1%) or Type-2 (*n* = 60/151, 39.7%), and all (*n* = 35/35) isolates recovered from ayu belonged to Type-3 (Rochat et al. 2017). Saticioglu et al. (2018) serotyped 25 *F. psychrophilum* isolates recovered from rainbow trout in Turkey, finding most isolates belonged to Type-1 (*n* = 8/25, 32%) or Type-2 (*n* = 10, 40%), but a few belonged to Type-0 (*n* = 3, 12%) or Type-3 (*n* = 3, 12%), while one was untypable. Avendaño-Herrera et al. (2020) serotyped 118 *F. psychrophilum* isolates recovered from rainbow trout (*n* = 85), Atlantic salmon (*n* = 32), and coho salmon (*n* = 1) from Chile, and identified another *O*-antigen gene conserved among some *F. psychrophilum* isolates (e.g., Type-4). Using this updated molecular serotyping scheme, most Chilean isolates recovered from rainbow trout belonged to Type-2 (*n* = 50/85, 58.8%) followed by Type-4 (*n* = 17/85, 20.0%), Type-1 (*n* = 14/85, 16.5%), and Type-0 (*n* = 4/85, 4.7%), whereas most isolates recovered from Atlantic salmon belonged to Type-4 (*n* = 29/32, 90.6%), followed by Type-0 (*n* = 2, 6.3%), and Type-1 (*n* = 1/32, 3.1%). The isolate recovered from coho salmon belonged to Type-2. In China, Li et al. (2021) serotyped eight *F. psychrophilum* isolates recovered from rainbow trout, finding all belonged to Type-1. In

the same year, Calvez et al. (2021) serotyped 31 *F. psychrophilum* isolates recovered from rainbow trout in France, revealing most belonged to Type-2 (n = 14/31, 45.2%), followed by Type-0 (n = 9, 29.0%) and Type-1 (n = 8, 25.8%). In the USA, Knupp et al. 2021 serotyped one *F. psychrophilum* isolate recovered from rainbow trout and one isolate recovered from coho salmon, finding they belonged to Type-2 and Type-0, respectively.

Collectively, these studies provide evidence that rainbow trout are seemingly most affected by Type-1 and Type-2 *F. psychrophilum* isolates, and to a lesser extent, Type-0, Type-3, and Type-4. At present, Atlantic salmon seem to be most affected by Type-2 and Type-4, and sometimes Type-0, Type-1, and Type-3. Coho salmon are most affected by Type-0 and, infrequently, Type-2. Although observations suggest molecular serotypes may have host associations, in vivo evidence for such associations are lacking. Indeed, if some *F. psychrophilum* serotypes are associated with specific host species this could have important implications, such as for strain selection for BCWD vaccines and selective breeding programs.

1.2.6. Detection methods from fish

Pathogen detection is essential to disease diagnosis, which in turn informs management and treatment strategies. These strategies are designed to mitigate associated losses and prevent further transmission of the pathogen. In this context, multiple molecular assays have been developed to detect *F. psychrophilum* in fish tissue, including conventional, nested, multiplex, and quantitative PCRs targeting single (e.g., *rpoC*, *gyrB*, *parE*, RFPS00910; Izumi et al. 2005; Marancik and Wiens, 2013; Strepparava et al. 2014) and multicopy genes (e.g., 16S rRNA Nakagawa and Yamasota, 1993; Toyama et al. 1994; Urdaci et al. 1998). Likewise, multiple serological assays, including agglutination (Nagai and Nakai, 2011), immunofluorescent antibody technique (Aoki et al. 2005; Vatsos et al. 2006), immunohistochemistry (Lorenzen and

Karas, 1992; Evensen and Lorenzen, 1997; Madetoja et al. 2000), and enzyme-linked immunosorbent assay (Mata and Santos, 2001; Crump et al. 2003; Lindstrom et al. 2009) have been developed to directly detect *F. psychrophilum* in fish tissue. Although these assays are invaluable tools for detecting this fish pathogen and facilitate a timely diagnosis, a primary limitation is their inability to recover viable *F. psychrophilum* isolates, which are needed for antibiotic susceptibility testing, conducting molecular typing assays, developing vaccines, and for further research (Van Vliet et al. 2017; Ma et al. 2019; Knupp et al. 2019). These needs, coupled with the fact that most of these assays do not readily differentiate between live and dead *F. psychrophilum* cells, contribute to the reason why culture-based *F. psychrophilum* diagnostics are considered the gold-standard for detecting and identifying *F. psychrophilum*. In fact, the American Fisheries Society – Fish Health Section (AFS-FHS) Blue Book identifies culture as a prerequisite step to confirmatory diagnosis of BCWD (AFS-FHS 2020). In this context, many culture media have been used for *F. psychrophilum* isolation and identification, and although variation in employed medium occurs by country/region, tryptone yeast extract salts medium (TYES; Holt, 1987) is among the most widely used and thus could be considered the current gold-standard medium. However, TYES was developed before we had a more complete understanding of *F. psychrophilum* diversity, and ongoing research suggests it may not be ideally-suited for the recovery of all *F. psychrophilum* variants.

1.2.7. Bacterial recovery on solid media

1.2.7.1. Names and formulations

Anacker and Ordal (AO) medium, also referred to as Cytophaga agar, was developed by Anacker and Ordal (1959) for *F. psychrophilum* recovery and is formulated with tryptone, beef extract, yeast extract, and sodium acetate (Table 1.1). Since its development, at least 9

derivations of AO, typically referred to as modified or enriched AO (mAO or EAO), have been used for *F. psychrophilum* recovery (Table 1.1). Most derivations have retained tryptone, beef extract, yeast extract, and sodium acetate but have modified their concentration and/or added medium components, including $CaCl_2$, fetal bovine serum, horse serum, skimmed milk, fish or horse blood, carbohydrates (e.g., glucose, galactose, rhamnose), activated charcoal, and/or aromatic compounds (e.g., L-Tyrosine, L-Phenylalanine, L-Tryptophan, 4-Aminobenzoic acid, 4-Hydroxybenzoic acid, and 2,3-Dihydroxybenzoic acid). In addition, some derivations removed tryptone and beef extract and replaced them with a different protein source (e.g., peptone) or removed sodium acetate (Table 1.1).

Tryptone yeast extract salts medium (TYES) was developed by Holt (1987) for *F. psychrophilum* recovery and is formulated with tryptone, yeast extract, $CaCl_2$, and $MgSO_4$ (Table 1.2). Since its development, at least three derivations of TYES have been used for *F. psychrophilum* recovery, which have adjusted the concentration of one (e.g., $CaCl_2$; *F. psychrophilum* agar, FLPA; Cepeda et al. 2004) or two components (e.g., yeast extract and agar; no change to medium name; Madetoja et al. 2000) and/or added new components, including horse serum (modified TYES, mTYES; Knupp et al. 2021) or glucose (FLPA; Table 1.2).

Besides TYES, AO, and their derivations, other media have been used to recover *F. psychrophilum*. These media include nutrient agar (Secades et al. 2001), Shieh's medium (Holt, 1987), Hsu-Shotts agar (Cipriano and Holt, 2005), and modified veggietone (Ngo et al. 2017; Table 1.3).

1.2.7.2. Comparative studies

1.2.7.2.1. Michel et al. (1999)

In a study by Michel et al. (1999), five *F. psychrophilum* isolates recovered from rainbow trout in France (n = 3), Idaho (n = 1), and Denmark (n = 1) were grown in four different broth solutions. Then, bacterial yields (i.e., measured in colony forming unit; cfu) on EAO [(Bernardet and Kerouault (1989) formulation; Table 1.1] and TYES [Holt (1987) formulation with 1% skimmed milk; Table 1.2) with and without 10% fetal calf serum (FCS) were compared. These comparisons showed that TYES alone did not recover *F. psychrophilum*, but TYES+FCS recovered <1 cfu, on average, from 1/4 broth solutions. In contrast, EAO recovered 0.66 – 10.3 mean *F. psychrophilum* cfus across all broth solutions, and EAO+FCS improved recovery further by obtaining 16.3 – 84 mean cfus. In subsequent trials, where FCS was replaced by horse serum (HS, 5 or 10% concentration), it was observed that HS improved recovery over FCS by 18.3%. Thus, the authors suggested that EAO should be reformulated to include 5% HS.

Michel et al. (1999) presented an improved *F. psychrophilum* recovery medium; however, it is unclear if all five isolates were recovered similarly (i.e., cfus were reported as means without standard deviations). Furthermore, replication and statistical analyses were not reported, thus it is difficult to determine if this modified EAO yielded significantly more cfus in comparison to the original base media.

1.2.7.2.2. Crump et al. (2001)

Although not the primary goal of Crump et al. (2001), *F. psychrophilum* recovery was compared between mAO (Bernardet and Kerouault, 1989; Table 1.1) and mAO with 3% fish or horse blood using four *F. psychrophilum* isolates recovered from the USA (n = 2), England (n =

1), and Denmark (*n* = 1). As a result, mAO with blood was observed to enhance *F. psychrophilum* recovery, though to what extent was not reported.

1.2.7.2.3. Cepeda et al. (2004)

In a study by Cepeda et al. (2004), recovery of 13 *F. psychrophilum* isolates recovered from rainbow trout (*n* = 8), coho salmon (*n* = 3), European eel (*n* = 1), and common carp (*n* = 1) in Spain (*n* = 5), France (*n* = 3), the USA (*n* = 2), the U.K. (*n* =1), and Japan (*n* = 1) were compared on five solid media, including MAO (Toranzo and Barja, 1993; Table 1.1), MAO with glucose (MAOG), TYES (Cepeda et al. 2004 formulation), FLPA (this study; Table 1.2), and AO (Anacker and Ordal, 1959; Table 1.1) agar with carbohydrates (e.g., glucose, galactose, and rhamnose) and skimmed milk (CACM). Following these comparisons, FLPA recovered the most *F. psychrophilum* cfus overall, followed by TYES and CACM (i.e., tryptone-containing media), and recovery was reportedly worse in the peptone-containing media (e.g., mAO and MAOG). Moreover, and as noted in this study, recovery varied according to *F. psychrophilum* isolate, although the extent to which this occurred was not discussed.

In addition to comparing *F. psychrophilum* recovery in the laboratory, Cepeda et al. (2004) compared media (e.g., MAO, MAOG, TYES, FLPA, and CACM) as to their ability to recover *F. psychrophilum* from the kidney and spleen of naturally infected, farm-reared "trout" via streak plating (*n* = 17 cultures over two years). Here too, FLPA was most effective at *F. psychrophilum* recovery, and recovered the bacterium in pure and mixed (e.g., with *Aeromonas* and *Enterobacter* spp.) cultures.

1.2.7.2.4. Álvarez and Guijarro (2007)

In a study by Álvarez and Guijarro (2007), the recovery of one *F. psychrophilum* isolate recovered in the USA (e.g., THC02-90, MLST ST9 in CC-ST9; Nicolas et al. 2008) was

compared on six media, including nutrient agar (NA), NA with activated charcoal (NAC), EAO with 5% horse serum (EAOS; Michel et al. 1999), EAOS with aromatic compounds (e.g., L-Tyrosine, L-Phenylalanine, L-Tryptophan, 4-Aminobenzoic acid, 4-Hydroxybenzoic acid, and 2,3-Dihydroxybenzoic acid; EAOSa), EAOS with activated charcoal (EAOC), and EAOC with aromatic compounds (EAOCa). Results showed that the addition of charcoal significantly increased *F. psychrophilum* recovery across media. Moreover, and although NAC yielded the most colonies overall, *F. psychrophilum* recovery on EAOCa was less variable and thus ultimately the recommended medium for future studies.

1.2.7.2.5. Oplinger and Wagner (2012)

In a study by Oplinger and Wagner (2012), *F. psychrophilum* growth was compared in broth, but because the newly optimized medium developed by the authors was also shown to support *F. psychrophilum* recovery, it has been included in this section. The growth of one *F. psychrophilum* isolate recovered in France (e.g., ATCC FP49510) was initially compared in six iterations of EAO (Bernardet and Kerouault, 1989; Table 1.1). Within iterations, individual medium component (tryptone, yeast extract, beef extract, sodium acetate, skimmed milk, maltose, horse serum, $CaCl_2$, and $MgSO_4$) concentrations were varied (2 – 4 concentrations per medium component). Overall, tryptone, yeast extract, beef extract, skimmed milk, and horse serum were reported to benefit *F. psychrophilum* growth, and thus were included in a newly developed medium. The newly optimized medium was compared to EAO, TYES, and MAT (TYES with 1% maltose) using the same *F. psychrophilum* isolate ATCC FP49510, as well as CSF259-93 (MLST ST10 in CC-ST10; Nicolas et al. 2008), revealing that growth was best in the newly developed medium (OW), followed by MAT, EAO, and TYES.

Although these studies each reported an improved *F. psychrophilum* recovery medium, most only tested 1 – 5 isolates. Notably, however, in the one study (Cepeda et al. 2004) that used 13 *F. psychrophilum* isolates recovered from multiple host species, noticeable variation in cfu yields were described. Thus, future research should prioritize incorporating a diversity of *F. psychrophilum* isolates to produce an optimized recovery medium.

1.2.8. Detection and quantification from water

Detection and quantification of *F. psychrophilum* from water containing fish has been conducted via culture and immunofluorescence antibody technique (Madetoja et al. 2000; Madetoja and Wiklund, 2002; Madetoja et al. 2003), both of which are sensitive (e.g., detection limit of ~10^1 - 10^2 cfus/mL) but time consuming and vary in specificity. One quantitative PCR (qPCR) has been reported in the published literature for detecting and quantifying *F. psychrophilum* from water (Strepparava et al. 2014; Nguyen et al. 2018) and although sensitive (detection limit of ~10^1 gene copies) and specific, its quantification limit was relatively high (e.g., ~10^3 *F. psychrophilum* cells/mL; Strepparava et al. 2014). Of note, some laboratories have noticed non-specific amplification with this assay (Loch and Soto, unpublished). Indeed, the development or optimization of a qPCR assay to detect *F. psychrophilum* from water could be useful for deepening our understanding of transmission dynamics and may be useful for early *F. psychrophilum* detection in fish farms and hatcheries, thereby potentially mitigating losses.

1.2.9. Survival outside of fish

The widespread distribution and apparent success of *F. psychrophilum* may be partially related to its ability to survive outside its host. For example, Vatsos et al. (2003) found *F. psychrophilum* could survive for 133 days in sterile stream water. A lengthy survival time in water was also noted by Madetoja et al. (2003), whereby *F. psychrophilum* remained viable in

sterile lake water for 300 days. Although these studies are invaluable for clarifying *F. psychrophilum* persistence outside of a fish host, they were conducted using a total of three *F. psychrophilum* isolates (e.g., NCIMB 1947^{T}, ST9 in CC-ST9; V9/93, MLST variant unknown; B97026, MLST variant unknown) and represent the totality of our knowledge on this subject. Thus, it is still unknown whether the ability to persist long term in environments with limited nutrients is a widespread trait among *F. psychrophilum* variants. Madetoja et al. (2003) also found that *F. psychrophilum* survival could be improved (e.g., cells were recovered over longer period and at higher concentrations) if lake water also contained natural beach sand. Although beach sand is not commonly found in fish farm and hatchery rearing units, other sediments are, like detritus and uneaten fish food (Schumann, 2021). However, *F. psychrophilum* survival in these rearing unit microenvironments has yet to be explored and could partially explain why some MLST variants (e.g., ST10, ST253) are repeatedly recovered (i.e., over multiple years) from the same facility (Knupp et al. 2019). Indeed, by improving our understanding of *F. psychrophilum* environmental persistence strategies within fish farm/hatchery environments, we may be able to disrupt *F. psychrophilum* transmission pathways and mitigate the spread and losses caused by this bacterium.

1.2.10. Bacterial inoculum preparations

Various liquid culture media, such as TYES, iron-limited TYES, TYES with maltose or horse serum, Shieh (with or without iron), EAO, and modified EAO have been employed for the in vitro growth of *F. psychrophilum* prior to experimental challenge (Madsen and Dalsgaard, 1999; Decostere et al. 2000; Garcia et al. 2000; Madetoja et al. 2000; Aoki et al. 2005; Long et al. 2014; Sundell et al. 2019; Knupp et al. 2021). Similarly, a range of incubation conditions, including varying temperatures (e.g., 15 – 18 °C), durations (e.g., 18, 24, 48, or 72 hours), with

or without agitation, and different methods of estimating inoculum size prior to infection (e.g., correlating incubation time to growth phase or using optical density to correlate with viable cell counts) have been used (Madsen and Dalsgaard, 1999; Decostere et al. 2000; Garcia et al. 2000; Madetoja et al. 2000; Aoki et al. 2005; Long et al. 2014; Sundell et al. 2019; Knupp et al. 2021). Importantly, this variation in *F. psychrophilum* inoculum preparation affects study outcomes, thereby impeding comparability of findings among laboratories. For example, Aoki et al. (2005) found *F. psychrophilum* was more virulent when used in logarithmic phase in comparison to other culture phases. Thus, additional studies comparing the virulence of different *F. psychrophilum* variants following different culture conditions are warranted and may result in a reliable and standardized approach to inoculum preparation.

1.2.11. Experimental challenge models

Experimental challenge models are not only crucial for fulfilling Koch's postulates (Walker, 2006), but are invaluable for investigating host-pathogen interactions, including pathogenesis, virulence mechanisms, host immune response and susceptibility, and for assessing the efficacy of BCWD prevention (e.g., prophylactics and vaccines) and control (e.g., bacteriophages and antimicrobials) measures (Ekman and Norrgren, 2003; Van Vliet et al. 2017; Perez-Pascual et al. 2017; Rochat et al. 2019; Ma et al. 2019; Sundell et al. 2020; Semple et al. 2020; Deng et al. 2022; Huyben et al. 2023). To this end, various infection routes, including subcutaneous, intraperitoneal, and intramuscular injection (Holt, 1987; Obach and Laurencin, 1991; Fredriksen et al. 2016), oral and anal intubation, cohabitation of naïve with infected fish, and bath immersion methods have been used with varying success and reproducibility (Lorenzen et al. 1991; Decostere et al. 2000; Madetoja et al. 2000; Knupp et al. 2021).

Injection methods (e.g., subcutaneous, intraperitoneal, intramuscular) are generally considered to be the most reproducible exposure route as they generate consistent mortality among experimentally challenged fish (Madsen and Dalsgaard, 1999; Garcia et al. 2000). Moreover, some studies suggest intramuscular injection may be most advantageous, especially among larger (e.g., >5g) fish (Garcia et al. 2000; Fredriksen et al. 2013). For example, Fredriksen et al. (2013) used intramuscular injection to cause mortality among ~36.6g rainbow trout but was unable to produce mortality via intraperitoneal injection, even at a ~10-fold higher dose. Likewise, Holt (1987) found that to elicit comparable mortality in coho salmon, a substantially higher dose was required for intraperitoneal injection in comparison to intramuscular injection. Although injection methods may be preferred under specific circumstances, they inherently bypass natural barriers to infection, such as skin, mucus, lysozyme, complement, heat shock proteins, and immunoglobulins (Fraslin et al. 2018). For these reasons, injection methods may confound study findings, especially those influenced by host defenses. Indeed, injection could partially explain why some *F. psychrophilum* isolates belonging to MLST variants with apparent host-associations were able to cause mortality in multiple "unnatural" host species. For example, Holt (1987) found *F. psychrophilum* isolate SH3-81, which was recovered from coho salmon and belongs to CC-ST9 (i.e., a widespread, coho salmon-associated CC; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Knupp et al. 2019) was virulent via injection to not only coho salmon but chinook salmon and rainbow trout. Likewise, Bruce et al. (2021) found *F. psychrophilum* isolate CSF259-93, which was recovered from rainbow trout and belongs to CC-ST10 (i.e., the largest and most widespread rainbow trout-associated CC; Nicolas et al. 2008; Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014; Knupp et al. 2019) was virulent via injection to Atlantic

salmon and brook trout. However, it is also important to consider the true host specific nature of these isolates are unknown and may also contribute to these findings.

The prevalent use of injection methods in experimental challenges may inadvertently be shaping our understanding of *F. psychrophilum*-host interactions and consequently could significantly impact the development of BCWD prevention and control strategies. If our understanding of *F. psychrophilum* host associations and host response is primarily informed by injection studies, it may lead to narrow focus on specific *F. psychrophilum* variants while overlooking the substantial intraspecific diversity present in this species. Consequently, we risk developing narrow-spectrum vaccines or salmonid genetic lines resistant to only a limited set of *F. psychrophilum* variants. Thus, it is important to further evaluate *F. psychrophilum* host associations and host responses using more natural exposure routes that better capture the intricacies of host-pathogen interactions.

In this context, immersion studies, which involve immersing fish in suspensions of *F. psychrophilum*, more accurately simulate natural exposure. However, achieving reproducibility often necessitates disturbing a portion of the skin/mucus layer of the fish using mechanical or chemical methods (Madsen and Dalsgaard 1999, Garcia et al. 2000, Madetoja et al. 2000, Henriksen et al. 2013, Long et al. 2013; Macchia et al. 2022). The ideal approach would minimize this disruption, thereby maintaining the integrity of the fish's innate defenses. In this context, Madetoja et al. (2000) found that creating a 1 – 2-mm incision below the dorsal fin was sufficient for *F. psychrophilum* to invade and cause >80% mortality in rainbow trout. Long et al. (2013) described nearly identical findings in rainbow trout by using the same method. However, this wound type is not representative of those naturally incurred by fish in farms/hatcheries. Thus, Long et al. (2013) also attempted to induce mortality in rainbow trout by removing the

adipose fin, which is a common practice used in salmonid hatcheries for delineating hatchery fish from wild fish (Auld et al. 2019). In this context, adipose fin clipping prior to *F. psychrophilum* exposure led to successful bacterial invasion and resulted in >80% mortality. Besides rainbow trout, adipose fin-clipping prior to immersion has been successful in causing mortality among coho salmon (Holt, 1987; Knupp et al. 2021). Whether this exposure method is suitable for other intensively reared and economically important salmonid species (e.g., Atlantic salmon) remains to be determined but warrants further investigation. Indeed, an effective immersion challenge model for multiple salmonid species would be instrumental in examining *F. psychrophilum*-host interactions and testing the efficacy of BCWD vaccines.

1.2.12. Gross signs of BCWD in rainbow trout (*Oncorhynchus mykiss*), coho salmon (*O. kisutch*), and Atlantic salmon (*Salmo salar*)

1.2.12.1. Rainbow trout

External BCWD signs in naturally infected rainbow trout include lethargy, anorexia, distended abdomen, deep, focally extensive hemorrhagic ulceration on the dorsal aspect of the caudal peduncle, complete erosion of the caudal fin/peduncle, exophthalmia, pale gills, and dark skin pigmentation (Nematollahi et al. 2003; Nilsen et al. 2011a; Starliper et al. 2011; Li et al. 2021). Internally, naturally infected rainbow trout present with signs of anemia (e.g., pale liver and kidney), and have a swollen spleen and/or enteritis (Nematollahi et al. 2003; Nilsen et al. 2011a; Starliper et al. 2011; Li et al. 2021). Following experimental immersion challenge, identical external and internal signs of BCWD have been observed, along with an additional sign - erosion of the mouth (Aoki et al. 2005; Henriksen et al. 2013; Hoare et al. 2017).

1.2.12.2. Coho salmon

External BCWD signs in naturally infected coho salmon include erratic swimming, ulcerations on the caudal peduncle, trunk, jaw, and/or skull, dorsal swelling posterior to the skull, and dark skin pigmentation (Davis, 1946; Wood, 1974; Holt, 1987; Cipriano and Holt, 2005). Internally, acute septicemia has been described (Davis, 1946; Wood, 1974; Holt, 1987; Cipriano and Holt, 2005). Despite the known susceptibility of coho salmon to BCWD, their critical importance in conservation and stock enhancement efforts, and their prominent role in aquaculture (Nematollahi et al. 2003; Fujiwara-Nagata et al. 2013; Avendano-Herrera et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019; FAO, 2022), few studies have examined *F. psychrophilum*-coho salmon interactions via injection or immersion studies. In this context, Holt (1987) successfully caused mortality in juvenile coho salmon via immersion; however, the resultant gross disease signs were not reported. Knupp et al. (2021) immersion challenged coho salmon and reported multifocal ulcerations on the caudal peduncle that eventually exposed the spinal processes. Moreover, coho salmon had focally extensive ulcerations of the rostrum, in addition to diffuse ecchymoses and petechiae of the gills and intraocular focal ecchymosis. Internally, coho salmon had organ pallor, splenic swelling, perisplenic hemorrhage, multifocal hepatic ecchymoses, and hemorrhage within the pyloric caeca and the surrounding adipose tissue.

1.2.12.3. Atlantic salmon

Published reports of gross BCWD signs in Atlantic salmon are less frequent, but appear to be similar to rainbow trout. Externally, lethargy, erratic swimming or resting at the bottom of tanks, exophthalmia, swelling and/or ulceration with hemorrhage on the caudal peduncle are present (Nilsen et al. 2011b). Petechiae on the abdomen have also been reported (Nilsen et al.

2011b). Internally, Atlantic salmon display mottled liver, swollen and dark spleen, serohemorrhagic ascites, hemorrhage in the abdominal fat and skeletal muscle (Nilsen et al. 2011b). Although immersion challenges have used Atlantic salmon and caused mortality, gross disease signs have not always manifested or haven't been reported (Martinez et al. 2004; Macchia et al. 2022).

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APPENDIX

Table 1.1. Formulation (per 1L H_2O) of *Flavobacterium psychrophilum* recovery medium Anacker and Ordal (AO; Anacker and Ordal, 1959) and its derivatives, as reported in the literature.

		Medium									
Medium component	Unit	AO	mAO[a]	mAO[b]	mAO[c]	mAO[d]	mAO[e]	mAO[f]	mAO[g]	EAOCa[h]	OW[i]
Agar	g	11.0	11.0	9.0	15.0	11.0	15.0	10.0	15.0	15.0	10.0
Tryptone	g	0.5	5.0	0.5		0.5	2.0	0.5	5.0	5.0	5.0
Peptone	g				5.0						
Beef extract	g	0.2	0.2	0.2		0.2	0.2	0.2	0.2	0.2	0.2
Yeast extract	g	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sodium acetate	g	0.2	0.2	0.2	0.01	0.2	0.2	0.2	0.2	0.2	
$CaCl_2$	g						0.2				
Fetal bovine serum	%					10.0					
Horse serum	%							5.0		5.0	1.0
Skimmed milk	%			5.0							0.2
Fish blood	%								5.0		
Horse blood	%								5.0		
Glucose	g			0.5							
Galactose	g			0.5							
Rhamnose	g			0.5							
Activated charcoal	g									0.5	
L-Tyrosine	µmol									10.0	
L-Phenylalanine	µmol									10.0	
L-Tryptophan	µmol									10.0	
4-Aminobenzoic acid	µmol									10.0	
4-Hydroxybenzoic acid	µmol									10.0	
2,3-Dihydroxybenzoic acid	µmol									10.0	

[a] Modified AO (mAO), reported in Bernardet and Kerouault (1989).
[b] Reported in Daskalov et al. (1999).
[c] Reported in Toranzo and Barja (1993).
[d] Reported in Obach and Laurencin (1991).
[e] Reported in Wakabayashi and Egusa (1974).
[f] Reported in Michel et al. (1999).
[g] Reported in Crump et al. (2001).
[h] Enriched AO supplemented with charcoal and aromatic compounds, reported in Alvarez and Guijarro (2007).
[i] Oplinger and Wagner (OW) medium, reported in Oplinger and Wagner (2012).

Table 1.2. Formulation (per 1L H_2O) of *Flavobacterium psychrophilum* recovery medium tryptone yeast extract salts (TYES; Holt, 1987) agar and its derivatives, as reported in the literature.

		Medium			
Medium component	Unit	TYES	mTYES[a]	TYES[b]	FLPA[c]
Agar	G	10.0	10.0	15.0	10.0
Tryptone	G	4.0	4.0	4.0	4.0
Yeast extract	G	0.4	0.4	0.5	0.4
$CaCl_2$	G	0.5	0.5	0.5	0.2
$MgSO_4$	G	0.5	0.5	0.5	0.5
Glucose	G				0.5
Skimmed milk	%				
Horse serum	%		5.0		

[a] Modified TYES (mTYES), reported in Knupp et al. (2021).

[b] Reported in Madetoja et al. (2000).

[c] *Flavobacterium psychrophilum* agar (FLPA), reported in Cepeda et al. (2004). Later referred to as TYESG (i.e., TYES + glucose) in Barbier et al. (2020).

Table 1.3. Formulation (per 1L H_2O) of less frequently used (i.e., reported) *Flavobacterium psychrophilum* recovery media.

		Medium			
Medium component	Unit	Nutrient agar[a]	Shieh's[b]	Modified veggietone[c]	Hsu-Shotts[d]
Agar	G	Not reported	15.0	15.0	9.0 -15.0
Tryptone	G				0.20
Casitone	G				0.30
Veggitones soya peptone	G			5.0	
Beef extract	G	3.0			
Peptone	G	5.0	5.0		
Yeast extract	G		0.5	0.5	0.05
Sodium acetate	G		0.01		
$CaCl_2$	G		0.0067	0.2	0.03
$MgSO_4$	G		0.3	0.5	
Glucose	G		1.0	2.0	
KH_2PO_4	G		0.05		
$NaHCO_3$	G		0.05		
Sodium pyruvate	G		0.1		
K_2HPO_4	G		0.1		
$FeSO_4$	G		0.001		
$BaCl_2$	G		0.1		

[a] Reported in Secades et al. (2001).
[b] Reported in Holt (1987).
[c] Reported in Ngo et al. (2017).
[d] Reported in Cipriano and Holt (2005).

Chapter 2:

Enhanced culture media for the recovery of *Flavobacterium psychrophilum*, causative agent of bacterial coldwater disease and rainbow trout fry syndrome

2.1. Abstract

Flavobacterium psychrophilum causes bacterial coldwater disease (BCWD) in salmon and trout, resulting in significant economic losses worldwide. Bacterial culture remains a gold-standard method for detecting *F. psychrophilum* and is a core component of many BCWD research studies; however, observations suggest some variants may require an improved recovery medium. Therefore, this study sought to develop a culture medium that enhanced the recovery of a wide diversity of *F. psychrophilum* multilocus sequence typing (MLST) variants. Initially, the recovery of 165 geographically, temporally, and genetically diverse *F. psychrophilum* isolates was compared on three published *F. psychrophilum* culture media (e.g., tryptone yeast extract salts agar; TYES, Oplinger and Wagner medium, and enriched Anacker and Ordal medium supplemented with charcoal and aromatic compounds). The medium recovering the most colony forming units for the greatest number of isolates (e.g., TYES) was then modified following a Plackett-Burman experimental design, in which eleven nitrogen and two salt sources were assayed for recovery effects. Five compounds (e.g., $CaCl_2$, $MgSO_4$, casamino acids, tryptose, and fetal bovine serum) significantly influenced *F. psychrophilum* recovery. Guided by these findings, two new culture media (*Flavobacterium psychrophilum* medium-A and -B; FPM-A and FPM-B) were formulated, both of which significantly increased recovery (e.g., >141% increase in yielded colony forming units) compared to TYES. Next, the *F. psychrophilum* detection capabilities of the new media were compared to TYES during surveillance at four Michigan salmonid spawning-sites. In total, 300 spawning-age salmonids belonging to four different species (e.g., Atlantic salmon, *Salmo salar*; Chinook salmon, *Oncorhynchus tshawytscha*; coho salmon, *O. kisutch*; and rainbow trout, *O. mykiss*) were sampled and overall, the new media recovered *F. psychrophilum* from 7.9 - 8.7% more fish than TYES. Collectively, results

demonstrate that FPM-A and FPM-B support the recovery of many *F. psychrophilum* genetic variants from cryostock, improve *F. psychrophilum* recovery from naturally infected fish, and will therefore serve as a resource for enhancing ongoing and future BCWD research and diagnostic efforts.

2.2. Introduction

Flavobacterium psychrophilum (Family *Flavobacteriaceae*; Phylum Bacteroidetes), causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), is a Gram-negative fish-pathogenic bacterium that causes considerable economic losses in hatcheries and fish farms, particularly those raising salmonids (Family *Salmonidae*; Loch and Faisal, 2017). The current gold standard for detecting *F. psychrophilum* in infected fish is via in vitro isolation on nutrient deplete culture media (Nematollahi et al. 2003; Alvarez and Guijarro, 2007; Barnes and Brown, 2011). In contrast to most molecular and serological techniques, culture-based methods provide evidence for active infections via recovery of live cells, and give diagnosticians access to isolates from disease outbreaks, enabling ancillary tests necessary for guiding outbreak-specific treatment recommendations and/or to produce bacterins. Moreover, live isolates are required for many different BCWD experiments, including those leading to the development of efficacious BCWD prevention and control strategies. For instance, live *F. psychrophilum* has been used to test the efficacy of fish egg disinfectants (Brown et al. 1997; Kumagai et al. 1998), antimicrobials (Bruun, et al. 2000; Van Vliet et al. 2016; Li et al. 2021), and vaccines (Gliniewicz et al. 2012; Ma et al. 2019a). However, *F. psychrophilum* culture-based detection methods are not without challenges.

Flavobacterium psychrophilum is fastidious and relatively slow growing, with a reported generation time of ~2 hours at 15ºC (Holt, 1987). Likewise, the bacterium does not grow well on

commercially available culture media routinely used for growing other bacterial fish pathogens (Nematollahi et al. 2003). In this context, several culture media have been developed for the primary isolation and cultivation of *F. psychrophilum* (Anacker and Ordal, 1959; Bernardet and Kerouault, 1989; Bernardet and Bowman, 2006; Austin and Austin, 2007; Starliper, 2011), which are formulated with low nutrients and contain a protein source and salts. Some of the most common culture media for *F. psychrophilum* recovery are Cytophaga agar (Anacker and Ordal, 1959), tryptone enriched Cytophaga agar (Bernardet and Kerouault, 1989), and tryptone yeast extract salts medium (TYES; Holt, 1987); however, several studies have attempted to improve upon these formulations by adding different carbohydrates (Daskalov et al. 1999; MacLean et al. 2001; Cepeda et al. 2004; Hoare et al. 2019), proteins (Obach and Baudin-Laurencin, 1991; Lorenzen and Olesen, 1997; Daskalov et al. 1999; Michel et al. 1999; MacLean et al. 2001; Cepeda et al. 2004) and/or a detoxifier (Álvarez and Guijarro, 2007). Although some studies provided evidence these formulations improved *F. psychrophilum* growth in the laboratory and/or its recovery from infected fishes compared to other media (Daskalov et al. 1999; Michel et al. 1999; Cepeda et al. 2004; Álvarez and Guijarro, 2007), others found some incorporated medium components had neutral or negative effects on *F. psychrophilum* growth (Oplinger and Wagner, 2012). For instance, although Holt (1987) found the addition of $CaCl_2$ and $MgSO_4$ improved *F. psychrophilum* growth, Oplinger and Wagner (2012) reported these salts stunted growth.

The variability in formulations and performance among different *F. psychrophilum* culture media may be partially caused by this species' intraspecific diversity. Indeed, substantial genetic, serotypic, and phenotypic heterogeneity has been unearthed among different *F. psychrophilum* isolates (Wakabayashi et al. 1994; Lorenzen and Olesen, 1997; Madsen and

Dalsgaard, 1998; Madetoja et al. 2001; Nicolas et al. 2008; Högsfors-Rönnholm and Wiklund, 2010). Currently, the most widely-adopted technique for characterizing *F. psychrophilum* genetic diversity is multilocus sequence typing (MLST), which has been applied to >1500 isolates from 19 countries, resulting in the generation of >260 sequence types (STs; https://pubmlst.org/organisms/flavobacterium-psychrophilum). Most *F. psychrophilum* STs (i.e., variants) found in the U.S. are genetically distinct from those reported in other countries, and some also appear to vary in virulence, apparent host-associations, antibiotic susceptibility, and proteolytic activity (Van Vliet et al. 2016; Van Vliet et al. 2017; Sebastião et al. 2020; Li et al. 2021; Knupp et al. 2021a; Knupp et al. 2021b; Harrison et al. 2022). Likewise, ongoing studies suggest some U.S. *F. psychrophilum* variants may also differ in nutritional requirements, as evidenced by difficulties in primary isolation (Loch and Knupp, unpublished). In reviewing published studies that aimed to improve *F. psychrophilum* recovery, most studies used ≤5 isolates and found universal improvement. (Daskalov et al. 1999; Michel et al. 1999; Alvarez and Guijarro, 2007; Oplinger and Wagner, 2012). In contrast, another study using a greater number of *F. psychrophilum* isolates (e.g., 13) found recovery differed among isolates (Cepeda et al. 2004).

Given the increasing recognition of *F. psychrophilum* intraspecific diversity and the inconsistencies among in vitro culture findings, this study was designed to develop a culture medium that would enhance recovery and in vitro culture of a range of *F. psychrophilum* variants, thereby supporting future BCWD research and diagnostic efforts. To accomplish this goal, the recovery of a large collection of diverse *F. psychrophilum* isolates from cryostock was initially compared on three published media, including TYES (Holt, 1987), Oplinger and Wagner medium (OW; Oplinger and Wagner, 2012), and enriched Anacker and Ordal medium

supplemented with activated charcoal and aromatic compounds (EAOCa; Alvarez and Guijarro, 2007), which are formulated with different proteins, salts, and other nutrients. Next, findings were used to select a basal medium and formulate two new media via a Plackett-Burman experimental design (Plackett and Burman, 1944). The newly developed culture media and the original medium were then compared for their ability to recover *F. psychrophilum* from cryostock and naturally infected fish at several Michigan gamete collection sites.

2.3. Materials and Methods

2.3.1. *Flavobacterium psychrophilum* isolates

A total of 165 *F. psychrophilum* isolates, recovered from 1981 to 2020 and originating from 19 U.S. states, one Canadian province, and the countries of Chile and Denmark, were used in this study (Table 2.1, Table 2.2). The isolates were recovered from external and internal organs of captive or wild/feral fish belonging to five genera (within the families *Salmonidae* and *Acipenseridae*) and 12 species, including rainbow/steelhead trout (*Oncorhynchus mykiss*; n = 96), Chinook salmon (*O. tshawytscha*; n = 25), coho salmon (*O. kisutch*; n = 17), brown trout (*Salmo trutta*; n = 9), Atlantic salmon (*S. salar*; n = 7), lake whitefish (*Coregonus clupeaformis*; n = 3), brook trout (*S. fontinalis*; n = 2), lake trout (*S. namaycush*; n = 2), cutthroat trout (*O. clarkii*; n = 1), sockeye salmon (*O. nerka*; n = 1), splake (*S. fontinalis* x *S. namaycush*; n = 1), and white sturgeon (*Acipenser transmontanus*; n =1) at various life-stages (Table 2.1, Table 2.2). The isolates were genetically diverse according to multilocus sequence typing (MLST), whereby most (n = 153) had been previously genotyped (Van Vliet et al. 2016; Knupp et al. 2019; Ma et al. 2019a; Li et al. 2021; Harrison et al. 2022) but several (n = 12) were newly genotyped in the current study following published protocols (Knupp et al. 2019; Table 2.2). In total, isolates

belonged to 105 MLST sequence types (STs) that were either assigned to one of eighteen clonal complexes (CCs) or were singletons (Table 2.1, Table 2.2).

2.3.2. Basal medium experiment

2.3.2.1. Media selection

A total of three previously reported media, including tryptone yeast extract salts agar (TYES; Holt, 1987), Oplinger and Wagner medium (OW; Oplinger and Wagner, 2012), and enriched Anacker and Ordal medium supplemented with activated charcoal and aromatic compounds (EAOCa; Alvarez and Guijarro, 2007) were compared for *F. psychrophilum* recovery [e.g., colony forming unit (cfu) yield] from cryostock. The three media (e.g., TYES, OW, and EAOCa) were selected for their widespread geographical and/or recommended use (Barnes and Brown, 2011), differing formulations (Table 2.3), and/or reported ability to improve *F. psychrophilum* growth in comparison to other media recommended for *F. psychrophilum* culture (Holt, 1987; Alvarez and Guijarro, 2007; Oplinger and Wagner, 2012).

2.3.2.2. Bacterial preparation

For this experiment, 165 cryopreserved *F. psychrophilum* isolates were revived onto a medium formulated herein that would not favor one medium over the others (termed "neutral medium"), which consisted of (per liter): 10 g bacteriological agar (ThermoFisher Scientific), 4.5 g tryptone (ThermoFisher Scientific), and 0.45 g yeast extract (ThermoFisher Scientific; Table 2.3). Following inoculation of cryostock onto the neutral medium, plates were incubated at 15 ºC for 72 hours, after which cultures were visually inspected for purity. A 1-µl loopful of each isolate was inoculated into 40 mL of analogous neutral medium broth and incubated at 15 ºC with constant shaking at 180 rpm for 48 hours. Bacteria were harvested from broth via centrifugation (2,571 × *g*, 10 min) and then adjusted to a standardized optical density at 600-nm

(OD_{600} = 2.0) using a Biowave CO8000 Cell Density Meter (i.e., spectrophotometer; WPA Inc.) and sterile 0.65% saline. To compare *F. psychrophilum* recovery, serial dilutions in 10-fold increments (diluted up to 100,000,000-fold) were plated using 10-µl drops on TYES, OW, and EAOCa, in duplicate, and then incubated at 15 ºC for seven days, after which final colony counts were performed.

2.3.2.3. Data analysis

The goal of this experiment was to determine which medium (e.g., TYES, OW, or EAOCa) would be used as the base formulation for the Plackett-Burman experiment. To do so, the medium that recovered the most cfus of *F. psychrophilum* for the greatest number of isolates was selected. In this context, a linear mixed model was used to quantify the effect (log_{10} cfu yield) of each medium on the 165 *F. psychrophilum* isolates. The model included medium, isolate, and the interaction between medium and isolate as fixed effects. Replicates nested within isolates were treated as random effects to account for the variability among replicates within each isolate and allow for the inference about the variability of the response variable (e.g., log_{10} cfu yield) for different isolates not specifically measured in this study. Degrees of freedom for fixed effects were calculated using the Kenward-Roger method. Custom hypothesis tests as to differences in overall mean log_{10} cfu yield between media (e.g., EAOCa vs. OW, EAOCa vs. TYES, and EAOCa vs. OW) and between media for each isolate, were evaluated through pairwise comparisons of least-square means and adjusted for multiple comparisons using the Tukey-Kramer method ($\alpha = 0.05$). Analyses were performed using PROC MIXED in SAS® Version 9.4; custom hypothesis testing was performed using the LSMEANS statement and pdiff option.

2.3.3. Plackett-Burman experiment

2.3.3.1. Experimental design

A Plackett-Burman experimental design (Plackett and Burman, 1944) was used to screen 11 independent variables (i.e., medium components) at a low (-1) and high (+1) concentration for their main effect on *F. psychrophilum* recovery (Table 2.4, Table 2.5). Of the 11 tested medium components, eight were nitrogen sources, as metabolic reconstruction of *F. psychrophilum* following whole-genome sequencing indicated that proteins and amino acids are likely the main energy sources for *F. psychrophilum* (Duchaud et al. 2007). In this context, tryptone, yeast extract, beef extract, casamino acids, tryptose, L-aspartic acid, horse serum, and fetal bovine serum (FBS) were evaluated (Table 2.4). Additionally, two salts (e.g., calcium chloride dihydrate, $CaCl_2$ and magnesium sulfate heptahydrate, $MgSO_4$) that were reported to improve *F. psychrophilum* growth (Holt, 1987), and rainbow trout blood, which is a source of multiple host factors, including iron, an essential element for bacterial growth and virulence (Ratledge and Dover, 2000; Table 2.4), were also evaluated. All medium components were obtained from ThermoFisher Scientific or Millipore Sigma as detailed in Table 2.4, except rainbow trout blood, which was obtained from yearling rainbow trout reared under quarantine at the Michigan State University (MSU) – University Research Containment Facility in accordance with the MSU – Institutional Animal Care and Use Committee (AUF:201900312). Briefly, rainbow trout were euthanized with an overdose (250 mg/L) of sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; Syndel) and then blood was collected by caudal venipuncture, which was immediately combined with an equal volume of Alsever's solution (Millipore Sigma) and transported on ice for immediate use. In total, 12 media types were formulated according to the Plackett-Burman design matrix (Table 2.5), whereby each media type incorporated six medium

components at the high concentration and five at the low concentration, except for the first medium, which incorporated all medium components at the low concentration. Across the 12 media types, each medium component was incorporated six times at the high and low concentrations (see columns in Table 2.5). Medium component concentrations were selected to be at least 25% below and 50% above those used in EAOCa, OW, and TYES, while medium components not used EAOCa, OW, and TYES were modeled similarly.

2.3.3.2. Media preparation

Each media type was prepared identically as follows: bacteriological agar, $CaCl_2$ and $MgSO_4$ were weighed and added to ultrapure water that was subsequently brought to a boil, and then sterilized by autoclaving for 15 min at 121 ºC. Meanwhile, a proteinaceous solution composed of tryptone, yeast extract, beef extract, casamino acids, tryptose, and L-aspartic acid was prepared and adjusted to a pH of 7.20 ± 0.01 using potassium hydroxide. The proteinaceous solution was then filter sterilized using a 0.22-µm filter flask with a polyethersulfone filter membrane (Santa Cruz Biotechnology, Dallas, TX, USA) to prevent protein denaturation that occurs with autoclave sterilization (Taha and Mohamed, 2003). The sterilized proteinaceous solution, horse serum, FBS, and rainbow trout blood were added to the autoclaved solution (cooled to 55 ºC), and then continuously mixed via a stir plate while 24mL of media was dispensed into Petri dishes (100-mm in diameter x 15-mm in height; VWR, Radnor, PA, USA). Once the media solidified, the petri dishes were immediately stored at 4 ºC and used within seven days.

2.3.3.3. Bacteria preparation

For this experiment, 50 cryo-preserved *F. psychrophilum* isolates representing 49 different MLST STs (Table 2.2) were revived, cultured in broth, adjusted to a standardized OD_{600}, and then inoculated onto the 12 media types as described in section 2.3.2.2.

2.3.3.4. Data analysis

The Plackett-Burman experimental design consisted of 11 factors (i.e., medium components), 12 runs (i.e., media types), and 50 replicates (i.e., *F. psychrophilum* isolates), equating to 600 total runs. The effect of each medium component was calculated with the following equation:

$$E(x_i) = \frac{2(\sum C_i^+ - C_i^-)}{N}$$

where $E(x_i)$ is the concentration effect of the tested medium component, C_i^+ and C_i^- are the *F. psychrophilum* cfus recovered from the runs where the medium component (x_i) was at the high and low concentrations, respectively, and N is the number of runs (600). The significance of each medium component's concentration was determined using a one-sample, two-tailed Student's t test ($H_0 = 0$, $\alpha = 0.05$):

$$t(x_i) = \frac{E(x_i)}{SE}$$

where $E(x_i)$ is the concentration effect of the tested medium component x_i. The development of the Plackett-Burman experimental design and generation of the normal plot of the standardized effects and Pareto chart was completed in Minitab 21 Trial (Minitab Inc., USA).

2.3.4. Comparison of newly formulated media to TYES

2.3.4.1. Formulation of new media

Based upon results from the Plackett-Burman experiment, two new media types (e.g., *F. psychrophilum* medium-A, FPM-A; *F. psychrophilum* medium-B, FPM-B), were formulated and then compared to TYES for *F. psychrophilum* growth. *Flavobacterium psychrophilum* medium-A consisted of (per L): 0.75 g tryptone, 0.075 g yeast extract, 0.75 g $CaCl_2$, 1.11 g $MgSO_4$, 0.075 g beef extract, 0.75 g L-aspartic acid, and 7.5 mL horse serum. *Flavobacterium psychrophilum*-B consisted of (per L): 0.75 g Tryptone, 0.075 g yeast extract, 0.75 g $CaCl_2$, 1.11 g $MgSO_4$, 0.075 g beef extract, 0.75 g casamino acids, 0.75 g tryptose, 0.75 g L-aspartic acid, 7.5 mL horse serum, and 7.5 mL FBS. Both FPM-A and FPM-B were prepared as described in section 2.3.3.2.

2.3.4.2. Bacterial preparation

In this experiment, 165 *F. psychrophilum* isolates (Table 2.1, Table 2.2) were revived, cultured in broth, adjusted to a standardized OD_{600}, and then inoculated onto TYES, FPM-A, and FPM-B as described in section 2.3.2.2.

2.3.4.3. Data analysis

A linear mixed model was used to quantify the effect (cfu yield) of each medium on the 165 *F. psychrophilum* isolates. The model included medium, isolate, and the interaction between medium and isolate as fixed effects. Replicates nested within isolates were treated as random effects to account for the variability among replicates within each isolate and allows for the inference about the variability of the response variable (e.g., cfu yield) for different isolates not specifically measured in this study. Degrees of freedom for fixed effects were calculated using the Kenward-Roger method. Custom hypothesis tests as to differences in overall mean cfu yield among media (e.g., FPM-A vs. TYES, FPM-B vs. TYES, and FPM-A vs. FPM-B) were

evaluated through pairwise comparisons of least-square means and adjusted for multiple comparisons using the Tukey-Kramer method ($\alpha = 0.05$). Analyses were performed using PROC MIXED in SAS® Version 9.4; custom hypothesis testing was performed using the LSMEANS statement and pdiff option.

In addition, overall percent change in *F. psychrophilum* cfus was calculated for each of the three comparisons using the following equation:

$$Percent\ change = \left(\frac{Mean\ cfus\ on\ Medium\ X - Mean\ cfus\ on\ Medium\ Y}{Mean\ cfus\ on\ Medium\ X}\right) * 100$$

All computations were performed in SAS® Version 9.4.

2.3.5. Comparison of new media to TYES for the recovery of *Flavobacterium psychrophilum* from naturally infected fish

2.3.5.1. *Flavobacterium psychrophilum* surveillance in Michigan salmonid broodstock

To compare the *F. psychrophilum* detection capabilities of FPM-A, FPM-B, and TYES, each media type was used in the fall of 2021 to test for *F. psychrophilum* infections at four salmonid spawning sites (i.e., weirs) in Michigan (Figure 2.1), where systemic infections are prevalent in most years (Van Vliet et al. 2015; Knupp et al. 2019). Spawning phase Chinook salmon (n = 30 males and n = 30 females per site) were collected at the Little Manistee River Weir (LMRW) and Swan River Weir (SRW), whereas coho salmon and Atlantic salmon (n = 30 males and n = 30 females per species) were obtained at Platte River Weir (PRW) and St. Mary's River (SMR), respectively. Similarly, in the spring of 2022, FPM-A was used alongside TYES at LMRW, to test for *F. psychrophilum* infections in 30 male and 30 female spawning-phase steelhead trout that were captured. All fish were collected and processed for bacterial isolation as previously described (Loch et al. 2012; Van Vliet et al. 2015). Briefly, fish were euthanized on site using a pneumatic stunner (Seafood Innovations International, Queensland, Australia) and

then necropsied, whereby fish were surfaced disinfected with 70% ethanol and then the coelomic cavities and renal capsule were opened using sterile forceps and scissors. A sterile 10-µl loop was passed through the entire length of the kidney multiple times and then tissues streaked directly onto each media type, all of which contained 4 mg/L of neomycin sulfate (Millipore Sigma). All media types were subsequently incubated at 15 ºC for seven days, after which plates were examined for yellow-pigmented bacterial growth.

All yellow-pigmented bacteria recovered on each media type were sub-cultured, and following verification of culture purity, bacterial genomic DNA was extracted, quantified, and diluted to 20 ng/µl as described previously (Knupp et al. 2019). To determine if the identity of the recovered bacterial isolate was *F. psychrophilum*, the conventional PCR assay of Toyama et al. (1994) was employed as detailed previously (Van Vliet et al. 2015). The proportion of fish with *F. psychrophilum* (i.e., infection prevalence) is reported.

2.4. Results

2.4.1. Selection of basal medium

Of the three previously published and tested media (e.g., TYES, OW, and EAOCa), TYES recovered, on average, significantly more *F. psychrophilum* $\log_{10}$ cfus (e.g., 8.73 ± 0.013) than OW (e.g., 8.32 ± 0.013; $t = 23.49$, $df = 330$, P-value < 0.0001) and EAOCa (7.36 ± 0.013; $t = 79$, $df = 330$, P-value < 0.0001), and OW recovered significantly more *F. psychrophilum* cfus than EAOCa (e.g., 8.32 ± 0.013 vs. 7.36 ± 0.013; $t = 55.51$, $df = 330$, P-value < 0.0001).

Additionally, pairwise comparisons between media types for individual *F. psychrophilum* isolates showed TYES recovered the most cfus for the greatest number of isolates ($n = 7/165$ isolates, 4.24%; P-values < 0.05; Table 2.6) or recovered a similar number of cfus (i.e., no significant difference; P-values > 0.05) as OW ($n = 84/165$ isolates, 50.91%), EAOCa ($n = 8/165$

isolates, 4.85%), or OW and EAOCa (n = 64/165 isolates, 38.79%). In contrast to TYES, EAOCa achieved the most growth for one isolate (e.g., US151), or OW and EAOCa recovered a similar (i.e., no significant difference) number of cfus for one isolate (e.g., US487; Table 2.6). Based on these results, TYES was selected as the medium to improve upon as guided by the Plackett-Burman experiment.

2.4.2. Plackett-Burman experiment

The Plackett-Burman experiment was designed to screen 11 medium components for their effect on *F. psychrophilum* recovery. Of these, five medium components (e.g., $CaCl_2$, $MgSO_4$, casamino acids, tryptose, and FBS) had a significant effect on *F. psychrophilum* recovery (P-values < 0.0001), whereas six medium components did not (P-values > 0.05; Table 2.7). Normal plot of the standardized effects showed that of the five statistically significant medium components, two (e.g., $CaCl_2$ and $MgSO_4$) had a significant positive effect on *F. psychrophilum* recovery (i.e., increase of component concentration yielded more cfus), whereas the remaining three medium components (e.g., casamino acids, tryptose, and FBS) had a significant negative effect on *F. psychrophilum* recovery (i.e., increase of component concentration yielded less cfus; Figure 2.2). Overall, casamino acids affected *F. psychrophilum* recovery the most, followed by tryptose, $CaCl_2$, $MgSO_4$, FBS, L-aspartic acid, tryptone, beef extract, *O. mykiss* blood, horse serum, and yeast extract (Figure 2.3).

As a result of the Plackett-Burman experiment, two new media were formulated (see section 2.3.4) and named FPM-A and FPM-B (*F. psychrophilum* medium-A and -B). FPM-A contained the statistically significant medium components that had a positive effect on *F. psychrophilum* recovery with increasing concentration (e.g., $CaCl_2$ and $MgSO_4$) in addition to most of the statistically insignificant medium components (e.g., tryptone, yeast extract, beef

extract, L-aspartic acid, and horse serum), but omitted the statistically significant medium components that had a negative effect on *F. psychrophilum* recovery with increasing concentration (e.g., casamino acids, tryptose, and FBS) in addition to *O. mykiss* blood, as it did not significantly affect *F. psychrophilum* recovery, has a short shelf-life, and is difficult to source. FPM-B was formulated with all tested medium components except *O. mykiss* blood for the same rationale stated previously. The medium components for both new media types were incorporated according to their concentration effect in Table 2.7. More specifically, tryptone, yeast extract, beef extract, casamino acids, tryptose, horse serum, and FBS were incorporated at the low concentration, whereas $CaCl_2$, $MgSO_4$, and L-aspartic acid were incorporated at the high concentration (Table 2.4).

2.4.3. Comparisons of *Flavobacterium psychrophilum* growth on FPM-A, FPM-B, and TYES

Of the three media (e.g., FPM-A, FPM-B, and TYES) compared for *F. psychrophilum* recovery, FPM-A recovered, on average, significantly more *F. psychrophilum* cfus than TYES (e.g., $2.67 \times 10^9 \pm 1.10 \times 10^8$ vs. $1.07 \times 10^9 \pm 1.10 \times 10^8$; 149.5% increase; $t = 10.27$, $df = 495$, P-value < 0.0001) but not FPM-B (e.g., $2.67 \times 10^9 \pm 1.10 \times 10^8$ vs. $2.58 \times 10^9 \pm 1.10 \times 10^8$; 3.5% increase; $t = 0.59$, $df = 495$, P-value = 0.8236). Similarly, FPM-B recovered significantly more *F. psychrophilum* cfus than TYES ($2.58 \times 10^9 \pm 1.10 \times 10^8$ vs. $1.07 \times 10^9 \pm 1.10 \times 10^8$; 141.1% increase; $t = 9.67$, $df = 495$, P-value < 0.0001).

2.4.4. FPM-A, FPM-B, and TYES *Flavobacterium psychrophilum* detection comparison

Flavobacterium psychrophilum was recovered from each of the four sampling locations and salmonid species (Figure 2.4), where Chinook salmon from SRW had the lowest infection prevalence (0 - 1.7%) and Atlantic salmon from SMR had the highest (73.3 - 86.7%; Figure 2.4).

When comparing the three media across the four sampling events during which they were used simultaneously, FPM-A recovered *F. psychrophilum* at the highest prevalence overall (86/240 fish, 35.8%), followed by FPM-B (84/240 fish, 35.0%) and then TYES (65/240 fish, 27.1%). When comparing systemic *F. psychrophilum* infection prevalence as detected on each medium and at each individual site, prevalence ranged from 11.7% with TYES to 25.0% with FPM-A at LMRW with Chinook salmon; 73.3% (TYES) - 86.7% (FPM-B) at SMR with Atlantic salmon; 23.3% (TYES) - 35.0% (FPM-A) at PRW with coho salmon, and 0.0% (TYES and FPM-A) - 1.7% (FPM-B) at SRW with Chinook salmon (Figure 2.4). For steelhead collected from the LMRW*, F.* psychrophilum infection prevalence on FPM-A and TYES was 35.0% and 26.7%, respectively (Figure 2.4).

2.5. Discussion

Although several previously published *F. psychrophilum* culture media exist, ongoing research, deepening insight into the potential implications of *F. psychrophilum* intraspecific diversity, and the need for enhanced culture media to support BCWD vaccine development collectively necessitated the need for an evaluation and comparison of contemporary culture media, with the intent of further improvement. Accordingly, two new *F. psychrophilum* culture media (e.g., FPM-A and FPM-B) were developed, both of which significantly increased (e.g., by 141.1 – 149.5%) *F. psychrophilum* recovery in the laboratory when compared to the widely used and original basal medium, TYES. Indeed, the robust performance of FPM-A and FPM-B suggests that these media will be instrumental to future BCWD research studies with a culture component, which is a prevalent feature across multiple study areas. For example, culture is used to study *F. psychrophilum* virulence mechanisms, such as proteolytic activity, biofilm, and motility (Levipan and Avendaño-Herrera, 2017; Perez-Pascual et al. 2017; Rochat et al. 2019),

and host-pathogen interactions including virulence, host specificity, comorbidity, and transmission (Madetoja et al. 2000; Ma et al. 2019b; Knupp et al. 2021a; Knupp et al. 2021b; Li et al. 2021; Bruce et al., 2021). Likewise, culture is necessary for the development and testing of BCWD prevention (e.g., vaccines, phage-therapy; ultraviolet light susceptibility; Christiansen et al. 2014; Ma et al. 2019a; Donati et al. 2021; Knupp et al. 2023, in press) and control (e.g., antimicrobial susceptibility; Miranda et al. 2016; Saticioglu et al. 2019; Sebastiao et al. 2020) strategies. Thus, FPM-A and FPM-B are promising tools for improving the efficiency of many important facets of BCWD research designed to reduce economic losses.

Another important outcome of this study is the increased recovery of *F. psychrophilum* from naturally infected wild/feral salmonid broodstock populations on the new media when compared to TYES. Notably, the recovery of non-target bacteria was minimal (data not shown), although whether this reflects the specificity of the new media, or the infection status of the host requires further investigation. The improved detection capability not only underscores the usefulness of the new media under field conditions, but also promises more effective *F. psychrophilum* surveillance in these populations, which can guide the development of improved management strategies potentially leading to healthier broodstock and their progeny. Likewise, and although not studied herein, early detection in captive salmonid broodstock or their progeny could also prevent future losses. Similarly, if *F. psychrophilum* infections go undetected during BCWD epizootics, treatments may be delayed subsequently resulting in avoidable losses. In this context, FPM-A and TYES were deployed side-by-side to detect *F. psychrophilum* in hatchery-reared Atlantic salmon with gross signs of BCWD (data not shown). Consequently, only FPM-A recovered *F. psychrophilum*, which led to management and treatment actions that prevented additional losses. An additional outcome of the improved *F. psychrophilum* recovery from

naturally infected fish is that these new media provided isolates that will aid in deepening our understanding of *F. psychrophilum* molecular and serological diversity, the results of which can continue to inform BCWD vaccine development and testing (Hoare et al. 2017; Ma et al. 2019a). Collectively, results provide evidence that the new media are effective at detecting *F. psychrophilum* from a range of naturally infected salmonids and thus will serve as a critical resource for fisheries personnel and diagnosticians alike.

The improved recovery of *F. psychrophilum* by FPM-A and FPM-B likely resulted from the inclusion of essential medium components at favorable concentrations. For instance, calcium has been previously shown to contribute to several microbial cellular processes, including growth (Herbaud et al. 1998; Wang et al. 2019). In the present study, calcium was found to significantly enhance bacterial recovery with increasing concentration, which may have resulted from increased metalloprotease activity, considering some *F. psychrophilum* metalloproteases (e.g., Fpp1) are calcium-dependent (Secades et al. 2001). Like calcium, magnesium also significantly improved *F. psychrophilum* recovery and although results imply a role in cell growth, its function in *F. psychrophilum* cellular processes remains to be determined. In contrast to the improvements made by calcium and magnesium and despite representing primary *F. psychrophilum* energy sources (e.g., amino acids/proteins; Duchaud et al. 2007), tryptose, casamino acids, and FBS negatively impacted *F. psychrophilum* recovery. In view of this, tryptose may be associated with a higher metabolic cost, considering this product is comprised of mostly polypeptides (i.e., long amino acid chains with many peptide bonds). Casamino acids, while mostly free amino acids (i.e., a seemingly low metabolic cost), also contains low levels of cystine, maltose, iron, and sodium chloride, some, or all of which may have reduced *F. psychrophilum* recovery. Lastly, FBS, which has been used to enrich *F. psychrophilum* culture

media (Obach & Baudin-Laurencin, 1991; Lorenzen, 1993; Michel et al. 1999), appears to have an inhibitory effect on this species under some conditions, possibly due to a deficiency of essential nutrients or presence of inhibitory host factors. In this context, future studies assessing *F. psychrophilum* metabolism may lead to further culture media improvements.

To develop FPM-A and FPM-B, *F. psychrophilum* recovery was originally compared between three previously published *F. psychrophilum* culture media (e.g., EAOCa, OW, and TYES). This comparison revealed that TYES outperformed both EAOCa and OW, yielding the most *F. psychrophilum* cfus overall. However, and despite TYES being the best basal medium, EAOCa and/or OW collectively recovered two *F. psychrophilum* isolates (e.g., US151 and US487) at significantly higher yields compared to TYES. Similarly, FPM-A and FPM-B outperformed TYES in terms of overall cfu yield; however, 11.5 – 13.9% of isolates (in 26 STs and recovered from eight host species) yielded more cfus on TYES, although these yields were not significantly greater (data not shown). Although current observations suggest that the new media are not selective for specific *F. psychrophilum* genetic variants or host species, further investigation with a larger sample size per variant and host is warranted to validate this finding. Collectively, these observations not only demonstrate that *F. psychrophilum* diversity affects its recovery but also underscores the importance of considering a wide array of isolates in future studies. Indeed, incorporating such diversity into study design is crucial for ensuring robust results and a comprehensive understanding of *F. psychrophilum* behavior.

This study employed a Plackett-Burman experimental design (Plackett and Burman, 1944), an approach to culture medium development that has been used to improve bacterial recovery (Stevens, 1995; Bhattacharjee and Joshi, 2016), increase bacterial biomass in vitro (Waśko et al. 2010), and enhance the production of bacterial products for biotechnological

purposes (Zeinab et al. 2015; Ekpenyong et al. 2017; El-Shanshoury et al. 2018). However, and prior to this study, a Plackett-Burman design approach had not been used for the development of *F. psychrophilum* culture media. When developing the OW medium, Oplinger and Wagner (2012) began with a single base medium and took an iterative approach to optimization by adding or subtracting a few medium components, keeping those that improved *F. psychrophilum* growth. In contrast, Cepeda et al. (2004) and Álvarez and Guijarro (2007) both began with two or three basal media, added one or more medium components at a single concentration, and then compared *F. psychrophilum* growth and/or recovery. Similarly, Michel et al. (1999) began with two basal media and added three medium components at one or more concentrations and in different combinations. The Plackett-Burman approach to culture medium development used herein is an improvement over previous methods as it allows for the simultaneous screening of many medium components at two concentrations and is inherently designed so that the concentration effect of each component can be assessed, which ultimately guided the development of the two new and improved *F. psychrophilum* media.

In conclusion, BCWD and RTFS, although not regulatory diseases, remain substantial impediments to the production and health of salmonids being raised for food and/or conservation purposes, and bacterial culture remains the gold-standard for its detection and diagnosis. Crucially, culture-derived outbreak isolates not only guide the immediate treatment strategies to minimize losses but also serve as invaluable resources for future research targeting the development of BCWD prevention and control strategies. Herein, two new culture media (e.g., FPM-A and FPM-B) were developed, both of which not only yielded significantly more *F. psychrophilum* for a wide diversity of isolates, but were also more capable of recovering *F. psychrophilum* from multiple naturally infected salmonid species in comparison to the current

gold standard medium, TYES. Thus, these media set the foundation for more effective BCWD detection and provide an improved platform for subsequent studies, ultimately paving the way towards more robust prevention and control measures against this devastating fish pathogen.

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APPENDIX

Table 2.1. Metadata for the 165 *Flavobacterium psychrophilum* isolates used in this study, including multilocus sequence typing sequence type (ST), clonal complex (CC), isolation location, and host of origin. Information is presented in order by CC then ST.

ST (CC)	No. of isolates	U.S. state, Canadian province, or country of isolation[a]	Host of origin[b]
ST9 (CC-ST9)[c]	4	BC (Canada), OR, UT, WA	COS, RBT
ST13 (CC-ST9)[c, d, e]	3	MI, WA	COS
ST2 (CC-ST10)[f]	1	Denmark	RBT
ST10 (CC-ST10)[c]	14	CA, CO, ID, MD, MI, MT, NC, NM, OR, PA, SD, UT, WA	CHS, RBT, WHT
ST78 (CC-ST10)[c, d, g]	10	CA, CO, ID, MI, NC, NM, UT, WA, WY	RBT
ST79 (CC-ST10)[f]	1	Chile	ATS
ST84 (CC-ST10)[c]	1	NC	RBT
ST85 (CC-ST10)[c, e]	2	CA, WA	RBT
ST86 (CC-ST10)[c]	2	UT, WA	RBT
ST275 (CC-ST10)[c]	6	MI, NC, PA, VA, WV	RBT
ST294 (CC-ST10)[c]	1	WA	RBT
ST300 (CC-ST10)[c]	2	ID, MT	RBT
ST303 (CC-ST10)[c]	1	ID	RBT
ST304 (CC-ST10)[c]	1	ID	RBT
ST305 (CC-ST10)[c]	1	ID	RBT
ST306 (CC-ST10)[c]	1	ID	RBT
ST316 (CC-ST10)[c]	1	WA	RBT
ST317 (CC-ST10)[c]	1	ID	RBT
ST319 (CC-ST10)[e]	1	CA	RBT
ST341 (CC-ST10)[e]	1	MI	RBT
ST342 (CC-ST10)[g]	2	MI	RBT
ST11 (CC-ST11)[c]	1	OR	RBT
ST262 (CC-ST11)[d]	1	MI	CHS
ST29 (CC-ST29)[d]	1	MI	CHS
ST308 (CC-ST29)[c]	1	OR	CHS
ST28 (CC-ST31)[c]	1	OR	CUT
ST31 (CC-ST31)[d]	1	MI	RBT
ST70 (CC-ST124)[d]	1	WA	ATS
ST267 (CC-ST191)[c, d]	2	MI	RBT
ST301 (CC-ST191)[c]	3	ID, PA, WV	RBT
ST277 (CC-ST232)[c]	1	MI	ATS
ST252 (CC-ST256)[d]	1	MI	COS
ST256 (CC-ST256)[c, d, g]	4	MI, WI	COS, CHS, RBT
ST257 (CC-ST276)[c, d]	2	MI	RBT
ST276 (CC-ST276)[c]	2	PA	RBT
ST279 (CC-ST281)[c]	1	PA	RBT
ST281 (CC-ST281)[c]	1	PA	RBT
ST331 (CC-ST281)[d]	1	PA	RBT
ST332 (CC-ST281)[d]	1	PA	RBT
ST286 (CC-ST286)[g]	1	MI	BNT
ST349 (CC-ST286)[g]	1	MI	RBT
ST371 (CC-ST286)[h]	1	MI	ATS
ST375 (CC-ST286)[h]	2	MI	ATS, RBT

Table 2.1. (cont'd)

ST (CC)	No. of isolates	U.S. state, Canadian province, or country of isolation[a]	Host of origin[b]
ST254 (CC-ST287)[d]	1	MI	CHS
ST280 (CC-ST287)[c]	1	WI	SPL
ST287 (CC-ST287)[c]	1	MI	COS
ST354 (CC-ST287)[g]	1	MI	RBT
ST288 (CC-ST288)[c]	1	MI	CHS
ST289 (CC-ST288)[c]	1	MI	CHS
ST347 (CC-ST288)[g]	1	MI	CHS
ST265 (CC-ST296)[d]	1	MI	CHS
ST296 (CC-ST296)[c]	1	WA	SOC
ST299 (CC-ST296)[c]	1	WA	RBT
ST291 (CC-ST310)[c]	3	CA, ID, NC	RBT
ST310 (CC-ST310)[c]	1	ID	RBT
ST311 (CC-ST310)[c]	1	ID	RBT
ST318 (CC-ST318)[c]	1	CA	RBT
ST330 (CC-ST318)[c]	1	Unknown	RBT
ST343 (CC-ST343)[g]	1	NJ	RBT
ST344 (CC-ST344)[g]	1	NJ	RBT
ST27[c]	1	CA	RBT
ST30[c]	1	OR	COS
ST74[c]	1	OR	COS
ST76[c]	1	WA	CHS
ST250[d]	1	MI	CHS
ST251[d]	1	MI	CHS
ST253[c]	2	MI	BNT
ST255[d]	2	MI	CHS
ST258[c, g]	3	MI	COS, RBT
ST259[d]	1	MI	CHS
ST260[d]	1	MI	CHS
ST261[d, h]	2	MI	CHS, RBT
ST263[d]	1	MI	CHS
ST264[d]	1	MI	COS
ST266[d]	1	MI	CHS
ST278[c, h]	2	MI	ATS, LAT
ST282[c]	1	MI	ATS
ST284[c]	1	PA	BNT
ST290[c]	1	MI	CHS
ST292[c]	5	NC	RBT
ST293[c]	1	NC	RBT
ST295[c]	1	WA	CHS
ST297[c]	1	OR	COS
ST298[c]	1	ID	RBT
ST302[c]	1	ID	RBT
ST307[c]	1	OR	RBT
ST309[c]	1	ID	RBT
ST312[c]	1	ID	RBT
ST313[c]	1	MT	RBT
ST314[c]	1	NC	RBT
ST315[c]	1	NC	RBT
ST320[c]	1	AK	COS
ST333[e]	1	CA	RBT
ST345[g]	1	MI	CHS

Table 2.1. (cont'd)

ST (CC)	No. of isolates	U.S. state, Canadian province, or country of isolation[a]	Host of origin[b]
ST346[g]	1	MI	CHS
ST350[g]	2	MI	ATS, COS
ST351[g]	1	WI	BKT
ST352[g]	1	WI	BNT
ST353[g]	2	MI	BKT, LAT
ST369[h]	1	MI	RBT
ST373[h]	1	MI	BNT
ST374[i]	1	MI	LWF
ST376[h]	1	MI	BNT
ST377[i]	1	MI	LWF
ST378[i]	1	WI	LWF
Unknown[h]	1	MI	BNT

[a] Isolates were recovered from 19 U.S. states, one Canadian province, and the countries of Chile and Denmark. AK, Alaska; BC, British Columbia; CA, California; CO, Colorado; ID, Idaho; MD, Maryland; MI, Michigan; MT, Montana; NC, North Carolina; NJ, New Jersey; NM, New Mexico; OR, Oregon; PA, Pennsylvania; SD, South Dakota; UT, Utah; VA, Virginia; WA, Washington; WI, Wisconsin; WV, West Virginia; WY, Wyoming.

[b] Isolates were recovered from 12 fish species. ATS, Atlantic salmon (*Salmo salar*); BKT, brook trout (*Salvelinus fontinalis*); BNT, brown trout (*S. trutta*); CHS, Chinook salmon (*Oncorhynchus tshawytscha*); COS, coho salmon (*O. kisutch*); CUT, cutthroat trout (*O. clarkii*); LAT, lake trout (*S. namaycush*); LWF, lake whitefish (*Coregonus clupeaformis*); RBT, rainbow trout (*O. mykiss*); SOC, sockeye salmon (*O. nerka*); SPL, splake (*S. namaycush* x *S. fontinalis*); WHT, white sturgeon (*Acipenser transmontanus*).

[c] Published in Knupp et al. (2019).

[d] Published in Van Vliet et al. (2016).

[e] Published at https://pubmlst.org/fpsychrophilum/

[f] Published in Ma et al. (2019) and Madsen and Dalsgaard (1999).

[g] Published in Li et al. (2021).

[h] Published in this study.

[i] Published in Harrison et al. (2022).

Table 2.2. Additional meta data of the 165 *Flavobacterium psychrophilum* isolates used in this study, including isolate identifier (ID), year of isolation, location of isolation, host of origin, tissue of origin, life history (e.g., wild/feral or captive), life stage, and multilocus sequencing typing sequence type (ST) and clonal complex (CC). All 165 *F. psychrophilum* isolates were used to select the best basal medium (e.g., tryptone yeast extract agar; TYES) and in the comparison of the new media (*F. psychrophilum* medium-A and -B) to TYES. Fifty *F. psychrophilum* isolates were used in the Plackett-Burman experiment (denoted here with [h]). The information is presented in order by multi-locus sequence typing (MLST) clonal complex (CC), then MLST sequence type (ST).

Isolate ID	Year of isolation	Location of isolation[i]	Host of origin	Isolation tissue	Wild/feral or captive	Life stage	ST	CC
US161[a]	1990	OR	*O. kisutch*	Unknown	Unknown	Unknown	ST9	CC-ST9
US165[a]	1984	BC	*O. kisutch*	Unknown	Unknown	Unknown	ST9	CC-ST9
US254[a,h]	Unknown	UT	*O. mykiss*	Spleen	Unknown	Unknown	ST9	CC-ST9
US256[a]	Unknown	WA	*O. kisutch*	Unknown	Unknown	Unknown	ST9	CC-ST9
US019[b,h]	2010	MI	*O. kisutch*	Kidney	Wild/feral	Adult	ST13	CC-ST9
US155[a]	Unknown	WA	*O. kisutch*	Unknown	Unknown	Unknown	ST13	CC-ST9
US458[e]	2019	MI	*O. kisutch*	Kidney	Captive	Juvenile	ST13	CC-ST9
900406-1/3[f,h]	1990	Denmark	*O. mykiss*	Kidney	Unknown	Juvenile	ST2	CC-ST10
US075[a,h]	2016	PA	*O. mykiss*	Kidney	Captive	Broodstock	ST10	CC-ST10
US133[a]	2012	SD	*O. mykiss*	Pooled tissue	Captive	Unknown	ST10	CC-ST10
US148[a]	Unknown	CA	*A. transmontanus*	Kidney	Unknown	Unknown	ST10	CC-ST10
US162[a]	1990	OR	*O. mykiss*	Unknown	Unknown	Unknown	ST10	CC-ST10
US188[a]	2016	MD	*O. mykiss*	Unknown	Captive	Fingerling	ST10	CC-ST10
US226[a]	2013	NC	*O. mykiss*	Lesion	Captive	Fingerling	ST10	CC-ST10
US236[a]	2011	UT	*O. mykiss*	Spleen	Captive	Fingerling	ST10	CC-ST10
US250[a]	2000	WA	*O. tshawytscha*	Ovarian fluid	Unknown	Unknown	ST10	CC-ST10
US253[a]	Unknown	CO	*O. mykiss*	Unknown	Unknown	Unknown	ST10	CC-ST10
US305[a,h]	2011	UT	*O. mykiss*	Spleen	Captive	Fry	ST10	CC-ST10
US312[a]	2010	MT	*O. mykiss*	Kidney	Captive	Fingerling	ST10	CC-ST10
US351[a]	2015	ID	*O. mykiss*	Spleen	Captive	Juvenile	ST10	CC-ST10
US352[a]	2010	MI	*O. mykiss*	Kidney	Captive	Fingerling	ST10	CC-ST10
US368[a]	2002	NM	*O. mykiss*	Kidney	Unknown	Unknown	ST10	CC-ST10
US042[b]	2011	MI	*O. mykiss*	Kidney	Captive	Juvenile	ST78	CC-ST10
US053[b,h]	2011	MI	*O. mykiss*	ext. lesion	Captive	Juvenile	ST78	CC-ST10
US118[a]	2013	NC	*O. mykiss*	Unknown	Captive	Fingerling	ST78	CC-ST10
US172[a]	2014	WA	*O. mykiss*	Kidney	Captive	Yearling	ST78	CC-ST10
US241[a]	2013	ID	*O. mykiss*	Unknown	Captive	Juvenile	ST78	CC-ST10
US251[a]	2002	NM	*O. mykiss*	Kidney	Unknown	Unknown	ST78	CC-ST10
US252[a]	Unknown	CO	*O. mykiss*	Kidney	Unknown	Unknown	ST78	CC-ST10
US361[a]	2017	CA	*O. mykiss*	Unknown	Unknown	Unknown	ST78	CC-ST10
US367[a]	2008	UT	*O. mykiss*	Spleen	Captive	Fry	ST78	CC-ST10

Table 2.2. (cont'd)

Isolate ID	Year of isolation	Location of isolation[i]	Host of origin	Isolation tissue	Wild/feral or captive	Life stage	ST	CC
US459[c]	Unknown	WY	Unknown	Unknown	Unknown	Unknown	ST78	CC-ST10
622-97[f]	Unknown	Chile	*S. salar*	Unknown	Unknown	Unknown	ST79	CC-ST10
US167[a]	2009	NC	*O. mykiss*	Spleen	Captive	Fingerling	ST84	CC-ST10
US364[a]	2014	WA	*O. mykiss*	Heart	Captive	Yearling	ST85	CC-ST10
US392[e,h]	2017	CA	*O. mykiss*	Unknown	Unknown	Unknown	ST85	CC-ST10
US073[a,h]	2008	UT	*O. mykiss*	Spleen	Captive	Fry	ST86	CC-ST10
US354[a]	2015	WA	*O. mykiss*	Skin	Captive	Yearling	ST86	CC-ST10
US057[a]	2014	PA	*O. mykiss*	Kidney	Captive	Fingerling	ST275	CC-ST10
US087[a]	2016	MI	*O. mykiss*	Ext. lesion	Captive	Fingerling	ST275	CC-ST10
US104[a,h]	2016	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST275	CC-ST10
US116[a]	2013	NC	*O. mykiss*	Spleen	Captive	Fingerling	ST275	CC-ST10
US119[a]	2013	WV	*O. mykiss*	Spleen	Captive	Fingerling	ST275	CC-ST10
US171[a]	2014	VA	*O. mykiss*	Spleen	Captive	Fingerling	ST275	CC-ST10
US151[a,h]	Unknown	WA	*O. mykiss*	Unknown	Unknown	Unknown	ST294	CC-ST10
US178[a,h]	2015	ID	*O. mykiss*	Spleen	Captive	Unknown	ST300	CC-ST10
US314[a]	2011	MT	*O. mykiss*	Kidney	Captive	Fingerling	ST300	CC-ST10
US323[a]	2013	ID	*O. mykiss*	Spleen	Captive	Juvenile	ST303	CC-ST10
US205[a]	2015	ID	*O. mykiss*	Kidney	Captive	Juvenile	ST304	CC-ST10
US245[a]	2011	ID	*O. mykiss*	Kidney	Captive	Juvenile	ST305	CC-ST10
US259[a]	1990	ID	*O. mykiss*	Unknown	Unknown	Unknown	ST306	CC-ST10
US355[a]	2015	WA	*O. mykiss*	Unknown	Captive	Yearling	ST316	CC-ST10
US356[a]	2016	ID	*O. mykiss*	Spleen	Captive	Juvenile	ST317	CC-ST10
US390[e]	2017	CA	*O. mykiss*	Unknown	Unknown	Unknown	ST319	CC-ST10
US399[e]	2018	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST341	CC-ST10
US400[c]	2018	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST342	CC-ST10
US465[g]	2019	MI	*O. mykiss*	Kidney	Captive	Juvenile	ST342	CC-ST10
US163[a,h]	1984	OR	*O. mykiss*	Unknown	Unknown	Unknown	ST11	CC-ST11
US033[b,h]	2010	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST262	CC-ST11
US025[b,h]	2011	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST29	CC-ST29
US261[a,h]	1981	OR	*O. tshawytscha*	Unknown	Unknown	Unknown	ST308	CC-ST29
US164[a]	1986	OR	*O. clarkii*	Unknown	Unknown	Unknown	ST28	CC-ST31
US028[b,h]	2010	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST31	CC-ST31
US149[a,h]	Unknown	WA	*S. salar*	Unknown	Unknown	Unknown	ST70	CC-ST124
US054[b]	2013	MI	*O. mykiss*	Kidney	Captive	Juvenile	ST267	CC-ST191
US215[a,h]	2017	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST267	CC-ST191
US181[a,h]	2015	PA	*O. mykiss*	Unknown	Captive	Unknown	ST301	CC-ST191
US277[a]	2015	ID	*O. mykiss*	Spleen	Captive	Juvenile	ST301	CC-ST191

Table 2.2. (cont'd)

Isolate ID	Year of isolation	Location of isolation[i]	Host of origin	Isolation tissue	Wild/feral or captive	Life stage	ST	CC
US343[a]	2014	WV	*O. mykiss*	Caudal Fin	Captive	Fingerling	ST301	CC-ST191
US062[a,h]	2012	MI	*S. salar*	Ext. lesion	Wild/feral	Adult	ST277	CC-ST232
US008[b,h]	2011	MI	*O. kisutch*	Kidney	Wild/feral	Adult	ST252	CC-ST256
US047[b]	2011	MI	*O. tshawytscha*	egg	Wild/feral	NA	ST256	CC-ST256
US101[a]	2014	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST256	CC-ST256
US217[a,h]	2017	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST256	CC-ST256
US445[c]	2018	WI	*O. kisutch*	Unknown	Unknown	Unknown	ST256	CC-ST256
US016[b]	2013	MI	*O. mykiss*	Kidney	Captive	Juvenile	ST257	CC-ST276
US095[a,h]	2016	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST257	CC-ST276
US061[a,h]	2014	PA	*O. mykiss*	Kidney	Captive	Fingerling	ST276	CC-ST276
US200[a]	2016	PA	*O. mykiss*	Ulcer	Captive	Broodstock	ST276	CC-ST276
US065[a,h]	2016	PA	*O. mykiss*	Kidney	Wild/feral	Adult	ST279	CC-ST281
US067[a,h]	2016	PA	*O. mykiss*	Kidney	Captive	Fry	ST281	CC-ST281
US379[a,h]	2017	PA	*O. mykiss*	Kidney	Wild/feral	Adult	ST331	CC-ST281
US380[a,h]	2017	PA	*O. mykiss*	Kidney	Wild/feral	Adult	ST332	CC-ST281
US461[c]	2019	MI	*S. trutta*	Gill	Captive	Juvenile	ST286	CC-ST286
US439[c,h]	2018	MI	*O. mykiss*	Kidney	Captive	Juvenile	ST349	CC-ST286
US478[g,h]	2020	MI	*S. salar*	Kidney	Captive	Juvenile	ST371	CC-ST286
US493[g]	2020	MI	*S. salar*	Pectoral fin	Captive	Juvenile	ST375	CC-ST286
US502[g]	2020	MI	*O. mykiss*	Caudal ped.	Captive	Broodstock	ST375	CC-ST286
US012[b]	2013	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST254	CC-ST287
US066[a,h]	2016	WI	*S. namaycus x S. fontinalis*	Unknown	Captive	Unknown	ST280	CC-ST287
US096[a,h]	2013	MI	*O. kisutch*	Dorsal fin	Captive	Yearling	ST287	CC-ST287
US455[c]	2019	MI	*O. mykiss*	Caudal ped.	Captive	Juvenile	ST354	CC-ST287
US108[a,h]	2016	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST288	CC-ST288
US109[a,h]	2016	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST289	CC-ST288
US414[c,h]	2018	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST347	CC-ST288
US037[b]	2012	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST265	CC-ST296
US153[a,h]	Unknown	WA	*O. nerka*	Unknown	Unknown	Unknown	ST296	CC-ST296
US176[a]	2014	WA	*O. mykiss*	Kidney	Captive	Yearling	ST299	CC-ST296
US122[a]	2011	NC	*O. mykiss*	Brain	Captive	Fingerling	ST291	CC-ST310
US331[a]	2014	ID	*O. mykiss*	Kidney	Captive	Juvenile	ST291	CC-ST310
US359[a]	2017	CA	*O. mykiss*	Unknown	Unknown	Unknown	ST291	CC-ST310
US329[a,h]	2014	ID	*O. mykiss*	Kidney	Captive	Juvenile	ST310	CC-ST310
US328[a]	2014	ID	*O. mykiss*	Spleen	Captive	Juvenile	ST311	CC-ST310
US357[a]	2016	CA	*O. mykiss*	Unknown	Unknown	Unknown	ST318	CC-ST318

Table 2.2. (cont'd)

Isolate ID	Year of isolation	Location of isolation[i]	Host of origin	Isolation tissue	Wild/feral or captive	Life stage	ST	CC
US374[a,h]	Unknown	Unknown	*O. mykiss*	Unknown	Unknown	Unknown	ST330	CC-ST318
US403[c,h]	2018	NJ	*O. mykiss*	Kidney	Captive	Juvenile	ST343	CC-ST343
US404[c,h]	2018	NJ	*O. mykiss*	Kidney	Captive	Juvenile	ST344	CC-ST343
US191[a,h]	2016	CA	*O. mykiss*	Spleen	Captive	Unknown	ST27	
US156[a]	1981	OR	*O. kisutch*	Unknown	Unknown	Unknown	ST30	
US255[a]	1981	OR	*O. kisutch*	Unknown	Unknown	Unknown	ST74	
US249[a,h]	2000	WA	*O. tshawytscha*	Ovarian fluid	Unknown	Unknown	ST76	
US005[b]	2011	MI	*O. tshawytscha*	Egg	Wild/feral	Unknown	ST250	
US006[b]	2011	MI	*O. tshawytscha*	Egg	Wild/feral	Unknown	ST251	
US060[a]	2010	MI	*S. trutta*	Ascites	Captive	Adult	ST253	
US098[a]	2013	MI	*S. trutta*	Spleen	Captive	Fingerling	ST253	
US013[b]	2012	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST255	
US031[b]	2012	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST255	
US099[a]	2013	MI	*O. kisutch*	Gill	Captive	Yearling	ST258	
US218[a]	2017	MI	*O. mykiss*	Kidney	Wild/feral	Adult	ST258	
US454[c]	2018	MI	*O. kisutch*	Kidney	Wild/feral	Adult	ST258	
US023[b]	2013	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST259	
US056[b]	2010	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST260	
US024[b]	2009	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST261	
US479[g]	2020	MI	*O. mykiss*	Eye	Captive	Juvenile	ST261	
US034[b]	2012	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST263	
US035[b]	2011	MI	*O. kisutch*	Kidney	Wild/feral	Adult	ST264	
US036[b]	2008	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST266	
US063[a]	2012	MI	*S. namaycus*	Fin	Captive	Fingerling	ST278	
US508[g]	2020	MI	*S. salar*	Kidney	Wild/feral	Adult	ST278	
US083[a]	2016	MI	*S. salar*	Eye	Wild/feral	Adult	ST282	
US088[a,h]	2016	PA	*S. trutta*	Lesion	Captive	Adult	ST284	
US110[a]	2016	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST290	
US139[a]	2013	NC	*O. mykiss*	Spleen	Captive	Fingerling	ST292	
US141[a]	2011	NC	*O. mykiss*	Kidney	Captive	Fingerling	ST292	
US225[a]	2013	NC	*O. mykiss*	Spleen	Captive	Fingerling	ST292	
US232[a]	2011	NC	*O. mykiss*	Kidney	Captive	Fingerling	ST292	
US244[a,h]	2011	NC	*O. mykiss*	Spleen	Captive	Fingerling	ST292	
US142[a]	2011	NC	*O. mykiss*	Brain	Captive	Fingerling	ST293	
US152[a]	Unknown	WA	*O. tshawytscha*	Unknown	Unknown	Unknown	ST295	
US158[a,h]	1989	OR	*O. kisutch*	Unknown	Unknown	Unknown	ST297	
US168[a,h]	2014	ID	*O. mykiss*	Kidney	Captive	Juvenile	ST298	

Table 2.2. (cont'd)

Isolate ID	Year of isolation	Location of isolation[i]	Host of origin	Isolation tissue	Wild/feral or captive	Life stage	ST	CC
US187[a]	2014	ID	*O. mykiss*	Spleen	Captive	Unknown	ST302	
US260[a]	1985	OR	*O. mykiss*	Unknown	Unknown	Unknown	ST307	
US265[a]	2014	ID	*O. mykiss*	Kidney	Captive	Juvenile	ST309	
US283[a]	2014	ID	*O. mykiss*	Spleen	Captive	Juvenile	ST312	
US310[a,h]	2010	MT	*O. mykiss*	Kidney	Captive	Fingerling	ST313	
US324[a]	2014	NC	*O. mykiss*	Spleen	Captive	Fry	ST314	
US325[a]	2014	NC	*O. mykiss*	Spleen	Captive	Fry	ST315	
US372[a,h]	2011	AK	*O. kisutch*	Kidney	Captive	Fingerling	ST320	
US394[e]	2017	CA	*O. mykiss*	Unknown	Unknown	Unknown	ST333	
US411[c]	2018	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST345	
US413[c]	2018	MI	*O. tshawytscha*	Kidney	Wild/feral	Adult	ST346	
US442[c]	2018	MI	*S. salar*	Kidney	Wild/feral	Adult	ST350	
US443[c]	2019	MI	*O. kisutch*	Kidney	Captive	Juvenile	ST350	
US444[c,h]	2018	WI	*S. fontinalis*	Unknown	Unknown	Unknown	ST351	
US449[c]	2018	WI	*S. trutta*	Unknown	Unknown	Unknown	ST352	
US450[c]	2019	MI	*S. namaycus*	Dorsal fin	Captive	Juvenile	ST353	
US487[g]	2020	MI	*S. fontinalis*	Dorsal fin	Captive	Broodstock	ST353	
US476[g]	2020	MI	*O. mykiss*	Pectoral fin	Captive	Juvenile	ST369	
US485[g]	2020	MI	*S. trutta*	Kidney	Captive	Juvenile	ST373	
US490[d,h]	2019	MI	*C. clupeaformis*	Kidney	Wild/feral	Adult	ST374	
US505[g]	2020	MI	*S. trutta*	Caudal ped.	Captive	Adult	ST376	
US488[d]	2019	MI	*C. clupeaformis*	Kidney	Wild/feral	Adult	ST377	
US489[d]	2019	WI	*C. clupeaformis*	Kidney	Wild/feral	Adult	ST378	
US460[g]	2019	MI	*S. trutta*	Kidney	Captive	Juvenile	NA	

[a] Published in Knupp et al. (2019)
[b] Published in Van Vliet et al. (2016)
[c] Published in Li et al. (2021)
[d] Published in Harrison et al. (2022)
[e] Published at https://pubmlst.org/fpsychrophilum/
[f] Published in Ma et al. (2019) and Madsen and Dalsgaard (1999)
[g] Published in this study
[h] Isolate used in Plackett-Burman experiment
[i] Key to location abbreviations: AK=Alaska; BC=British Columbia; CA=California; CO=Colorado; ID=Idaho; MD=Maryland; MI=Michigan; MT=Montana; NC=North Carolina; NJ=New Jersey; NM=New Mexico; OR=Oregon; PA=Pennsylvania; SD=South Dakota; UT=Utah; VA=Virginia; WA=Washington; WI=Wisconsin; WV=West Virginia; WY=Wyoming

Table 2.3. Formulations (per 1L H_2O) of media used in basal medium experiment, including tryptone yeast extract salts agar (TYES; Holt 1987), Oplinger and Wagner medium (OW; Oplinger and Wagner, 2012), enriched Anacker and Ordal medium supplemented with activated charcoal and aromatic compounds (EAOCa; Alvarez and Guijarro, 2007), and neutral medium (NM; this study).

		Medium				
Medium component	Unit	TYES	OW	EAOCa	NM	Vendor
Bacteriological agar	g	10	10	15	10	Thermofisher Scientific
Tryptone	g	4.0	5.0	5.0	4.5	Thermofisher Scientific
Yeast extract	g	0.4	0.5		0.45	Thermofisher Scientific
$CaCl_2 \cdot 2H_2O$	g	0.5				Thermofisher Scientific
$MgSO_4 \cdot 7H_2O$	g	0.5				Thermofisher Scientific
Beef extract	g		0.2	0.2		Millipore Sigma
Horse serum	%		1.0	5.0		Thermofisher Scientific
Skim milk powder	%		0.2			Thermofisher Scientific
Activated charcoal	g			0.5		Millipore Sigma
Sodium acetate	g			0.2		Millipore Sigma
L-tyrosine	µmol			10		Millipore Sigma
L-phenylalanine	µmol			10		Millipore Sigma
L-tryptophan	µmol			10		Millipore Sigma
p-aminobenzoic acid	µmol			10		Millipore Sigma
p-hydroxybenzoic acid	µmol			10		Millipore Sigma
2,3-di-hydroxybenzoic acid	µmol			10		Millipore Sigma

Table 2.4. Medium components tested as part of Plackett-Burman experimental design, including tested quantities per 1L H_2O.

Medium component	Unit	Component symbol	Low value (-1)	High value (+1)	Brief rationale	Vendor	Catalog number
Tryptone	g	X1	0.750	7.50	Amino acid source	ThermoFisher Scientific	LP0042B
Yeast extract	g	X2	0.075	0.75	Vitamin and nitrogen source	ThermoFisher Scientific	Y1625
$CaCl_2 \cdot 2H_2O$	g	X3	0.075	0.75	Ca^{++} source	Millipore Sigma	C3306
$MgSO_4 \cdot 7H_2O$	g	X4	0.111	1.11	Mg^{++} source	Millipore Sigma	63138
Beef extract	g	X5	0.075	0.75	Amino acid source	Millipore Sigma	B4888
Casamino acids	g	X6	0.750	7.50	Amino acid source	ThermoFisher Scientific	223050
Tryptose	g	X7	0.750	7.50	Amino acid source	Millipore Sigma	70937
L-aspartic acid	g	X8	0.075	0.75	A main amino acid in *O. mykiss* muscle tissue	Millipore Sigma	A9256
Horse serum	%	X9	0.750	7.50	Source of proteins and other growth factors	ThermoFisher Scientific	26050088
Fetal bovine serum	%	X10	0.750	7.50	Source of proteins and other growth factors	ThermoFisher Scientific	100500
O. mykiss blood	%	X11	0.750	7.50	Source of iron and other host factors	This study	

Table 2.5. Plackett-Burman experimental design matrix, consisting of 11 tested medium components (e.g., X1-X11) resulting in 12 media formulations. Medium components incorporated at their low or high concentration are denoted with -1 and +1, respectively.

	Medium Component										
Medium Formulation	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
2	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1
3	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1
4	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1
5	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1
6	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1
7	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1
8	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1
9	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1
10	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1
11	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1
12	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1

Table 2.6. Differences of least square mean estimates ± standard error (SE) for the interaction between medium (e.g., EAOCa, OW, and TYES) and 165 *Flavobacterium psychrophilum* isolates. Pairwise comparisons between media for each isolate are provided. Tukey-Kramer adjusted *P*-values for multiple comparisons are shown (α = 0.05). Table is ordered by isolate.

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	FP622-97	OW	FP622-97	-0.6505	0.2224	330	-2.93	1
EAOCa	FP622-97	TYES	FP622-97	-0.9515	0.2224	330	-4.28	0.4881
OW	FP622-97	TYES	FP622-97	-0.301	0.2224	330	-1.35	1
EAOCa	FP900406	OW	FP900406	-6.88E-15	0.2224	330	0	1
EAOCa	FP900406	TYES	FP900406	-1.2386	0.2224	330	-5.57	0.0046
OW	FP900406	TYES	FP900406	-1.2386	0.2224	330	-5.57	0.0046
EAOCa	US005	OW	US005	-1.787	0.2224	330	-8.04	<.0001
EAOCa	US005	TYES	US005	-2.088	0.2224	330	-9.39	<.0001
OW	US005	TYES	US005	-0.301	0.2224	330	-1.35	1
EAOCa	US006	OW	US006	-1.0945	0.2224	330	-4.92	0.0734
EAOCa	US006	TYES	US006	-1.5104	0.2224	330	-6.79	<.0001
OW	US006	TYES	US006	-0.4158	0.2224	330	-1.87	1
EAOCa	US008	OW	US008	-0.3099	0.2224	330	-1.39	1
EAOCa	US008	TYES	US008	-0.7955	0.2224	330	-3.58	0.9786
OW	US008	TYES	US008	-0.4856	0.2224	330	-2.18	1
EAOCa	US012	OW	US012	-1.3288	0.2224	330	-5.98	0.0006
EAOCa	US012	TYES	US012	-1.2407	0.2224	330	-5.58	0.0043
OW	US012	TYES	US012	0.08805	0.2224	330	0.4	1
EAOCa	US013	OW	US013	-2.2218	0.2224	330	-9.99	<.0001
EAOCa	US013	TYES	US013	-3.0713	0.2224	330	-13.81	<.0001
OW	US013	TYES	US013	-0.8495	0.2224	330	-3.82	0.8924
EAOCa	US016	OW	US016	-1.4225	0.2224	330	-6.4	<.0001
EAOCa	US016	TYES	US016	-1.6109	0.2224	330	-7.24	<.0001
OW	US016	TYES	US016	-0.1884	0.2224	330	-0.85	1
EAOCa	US019	OW	US019	-1.5812	0.2224	330	-7.11	<.0001
EAOCa	US019	TYES	US019	-2.2211	0.2224	330	-9.99	<.0001
OW	US019	TYES	US019	-0.6399	0.2224	330	-2.88	1
EAOCa	US023	OW	US023	1.1505	0.2224	330	5.17	0.0271
EAOCa	US023	TYES	US023	-1.0256	0.2224	330	-4.61	0.2095
OW	US023	TYES	US023	-2.1761	0.2224	330	-9.79	<.0001
EAOCa	US024	OW	US024	0.1823	0.2224	330	0.82	1
EAOCa	US024	TYES	US024	-0.3741	0.2224	330	-1.68	1
OW	US024	TYES	US024	-0.5564	0.2224	330	-2.5	1
EAOCa	US025	OW	US025	-0.3656	0.2224	330	-1.64	1
EAOCa	US025	TYES	US025	-0.554	0.2224	330	-2.49	1
OW	US025	TYES	US025	-0.1884	0.2224	330	-0.85	1
EAOCa	US028	OW	US028	-0.699	0.2224	330	-3.14	0.9999
EAOCa	US028	TYES	US028	-1.9375	0.2224	330	-8.71	<.0001
OW	US028	TYES	US028	-1.2386	0.2224	330	-5.57	0.0046
EAOCa	US031	OW	US031	-1.2614	0.2224	330	-5.67	0.0028
EAOCa	US031	TYES	US031	-1.588	0.2224	330	-7.14	<.0001
OW	US031	TYES	US031	-0.3266	0.2224	330	-1.47	1
EAOCa	US033	OW	US033	-0.7992	0.2224	330	-3.59	0.9755
EAOCa	US033	TYES	US033	-1.6109	0.2224	330	-7.24	<.0001
OW	US033	TYES	US033	-0.8117	0.2224	330	-3.65	0.9625
EAOCa	US034	OW	US034	-2.713	0.2224	330	-12.2	<.0001

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
OW	US035	TYES	US035	0.03959	0.2224	330	0.18	1
EAOCa	US036	OW	US036	-2	0.2224	330	-8.99	<.0001
EAOCa	US036	TYES	US036	-2.199	0.2224	330	-9.89	<.0001
OW	US036	TYES	US036	-0.199	0.2224	330	-0.89	1
EAOCa	US037	OW	US037	-0.6109	0.2224	330	-2.75	1
EAOCa	US037	TYES	US037	-0.6109	0.2224	330	-2.75	1
OW	US037	TYES	US037	-1.17E-15	0.2224	330	0	1
EAOCa	US042	OW	US042	-0.1505	0.2224	330	-0.68	1
EAOCa	US042	TYES	US042	-0.5	0.2224	330	-2.25	1
OW	US042	TYES	US042	-0.3495	0.2224	330	-1.57	1
EAOCa	US047	OW	US047	-0.9621	0.2224	330	-4.33	0.4413
EAOCa	US047	TYES	US047	-0.8751	0.2224	330	-3.93	0.815
OW	US047	TYES	US047	0.08708	0.2224	330	0.39	1
EAOCa	US053	OW	US053	-2.1505	0.2224	330	-9.67	<.0001
EAOCa	US053	TYES	US053	-2.9375	0.2224	330	-13.21	<.0001
OW	US053	TYES	US053	-0.787	0.2224	330	-3.54	0.9845
EAOCa	US054	OW	US054	-4.1109	0.2224	330	-18.49	<.0001
EAOCa	US054	TYES	US054	-5.0089	0.2224	330	-22.52	<.0001
OW	US054	TYES	US054	-0.8979	0.2224	330	-4.04	0.7273
EAOCa	US056	OW	US056	-0.4543	0.2224	330	-2.04	1
EAOCa	US056	TYES	US056	0.1672	0.2224	330	0.75	1
OW	US056	TYES	US056	0.6215	0.2224	330	2.79	1
EAOCa	US057	OW	US057	-1.412	0.2224	330	-6.35	<.0001
EAOCa	US057	TYES	US057	-2.301	0.2224	330	-10.35	<.0001
OW	US057	TYES	US057	-0.8891	0.2224	330	-4	0.763
EAOCa	US060	OW	US060	-3.273	0.2224	330	-14.72	<.0001
EAOCa	US060	TYES	US060	-2.7959	0.2224	330	-12.57	<.0001
OW	US060	TYES	US060	0.4771	0.2224	330	2.15	1
EAOCa	US061	OW	US061	-2.7461	0.2224	330	-12.35	<.0001
EAOCa	US061	TYES	US061	-2.7461	0.2224	330	-12.35	<.0001
OW	US061	TYES	US061	-1.78E-15	0.2224	330	0	1
EAOCa	US062	OW	US062	-0.4771	0.2224	330	-2.15	1
EAOCa	US062	TYES	US062	-0.3891	0.2224	330	-1.75	1
OW	US062	TYES	US062	0.08805	0.2224	330	0.4	1
EAOCa	US063	OW	US063	-2.4098	0.2224	330	-10.84	<.0001
EAOCa	US063	TYES	US063	-2.301	0.2224	330	-10.35	<.0001
OW	US063	TYES	US063	0.1087	0.2224	330	0.49	1
EAOCa	US065	OW	US065	-1	0.2224	330	-4.5	0.2909
EAOCa	US065	TYES	US065	-2.2386	0.2224	330	-10.07	<.0001
OW	US065	TYES	US065	-1.2386	0.2224	330	-5.57	0.0046
EAOCa	US066	OW	US066	-2.772	0.2224	330	-12.46	<.0001
EAOCa	US066	TYES	US066	-4.1505	0.2224	330	-18.66	<.0001
OW	US066	TYES	US066	-1.3785	0.2224	330	-6.2	0.0002
EAOCa	US067	OW	US067	-2.8406	0.2224	330	-12.77	<.0001
EAOCa	US067	TYES	US067	-3.1751	0.2224	330	-14.28	<.0001
OW	US067	TYES	US067	-0.3345	0.2224	330	-1.5	1
EAOCa	US073	OW	US073	-3.4375	0.2224	330	-15.46	<.0001
EAOCa	US073	TYES	US073	-3.7526	0.2224	330	-16.87	<.0001
OW	US073	TYES	US073	-0.315	0.2224	330	-1.42	1
EAOCa	US075	OW	US075	-0.2849	0.2224	330	-1.28	1

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US075	TYES	US075	-0.728	0.2224	330	-3.27	0.9992
OW	US075	TYES	US075	-0.443	0.2224	330	-1.99	1
EAOCa	US083	OW	US083	-0.8391	0.2224	330	-3.77	0.9168
EAOCa	US083	TYES	US083	-0.801	0.2224	330	-3.6	0.9739
OW	US083	TYES	US083	0.03808	0.2224	330	0.17	1
EAOCa	US087	OW	US087	-2.2218	0.2224	330	-9.99	<.0001
EAOCa	US087	TYES	US087	-2.5625	0.2224	330	-11.52	<.0001
OW	US087	TYES	US087	-0.3406	0.2224	330	-1.53	1
EAOCa	US088	OW	US088	-1.7614	0.2224	330	-7.92	<.0001
EAOCa	US088	TYES	US088	-2.014	0.2224	330	-9.06	<.0001
OW	US088	TYES	US088	-0.2526	0.2224	330	-1.14	1
EAOCa	US095	OW	US095	-1.8495	0.2224	330	-8.32	<.0001
EAOCa	US095	TYES	US095	-2.088	0.2224	330	-9.39	<.0001
OW	US095	TYES	US095	-0.2386	0.2224	330	-1.07	1
EAOCa	US096	OW	US096	-0.6972	0.2224	330	-3.13	0.9999
EAOCa	US096	TYES	US096	-0.8081	0.2224	330	-3.63	0.9667
OW	US096	TYES	US096	-0.1109	0.2224	330	-0.5	1
EAOCa	US098	OW	US098	-1.9208	0.2224	330	-8.64	<.0001
EAOCa	US098	TYES	US098	-2.2218	0.2224	330	-9.99	<.0001
OW	US098	TYES	US098	-0.301	0.2224	330	-1.35	1
EAOCa	US099	OW	US099	-0.8406	0.2224	330	-3.78	0.9135
EAOCa	US099	TYES	US099	-0.9031	0.2224	330	-4.06	0.7056
OW	US099	TYES	US099	-0.06247	0.2224	330	-0.28	1
EAOCa	US101	OW	US101	0.3702	0.2224	330	1.66	1
EAOCa	US101	TYES	US101	-0.2803	0.2224	330	-1.26	1
OW	US101	TYES	US101	-0.6505	0.2224	330	-2.93	1
EAOCa	US104	OW	US104	-0.301	0.2224	330	-1.35	1
EAOCa	US104	TYES	US104	-0.7197	0.2224	330	-3.24	0.9995
OW	US104	TYES	US104	-0.4186	0.2224	330	-1.88	1
EAOCa	US108	OW	US108	-0.7614	0.2224	330	-3.42	0.9949
EAOCa	US108	TYES	US108	-0.772	0.2224	330	-3.47	0.9917
OW	US108	TYES	US108	-0.01059	0.2224	330	-0.05	1
EAOCa	US109	OW	US109	-0.7261	0.2224	330	-3.27	0.9993
EAOCa	US109	TYES	US109	-1.184	0.2224	330	-5.32	0.0141
OW	US109	TYES	US109	-0.4578	0.2224	330	-2.06	1
EAOCa	US110	OW	US110	-2.6215	0.2224	330	-11.79	<.0001
EAOCa	US110	TYES	US110	-2.588	0.2224	330	-11.64	<.0001
OW	US110	TYES	US110	0.03347	0.2224	330	0.15	1
EAOCa	US116	OW	US116	-0.6422	0.2224	330	-2.89	1
EAOCa	US116	TYES	US116	-0.6611	0.2224	330	-2.97	1
OW	US116	TYES	US116	-0.01889	0.2224	330	-0.08	1
EAOCa	US118	OW	US118	-1.5379	0.2224	330	-6.92	<.0001
EAOCa	US118	TYES	US118	-1.3645	0.2224	330	-6.14	0.0003
OW	US118	TYES	US118	0.1734	0.2224	330	0.78	1
EAOCa	US119	OW	US119	-1.8724	0.2224	330	-8.42	<.0001
EAOCa	US119	TYES	US119	-2.6109	0.2224	330	-11.74	<.0001
OW	US119	TYES	US119	-0.7386	0.2224	330	-3.32	0.9985
EAOCa	US122	OW	US122	2.0256	0.2224	330	9.11	<.0001
EAOCa	US122	TYES	US122	0.2641	0.2224	330	1.19	1
OW	US122	TYES	US122	-1.7614	0.2224	330	-7.92	<.0001

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US133	OW	US133	-0.9269	0.2224	330	-4.17	0.6
EAOCa	US133	TYES	US133	-0.5775	0.2224	330	-2.6	1
OW	US133	TYES	US133	0.3495	0.2224	330	1.57	1
EAOCa	US139	OW	US139	-2.8335	0.2224	330	-12.74	<.0001
EAOCa	US139	TYES	US139	-3.5229	0.2224	330	-15.84	<.0001
OW	US139	TYES	US139	-0.6894	0.2224	330	-3.1	0.9999
EAOCa	US141	OW	US141	-1.3099	0.2224	330	-5.89	0.0009
EAOCa	US141	TYES	US141	-2.4604	0.2224	330	-11.06	<.0001
OW	US141	TYES	US141	-1.1505	0.2224	330	-5.17	0.0271
EAOCa	US142	OW	US142	-1.912	0.2224	330	-8.6	<.0001
EAOCa	US142	TYES	US142	-3.7386	0.2224	330	-16.81	<.0001
OW	US142	TYES	US142	-1.8266	0.2224	330	-8.21	<.0001
EAOCa	US148	OW	US148	1.057	0.2224	330	4.75	0.1336
EAOCa	US148	TYES	US148	0.01109	0.2224	330	0.05	1
OW	US148	TYES	US148	-1.0459	0.2224	330	-4.7	0.1575
EAOCa	US149	OW	US149	0.07306	0.2224	330	0.33	1
EAOCa	US149	TYES	US149	0.03347	0.2224	330	0.15	1
OW	US149	TYES	US149	-0.03959	0.2224	330	-0.18	1
EAOCa	US151	OW	US151	3.3684	0.2224	330	15.15	<.0001
EAOCa	US151	TYES	US151	1.787	0.2224	330	8.04	<.0001
OW	US151	TYES	US151	-1.5814	0.2224	330	-7.11	<.0001
EAOCa	US152	OW	US152	-2.2614	0.2224	330	-10.17	<.0001
EAOCa	US152	TYES	US152	-2.7386	0.2224	330	-12.31	<.0001
OW	US152	TYES	US152	-0.4771	0.2224	330	-2.15	1
EAOCa	US153	OW	US153	1.3891	0.2224	330	6.25	0.0001
EAOCa	US153	TYES	US153	0.1505	0.2224	330	0.68	1
OW	US153	TYES	US153	-1.2386	0.2224	330	-5.57	0.0046
EAOCa	US155	OW	US155	0.7245	0.2224	330	3.26	0.9993
EAOCa	US155	TYES	US155	0.1225	0.2224	330	0.55	1
OW	US155	TYES	US155	-0.6021	0.2224	330	-2.71	1
EAOCa	US156	OW	US156	-0.2218	0.2224	330	-1	1
EAOCa	US156	TYES	US156	-0.9208	0.2224	330	-4.14	0.6277
OW	US156	TYES	US156	-0.699	0.2224	330	-3.14	0.9999
EAOCa	US158	OW	US158	1.0986	0.2224	330	4.94	0.0685
EAOCa	US158	TYES	US158	-0.9894	0.2224	330	-4.45	0.3297
OW	US158	TYES	US158	-2.088	0.2224	330	-9.39	<.0001
EAOCa	US161	OW	US161	-3.5542	0.2224	330	-15.98	<.0001
EAOCa	US161	TYES	US161	-3.3891	0.2224	330	-15.24	<.0001
OW	US161	TYES	US161	0.1651	0.2224	330	0.74	1
EAOCa	US162	OW	US162	-0.4515	0.2224	330	-2.03	1
EAOCa	US162	TYES	US162	-0.7386	0.2224	330	-3.32	0.9985
OW	US162	TYES	US162	-0.287	0.2224	330	-1.29	1
EAOCa	US163	OW	US163	-1.199	0.2224	330	-5.39	0.0104
EAOCa	US163	TYES	US163	-2.1505	0.2224	330	-9.67	<.0001
OW	US163	TYES	US163	-0.9515	0.2224	330	-4.28	0.4881
EAOCa	US164	OW	US164	1.3266	0.2224	330	5.97	0.0006
EAOCa	US164	TYES	US164	0.2386	0.2224	330	1.07	1
OW	US164	TYES	US164	-1.088	0.2224	330	-4.89	0.0818
EAOCa	US165	OW	US165	-0.1722	0.2224	330	-0.77	1
EAOCa	US165	TYES	US165	-1.1228	0.2224	330	-5.05	0.045

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US133	OW	US133	-0.9269	0.2224	330	-4.17	0.6
EAOCa	US133	TYES	US133	-0.5775	0.2224	330	-2.6	1
OW	US133	TYES	US133	0.3495	0.2224	330	1.57	1
EAOCa	US139	OW	US139	-2.8335	0.2224	330	-12.74	<.0001
EAOCa	US139	TYES	US139	-3.5229	0.2224	330	-15.84	<.0001
OW	US139	TYES	US139	-0.6894	0.2224	330	-3.1	0.9999
EAOCa	US141	OW	US141	-1.3099	0.2224	330	-5.89	0.0009
EAOCa	US141	TYES	US141	-2.4604	0.2224	330	-11.06	<.0001
OW	US141	TYES	US141	-1.1505	0.2224	330	-5.17	0.0271
EAOCa	US142	OW	US142	-1.912	0.2224	330	-8.6	<.0001
EAOCa	US142	TYES	US142	-3.7386	0.2224	330	-16.81	<.0001
OW	US142	TYES	US142	-1.8266	0.2224	330	-8.21	<.0001
EAOCa	US148	OW	US148	1.057	0.2224	330	4.75	0.1336
EAOCa	US148	TYES	US148	0.01109	0.2224	330	0.05	1
OW	US148	TYES	US148	-1.0459	0.2224	330	-4.7	0.1575
EAOCa	US149	OW	US149	0.07306	0.2224	330	0.33	1
EAOCa	US149	TYES	US149	0.03347	0.2224	330	0.15	1
OW	US149	TYES	US149	-0.03959	0.2224	330	-0.18	1
EAOCa	US151	OW	US151	3.3684	0.2224	330	15.15	<.0001
EAOCa	US151	TYES	US151	1.787	0.2224	330	8.04	<.0001
OW	US151	TYES	US151	-1.5814	0.2224	330	-7.11	<.0001
EAOCa	US152	OW	US152	-2.2614	0.2224	330	-10.17	<.0001
EAOCa	US152	TYES	US152	-2.7386	0.2224	330	-12.31	<.0001
OW	US152	TYES	US152	-0.4771	0.2224	330	-2.15	1
EAOCa	US153	OW	US153	1.3891	0.2224	330	6.25	0.0001
EAOCa	US153	TYES	US153	0.1505	0.2224	330	0.68	1
OW	US153	TYES	US153	-1.2386	0.2224	330	-5.57	0.0046
EAOCa	US155	OW	US155	0.7245	0.2224	330	3.26	0.9993
EAOCa	US155	TYES	US155	0.1225	0.2224	330	0.55	1
OW	US155	TYES	US155	-0.6021	0.2224	330	-2.71	1
EAOCa	US156	OW	US156	-0.2218	0.2224	330	-1	1
EAOCa	US156	TYES	US156	-0.9208	0.2224	330	-4.14	0.6277
OW	US156	TYES	US156	-0.699	0.2224	330	-3.14	0.9999
EAOCa	US158	OW	US158	1.0986	0.2224	330	4.94	0.0685
EAOCa	US158	TYES	US158	-0.9894	0.2224	330	-4.45	0.3297
OW	US158	TYES	US158	-2.088	0.2224	330	-9.39	<.0001
EAOCa	US161	OW	US161	-3.5542	0.2224	330	-15.98	<.0001
EAOCa	US161	TYES	US161	-3.3891	0.2224	330	-15.24	<.0001
OW	US161	TYES	US161	0.1651	0.2224	330	0.74	1
EAOCa	US162	OW	US162	-0.4515	0.2224	330	-2.03	1
EAOCa	US162	TYES	US162	-0.7386	0.2224	330	-3.32	0.9985
OW	US162	TYES	US162	-0.287	0.2224	330	-1.29	1
EAOCa	US163	OW	US163	-1.199	0.2224	330	-5.39	0.0104
EAOCa	US163	TYES	US163	-2.1505	0.2224	330	-9.67	<.0001
OW	US163	TYES	US163	-0.9515	0.2224	330	-4.28	0.4881
EAOCa	US164	OW	US164	1.3266	0.2224	330	5.97	0.0006
EAOCa	US164	TYES	US164	0.2386	0.2224	330	1.07	1
OW	US164	TYES	US164	-1.088	0.2224	330	-4.89	0.0818
EAOCa	US165	OW	US165	-0.1722	0.2224	330	-0.77	1
EAOCa	US165	TYES	US165	-1.1228	0.2224	330	-5.05	0.045

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
OW	US165	TYES	US165	-0.9506	0.2224	330	-4.27	0.4925
EAOCa	US167	OW	US167	-1.1365	0.2224	330	-5.11	0.0351
EAOCa	US167	TYES	US167	-1.3601	0.2224	330	-6.12	0.0003
OW	US167	TYES	US167	-0.2236	0.2224	330	-1.01	1
EAOCa	US168	OW	US168	-1.145	0.2224	330	-5.15	0.03
EAOCa	US168	TYES	US168	-1.1422	0.2224	330	-5.14	0.0316
OW	US168	TYES	US168	0.002802	0.2224	330	0.01	1
EAOCa	US171	OW	US171	0.2526	0.2224	330	1.14	1
EAOCa	US171	TYES	US171	-0.3601	0.2224	330	-1.62	1
OW	US171	TYES	US171	-0.6127	0.2224	330	-2.75	1
EAOCa	US172	OW	US172	-0.456	0.2224	330	-2.05	1
EAOCa	US172	TYES	US172	0.03959	0.2224	330	0.18	1
OW	US172	TYES	US172	0.4956	0.2224	330	2.23	1
EAOCa	US176	OW	US176	-2.912	0.2224	330	-13.09	<.0001
EAOCa	US176	TYES	US176	-3.2218	0.2224	330	-14.49	<.0001
OW	US176	TYES	US176	-0.3099	0.2224	330	-1.39	1
EAOCa	US178	OW	US178	0.1505	0.2224	330	0.68	1
EAOCa	US178	TYES	US178	-6.22E-15	0.2224	330	0	1
OW	US178	TYES	US178	-0.1505	0.2224	330	-0.68	1
EAOCa	US181	OW	US181	-1.316	0.2224	330	-5.92	0.0008
EAOCa	US181	TYES	US181	-1.2636	0.2224	330	-5.68	0.0026
OW	US181	TYES	US181	0.05237	0.2224	330	0.24	1
EAOCa	US187	OW	US187	-2.4604	0.2224	330	-11.06	<.0001
EAOCa	US187	TYES	US187	-2.4604	0.2224	330	-11.06	<.0001
OW	US187	TYES	US187	1.28E-15	0.2224	330	0	1
EAOCa	US188	OW	US188	-0.617	0.2224	330	-2.77	1
EAOCa	US188	TYES	US188	-0.8785	0.2224	330	-3.95	0.8029
OW	US188	TYES	US188	-0.2614	0.2224	330	-1.18	1
EAOCa	US191	OW	US191	-1.301	0.2224	330	-5.85	0.0011
EAOCa	US191	TYES	US191	-1.7782	0.2224	330	-8	<.0001
OW	US191	TYES	US191	-0.4771	0.2224	330	-2.15	1
EAOCa	US200	OW	US200	-2.23	0.2224	330	-10.03	<.0001
EAOCa	US200	TYES	US200	-2.5311	0.2224	330	-11.38	<.0001
OW	US200	TYES	US200	-0.301	0.2224	330	-1.35	1
EAOCa	US205	OW	US205	-1.213	0.2224	330	-5.45	0.0078
EAOCa	US205	TYES	US205	-1.6021	0.2224	330	-7.2	<.0001
OW	US205	TYES	US205	-0.3891	0.2224	330	-1.75	1
EAOCa	US215	OW	US215	-0.8495	0.2224	330	-3.82	0.8924
EAOCa	US215	TYES	US215	-0.8495	0.2224	330	-3.82	0.8924
OW	US215	TYES	US215	-3.55E-15	0.2224	330	0	1
EAOCa	US217	OW	US217	-1.6109	0.2224	330	-7.24	<.0001
EAOCa	US217	TYES	US217	-1.8495	0.2224	330	-8.32	<.0001
OW	US217	TYES	US217	-0.2386	0.2224	330	-1.07	1
EAOCa	US218	OW	US218	-0.7557	0.2224	330	-3.4	0.9962
EAOCa	US218	TYES	US218	-0.4062	0.2224	330	-1.83	1
OW	US218	TYES	US218	0.3495	0.2224	330	1.57	1
EAOCa	US225	OW	US225	-2.2297	0.2224	330	-10.03	<.0001
EAOCa	US225	TYES	US225	-2.3495	0.2224	330	-10.56	<.0001
OW	US225	TYES	US225	-0.1198	0.2224	330	-0.54	1
EAOCa	US226	OW	US226	-0.787	0.2224	330	-3.54	0.9845

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US226	TYES	US226	-1.1594	0.2224	330	-5.21	0.0229
OW	US226	TYES	US226	-0.3724	0.2224	330	-1.67	1
EAOCa	US232	OW	US232	-2.1233	0.2224	330	-9.55	<.0001
EAOCa	US232	TYES	US232	-2.3363	0.2224	330	-10.51	<.0001
OW	US232	TYES	US232	-0.213	0.2224	330	-0.96	1
EAOCa	US236	OW	US236	0.3141	0.2224	330	1.41	1
EAOCa	US236	TYES	US236	-0.3717	0.2224	330	-1.67	1
OW	US236	TYES	US236	-0.6858	0.2224	330	-3.08	1
EAOCa	US241	OW	US241	-2.6184	0.2224	330	-11.77	<.0001
EAOCa	US241	TYES	US241	-2.588	0.2224	330	-11.64	<.0001
OW	US241	TYES	US241	0.03035	0.2224	330	0.14	1
EAOCa	US244	OW	US244	-1.0625	0.2224	330	-4.78	0.1229
EAOCa	US244	TYES	US244	-1.6734	0.2224	330	-7.52	<.0001
OW	US244	TYES	US244	-0.6109	0.2224	330	-2.75	1
EAOCa	US245	OW	US245	0.4287	0.2224	330	1.93	1
EAOCa	US245	TYES	US245	-0.04358	0.2224	330	-0.2	1
OW	US245	TYES	US245	-0.4722	0.2224	330	-2.12	1
EAOCa	US249	OW	US249	0.8662	0.2224	330	3.89	0.8445
EAOCa	US249	TYES	US249	-0.106	0.2224	330	-0.48	1
OW	US249	TYES	US249	-0.9722	0.2224	330	-4.37	0.3982
EAOCa	US250	OW	US250	-3.8116	0.2224	330	-17.14	<.0001
EAOCa	US250	TYES	US250	-3.9621	0.2224	330	-17.82	<.0001
OW	US250	TYES	US250	-0.1505	0.2224	330	-0.68	1
EAOCa	US251	OW	US251	-0.3251	0.2224	330	-1.46	1
EAOCa	US251	TYES	US251	-1.0485	0.2224	330	-4.71	0.1517
OW	US251	TYES	US251	-0.7234	0.2224	330	-3.25	0.9994
EAOCa	US252	OW	US252	0.5195	0.2224	330	2.34	1
EAOCa	US252	TYES	US252	0.4225	0.2224	330	1.9	1
OW	US252	TYES	US252	-0.09691	0.2224	330	-0.44	1
EAOCa	US253	OW	US253	-0.08805	0.2224	330	-0.4	1
EAOCa	US253	TYES	US253	-0.199	0.2224	330	-0.89	1
OW	US253	TYES	US253	-0.1109	0.2224	330	-0.5	1
EAOCa	US254	OW	US254	2	0.2224	330	8.99	<.0001
EAOCa	US254	TYES	US254	0.6109	0.2224	330	2.75	1
OW	US254	TYES	US254	-1.3891	0.2224	330	-6.25	0.0001
EAOCa	US255	OW	US255	0.04846	0.2224	330	0.22	1
EAOCa	US255	TYES	US255	-0.1672	0.2224	330	-0.75	1
OW	US255	TYES	US255	-0.2157	0.2224	330	-0.97	1
EAOCa	US256	OW	US256	-0.8099	0.2224	330	-3.64	0.9646
EAOCa	US256	TYES	US256	-0.5713	0.2224	330	-2.57	1
OW	US256	TYES	US256	0.2386	0.2224	330	1.07	1
EAOCa	US259	OW	US259	-1.1505	0.2224	330	-5.17	0.0271
EAOCa	US259	TYES	US259	-1.4771	0.2224	330	-6.64	<.0001
OW	US259	TYES	US259	-0.3266	0.2224	330	-1.47	1
EAOCa	US260	OW	US260	-2.0619	0.2224	330	-9.27	<.0001
EAOCa	US260	TYES	US260	-2.485	0.2224	330	-11.17	<.0001
OW	US260	TYES	US260	-0.4231	0.2224	330	-1.9	1
EAOCa	US261	OW	US261	0.7157	0.2224	330	3.22	0.9996
EAOCa	US261	TYES	US261	2.22E-15	0.2224	330	0	1
OW	US261	TYES	US261	-0.7157	0.2224	330	-3.22	0.9996

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US265	OW	US265	-0.713	0.2224	330	-3.21	0.9997
EAOCa	US265	TYES	US265	-1.2386	0.2224	330	-5.57	0.0046
OW	US265	TYES	US265	-0.5256	0.2224	330	-2.36	1
EAOCa	US277	OW	US277	-0.02558	0.2224	330	-0.12	1
EAOCa	US277	TYES	US277	-0.3205	0.2224	330	-1.44	1
OW	US277	TYES	US277	-0.2949	0.2224	330	-1.33	1
EAOCa	US283	OW	US283	-2.1505	0.2224	330	-9.67	<.0001
EAOCa	US283	TYES	US283	-3.1505	0.2224	330	-14.17	<.0001
OW	US283	TYES	US283	-1	0.2224	330	-4.5	0.2909
EAOCa	US305	OW	US305	-0.1127	0.2224	330	-0.51	1
EAOCa	US305	TYES	US305	-0.3513	0.2224	330	-1.58	1
OW	US305	TYES	US305	-0.2386	0.2224	330	-1.07	1
EAOCa	US310	OW	US310	0.08805	0.2224	330	0.4	1
EAOCa	US310	TYES	US310	-0.6109	0.2224	330	-2.75	1
OW	US310	TYES	US310	-0.699	0.2224	330	-3.14	0.9999
EAOCa	US312	OW	US312	-0.7793	0.2224	330	-3.5	0.9887
EAOCa	US312	TYES	US312	-1.8155	0.2224	330	-8.16	<.0001
OW	US312	TYES	US312	-1.0363	0.2224	330	-4.66	0.1807
EAOCa	US314	OW	US314	-1.5396	0.2224	330	-6.92	<.0001
EAOCa	US314	TYES	US314	-1.9031	0.2224	330	-8.56	<.0001
OW	US314	TYES	US314	-0.3635	0.2224	330	-1.63	1
EAOCa	US323	OW	US323	-1.0485	0.2224	330	-4.71	0.1517
EAOCa	US323	TYES	US323	-0.8979	0.2224	330	-4.04	0.7273
OW	US323	TYES	US323	0.1505	0.2224	330	0.68	1
EAOCa	US324	OW	US324	-1.3495	0.2224	330	-6.07	0.0004
EAOCa	US324	TYES	US324	-1.3495	0.2224	330	-6.07	0.0004
OW	US324	TYES	US324	-1.50E-15	0.2224	330	0	1
EAOCa	US325	OW	US325	-0.4287	0.2224	330	-1.93	1
EAOCa	US325	TYES	US325	-0.7157	0.2224	330	-3.22	0.9996
OW	US325	TYES	US325	-0.287	0.2224	330	-1.29	1
EAOCa	US328	OW	US328	-0.6761	0.2224	330	-3.04	1
EAOCa	US328	TYES	US328	-1.699	0.2224	330	-7.64	<.0001
OW	US328	TYES	US328	-1.0229	0.2224	330	-4.6	0.2173
EAOCa	US329	OW	US329	0.3099	0.2224	330	1.39	1
EAOCa	US329	TYES	US329	-0.6505	0.2224	330	-2.93	1
OW	US329	TYES	US329	-0.9604	0.2224	330	-4.32	0.4488
EAOCa	US331	OW	US331	-0.1505	0.2224	330	-0.68	1
EAOCa	US331	TYES	US331	-1	0.2224	330	-4.5	0.2909
OW	US331	TYES	US331	-0.8495	0.2224	330	-3.82	0.8924
EAOCa	US343	OW	US343	-2.8099	0.2224	330	-12.63	<.0001
EAOCa	US343	TYES	US343	-3.0485	0.2224	330	-13.71	<.0001
OW	US343	TYES	US343	-0.2386	0.2224	330	-1.07	1
EAOCa	US351	OW	US351	0.1505	0.2224	330	0.68	1
EAOCa	US351	TYES	US351	-0.2386	0.2224	330	-1.07	1
OW	US351	TYES	US351	-0.3891	0.2224	330	-1.75	1
EAOCa	US352	OW	US352	-0.9659	0.2224	330	-4.34	0.425
EAOCa	US352	TYES	US352	-0.8779	0.2224	330	-3.95	0.8051
OW	US352	TYES	US352	0.08805	0.2224	330	0.4	1
EAOCa	US354	OW	US354	-1.659	0.2224	330	-7.46	<.0001
EAOCa	US354	TYES	US354	-1.5938	0.2224	330	-7.17	<.0001

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
OW	US354	TYES	US354	0.06527	0.2224	330	0.29	1
EAOCa	US355	OW	US355	-2.3406	0.2224	330	-10.52	<.0001
EAOCa	US355	TYES	US355	-2.272	0.2224	330	-10.22	<.0001
OW	US355	TYES	US355	0.06859	0.2224	330	0.31	1
EAOCa	US356	OW	US356	0.699	0.2224	330	3.14	0.9999
EAOCa	US356	TYES	US356	-0.301	0.2224	330	-1.35	1
OW	US356	TYES	US356	-1	0.2224	330	-4.5	0.2909
EAOCa	US357	OW	US357	-1.1901	0.2224	330	-5.35	0.0125
EAOCa	US357	TYES	US357	-1.5	0.2224	330	-6.74	<.0001
OW	US357	TYES	US357	-0.3099	0.2224	330	-1.39	1
EAOCa	US359	OW	US359	-0.5853	0.2224	330	-2.63	1
EAOCa	US359	TYES	US359	-0.4348	0.2224	330	-1.96	1
OW	US359	TYES	US359	0.1505	0.2224	330	0.68	1
EAOCa	US361	OW	US361	-0.4793	0.2224	330	-2.16	1
EAOCa	US361	TYES	US361	-0.6694	0.2224	330	-3.01	1
OW	US361	TYES	US361	-0.1901	0.2224	330	-0.85	1
EAOCa	US364	OW	US364	-1.199	0.2224	330	-5.39	0.0104
EAOCa	US364	TYES	US364	-2.1505	0.2224	330	-9.67	<.0001
OW	US364	TYES	US364	-0.9515	0.2224	330	-4.28	0.4881
EAOCa	US367	OW	US367	1	0.2224	330	4.5	0.2909
EAOCa	US367	TYES	US367	-0.199	0.2224	330	-0.89	1
OW	US367	TYES	US367	-1.199	0.2224	330	-5.39	0.0104
EAOCa	US368	OW	US368	-0.897	0.2224	330	-4.03	0.7313
EAOCa	US368	TYES	US368	-1.699	0.2224	330	-7.64	<.0001
OW	US368	TYES	US368	-0.802	0.2224	330	-3.61	0.973
EAOCa	US372	OW	US372	1.9272	0.2224	330	8.67	<.0001
EAOCa	US372	TYES	US372	0.6797	0.2224	330	3.06	1
OW	US372	TYES	US372	-1.2474	0.2224	330	-5.61	0.0038
EAOCa	US374	OW	US374	0.6901	0.2224	330	3.1	0.9999
EAOCa	US374	TYES	US374	0.03959	0.2224	330	0.18	1
OW	US374	TYES	US374	-0.6505	0.2224	330	-2.93	1
EAOCa	US379	OW	US379	-0.3995	0.2224	330	-1.8	1
EAOCa	US379	TYES	US379	-1.0728	0.2224	330	-4.82	0.1045
OW	US379	TYES	US379	-0.6734	0.2224	330	-3.03	1
EAOCa	US380	OW	US380	-1.1065	0.2224	330	-4.98	0.0599
EAOCa	US380	TYES	US380	-1.412	0.2224	330	-6.35	<.0001
OW	US380	TYES	US380	-0.3054	0.2224	330	-1.37	1
EAOCa	US390	OW	US390	-0.6734	0.2224	330	-3.03	1
EAOCa	US390	TYES	US390	-0.912	0.2224	330	-4.1	0.6672
OW	US390	TYES	US390	-0.2386	0.2224	330	-1.07	1
EAOCa	US392	OW	US392	0.2474	0.2224	330	1.11	1
EAOCa	US392	TYES	US392	-0.1505	0.2224	330	-0.68	1
OW	US392	TYES	US392	-0.3979	0.2224	330	-1.79	1
EAOCa	US394	OW	US394	0.3116	0.2224	330	1.4	1
EAOCa	US394	TYES	US394	-0.07745	0.2224	330	-0.35	1
OW	US394	TYES	US394	-0.3891	0.2224	330	-1.75	1
EAOCa	US399	OW	US399	-0.01609	0.2224	330	-0.07	1
EAOCa	US399	TYES	US399	-0.443	0.2224	330	-1.99	1
OW	US399	TYES	US399	-0.4269	0.2224	330	-1.92	1
EAOCa	US400	OW	US400	1.1505	0.2224	330	5.17	0.0271

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US400	TYES	US400	0.2441	0.2224	330	1.1	1
OW	US400	TYES	US400	-0.9065	0.2224	330	-4.08	0.6912
EAOCa	US403	OW	US403	-3	0.2224	330	-13.49	<.0001
EAOCa	US403	TYES	US403	-3	0.2224	330	-13.49	<.0001
OW	US403	TYES	US403	1.44E-15	0.2224	330	0	1
EAOCa	US404	OW	US404	-1.3495	0.2224	330	-6.07	0.0004
EAOCa	US404	TYES	US404	-3.9515	0.2224	330	-17.77	<.0001
OW	US404	TYES	US404	-2.6021	0.2224	330	-11.7	<.0001
EAOCa	US411	OW	US411	-2.5018	0.2224	330	-11.25	<.0001
EAOCa	US411	TYES	US411	-2.4604	0.2224	330	-11.06	<.0001
OW	US411	TYES	US411	0.04139	0.2224	330	0.19	1
EAOCa	US413	OW	US413	-0.199	0.2224	330	-0.89	1
EAOCa	US413	TYES	US413	-0.9756	0.2224	330	-4.39	0.3843
OW	US413	TYES	US413	-0.7766	0.2224	330	-3.49	0.9899
EAOCa	US414	OW	US414	-1.228	0.2224	330	-5.52	0.0057
EAOCa	US414	TYES	US414	-1.529	0.2224	330	-6.88	<.0001
OW	US414	TYES	US414	-0.301	0.2224	330	-1.35	1
EAOCa	US439	OW	US439	-0.6795	0.2224	330	-3.06	1
EAOCa	US439	TYES	US439	-1.228	0.2224	330	-5.52	0.0057
OW	US439	TYES	US439	-0.5485	0.2224	330	-2.47	1
EAOCa	US442	OW	US442	-1.23	0.2224	330	-5.53	0.0055
EAOCa	US442	TYES	US442	-1.23	0.2224	330	-5.53	0.0055
OW	US442	TYES	US442	3.89E-16	0.2224	330	0	1
EAOCa	US443	OW	US443	-3.4096	0.2224	330	-15.33	<.0001
EAOCa	US443	TYES	US443	-2.9324	0.2224	330	-13.19	<.0001
OW	US443	TYES	US443	0.4771	0.2224	330	2.15	1
EAOCa	US444	OW	US444	-0.156	0.2224	330	-0.7	1
EAOCa	US444	TYES	US444	-0.5935	0.2224	330	-2.67	1
OW	US444	TYES	US444	-0.4375	0.2224	330	-1.97	1
EAOCa	US445	OW	US445	-0.3724	0.2224	330	-1.67	1
EAOCa	US445	TYES	US445	-0.383	0.2224	330	-1.72	1
OW	US445	TYES	US445	-0.01059	0.2224	330	-0.05	1
EAOCa	US449	OW	US449	-2.2386	0.2224	330	-10.07	<.0001
EAOCa	US449	TYES	US449	-2.7157	0.2224	330	-12.21	<.0001
OW	US449	TYES	US449	-0.4771	0.2224	330	-2.15	1
EAOCa	US450	OW	US450	-0.699	0.2224	330	-3.14	0.9999
EAOCa	US450	TYES	US450	-1.0485	0.2224	330	-4.71	0.1517
OW	US450	TYES	US450	-0.3495	0.2224	330	-1.57	1
EAOCa	US454	OW	US454	-0.4287	0.2224	330	-1.93	1
EAOCa	US454	TYES	US454	-0.5	0.2224	330	-2.25	1
OW	US454	TYES	US454	-0.07133	0.2224	330	-0.32	1
EAOCa	US455	OW	US455	-1.6734	0.2224	330	-7.52	<.0001
EAOCa	US455	TYES	US455	-1.5207	0.2224	330	-6.84	<.0001
OW	US455	TYES	US455	0.1527	0.2224	330	0.69	1
EAOCa	US458	OW	US458	-1.3363	0.2224	330	-6.01	0.0005
EAOCa	US458	TYES	US458	-1.9472	0.2224	330	-8.76	<.0001
OW	US458	TYES	US458	-0.6109	0.2224	330	-2.75	1
EAOCa	US459	OW	US459	-0.699	0.2224	330	-3.14	0.9999
EAOCa	US459	TYES	US459	-1.1215	0.2224	330	-5.04	0.046
OW	US459	TYES	US459	-0.4225	0.2224	330	-1.9	1

Table 2.6. (cont'd)

Medium 1	Isolate 1	Medium 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
EAOCa	US460	OW	US460	-0.9921	0.2224	330	-4.46	0.3196
EAOCa	US460	TYES	US460	-1.4147	0.2224	330	-6.36	<.0001
OW	US460	TYES	US460	-0.4225	0.2224	330	-1.9	1
EAOCa	US461	OW	US461	-1	0.2224	330	-4.5	0.2909
EAOCa	US461	TYES	US461	-1.1505	0.2224	330	-5.17	0.0271
OW	US461	TYES	US461	-0.1505	0.2224	330	-0.68	1
EAOCa	US465	OW	US465	-1.642	0.2224	330	-7.38	<.0001
EAOCa	US465	TYES	US465	-1.841	0.2224	330	-8.28	<.0001
OW	US465	TYES	US465	-0.199	0.2224	330	-0.89	1
EAOCa	US476	OW	US476	-2	0.2224	330	-8.99	<.0001
EAOCa	US476	TYES	US476	-3	0.2224	330	-13.49	<.0001
OW	US476	TYES	US476	-1	0.2224	330	-4.5	0.2909
EAOCa	US478	OW	US478	-0.6505	0.2224	330	-2.93	1
EAOCa	US478	TYES	US478	-1.1505	0.2224	330	-5.17	0.0271
OW	US478	TYES	US478	-0.5	0.2224	330	-2.25	1
EAOCa	US479	OW	US479	-0.3239	0.2224	330	-1.46	1
EAOCa	US479	TYES	US479	-0.5	0.2224	330	-2.25	1
OW	US479	TYES	US479	-0.1761	0.2224	330	-0.79	1
EAOCa	US485	OW	US485	-2.1901	0.2224	330	-9.85	<.0001
EAOCa	US485	TYES	US485	-1.8891	0.2224	330	-8.49	<.0001
OW	US485	TYES	US485	0.301	0.2224	330	1.35	1
EAOCa	US487	OW	US487	-0.199	0.2224	330	-0.89	1
EAOCa	US487	TYES	US487	2.801	0.2224	330	12.6	<.0001
OW	US487	TYES	US487	3	0.2224	330	13.49	<.0001
EAOCa	US488	OW	US488	-1.8266	0.2224	330	-8.21	<.0001
EAOCa	US488	TYES	US488	-2.6505	0.2224	330	-11.92	<.0001
OW	US488	TYES	US488	-0.8239	0.2224	330	-3.7	0.9453
EAOCa	US489	OW	US489	-1.1858	0.2224	330	-5.33	0.0136
EAOCa	US489	TYES	US489	-0.857	0.2224	330	-3.85	0.8721
OW	US489	TYES	US489	0.3288	0.2224	330	1.48	1
EAOCa	US490	OW	US490	-3.088	0.2224	330	-13.89	<.0001
EAOCa	US490	TYES	US490	-3.2849	0.2224	330	-14.77	<.0001
OW	US490	TYES	US490	-0.1969	0.2224	330	-0.89	1
EAOCa	US493	OW	US493	-1.1338	0.2224	330	-5.1	0.0369
EAOCa	US493	TYES	US493	-1.1338	0.2224	330	-5.1	0.0369
OW	US493	TYES	US493	4.00E-15	0.2224	330	0	1
EAOCa	US502	OW	US502	-0.8363	0.2224	330	-3.76	0.9226
EAOCa	US502	TYES	US502	-0.7343	0.2224	330	-3.3	0.9988
OW	US502	TYES	US502	0.1021	0.2224	330	0.46	1
EAOCa	US505	OW	US505	-2.0669	0.2224	330	-9.29	<.0001
EAOCa	US505	TYES	US505	-1.8239	0.2224	330	-8.2	<.0001
OW	US505	TYES	US505	0.243	0.2224	330	1.09	1
EAOCa	US508	OW	US508	-2.0969	0.2224	330	-9.43	<.0001
EAOCa	US508	TYES	US508	-2.3979	0.2224	330	-10.78	<.0001
OW	US508	TYES	US508	-0.301	0.2224	330	-1.35	1

Table 2.7. Medium component effect ± standard error (SE) on *Flavobacterium psychrophilum* recovery resulting from Plackett-Burman experiment as well as *t*-value and *p*-value. The symbol, *, indicates a medium component had a significant effect on *F. psychrophilum* recovery (α = 0.05).

Medium Component	Effect	SE	*t*-value	*P*-value
Tryptone	-0.0595	-0.0298	1.658	0.0978
Yeast extract	-0.0073	-0.0036	0.203	0.8392
$CaCl_2 \cdot 2H_2O$	0.3199	0.1600	8.916	<0.0001*
$MgSO_4 \cdot 7H_2O$	0.3051	0.1525	8.503	<0.0001*
Beef extract	-0.0399	-0.0199	1.112	0.2666
Casamino acids	-0.4181	-0.2091	11.65	<0.0001*
Tryptose	-0.3488	-0.1744	9.721	<0.0001*
L-aspartic acid	0.0618	0.0309	1.722	0.0856
Horse serum	-0.0121	-0.0061	0.337	0.7362
Fetal bovine serum	-0.2933	-0.1466	8.174	<0.0001*
O. mykiss blood	-0.0352	-0.0176	0.981	0.3270

Figure 2.1. Map of Michigan with *Flavobacterium psychrophilum* surveillance locations and salmonid species sampled (ATS = Atlantic salmon, *Salmo salar*; CHS = Chinook salmon, *Oncorhynchus tshawytscha*; COS = Coho salmon, *O. kisutch*; STT = Steelhead trout, *O. mykiss*).

Figure 2.2. Normal probability plot of the effect of different factors (i.e., medium components) on *Flavobacterium psychrophilum* growth (measured in colony forming units) in the Plackett-Burman design. Factors with a square symbol significantly affected *F. psychrophilum* recovery, whereas those with a circular symbol did not ($\alpha = 0.05$). Factors to the left of the line improved *F. psychrophilum* recovery when incorporated into media at their low concentration, whereas factors to the right of the line improved recovery when incorporated at their high concentration.

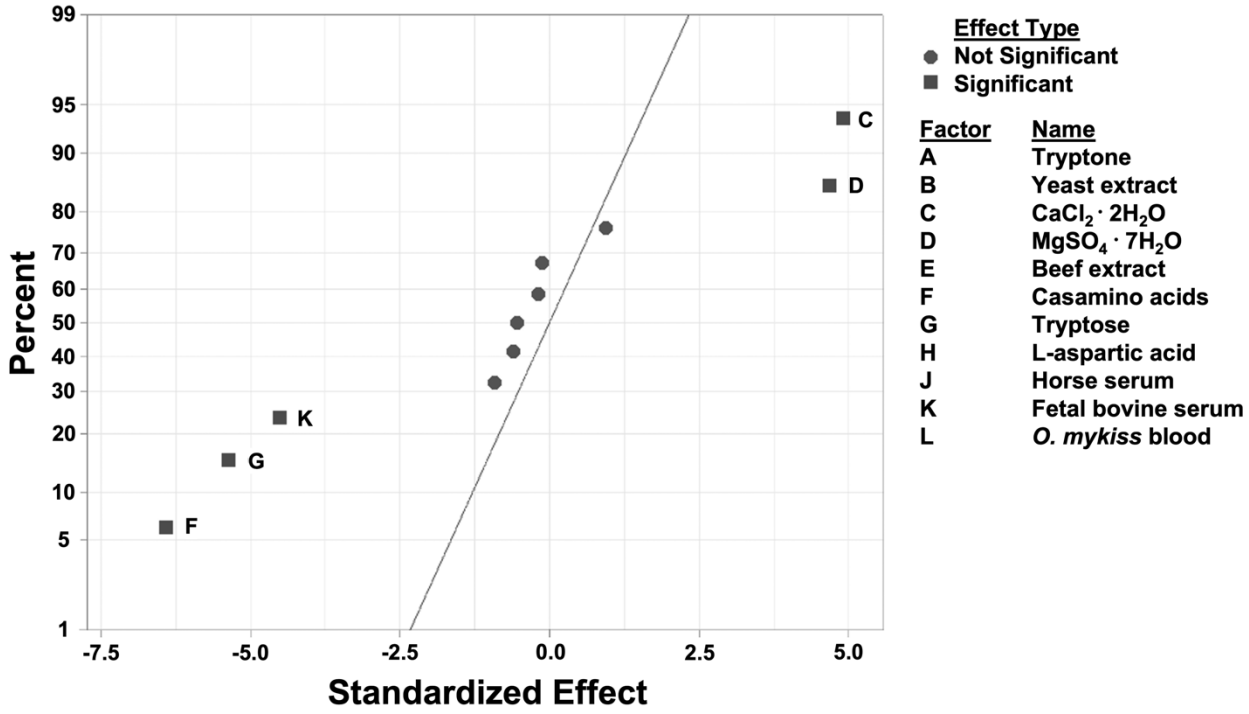


Figure 2.3. Pareto chart of the effect of different factors (i.e., medium components) on *Flavobacterium psychrophilum* growth (measured in colony forming units) in the Plackett-Burman design. The horizontal bars represent the ratio between the effects of the medium components and their standard error. The bars are ordered according to effect size, with the greatest effect on *F. psychrophilum* growth on top. The vertical dashed line illustrates the critical *t* value ($\alpha = 0.05$), meaning medium components crossing the line had a significant effect on *F. psychrophilum* growth.

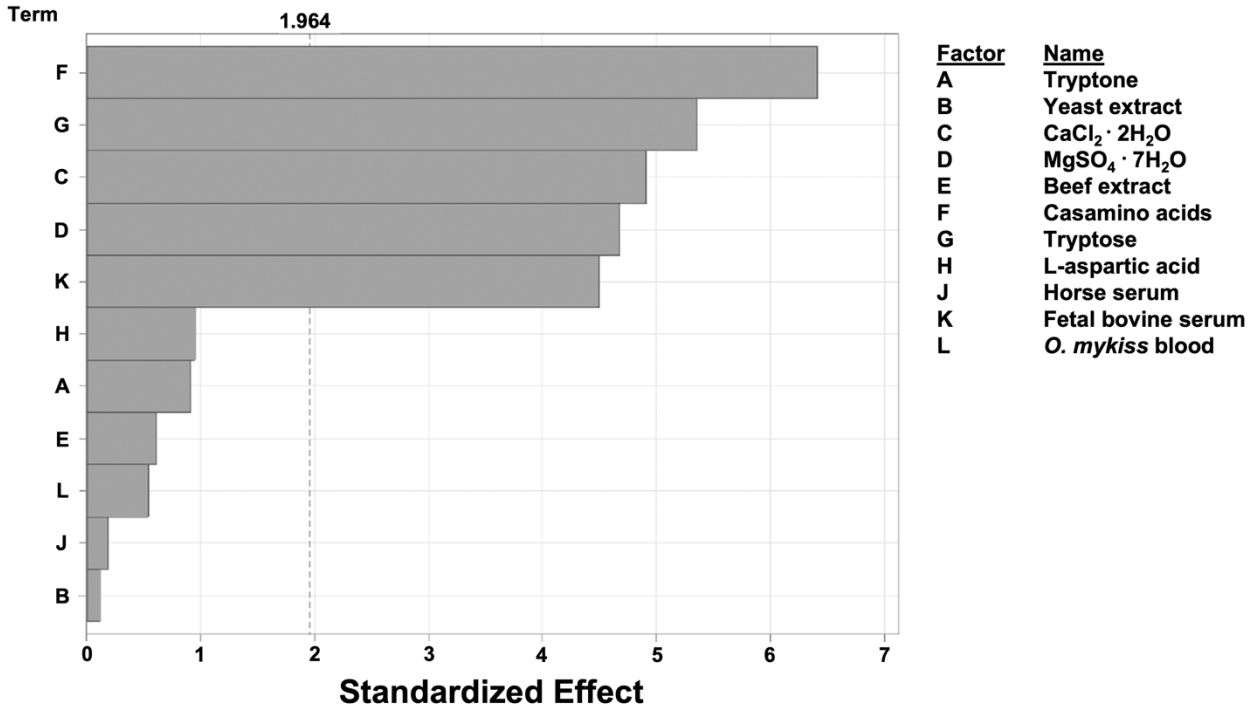
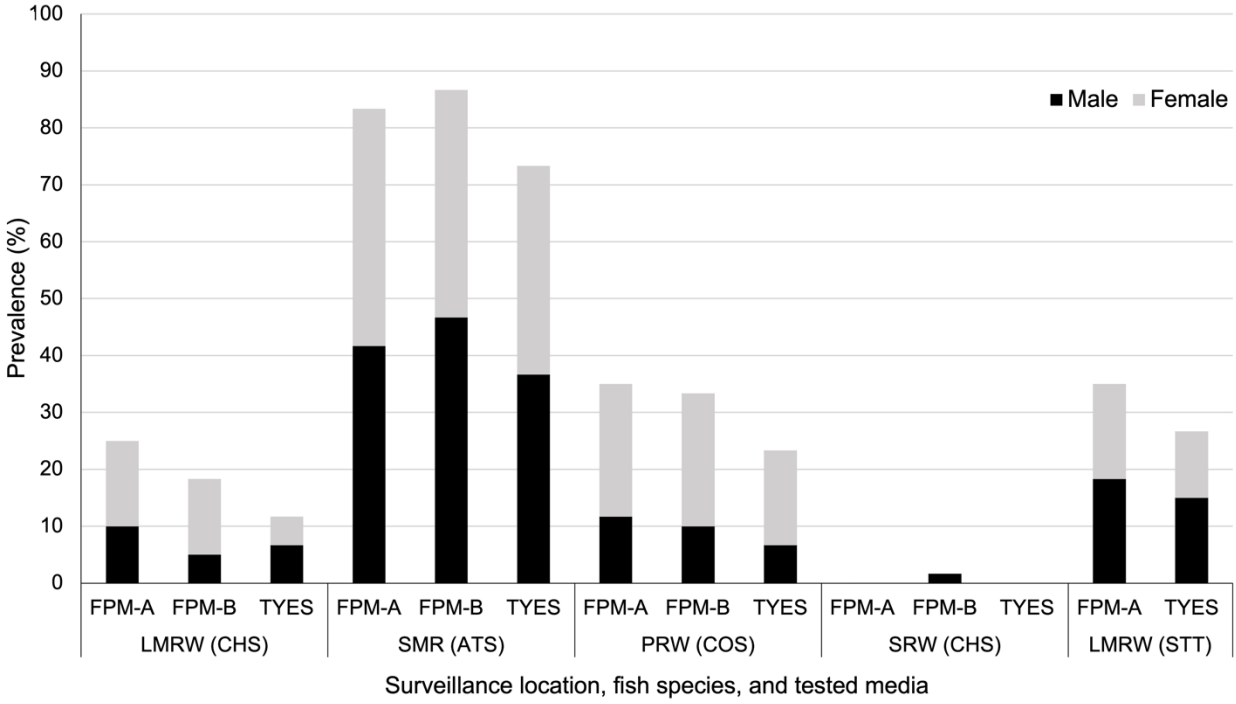


Figure 2.4. *Flavobacterium psychrophilum* infection prevalence among spawning phase salmonid (CHS, Chinook salmon, *Oncorhynchus tshawytscha*; ATS, Atlantic salmon, *Salmo salar*; COS, Coho salmon, *O. kisutch*; STT, Steelhead trout, *O. mykiss*) broodstock sampled at four Michigan gamete collection locations (Little Manistee River Weir, LMRW; St. Mary's River, SMR; Platte River Weir, PRW; Swan River Weir, SRW) according to three *F. psychrophilum* media (*F. psychrophilum* medium-A, FPM-A; *F. psychrophilum* medium-B, FPM-B; tryptone yeast extract salts medium, TYES).



Chapter 3:

Immersion challenge of three salmonid species (Family *Salmonidae*) with three heterologous *Flavobacterium psychrophilum* multilocus sequence typing variants provides further evidence of differential host-specificity

3.1. Abstract

Bacterial coldwater disease (BCWD), caused by *Flavobacterium psychrophilum*, results in globally significant losses amongst multiple salmonid (Family *Salmonidae*) species. Molecular epidemiology and serotyping studies have suggested that some variants are host specific; however, these associations have not been evaluated by cross-challenging fish species with putatively host-associated *F. psychrophilum* isolates via more natural (i.e., immersion) exposure routes. To this end, *F. psychrophilum* isolates US19-COS, US62-ATS, and US87-RBT, each originally recovered from diseased coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), or rainbow trout (*O. mykiss*), and belonging to a host-associated multilocus sequence typing clonal complex (CC; e.g., CC-ST9, CC-ST232, or CC-ST10), were PCR-serotyped, evaluated for proteolytic activity, and challenged against 4-month old Atlantic salmon, coho salmon, and rainbow trout via immersion. Findings showed US87-RBT caused disease and mortality only in rainbow trout (e.g., 56.7% survival probability vs. 100% in coho salmon and Atlantic salmon). US19-COS and US62-ATS caused more mortality in coho salmon and Atlantic salmon (i.e., their hosts of origin) but were also capable of causing disease in both other host species, albeit to a lesser extent. Observed differences in survival may be due to variant antigenic/virulence determinants as differences in serotype and proteolytic activity were discovered. Collectively, results highlight the intricacies of *F. psychrophilum*-host interactions and provide further in vivo evidence that some *F. psychrophilum* MLST variants are host specific, which may have implications for the development of BCWD prevention and control strategies.

3.2. Introduction

Flavobacterium psychrophilum, causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), causes substantial mortality and economic losses in farm and hatchery-reared salmonids (Family *Salmonidae*) worldwide (Loch and Faisal, 2017). Rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*O. kisutch*) are considered most susceptible (Holt, 1987), particularly at early life stages when mortality is highest (e.g., 50-90%; Barnes and Brown, 2011). Likewise, BCWD epizootics in farmed Atlantic salmon (*Salmo salar*) are also common (Nilsen et al. 2011; Avendaño-Herrera et al. 2020; Macchia et al. 2022).

Multilocus sequence typing (MLST) has become a widespread tool for molecular epidemiological studies on *F. psychrophilum* (Nicolas et al. 2008; Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Li et al. 2021). To date, >1500 isolates recovered in 18 countries on five continents have been genotyped via MLST, revealing the existence of >260 distinct sequence types (STs; https://pubmlst.org/fpsychrophilum). A common finding amongst MLST-based epidemiological studies is that some *F. psychrophilum* MLST clonal complexes (CCs) are most commonly associated with a single host species (Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019; Sebastiao et al. 2020; Li et al. 2021). For example, most *F. psychrophilum* isolates belonging to CC-ST10, which is the largest and most reported CC worldwide, are recovered from rainbow trout (Van Vliet et al. 2016; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Knupp et al. 2019; Li et al. 2021). Similarly, most *F. psychrophilum* isolates belonging to CC-ST9 are recovered from coho salmon, whereas all isolates in CC-ST232 have been recovered from Atlantic salmon (Fujiwara-Nagata et al. 2013; Avendaño-Herrera et al. 2014; Nilsen et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019).

In addition to being genetically diverse, *F. psychrophilum* also exhibits serotypic diversity (Pacha, 1968; Holt, 1987; Lorenzen and Olesen, 1997). Although classical serotyping methods remain in use, a multiplex PCR-based serotyping assay was recently developed by Rochat et al. (2017), which detects four molecular serotypes (e.g., Type-0 – Type-3) and that was later amended by Avendaño-Herrera et al. (2020) to detect a fifth molecular serotype (e.g., Type-4). This molecular serotyping assay has been applied to >350 *F. psychrophilum* isolates, and findings suggest some serotypes have host associations. For instance, *F. psychrophilum* isolates recovered from rainbow trout most commonly belong to Type-1 and Type-2, whereas isolates recovered from Atlantic salmon and coho salmon are most frequently associated with Type-2 and Type-4, or Type-0, respectively (Rochat et al. 2017; Sundell et al. 2019; Avendaño-Herrera et al. 2020; Li et al. 2021; Calvez et al. 2021; Knupp et al. 2021a). Likewise, *F. psychrophilum* isolates recovered from ayu (*Plecoglossus altivelis*) have been exclusively reported as Type-3 (Rochat et al. 2017).

The putative host-associations of some *F. psychrophilum* geno- and sero-variants are largely based on observational associations in naturally infected fish. However, several studies have directly or indirectly investigated such associations under in vivo laboratory conditions (Holt, 1987; Ekman and Norrgren, 2003; Fredriksen et al. 2016), though most have used a less natural exposure route (e.g., injection) that bypasses important immune defenses (Fast et al. 2002; Dash et al. 2018). In contrast, at least one study reported the virulence of two host-associated *F. psychrophilum* variants (e.g., US19-coho salmon in ST9 and US53-rainbow trout in ST78) in coho salmon following laboratory immersion exposure (Knupp et al. 2021a). This study revealed that only the coho salmon-recovered isolate caused disease and mortality in coho salmon, whereas the rainbow trout-recovered isolate did not, despite proving virulent to rainbow

trout in a previous study (Knupp et al. 2021b). However, a study has yet to simultaneously cross-challenge multiple salmonid species of a similar age with multiple putatively host specific *F. psychrophilum* variants, thereby leaving a gap of knowledge in BCWD ecology with potentially important implications for future prevention and control strategies. To further investigate *F. psychrophilum* host specificity, in vitro and in vivo experiments were designed to elucidate the interactions between *F. psychrophilum* and three salmonid species.

3.3. Materials and Methods

3.3.1. *Flavobacterium psychrophilum* isolates

Three *F. psychrophilum* isolates (e.g., US19, US62, and US87) belonging to three MLST STs (e.g., ST13, ST277, and ST275) within three MLST CCs (e.g., CC-ST9, CC-ST232, and CC-ST10) that are geographically widespread (i.e., detected on two to four continents; Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Knupp et al. 2019; Li et al. 2021) and exclusively or nearly exclusively recovered from one of three economically important salmonid species (e.g., coho salmon, rainbow trout, or Atlantic salmon) were selected from this study. In addition, recent studies have demonstrated US19 is virulent to coho salmon via immersion, and US87 to rainbow trout via injection (Knupp et al. 2021b).

3.3.2. Molecular serotyping

Like MLST ST, molecular serotype may also be indicative of some *F. psychrophilum* host associations (Rochat et al. 2017; Sundell et al. 2019; Avendaño-Herrera et al. 2020); therefore, the molecular serotypes of *F. psychrophilum* isolates US62-ATS and US87-RBT were determined using an adapted version of the mPCR-based serotyping approach as described by Knupp et al. (2021a). In brief, each 50-µl mPCR reaction contained 25 µl of 2X GoTaq® Green Master Mix (Promega), 20 ng of DNA template, 0.1 µM of each control primer, and 0.5 µM of

each primer targeting the molecular serotypes, with the remaining volume consisting of nuclease-free water. Sterile nuclease-free water functioned as a negative control, while *F. psychrophilum* isolates US19-COS, FP900406, CSF259-93, US104, and US515 acted as positive controls for Type-0, Type-1, Type-2, Type-3, and Type-4 respectively (Knupp et al. 2021; Loch and Knupp, unpublished). The mPCR cycling conditions outlined by Rochat et al. (2017) were employed using an Eppendorf Mastercycler pro thermal cycler. A 1.5% agarose gel containing 1X SYBR Safe DNA gel stain was used to separate 5 µl of the amplified PCR product via electrophoresis for 35 minutes at 100 V. A 1-Kb Plus DNA Ladder (ThermoFisher Scientific) served as the molecular size standard. The gel was examined under UV transillumination to estimate amplicon size and assign mPCR serotypes (e.g., Type-0, 188 bp; Type-1, 188 and 549 bp; Type-2, 188 and 841 bp; Type-3, 188 and 361 bp; and Type-4, 188 and 992 bp; Rochat et al., 2017; Avendaño-Herrera et al., 2020).

3.3.3. Characterization of proteolytic activity

Flavobacterium psychrophilum can proteolyze multiple components (e.g., casein, gelatin, elastin, and collagen) representative of host connective and muscle tissue, and therefore proteolytic activity has been suggested as a virulence determinant (Madsen and Dalsgaard, 1998; Nakayama et al. 2016; Rochat et al. 2019; Knupp et al. 2021a). Moreover, proteolysis of some components (e.g., elastin and collagen) may be more commonly associated with *F. psychrophilum* isolates with different host associations (e.g., Nakayama et al. 2016; Rochat et al. 2019; Knupp et al. 2021a). Therefore, the proteolytic activities of US62-ATS and US87-RBT were assessed on tryptone yeast extract salt medium (TYES; Holt, 1987) supplemented with casein, elastin, or gelatin as described previously (Sundell et al. 2019; Knupp et al. 2021a). The proteolytic activity of US19-COS has been previously assessed on these substrates (Knupp et al.

2021) but was included for comparison purposes. Additionally, collagenase activity was assessed by supplementing TYES with 5% (w/v) collagen from bovine Achilles tendon (ThermoFisher). Briefly, *F. psychrophilum* was revived from cryostock (maintained at -80 °C) on TYES, which was modified according to Michel et al. (1999), incubated for 48 hours at 15 °C, and then visually inspected for purity. Each *F. psychrophilum* isolate was inoculated into 80 mL of analogous broth and incubated with constant shaking (180 rpm) for 48 hours at 15 °C. Bacteria were harvested via centrifugation (2,571 × *g*, 15 min) and adjusted to an optical density at 600 nm (OD_{600}) corresponding to 1×10^9 cfu/mL using a spectrophotometer (WPA, Inc.) and sterile 0.65% saline. To quantify *F. psychrophilum* concentrations, serial dilutions in ten-fold increments (up to 100,000,000-fold) were plated on modified TYES in duplicate, and then incubated for seven days, after which final colony counts were performed. To determine proteolytic activity, 10 µL of each *F. psychrophilum* isolate was spotted in triplicate on the surface of the four media, allowed to dry, and then incubated for seven days at 15 °C. The colony and clear zone diameters were summed and then divided by the colony diameter to yield the clear zone ratio (CZR; Sundell et al. 2019).

3.3.4. In vivo virulence assessment of *Flavobacterium psychrophilum* isolates US19-COS, US62-ATS, and US87-RBT to Atlantic salmon, coho salmon, and rainbow trout

3.3.4.1. Origin of fish for challenge experiments

Embryonated Atlantic salmon and rainbow trout eggs were sourced from a commercial egg distributor, while embryonated coho salmon eggs were procured from Platte River State Fish Hatchery. Coordination occurred so that all eggs from the three species arrived at the Michigan State University – University Research Containment Facility on the same day. In brief and upon receipt, eggs were disinfected with 100 ppm iodophor solution (pH 7.30) for 10 minutes before

being placed in a vertical incubator supplied with UV-treated, sand-filtered well water maintained at 12 ºC ± 1 ºC until hatching. Sac-fry were then moved to aerated flow-through tanks (40 L; 12 ºC ± 1 ºC) and, once exogenous feeding commenced, were given a continuous supply of appropriately sized commercial trout food (Skretting, the Netherlands) via an automatic feeder. After eight weeks, fish were hand-fed twice daily and the water volume in the tanks was increased (400 L; 12 ºC ± 1 ºC). Tanks were cleaned and siphoned daily to remove waste and any uneaten food. Before the challenge experiment, a sample of fish from each species were cultured to screen for bacterial infections (Knupp et al. 2021a), including those caused by *F. psychrophilum*, and confirmed to be bacterial infection-free.

3.3.4.2. *Flavobacterium psychrophilum* inoculum preparation for immersion challenge

Flavobacterium psychrophilum isolates US19-COS, US62-ATS, and US87-RBT were revived from cryostock (maintained at -80 ºC) on *Flavobacterium psychrophilum* medium-A (FPM-A; Chapter 2), incubated for 48 hours at 15 ºC, and then visually inspected for purity. Each isolate was inoculated into 3 L of FPM-A broth, incubated, harvested, and adjusted to 10^9 cfu/mL using sterile 0.65% saline as described in section 3.3.3. *F. psychrophilum* concentrations were verified via plate counts.

3.3.4.3. Immersion challenge experiment

The ability of *Flavobacterium psychrophilum* isolates US19-COS, US62-ATS, and US87-RBT to infect and cause disease in four-month-old Atlantic salmon (mean weight 1.1g), coho salmon (mean weight 5.0g), and rainbow trout (mean weight 7.8g) was assesed via immersion exposure. One-hundred and twenty Atlantic salmon, coho salmon, and rainbow trout were anesthetized in sodium bicarbonate-buffered (200 mg/L) tricaine methanesulfonate (MS-222; Syndel) at a concentration of 100 mg/L, adipose fin-clipped using sharp sterile scissors

(Holt, 1987), and then allowed to recover in aerated water. Fish (n = 15, in duplicate) of each species (n = 3) were immersed for 30 min in aerated water (12 ± 1 ºC) containing 10^7 cfu/mL of US19-COS, US62-ATS, or US87-RBT. Control fish (n = 15, in duplicate) of each species (n = 3) were immersed in an identical volume of water only. After bacterial exposure, fish were transferred into aerated flow-through glass aquaria (37.8 L; n =15 fish per aquarium, in duplicate) supplied with ultraviolet light-treated, sand-filtered, pathogen-free well water (12 ± 1 ºC).

Fish were monitored daily for 25 days and cared for as described in section 3.3.4.1; mortalities were necropsied and clinically examined, and multiple tissues (e.g., external ulcers and kidney) were bacteriologically analyzed for *F. psychrophilum* on FPM-A. Surviving fish (e.g., 25 days post-exposure) were euthanized via MS-222 overdose (250 mg/L) and analyzed similarly. All challenge experiments were conducted in accordance with the MSU-Institutional Animal Care and Use Committee (AUF:201900312).

Representative isolates recovered from dead and surviving fish were confirmed as *F. psychrophilum* via endpoint PCR (Toyama et al. 1994; Van Vliet et al. 2015). Likewise, *F. psychrophilum* MLST STs of representative isolates were confirmed via PCR amplification and Sanger-sequencing of all seven *F. psychrophilum*-specific MLST loci as previously described (Knupp et al. 2019).

3.3.5. Data analysis

The Kruskal-Wallis test was used to examine median CZR differences among isolates (e.g., US19-COS, US62-ATS, and US87-RBT) for the tested media (e.g., caseinase, collagenase, elastase, and gelatinase). If the null hypothesis of no difference in median CZR among isolates was rejected, pairwise comparisons of median CZR between isolates were carried out using

Dunn's test and applying the Bonferroni correction for multiple comparisons ($\alpha = 0.05$). The Kruskal-Wallis tests and Dunn's tests were conducted using PROC npar1way and custom SAS code, respectively.

Kaplan-Meier plots (Kaplan and Meier, 1958) with 95% confidence intervals were generated using PROC LIFETEST and SGPLOT to visualize Atlantic salmon, coho salmon, and rainbow trout survival probabilities over time after exposure to either US19-COS, US62-ATS, or US87-RBT.

Relative risk of death among fish species exposed to each isolate was assessed using Cox proportional hazards regression models. Fish species and fish weight were treated as factor and continuous variables, respectively. Fish weight was included as a covariate as it can affect *F. psychrophilum*-induced mortality (Madsen and Dalsgaard, 1999). Comparisons of hazard ratios (i.e., risk of death) between fish species were evaluated for each isolate while accounting for the effect of fish weight. If mortality did not occur among experimental units (i.e., aquaria) for one or more fish species, the Cox proportional hazards regression model was replaced by pairwise comparisons of survival rate between fish species on day 25 (i.e., the end of the experiment) using two-sample *z*-tests. The Cox proportional hazards regression models and pairwise comparisons were conducted using PROC PHREG and custom code. All statistical analyses were performed using SAS® Version 9.4; ($\alpha = 0.05$).

3.4. Results

3.4.1. Molecular serotype

The three *F. psychrophilum* isolates each belonged to a different molecular serotype, whereby US19-COS, US62-ATS, and US87-RBT were identified as Type-0 (Knupp et al. 2021a), Type-1, and Type-2, respectively.

3.4.2. Proteolytic activity

Flavobacterium psychrophilum isolates US19-COS, US62-ATS, and US87-RBT all proteolyzed casein, collagen, and gelatin; however, only US87-RBT proteolyzed elastin (Table 3.1). The Kruskal-Wallis test indicated that there were overall significant differences among the isolates in median CZR for elastase ($\chi 2 = 8.00$; $df = 2$; P-value = 0.0183) and gelatinase ($\chi 2 = 7.71$; $df = 2$; P-value = 0.0211). However, the null hypothesis of no difference in median CZR among the isolates for caseinase ($\chi 2 = 5.84$; $df = 2$; P-value = 0.0538) and collagenase ($\chi 2 = 5.73$; $df = 2$; P-value = 0.0571) could not be rejected (i.e., no significant difference in caseinase and collagenase activity among the isolates). For elastase, the median CZR produced by US87-RBT was significantly greater than US19-COS (Z-value = 3.4641; P-value = 0.0008) and US62-ATS (Z-value = 3.4641; P-value = 0.0008; Table 3.1). For gelatinase, the median CZR produced by US19-COS and US87-RBT were both significantly greater than US62-ATS (US19-COS vs. US62-ATS: Z-value = 2.3094, P-value = 0.0314; US87-RBT vs. US62-ATS: Z-value = 3.4641, P-value = 0.0008; Table 3.1).

3.4.3. Virulence of *Flavobacterium psychrophilum* isolates US19-COS, US62-ATS, and US87-RBT to Atlantic salmon, coho salmon, and rainbow trout

3.4.3.1. Negative control Atlantic salmon, coho salmon, and rainbow trout

Throughout the course of the in vivo challenge experiments, no negative control fish died in any of the three fish species.

3.4.3.2. *Flavobacterium psychrophilum* isolate US87-RBT

Following immersion exposure to *F. psychrophilum* isolate US87-RBT, rainbow trout was the only fish species to develop gross signs of BCWD. Disease signs were evident as early as nine days post-exposure in the form of focally extensive dermal ulceration of the caudal

peduncle that penetrated the underlying musculature (Figure 3.1A). As disease progressed, the caudal peduncle ulceration deepened further into the underlying musculature, exposing the vertebral column (Figure 3.1B). Rainbow trout also exhibited uni- or bilateral exophthalmia with or without intraocular ecchymosis (Figure 3.1C-D; Table 3.2) and/or gill pallor with or without ecchymosis and/or petechiae (Figure 3.1E). Internally, rainbow trout infected with US87-RBT presented with visceral organ (e.g., heart, liver, and kidney) pallor, multifocal hepatic ecchymoses (Figure 3.2A), severe splenic swelling with perisplenic hemorrhage (Figure 3.2B), and severe intestinal hemorrhage with accompanying peri-intestinal hemorrhage (Figure 3.2C; Table 3.2). In a subset of surviving rainbow trout (i.e., euthanized 25 days post-exposure), unilateral exophthalmia and caudal peduncle ulceration was present. Internally, most surviving rainbow trout had moderate to severe splenic swelling. Surviving Atlantic salmon and coho salmon remained apparently healthy.

Overall survival was 56.7% in rainbow trout and 100% in Atlantic salmon and coho salmon (i.e., no Atlantic salmon or coho salmon died; Figure 3.3A). Rainbow trout mortality began nine days post-exposure and peaked on day 18 (Figure 3.3A). Because Atlantic salmon and coho salmon experienced no mortality, the Cox proportional hazards regression model could not be used to compare risk of death; however, the model did indicate that fish weight contributed significantly (P-value = 0.0036) to the risk of death among rainbow trout, whereby for each gram increase in weight, risk of death was reduced by 34.8% (hazard ratio = 0.652). Rainbow trout survival was significantly lower than both Atlantic salmon and coho salmon (Z-scores = 4.7897; P-values < 0.0001).

3.4.3.3. *Flavobacterium psychrophilum* isolate US19-COS

Atlantic salmon, coho salmon, and rainbow trout exposed to US19-COS (ST13, CC-ST9) developed similar gross signs of BCWD as early as four days post-exposure in the form of focally extensive dermal ulceration of the caudal peduncle that penetrated into the underlying muscle; however, and early in the disease course, ulcerations were shallower (i.e., not exposing the vertebral column) in Atlantic salmon compared to coho salmon and rainbow trout (Figure 3.4A-C). In rainbow trout only, the tissues surrounding the ulcer frequently had diffuse ecchymoses and petechiae (Figure 3.4C; Table 3.2). As disease progressed, caudal peduncle ulcerations deepened in all species, including Atlantic salmon (Figure 3.4D). Another external BCWD sign common to all species was gill pallor with or without ecchymoses and petechiae. In contrast, peri-oral ulceration was apparent in coho salmon and rainbow trout only (Figure 3.4E-F). Internally, most gross disease signs caused by US19-COS were similar among the three species, which included visceral organ (e.g., heart, liver, and kidney) pallor and hepatic and renal ecchymoses (Figure 3.5A-E). However, splenic swelling and intestinal hemorrhage was present only among coho salmon and rainbow trout (Figure 3.5B-C, F; Table 3.2). Surviving Atlantic salmon, coho salmon, and rainbow trout did not exhibit gross external or internal BCWD signs.

Overall, survival ranged from 6.7% in coho salmon to 50.0% to 53.3% in Atlantic salmon, and rainbow trout, respectively (Figure 3.3B). Mortality began three days post-exposure in all species, and peaked on days six, seven, and eight in Atlantic salmon, rainbow trout, and coho salmon, respectively (Figure 3.3B). The Cox proportional hazards regression model indicated fish species and weight significantly affected survival (fish species: Wald $\chi 2 = 44.78$, $df = 2$, P-value < 0.0001; fish weight: Wald $\chi 2 = 31.03$, $df = 1$, P-value < 0.0001); therefore, both variables were included in the model. As a result, coho salmon and rainbow trout were

significantly less likely (*P*-values < 0.0001) to survive than Atlantic salmon, whereas the risk of death among rainbow trout and coho salmon was not significantly different (*P*-value = 0.6102). For each gram increase in fish weight, the risk of death among all species decreased by 45.1% (hazard ratio = 0.549; *P*-value < 0.0001).

3.4.3.4. *Flavobacterium psychrophilum* isolate US62-ATS

Atlantic salmon, rainbow trout, and coho salmon exposed to US62-ATS (ST277, CC-ST232) developed gross BCWD signs within two, three, or four days, respectively. All species exhibited focally extensive dermal ulceration of the caudal peduncle (Figure 3.6A-C). Like rainbow trout exposed to US19-COS, rainbow trout ulcers were often accompanied by surrounding and severe diffuse ecchymotic hemorrhage that extended posteriorly into the caudal fin (Figure 3.6B; Table 3.2). Atlantic salmon also had caudal fin ecchymoses (Figure 3.6D). In contrast, gross hemorrhage of the caudal peduncle/fin was not present in any coho salmon. Other external BCWD signs common to all fish species included gill pallor with or without ecchymoses and petechiae. A disease sign unique to coho salmon was severe peri-oral ulceration (Figure 3.6E). Internal BCWD signs caused by US62-ATS were identical among all species and included visceral organ (e.g., heart, liver, and kidney) pallor and mild splenic swelling (Table 3.2). Some surviving Atlantic salmon, coho salmon, and rainbow trout showed evidence of a prior caudal peduncle ulceration, evidenced by incomplete or complete healing. Internally, surviving fish of all species appeared grossly normal.

Overall survival ranged from 3.3% to 13.3% in Atlantic salmon and rainbow trout, respectively, to 66.7% in coho salmon (Figure 3.3C). Mortality began one (Atlantic salmon), three (rainbow trout), and six (coho salmon) day(s) post-exposure and peaked on days four, eight, and 10 in Atlantic salmon, rainbow trout, and coho salmon, respectively (Figure 3.3C).

The Cox proportional hazards regression model indicated fish species and weight significantly affected survival (fish species: Wald $\chi 2 = 42.73$, $df = 2$, P-value < 0.0001; fish weight: Wald $\chi 2 = 23.27$, $df = 1$, P-value < 0.0001); therefore, both variables were included in the model. Atlantic salmon and rainbow trout were significantly less likely (P-values < 0.0001) to survive US62-ATS infection when compared to coho salmon. There was no significant difference in the risk of death among Atlantic salmon and rainbow trout (P-value = 0.5127). For each gram increase in fish weight, the risk of death among all species decreased by 34.1% (hazard ratio = 0.659; P-value < 0.0001).

3.4.4. Infection status in Atlantic salmon, coho salmon, and rainbow trout following immersion exposure to *Flavobacterium psychrophilum* isolates US19-COS, US62-ATS, and US87-RBT

3.4.4.1. Negative control Atlantic salmon, coho salmon, and rainbow trout

No bacteria were recovered from any negative control fish throughout these experiments.

3.4.4.2. *Flavobacterium psychrophilum* isolate US87-RBT

F. psychrophilum isolate US87-RBT was recovered in a pure form and as perfuse lawns (i.e., colony forming units, cfus, too numerous to count) from the caudal peduncle and kidney of all dead rainbow trout (n = 13). In surviving rainbow trout, pure cultures of US87-RBT were recovered from ~35.3% (n = 6/17) of the kidney cultures at intensities ranging from $10^0 – 10^1$ cfu/g (as determined by calibrated inoculating loops and colony counts) of tissue. Molecular analyses confirmed the recovered bacteria were *F. psychrophilum* and belonged to ST275 (data not shown).

3.4.4.3. *Flavobacterium psychrophilum* isolate US19-COS

F. psychrophilum isolate US19-COS was recovered in a pure form and as perfuse lawns from the caudal peduncle and kidney of all dead Atlantic salmon (n = 15), coho salmon (n = 28), and rainbow trout (n = 14). *F. psychrophilum* isolate US19-COS was not recovered from the external or internal tissues of any surviving fish. Molecular analyses confirmed the recovered bacteria were *F. psychrophilum* and belonged to ST13 (data not shown).

3.4.4.4. *Flavobacterium psychrophilum* isolate US62-ATS

F. psychrophilum isolate US62-ATS was recovered in a pure form and as perfuse lawns from the caudal peduncle ulcerations of all dead Atlantic salmon (n = 29) and rainbow trout (n = 26). Similarly, US62-ATS was recovered at intensities ranging from 10^3 cfu/g to perfuse lawns from the kidney of all dead rainbow trout and most (e.g., n = 23/29, 79%) dead Atlantic salmon and in a pure form. In contrast, pure cultures of US62-ATS were obtained from the caudal peduncle ulcer of 40% (n = 4/10) of dead coho salmon at intensities ranging from 10^3 cfu/g to perfuse lawns. Internally, US62-ATS was recovered in a pure form from the kidney of all dead coho salmon (n = 10), with intensities ranging from 10^3 cfu/g to perfuse lawns. *F. psychrophilum* isolate US62-ATS was not recovered from external or internal tissues of any surviving fish. Molecular analyses confirmed the recovered bacteria were *F. psychrophilum* and belonged to ST277 (data not shown).

3.5. Discussion

Herein, results provide evidence that some *F. psychrophilum* MLST variants are host specific, a matter that may affect the development of targeted BCWD prevention and control strategies. Indeed, US87-RBT (ST275, in CC-ST10) showed strong infection and disease-fidelity to rainbow trout, as evidenced by causing disease and subsequent mortality only in rainbow

trout, despite coho salmon and Atlantic salmon being at a significantly greater risk of death because of their smaller size. Knupp et al. (2021a) also suggested at least one other CC-ST10 variant (e.g., ST78) was rainbow trout-specific after proving avirulent to coho salmon following immersion challenge; however, this study did not also assess the virulence of this variant in rainbow trout. Likewise, Fredriksen et al. (2016) reported that a *F. psychrophilum* isolate highly virulent to rainbow trout was avirulent to Atlantic salmon via injection; however, the MLST genotype of this isolate was not reported. The in vivo findings from this study support the MLST-based observations that CC-ST10 appears to be rainbow trout-specific (Nicolas et al. 2008; Nilsen et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019). In fact, of the 851 *F. psychrophilum* isolates recovered from fish and belonging to CC-ST10 (https://pubmlst.org/fpsychrophilum), >95% were recovered from *O. mykiss*. Interestingly, the CC-ST10 isolates that were recovered from other fish species (e.g., white sturgeon, *Acipenser transmontanus*; chinook salmon, *O. tshawytscha*; brown trout, *S. trutta*; coho salmon, Atlantic salmon, and *Salvelinus* sp.) may be the result of tightly interconnected fish farming practices. For example, most fish farms in Chile simultaneously rear Atlantic salmon, coho salmon, and rainbow trout (Avendaño-Herrera et al. 2020) and thus may partially explain why some CC-ST10 isolates were recovered from species other than rainbow trout. In addition to being host specific, US87-RBT was the only isolate recovered 25 days post infection (i.e., the end of the experiment), possibly suggesting it has evolved to circumvent the rainbow trout immune response.

Like US87-RBT, US19-COS (ST13, in CC-ST9) and US62-ATS (ST277, in CC-ST232) also caused the most mortality in their host of origin (e.g., coho salmon and Atlantic salmon, respectively). However, and in contrast to US87-RBT, these *F. psychrophilum* isolates also

proved capable of causing disease and mortality in other salmonids, albeit to a lesser degree. Ekman and Norrgren (2003) noted similar findings, whereby an *F. psychrophilum* isolate (MLST variant unknown) recovered from Atlantic salmon caused the most mortality in Atlantic salmon but also caused mortality in rainbow trout and sea trout (*S. trutta* L.). Likewise, Holt (1987) found that although an *F. psychrophilum* isolate (e.g., SH3-81, in the same CC as US19-COS; Van Vliet et al. 2016) recovered from coho salmon caused the most mortality in coho salmon, it also caused mortality in Chinook salmon (*O. tshawytscha*) and rainbow trout. However, and notably, these previous studies were conducted via injection, which bypasses some host defenses (Fast et al. 2002; Dash et al. 2018) and thereby complicates assessment of the host specificity of the tested variants. Nevertheless, findings herein show that some *F. psychrophilum* variants may have a broader host range, which could have substantial implications for fish farms and hatcheries rearing multiple salmonid species. For instance, these facilities may be at greater risk for widespread losses in the event of a BCWD outbreak in comparison to an outbreak caused by a host specific variant (e.g., ST275). In this context, future studies assessing the transmission dynamics of these *F. psychrophilum* variants and salmonid species are warranted. Beyond the immediate risks, these findings may also affect the development of vaccines intended to protect multiple salmonid species.

Although the primary focus of this study was not to extensively examine the mechanisms underlying *F. psychrophilum* host specificity, observations suggest *O*-polysaccharide antigenic determinants may play a role. In this context, US87-RBT belonged to Type-2 (i.e., serotype Th; Lorenzen and Olesen, 1997; Rochat et al. 2017) and was strongly host specific to rainbow trout. Knupp et al. (2021a) suggested a Type-2 *F. psychrophilum* variant (e.g., ST78) was rainbow trout specific after proving avirulent to coho salmon via immersion. Indeed, many Type-2/Th *F.*

psychrophilum isolates are virulent to rainbow trout and/or recovered from this species (Lorenzen and Olesen, 1997; Sundell et al. 2019; Avendaño-Herrera et al. 2020). US19-COS belonged to Type-0 (i.e., serotype Fp^T; Lorenzen and Olesen, 1997; Rochat et al. 2017; Knupp et al. 2021a) and caused the most mortality in its host of origin (e.g., coho salmon). These findings are consistent with previous studies, whereby most Type-0/Fp^T *F. psychrophilum* isolates are recovered from coho salmon (Lorenzen and Olesen, 1997; Rochat et al. 2017). Notably, the *F. psychrophilum* type strain (NCIMB 1947^T) also belongs to Type-0/Fp^T (Lorenzen and Olesen, 1997; Rochat et al. 2017) and is considered avirulent to rainbow trout (Jarau et al. 2018; Madsen and Dalsgaard, 2000; Sundell et al. 2019); however, and given our findings, it seems NCIMB 1947^T may not be well-suited to infect rainbow trout. US62-ATS belonged to Type-1 (i.e., serotype Fd; Lorenzen and Olesen, 1997), which contrasts with most previous studies showing Atlantic salmon isolates most often belong to Type-2 or Type-4 (Rochat et al. 2017; Avendaño-Herrera et al. 2020). Indeed, most *F. psychrophilum* isolates belonging to Type-1/Fd are recovered from rainbow trout (Lorenzen and Olesen, 1997; Rochat et al. 2017; Saticioglu et al. 2018; Avendaño-Herrera et al. 2020), and many are virulent to this species via injection (Sundell et al. 2019). Thus, our finding that US62-ATS belonged to Type-1 may partially explain the virulence of this isolate to not only Atlantic salmon but rainbow trout. Cisar et al. (2019) reported that serogroup, rather than serotype, more accurately defines Fd, Th, and Fp^T, and thus also applies to the molecular serotypes. Collectively, previous findings and observations herein suggest additional studies characterizing the serotypes of *F. psychrophilum* are needed, a matter that could impact BCWD vaccine development and selective breeding programs.

Another mechanism potentially contributing to *F. psychrophilum* host specificity is proteolytic activity. Rainbow trout-specific isolate US87-RBT was the only tested isolate to

degrade elastin. Indeed, elastinolytic activity is common among isolates recovered from rainbow trout and belonging to CC-ST10 (Sundell et al. 2019). However, not all rainbow trout-recovered *F. psychrophilum* isolates possess this capability (Dalsgaard and Madsen, 2000; Soule et al. 2005; Sundell and Wiklund, 2015; Rochat et al. 2019). Thus, elastinolytic activity may only provide an advantage for some rainbow trout-associated isolates. The observation that US19-COS lacks elastinolytic activity is unsurprising given most *F. psychrophilum* isolates recovered from coho salmon and/or belonging to MLST CC-ST9 and/or serotype Fp^T lack this ability (Dalsgaard and Madsen, 2000; Soule et al. 2005; Rochat et al. 2019). Thus, other virulence determinants and/or proteases are sufficient for causing mortality in coho salmon. Indeed, Barbier et al. (2020) reported *F. psychrophilum* isolate OSU THCO2-90, which was recovered from diseased coho salmon and belongs to CC-ST9 (i.e., same CC as US19-COS; Nicolas et al. 2008), secretes at least 49 proteins, including multiple undescribed proteases. Studies assessing the elastinolytic activity of Atlantic salmon-recovered *F. psychrophilum* isolates have produced mixed results to date (Soule et al. 2005; Sundell and Wiklund, 2015; Rochat et al. 2019). Findings herein clearly show some Atlantic salmon-recovered *F. psychrophilum* isolates can cause high mortality without this ability. Whether the lack of this trait is common to most Atlantic salmon-associated *F. psychrophilum* isolates remains to be determined.

This study emphasized the potential role of pathogen specificity. However, disease outcomes result from complex interactions between the pathogen, its host, and its environment (Casadevall and Pirofski, 1999), and previous studies have shown host genetics play a role in BCWD resistance. For example, Leeds et al. (2010) demonstrated via laboratory challenges that selective breeding was effective at increasing BCWD resistance among rainbow trout. Moreover, multiple quantitative trait loci associated with BCWD resistance in rainbow trout have been

identified (Wiens et al. 2013; Vallejo et al. 2014; Palti et al. 2015). Host genetics may contribute to BCWD resistance via differences in immune response. For example, Lee et al. (2023) found that in comparison to a BCWD-susceptible rainbow trout line, a BCWD-resistant rainbow trout line had increased expression of M2 macrophages involved anti-inflammatory responses and tissue repair, and two Toll-like receptors responsible for pathogen detection and inflammatory response. Nagai and Nakai (2011) found ayu-recovered *F. psychrophilum* isolates could survive and grow in ayu serum, whereas isolates recovered from salmonids and cyprinids could not. Herein, rainbow trout was the only species with severe diffuse ecchymotic hemorrhage surrounding the caudal peduncle and exophthalmia. Whether the observed differences in diseases signs among species following exposure to US19-COS, US62-ATS, and US87-RBT is pathogen and/or host-derived remains to be determined.

In conclusion, we confirmed the MLST-based observations that some *F. psychrophilum* variants are host-specific, whereas others appear more generalistic. We posit the mechanisms driving these disparities are multifaceted, potentially influenced by not only *F. psychrophilum* serotype and secreted proteases but also host genetics and corresponding immune response. The implications of these findings are broad and may affect *F. psychrophilum* transmission dynamics, and the development of effective BCWD vaccines and BCWD-resistant salmonid lines. Thus, future studies evaluating *F. psychrophilum* host specificity, transmission, and the underlying mechanisms are warranted.

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APPENDIX

Table 3.1. *Flavobacterium psychrophilum* isolates used in this study for *in vivo* challenge experiments against Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) and for the assessment of proteolytic activity, which is presented as a ratio of the median clear zone diameter to the colony diameter (in mm) ± standard error (SE). Median clear zone ratios for a particular enzyme (e.g., caseinase, gelatinase, or elastinase) containing an identical symbol (e.g., *, **, ***) are not significantly different ($\alpha = 0.05$) and a ratio of 1.00 ± 0.00 indicates no protease activity.

				Protease clear zone ratio ± SE			
Isolate ID	Host of origin	ST[a]	CC[b]	Caseinase	Collagenase	Gelatinase	Elastinase
US19	Coho salmon	ST13	CC-ST9	3.00 (0.13)*	2.00 (0.13)*	2.31 (0.05)*	1.00 (0.00)*
US62	Atlantic salmon	ST277	CC-ST232	3.88 (0.30)*	2.00 (0.12)*	1.79 (0.00)**	1.00 (0.00)*
US87	Rainbow trout	ST275	CC-ST10	3.44 (0.00)*	3.44 (0.00)*	3.50 (0.00)***	2.00 (0.00)**

[a] Sequence type.
[b] Clonal complex.

Table 3.2. Proportion of dead Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) with a range of gross external and internal bacterial coldwater disease signs following exposure to *Flavobacterium psychrophilum* isolates US19-COS, US62-ATS, and US87-RBT.

		External disease signs		Internal disease signs		
Isolate	Host species[a]	Hemorrhage surrounding caudal peduncle ulcer	Exophthalmia	Visceral organ hemorrhage	Splenic swelling	Intestinal hemorrhage
US19-COS	Atlantic salmon	0/15	0/15	1/15	0/15	0/15
	Coho salmon	0/28	0/28	2/28	25/28	3/28
	Rainbow trout	7/14	0/14	1/14	14/14	3/14
US62-ATS	Atlantic salmon	0/29	0/29	0/29	27/29	0/29
	Coho salmon	0/10	0/10	0/10	10/10	0/10
	Rainbow trout	12/26	0/26	3/26	26/26	0/26
US87-RBT	Atlantic salmon	-	-	-	-	-
	Coho salmon	-	-	-	-	-
	Rainbow trout	0/13	3/13	6/13	13/13	3/13

[a] All dead fish with caudal peduncle ulceration, gill pallor, and visceral organ pallor.

Figure 3.1. Gross external lesions in rainbow trout (*O. mykiss*) following immersion challenge with *F. psychrophilum* isolate US87-RBT. (A) Focally extensive ulceration of the caudal peduncle that penetrated the underlying musculature. (B) Focally extensive ulceration of the caudal peduncle that penetrated the underlying musculature and exposed the vertebral column. (C) Bilateral exophthalmia. (D) Unilateral exophthalmia with intraocular ecchymosis (arrow). (E) Gill pallor with ecchymoses and petechiae (arrow).

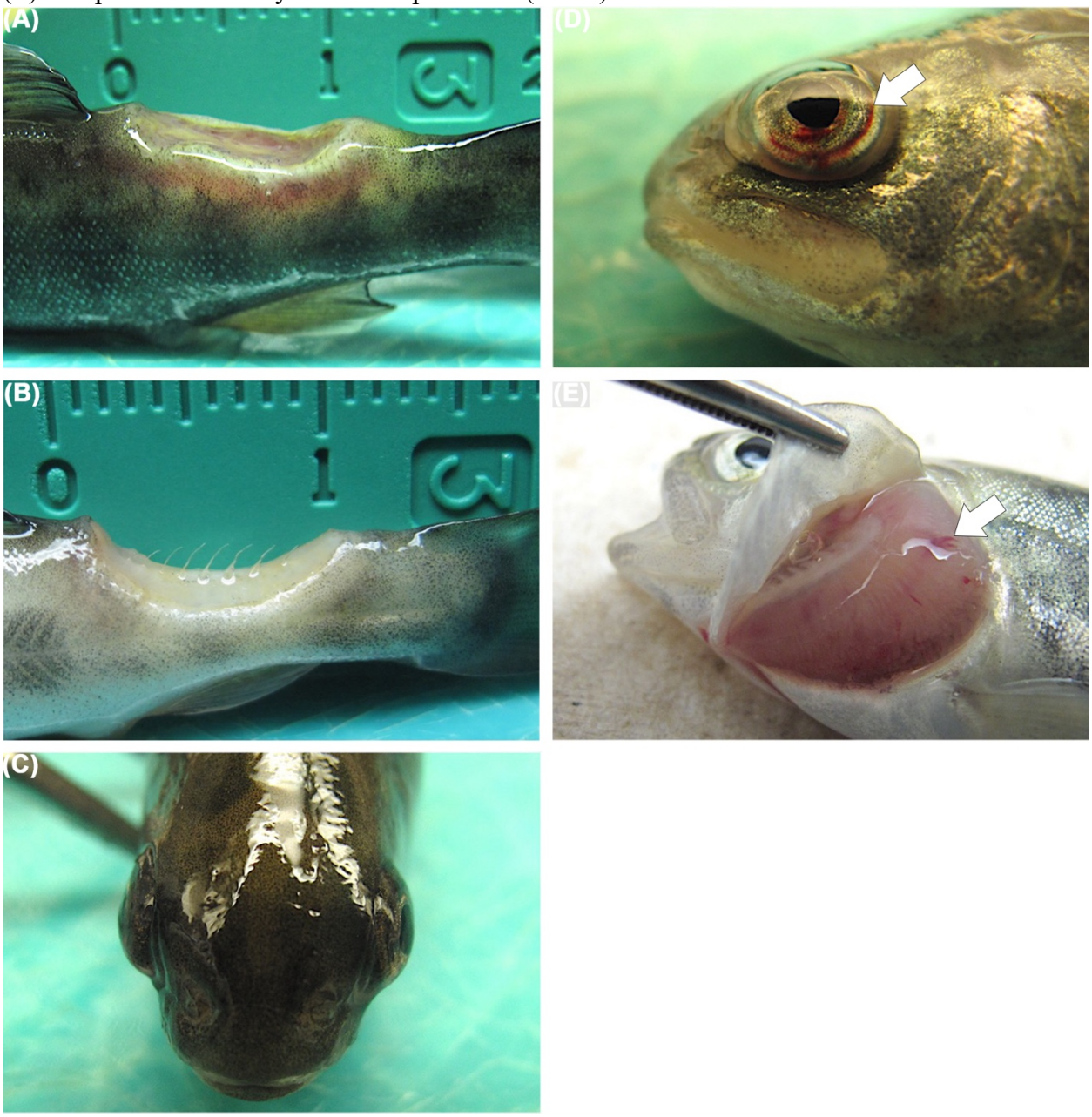


Figure 3.2. Gross internal lesions in rainbow trout (*O. mykiss*) following immersion challenge with *F. psychrophilum* isolate US87-RBT. (A) Pale liver with multifocal ecchymoses. (B) Severe splenic swelling with perisplenic hemorrhage. (C) Severe intestinal hemorrhage with accompanying peri-intestinal hemorrhage.

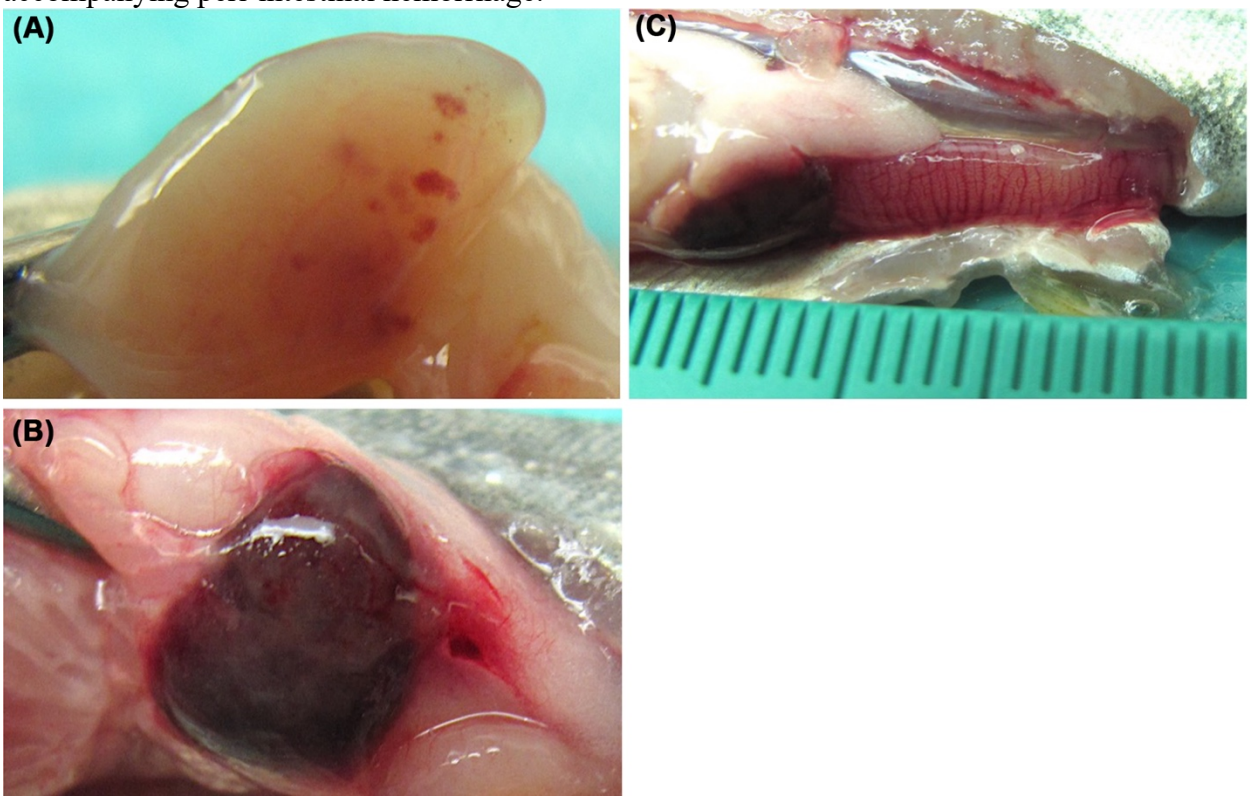


Figure 3.3. Kaplan-Meier survival probability curves of Atlantic salmon (*Salmo salar*; ATS), coho salmon (*Oncorhynchus kisutch*; COS), and rainbow trout (*O. mykiss*; RBT) over a 25-day period following immersion challenge with *Flavobacterium psychrophilum* isolates (A) US87-RBT, (B) US19-COS, and (C) US62-ATS. Shaded regions depict 95% confidence intervals. Lines with different symbols (e.g., *, **) indicate significant differences in survival (α = 0.05).

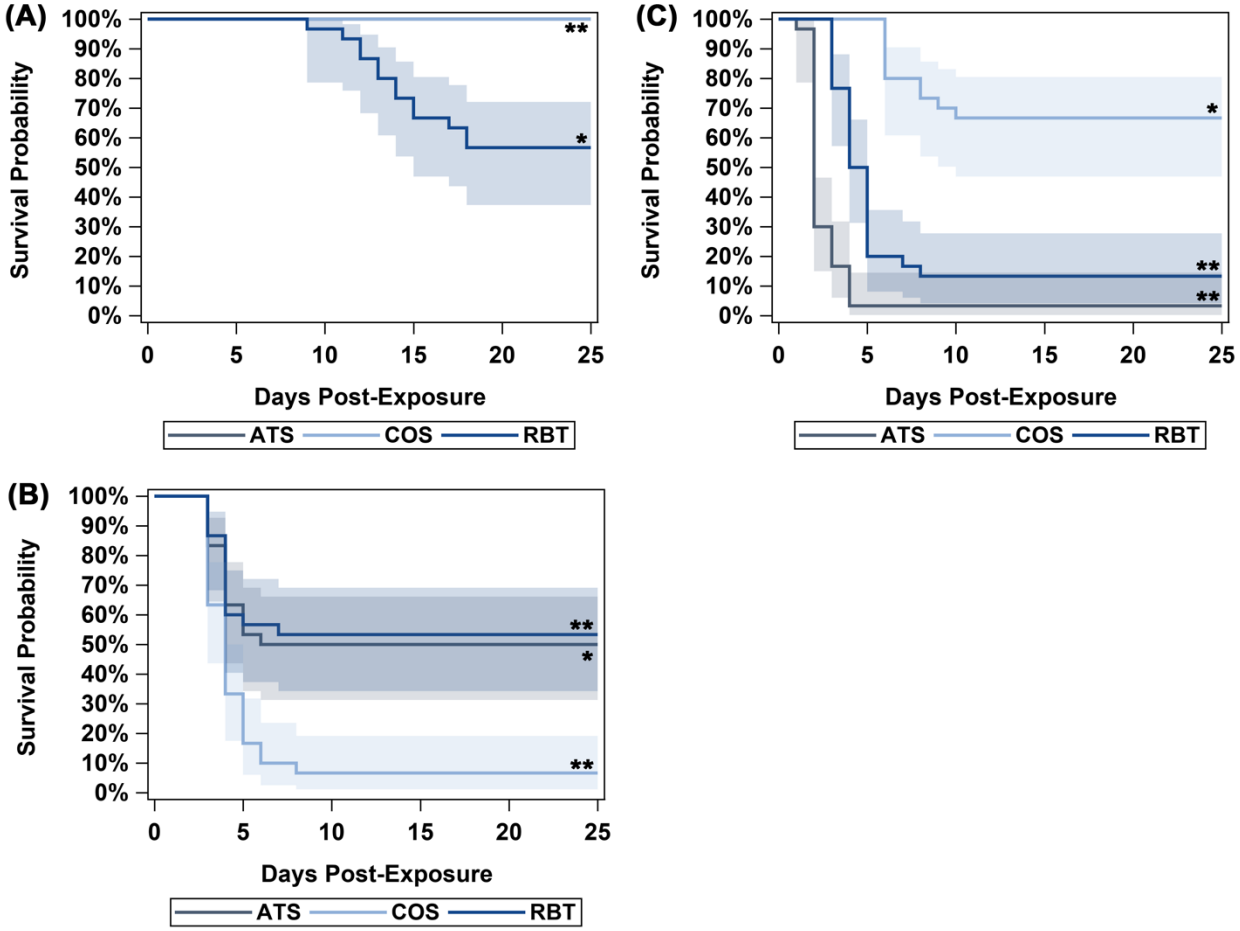


Figure 3.4. Gross external lesions in Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) following immersion challenge with *F. psychrophilum* isolate US19-COS. (A) Atlantic salmon with shallow focally extensive dermal ulceration of the caudal peduncle. (B) Coho salmon with focally extensive dermal ulceration of the caudal peduncle that penetrated the underlying musculature. (C) Rainbow trout with focally extensive dermal ulceration of the caudal peduncle that penetrated the underlying musculature. Diffuse ecchymoses and petechiae surround the ulcer. (D) Atlantic salmon with focally extensive dermal ulceration of the caudal peduncle, exposing the vertebral column. (E) and (F) Peri-oral ulceration in coho salmon (E) and rainbow trout (F).

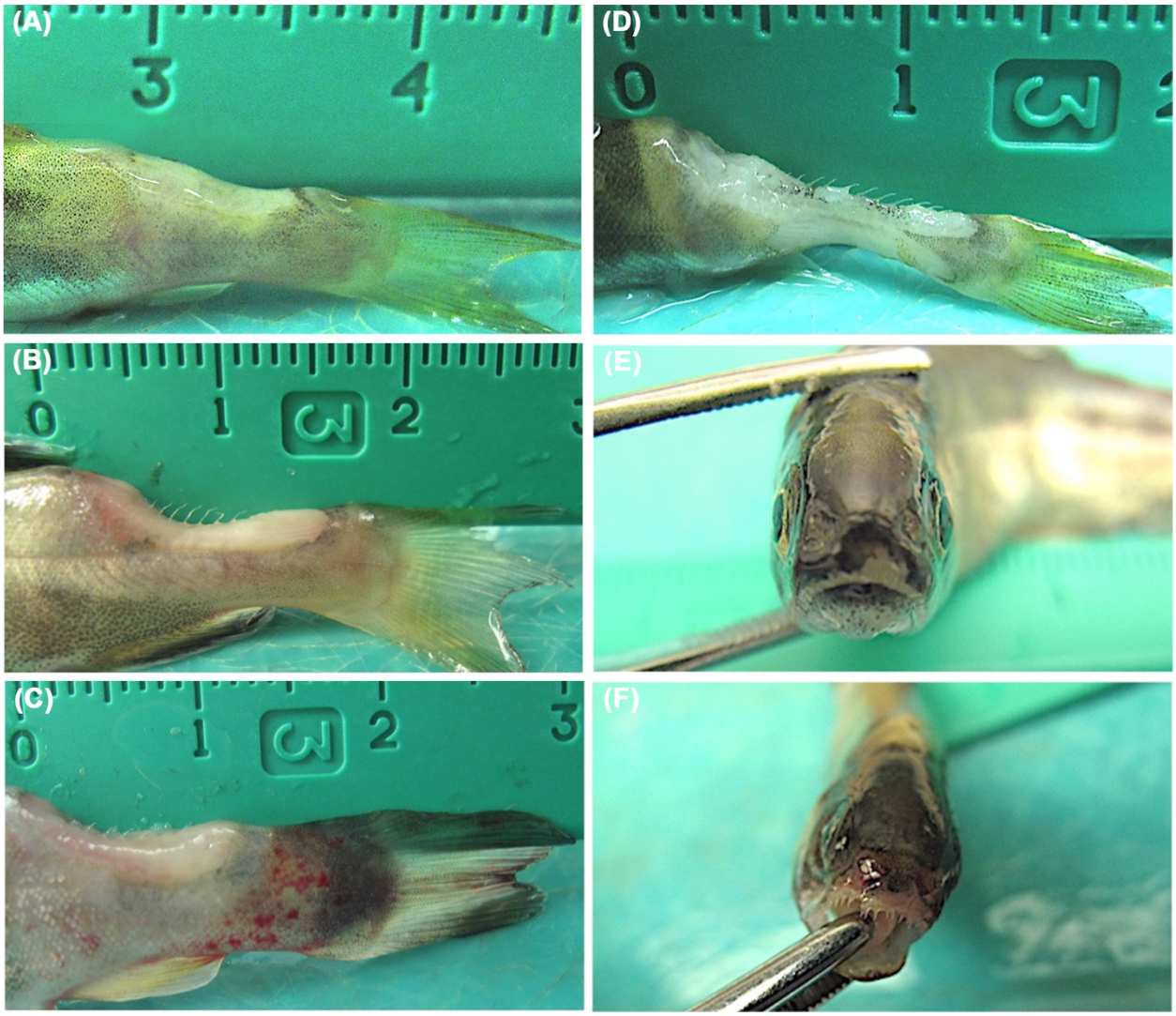


Figure 3.5. Gross internal lesions in Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) following immersion challenge with *F. psychrophilum* isolate US19-COS. (A) Atlantic salmon with liver pallor. (B) Coho salmon with pale liver and multifocal ecchymoses, splenic swelling, and intestinal hemorrhage. (C) Rainbow trout with liver pallor and splenic swelling. (D) Coho salmon with renal pallor. (E) Rainbow trout with renal pallor and diffuse ecchymoses. (F) Rainbow trout with intestinal hemorrhage.

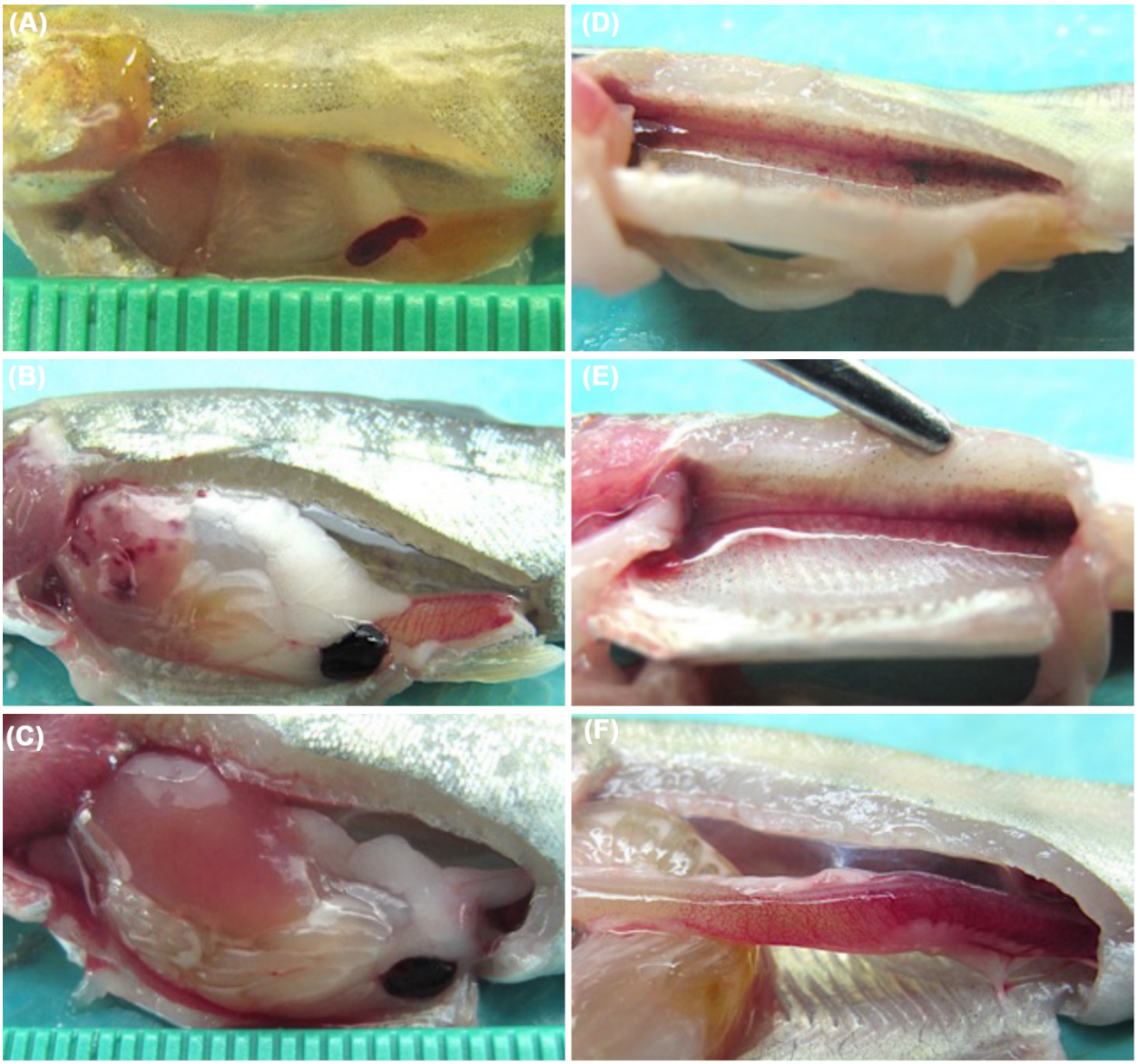
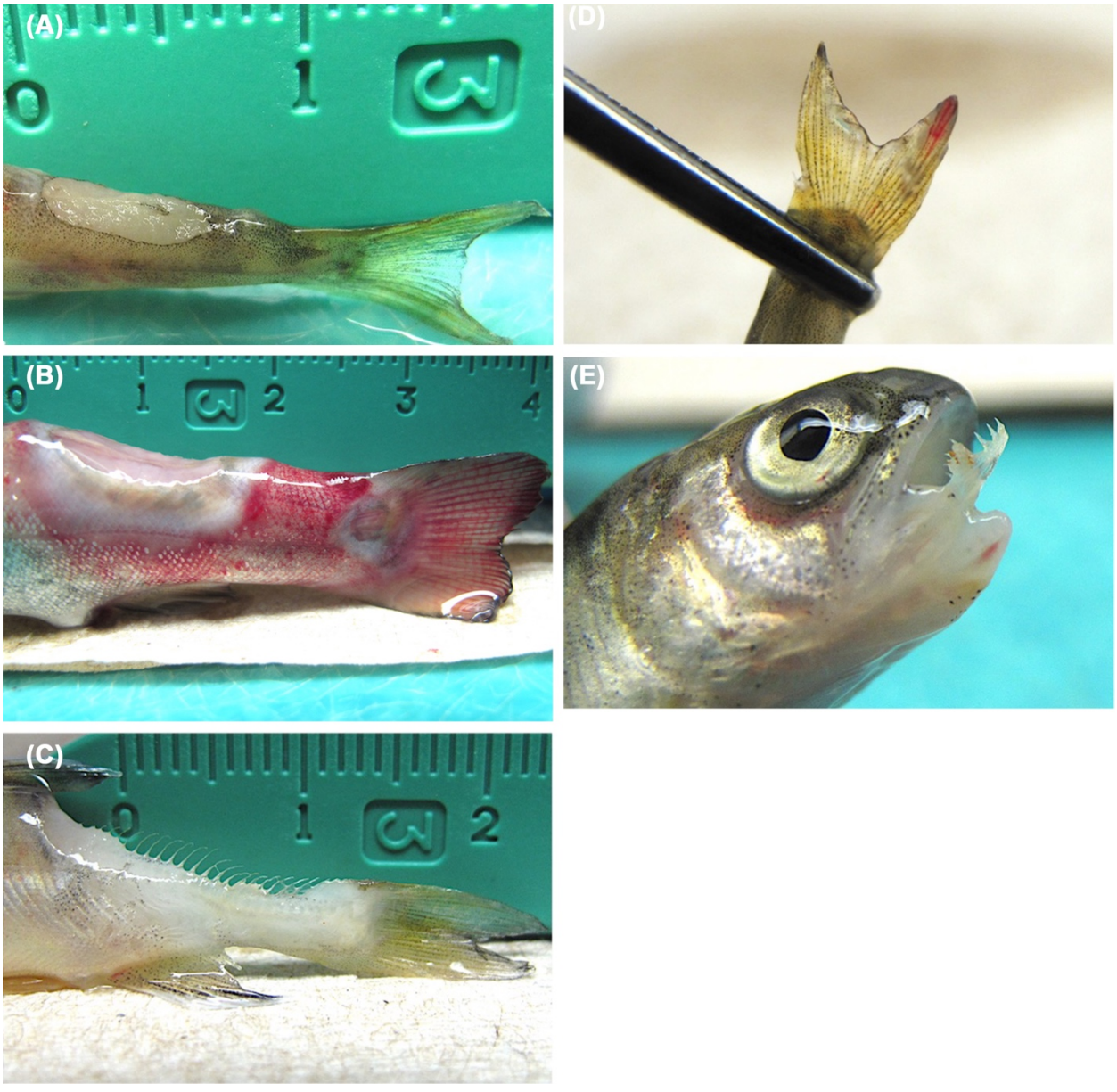


Figure 3.6. Gross external lesions in Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) following immersion challenge with *F. psychrophilum* isolate US62-ATS. (A) Atlantic salmon with focally extensive dermal ulceration of the caudal peduncle that penetrated the underlying musculature. (B) Rainbow trout with multiple, focally extensive dermal ulcerations of the caudal peduncle that penetrated the underlying musculature. Ulceration is surrounded by severe diffuse ecchymoses that extends posteriorly into the caudal fin. (C) Coho salmon with focally extensive ulceration of the caudal peduncle that penetrated the underlying musculature. (D) Atlantic salmon with focal ecchymosis of the caudal fin. (E) Coho salmon with severe peri-oral ulceration.



Chapter 4:

Varying *Flavobacterium psychrophilum* shedding dynamics in three bacterial coldwater disease-susceptible salmonid (Family *Salmonidae*) species

4.1. Abstract

Flavobacterium psychrophilum causes bacterial coldwater disease (BCWD) and is responsible for substantial losses in farm and hatchery-reared salmonids (Family *Salmonidae*). Although *F. psychrophilum* infects multiple economically important salmonid species and is efficiently transmitted horizontally, the extent of knowledge regarding *F. psychrophilum* shedding rates and duration is limited to rainbow trout (*Oncorhynchus mykiss*). Concurrently, hundreds of *F. psychrophilum* sequence types (STs; i.e., genetic variants) have been described using multilocus sequence typing (MLST) and evidence suggests that some variants have distinct phenotypes, including differences in host associations. Whether shedding strategies differ amongst *F. psychrophilum* variants and/or salmonid hosts remains unknown. To this end, three *F. psychrophilum* isolates (e.g., US19, US62, and US87) in three MLST STs (e.g., ST13, ST277, and ST275) with apparent host associations for either coho salmon (*O. kisutch*), Atlantic salmon (*Salmo salar*), or rainbow trout, were intramuscularly injected into each respective fish species, thereby ensuring a consistent initial dosage of bacteria. Shedding rates of live and dead fish were determined at regular intervals by quantifying *F. psychrophilum* loads in water via quantitative PCR. Both live and dead Atlantic and coho salmon shed *F. psychrophilum*, as did live and dead rainbow trout. Regardless of salmonid species, dead fish shed *F. psychrophilum* at higher rates (e.g., up to ~10^8 – 10^{10} cells/fish/hour) compared to live fish (up to ~10^7 - 10^9 cells/fish/hour), and for a longer duration (5 – 35 days vs. 98 days); however, shedding dynamics varied by *F. psychrophilum* variant and/or host species, a matter that may complicate BCWD management. Findings herein expand knowledge on *F. psychrophilum* shedding dynamics across multiple salmonid species and can be used to inform future BCWD management strategies.

4.2. Introduction

Flavobacterium psychrophilum, causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), causes substantial losses in farmed and hatchery-reared salmonid species (Family *Salmonidae*; Loch and Faisal, 2017). Many salmonid species are BCWD-susceptible (Starliper, 2011), and although rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*O. kisutch*) are generally considered most at risk (Holt, 1987), BCWD in Atlantic salmon (*Salmo salar*) is also common (Nilsen et al. 2011; Avendaño-Herrera et al. 2020; Macchia et al. 2022). Trout and salmon naturally infected by *F. psychrophilum* in their early life stages sustain substantial losses, with mortality rates ranging from 20 - 90% (Wood et al. 1974; Barnes and Brown, 2011). Although vertical transmission of *F. psychrophilum* plays an important role in the perpetuation of BCWD (Rangdale et al. 1996; Brown et al. 1997, Ekman 1999; Kumagai et al. 2001), horizontal transmission is also problematic (Starliper, 2011), facilitating the spread of *F. psychrophilum* within a population and exacerbating disease outbreaks.

Shedding of *F. psychrophilum* from infected fish into the water column is a primary factor driving horizontal transmission (Madetoja et al. 2000; Madetoja et al. 2002). Madetoja et al. (2000) showed that live rainbow trout shed up to ~10^6 cells/fish/hour for up to 21 days. Notably, the same authors also showed that dead rainbow trout shed *F. psychrophilum* at even higher rates (e.g., up to ~10^8 cells/fish/hour), and for a longer duration (e.g., up to 59 days longer; Madetoja et al. 2000). Although these studies provided invaluable data related to *F. psychrophilum* shedding dynamics (e.g., time to shedding, shedding rate and duration) in rainbow trout, they represent the totality of knowledge on this subject. Thus, it remains to be

determined if *F. psychrophilum* shedding dynamics in other BCWD-susceptible host species, such as Atlantic salmon and coho salmon, are similar.

In a similar context, whether *F. psychrophilum* shedding dynamics vary according to the *F. psychrophilum* multilocus sequence typing (MLST) variant causing the epizootic has yet to be determined. Multilocus sequencing typing of >1500 *F. psychrophilum* isolates has revealed >260 different sequence types (STs) worldwide (https://pubmlst.org/organisms/flavobacterium-psychrophilum), some of which differ in host species association (Nicolas et al. 2008; Knupp et al. 2019; Knupp et al. 2021a), virulence (Sundell et al. 2019; Knupp et al. 2021b), and prevalence in either hatchery-reared or wild/feral fish populations (Van Vliet et al. 2016; Knupp et al. 2019). Notably, most *F. psychrophilum* STs in North America differ from those that have been reported from other continents (e.g., Asia, Europe, South America; Nicolas et al. 2008; Siekoula-Nguedia et al. 2012; Fujiwara-Nagata et al. 2013; Strepparava et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019; Li et al. 2021), suggesting that the *F. psychrophilum* variant evaluated by Madetoja et al. (2000) is likely distinct from many causing losses in the USA.

The detection and quantification of *F. psychrophilum* from water containing fish has been attempted via culture and immunofluorescence antibody technique (Madetoja et al. 2000; Madetoja and Wiklund, 2002; Madetoja et al. 2003), both of which are sensitive (e.g., detection limit of ~10^1 - 10^2 cfus/mL) but time consuming and vary in specificity. An alternative that is highly sensitive, specific, and well-suited for *F. psychrophilum* quantification in water is quantitative PCR (qPCR). Strepparava et al. (2014) developed an *F. psychrophilum* qPCR for this purpose, and although sensitive (detection limit of ~10^1 gene copies) and specific, its quantification limit was reportedly high (e.g., ~10^3 *F. psychrophilum* cells/mL). A more sensitive

F. psychrophilum-specific qPCR exists (detection limit of ~10^0 gene copies; Marancik and Wiens, 2013), but in the published literature, this assay has not been used to quantify *F. psychrophilum* loads in water.

Towards improving our understanding of *F. psychrophilum* shedding dynamics in rainbow trout and, for the first time, Atlantic salmon and coho salmon, a series of experiments were devised to first optimize a previously developed *F. psychrophilum*-specific qPCR (Marancik and Wiens, 2013) for detection and quantification in water. Next, in vivo experiments were designed to elucidate *F. psychrophilum* shedding rates and durations in live and dead Atlantic salmon, coho salmon, and rainbow trout. Clarifying these aspects of BCWD ecology will offer insights into improved BCWD management strategies for multiple valuable salmonid species.

4.3. Materials and Methods

4.3.1. Marancik and Wiens (2013) qPCR

4.3.1.1. Reaction mixture and cycling parameters

The *F. psychrophilum*-specific qPCR developed by Marancik and Wiens (2013) was performed according to protocol with some modification. Briefly, each 15-µL reaction mixture contained 7.5-µL of TaqMan® Environmental Master Mix 2.0, 0.67 µM of forward and reverse primers, 0.17 µM of TaqMan® probe, 0.60-µL of VetMAX™ Xeno™ Internal Positive Control (IPC) Assay, and 1 µL of template, with nuclease-free water comprising the remainder. Reactions were run in MicroAmp™ Optical 96-Well Fast Reaction Plates (0.1-mL) covered with MicroAmp™ Optical Adhesive Film. A QuantStudio™ 3 real-time thermal cycler (ThermoFisher Scientific) was used to amplify the 77-bp target amplicon according to the cycling program of Marancik and Wiens (2013). All consumables were purchased through

ThermoFisher Scientific except the primers, which were obtained from Integrated DNA Technologies (IDT).

4.3.1.2. Preparation of standards

The qPCR target gene sequence was PCR amplified using previously extracted gDNA from *F. psychrophilum* isolate US53 (Van Vliet et al. 2016) and the same primers used for qPCR. Briefly, a 50-µL reaction mixture was prepared with 25-µL 2X GoTaq® Green Master Mix (Promega), 0.25 µM forward and reverse primers, 40 ng of US53 gDNA, with nuclease-free water comprising the remainder. A touchdown protocol consisting of initial denaturation for 2 min at 94 °C followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min was performed using an Eppendorf™ Mastercycler™ pro conventional thermal cycler (ThermoFisher Scientific). The PCR product was run through a 1.5% agarose gel prepared with 1X SYBR™ Safe DNA gel stain (ThermoFisher Scientific) for 40 min at 100 V, after which the gel was viewed under UV transillumination to confirm the presence of an appropriately sized band. The PCR product (57.1% guanine-cytosine content; molecular weight of 37,497.9 g mol^{-1}; Marancik and Wiens, 2013) was purified using the QIAquick® PCR Purification Kit (Qiagen) and then quantified using a Qubit™ fluorometer and the broad range Quant-iT™ dsDNA Assay Kit (Thermofisher Scientific). Gene copy concentration was calculated using a previously published formula (Standish et al. 2018):

$$\text{Gene copies}/\mu\text{L} = \frac{(\text{ngDNA } \mu\text{L}^{-1}) \times (6.022 \times 10^{23} \text{ copies mol}^{-1})}{(37497.9 \text{ g mol}^{-1}) \times (1 \times 10^{9} \text{ ng g}^{-1})}$$

Serial 10-fold dilutions of purified amplicon were made over nine orders of magnitude (e.g., 10^8 copies/µL – 10^0 copies/µL) using 1X IDTE solution (pH 8.0; Integrated DNA Technologies) supplemented with 100 ng/µL tRNA (Yeast tRNA; Thermofisher Scientific). Two standard curve assays were performed, each on individual 96-well plates using eight replicate reactions of

each dilution (i.e., qPCR standard); intra- and inter-assay variation was determined using mean quantification cycle (Cq), Cq standard deviation, and coefficient of variation (CV). Assay efficiency, slope, and the correlation coefficient (R^2) of each assay were also calculated; efficiency estimates between 90% and 110% were considered acceptable (Griffin et al. 2009; Griffin et al. 2011). These newly generated standards were used to determine *F. psychrophilum* DNA extraction efficiency, the limit of *F. psychrophilum* quantification and detection from spiked water (section 4.3.2.3), and to quantify *F. psychrophilum* loads in 50-mL water samples obtained during the in vivo shedding experiments (section 4.3.3.6).

4.3.2. Optimization of water sampling method and qPCR for *Flavobacterium psychrophilum* quantification from water

4.3.2.1. Preparation of mock water samples containing *Flavobacterium psychrophilum*

Flavobacterium psychrophilum isolate US53 was revived from cryostock (maintained at -80 ºC) on *F. psychrophilum* medium A (FPM-A; Chapter 2), incubated for 48 hours at 15 ºC, visually inspected for purity, inoculated into 250-mL of analogous broth, and incubated with constant shaking (180 rpm) for 48 hours at 15 ºC. Bacteria were harvested via centrifugation (2,571 × *g*, 15 minutes) and resuspended in 50-mL of ultraviolet light-treated, sand-filtered well water (i.e., the same water supplying the shedding experimental aquaria in section 4.3.3), which was then serially diluted up to 100,000,000-fold in ten-fold increments to create nine total mock water samples. To quantify bacteria in the most concentrated mock water sample, a 1-mL aliquot was serially diluted 100,000,000-fold in ten-fold increments and plated on FPM-A in duplicate, and then incubated for seven days, after which final colony counts were performed. All mock water samples were brought to a final volume of 50 mL to replicate the water sampling volume used during the shedding experiment.

4.3.2.2. Bacterial DNA extraction from water

Each mock water sample (n = 9; section 4.3.2.1) was vacuum filtered through a single sterile piece of Whatman® qualitative filter paper (grade 4, 70 mm in diameter; Millipore Sigma) that had been placed in a 70-mm diameter Büchner funnel. The filter paper was removed from the Büchner funnel and placed into a sterile Petri dish; sterile forceps were used to first fold the paper in half (the side receiving the bacterial suspension was facing inward) and then loosely roll it into a cylindrical shape. The rolled paper was placed inside a PowerBead Pro tube of the DNeasy® PowerSoil® Pro Kit (Qiagen) along with 20,000 copies of Xeno™ IPC to monitor inhibition; DNA was then extracted according to the manufacturers' protocol, resulting in 50-µL of eluted DNA per dilution.

4.3.2.3. DNA extraction efficiency and limit of quantification and detection

The qPCR standards (section 4.3.1.2) were used to simultaneously determine (i.e., on one 96-well plate) *F. psychrophilum* DNA extraction efficiency and the limit of detection (LOD) and quantification (LOQ). DNA extraction efficiency was measured as it is highly variable (e.g., 0.2% – 108.9%; Lebuhn et al. 2004; Slana et al. 2008; Stoeckel et al. 2009; Kralik et al. 2011; Van Tongeren et al. 2011; Ricchi et al. 2016), thereby potentially affecting estimation of the target microorganism in a sample (e.g., *F. psychrophilum* in water) and therefore the LOD/LOQ (Kralik et al. 2011).

Flavobacterium psychrophilum DNA extraction efficiency was calculated as the quotient of the mean qPCR-determined concentration of *F. psychrophilum* (in cells/mL) and the mean theoretical input (i.e., pre-DNA extraction) concentration of *F. psychrophilum* (Kralik and Ricchi et al. 2017). The input concentration of *F. psychrophilum* was considered theoretical, given that the most concentrated mock water sample alone was quantified via plate counts (section 4.3.2.1).

The median of the mean *F. psychrophilum* DNA extraction efficiency values was used as the universal DNA extraction efficiency (Kralik et al. 2011; Kralik and Ricchi et al. 2017); this number was used to apply a DNA extraction correction factor (DECF) to all qPCR-derived *F. psychrophilum* concentrations according to the formula:

$$\text{DECF} = \frac{100}{\text{Median of mean DNA extraction efficiency}}$$

The LOD was determined by running 10 replicate reactions theoretically containing 100, 10, and 1 *F. psychrophilum* cell(s)/mL and defined as the lowest mean qPCR-derived concentration of *F. psychrophilum* that could be detected in ≥95% of qPCR replicate reactions; this concentration was multiplied by the DECF to yield the LOD (Kralik and Ricchi et al. 2017).

The LOQ was determined by running triplicate reactions of eight qPCR standards (10^8 – 10^1 gene copies/reaction), triplicate reactions of six *F. psychrophilum* dilutions (1.00×10^8 cells/mL – 1.00×10^3 cells/mL), and 10 replicate reactions of each remaining dilution (e.g., 1×10^2 cells/mL – 1 cell/mL) and defined as the lowest mean qPCR-derived concentration of *F. psychrophilum* with a CV <25%; this concentration was multiplied by the DECF to yield the LOQ (Kralik and Ricchi et al. 2017).

4.3.3. In vivo assessment of shedding dynamics in Atlantic salmon, coho salmon, and rainbow trout

4.3.3.1. *Flavobacterium psychrophilum* isolate selection

Three *F. psychrophilum* variants, including US19 (ST13, CC-ST9; Van Vliet et al. 2016), US62 (ST277, CC-ST232; Knupp et al. 2019), and US87 (ST275, CC-ST10; Knupp et al. 2019), were selected for this study. Each CC is present across a wide geographic range, whereby CC-ST232 has been detected in two continents (Europe and North America; Nilsen et al. 2014; Knupp et al. 2019), and CC-ST9 and CC-ST10 have both been detected in four continents (e.g.,

Asia, Europe, North America, and South America; Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Knupp et al. 2019; Li et al. 2021). Moreover, each CC has been recovered almost exclusively from one salmonid species, including Atlantic salmon (CC-ST232), coho salmon (CC-ST9), and rainbow trout (CC-ST10).

4.3.3.2. Origin of fish for shedding experiment

Embryonated Atlantic salmon and rainbow trout eggs were sourced from a commercial egg distributor, while embryonated coho salmon eggs were procured from Platte River State Fish Hatchery. Coordination occurred so that all eggs from the three species arrived at the Michigan State University – University Research Containment Facility on the same day. In brief and upon receipt, eggs were disinfected with 100-ppm iodophor solution (pH 7.30) for 10 minutes before being placed in a vertical incubator supplied with UV-treated, sand-filtered well water maintained at 12 ºC ± 1 ºC until hatching. Sac-fry were then moved to aerated flow-through tanks (40 L; 12 ºC ± 1 ºC) and, once exogenous feeding commenced, were given a continuous supply of appropriately sized commercial trout food (Skretting, the Netherlands) via an automatic feeder. After eight weeks, fish were hand-fed twice daily and the water volume in the tanks was increased (400 L; 12 ºC ± 1 ºC). Tanks were cleaned and siphoned daily to remove waste and any uneaten food. Before the challenge experiment, a subset of fish from each species were cultured to screen for bacterial infections (Knupp et al. 2021a), including those caused by *F. psychrophilum*, and confirmed to be bacterial infection-free.

4.3.3.3. *Flavobacterium psychrophilum* inoculum preparation for shedding experiment

Flavobacterium psychrophilum variants US19, US62, and US87 were revived from cryostock and verified to be pure cultures as detailed in section 4.3.2.1. Each *F. psychrophilum* variant was inoculated into 250-mL of FPM-A broth and incubated with constant shaking (180

rpm) for 48 hours at 15 ºC. Bacteria were harvested via centrifugation (2,571 × *g*, 15 minutes) and adjusted to an optical density at 600-nm (OD_{600}) corresponding to 2.0×10^8 colony forming units (cfu)/mL using sterile 0.65% saline. Concentrations of each *F. psychrophilum* variant were determined as detailed in section 4.3.2.1.

4.3.3.4. Intramuscular challenge of fish

Each *F. psychrophilum* variant was inoculated into the salmonid species it is associated with, according to MLST. Thus, US19 was inoculated into coho salmon, whereas US62 and US87 were inoculated into Atlantic salmon and rainbow trout, respectively.

Atlantic salmon (*n* = 5, in duplicate; 8 months old; mean weight 18.1g), coho salmon (*n* = 5, in duplicate; 8 months old; mean weight 20.5g), and rainbow trout (*n* = 5, in duplicate; 8 months old; mean weight 25.1g) were anesthetized in sodium bicarbonate-buffered (200 mg/L) tricaine methanesulfonate (MS-222; Syndel) at a concentration of 100 mg/L. Then, fish were intramuscularly injected at the base of the dorsal fin with a 50-µL volume of *F. psychrophilum*, equating to a dose of 10^5 cfu/g, and then placed into aerated flow-through glass experimental aquaria (37.85 L; *n* = 2 aquaria per species, *n* = 5 fish per aquarium) supplied with ultraviolet light-treated, sand-filtered well water (12 ºC ± 1 ºC). Control fish (*n* = 5 per species, in duplicate aquaria) were treated identically except they were intramuscularly injected with an equal volume of 0.65% saline. The challenge experiment was conducted in accordance with the MSU-Institutional Animal Care and Use Committee (AUF:201900312).

4.3.3.5. Sampling of water containing live and dead fish

All live fish in a replicate experimental aquarium (i.e., main aquarium) were net transferred to a clean non-flow-through glass aquarium (i.e., shedding aquarium; 9.46 L) containing 3000-mL of fresh, ultraviolet light-treated, sand-filtered well water (12 ºC ± 1 ºC)

with constant aeration. The shedding aquarium was placed inside a larger, opaque, plastic aquarium that had flow-through water to maintain a water temperature of 12 ºC ± 1 ºC in the shedding aquarium; the plastic aquarium also had an opaque cover to minimize light-induced stress. After one hour, all fish were removed from the shedding aquarium and transferred back to the main aquarium. A 50-mL water sample was collected using a sterile 50-mL conical tube and then processed immediately as detailed in section 4.3.2.2. Water sampling of live fish (including negative control fish) occurred on every other day for the first week, twice per week during the second and third weeks, and then once a week until the end of the experiment (i.e., four weeks without detection of *F. psychrophilum*).

Up to two dead fish per replicate aquarium (i.e., ≤ 4 dead fish per species) were net transferred into a new flow-through glass aquarium (37.85 L, n = 1 dead fish per aquarium) supplied with ultraviolet light-treated, sand-filtered well water (12 ºC ± 1 ºC). After 1, 3, 5, 7, 14, 63, and 98-days post-death, fish were transferred to individual shedding aquaria and treated/sampled identically to the live fish. Fish that died that were not used for determining *F. psychrophilum* shedding rates were necropsied, clinically examined, and multiple tissues (e.g., external ulcers and kidney) were bacteriologically analyzed for *F. psychrophilum* on FPM-A. To disinfect shedding aquaria between samplings, aquaria were completely immersed in a 10% (v/v) bleach solution for ≥ 10 min, rinsed thoroughly with pathogen-free water, and then air dried.

4.3.3.6. Determination of *Flavobacterium psychrophilum* shedding rate from live and dead fish via qPCR

Flavobacterium psychrophilum shedding rates were determined via qPCR (section 4.3.1.1). Briefly, each 96-well qPCR plate consisted of triplicate reactions of eight qPCR standards (10^8 gene copies – 10^1 gene copies), duplicate reactions of template DNA, triplicate

reactions of no-template control (e.g., sterile, nuclease free water), and triplicate reactions of IPC amplification control (e.g., 1,000 copies of Xeno™ IPC). The shedding rate of *F. psychrophilum* from infected fish is reported as *F. psychrophilum* cells shed per fish per hour in 1-mL of water (i.e., *F. psychrophilum* cells/fish/hour; Madetoja et al. 2000) and was calculated using the following formula:

$$F.psychrophilum \text{ cells/fish/hour} = \frac{\text{Mean qPCR gene copies} \times \text{DECF} \times 3000 \text{ mL}}{\text{Number of fish in aquarium}}$$

4.4. Results

4.4.1. qPCR standards

All qPCR standards, which spanned nine orders of magnitude (10^8 gene copies/reaction – 1 gene copy/reaction), successfully amplified. Linear regression of C_q values versus the $\log_{10}$ target gene demonstrated acceptable correlation ($R^2 = 0.999 \pm 0.001$) with a slope and efficiency of -3.23 ± 0.01 and 104.01% ± 0.53%, respectively. The assay was repeatable within and between runs, as CV values were <2.5% within the linear range (10^8 gene copies/reaction – 1 gene copy/reaction) of the standard curve (Table 4.1). Consequently, and in accordance with Standish et al. (2018), these standards were used in all subsequent qPCR assays.

4.4.2. *Flavobacterium psychrophilum* DNA extraction efficiency and qPCR limit of detection and quantification

Mean *F. psychrophilum* concentrations of the nine mock water samples ranged from 1.00×10^8 to 1.00×10^0 cell(s)/mL and were compared to the mean qPCR-determined concentrations (Table 4.2). Mean DNA extraction efficiency ranged from 3.63% - 99.70%, with a median of 14.03% (Table 4.2); therefore, the DECF applied to all qPCR-derived concentrations was 7.128 (=100/14.03; Kralik et al. 2011).

The assay LOD and LOQ were calculated as 30.94 ± 6.84 cells/mL, whereby the DECF (7.128) was multiplied by the lowest mean qPCR-derived *F. psychrophilum* concentration meeting the qualifications for the LOD and LOQ (e.g., both 4.34 ± 0.96 cells/mL; Table 4.2). Additionally, amplification was achieved in 40% of the replicates at the next lowest concentration, which corresponded to 13.68 ± 7.09 cells/mL after correction for DNA extraction efficiency (=1.92 ± 0.99 cells/mL × 7.128).

The IPC DNA, which was added at the beginning of the DNA extraction process, successfully amplified in all replicate reactions of all but the most concentrated mock water sample (Table 4.2), which according to the VetMAX™ Xeno™ user guide was most likely caused by high target concentration. Nonetheless, of the eight mock water samples that had successful IPC amplification, C_q values ranged from 30.12 to 35.29 and all replicates were highly consistent (e.g., CV values ranged from 0.36% to 1.17%; Table 4.2).

4.4.3. *Flavobacterium psychrophilum* shedding dynamics in three salmonid species

4.4.3.1. Shedding rates of *Flavobacterium psychrophilum* from live fish

Following intramuscular injection of 10^5 cfu/g of *F. psychrophilum*, all tested species (e.g., Atlantic salmon, coho salmon, and rainbow trout) shed *F. psychrophilum* into the water over multiple days and/or prior to the occurrence of any mortality (Figure 4.1A-C).

Atlantic salmon shed 1.59×10^4 cells/fish/hour one day after inoculation; this shedding rate increased to 4.34×10^7 cells/fish/hour (i.e., >2,700-fold increase) by day five, which was two days prior to the first mortality (Figure 4.1A). Atlantic salmon shed the most *F. psychrophilum* (e.g., 6.37×10^7 cells/fish/hour) on day seven (i.e., the day of the first mortality; Figure 4.1A), with shedding rates decreasing thereafter on each subsequent sampling day (e.g., from 1.43×10^6 cells/fish/hour on day 11 to 4.30×10^3 cells/fish/hour on day 21; Figure 4.1A).

Cumulative mortality in Atlantic salmon peaked at 20% on day 9. Surviving Atlantic salmon were euthanized on day 49 (i.e., four weeks past the last detection of *F. psychrophilum*; Figure 4.1A), at which time *F. psychrophilum* infection status was examined (section 4.4.4).

Live rainbow trout began shedding *F. psychrophilum* into the water at a rate of 1.65×10^4 cells/fish/hour one day after inoculation; this shedding rate increased to 4.16×10^8 cells/fish/hour (i.e., >25,000-fold increase) by day seven, which was two days prior to the first mortality (Figure 4.1B). Rainbow trout shedding rates then increased to 4.18×10^9 cells/fish/hour on day 11 (50% cumulative mortality), decreased to 4.32×10^8 cells/fish/hour on day 14 (60% cumulative mortality) before increasing to 4.23×10^9 cells/fish/hour on day 18 (60% cumulative mortality; Figure 4.1B). On day 19, cumulative mortality increased to 80% and remained constant through day 35; during this time, rainbow trout shedding rates decreased from 5.98×10^7 cells/fish/hour (day 21) to 2.86×10^4 cells/fish/hour (day 35), after which *F. psychrophilum* was not detected in the water (Figure 4.1B). Cumulative mortality in rainbow trout reached its peak (e.g., 90%) on day 42 and the experiment ended on day 63 (Figure 4.1B).

Live coho salmon began shedding *F. psychrophilum* into the water at a rate of 1.04×10^5 cells/fish/hour one day after inoculation. Mortality began quickly in this species (e.g., on day two) and rapidly reached 100% by day seven. The highest detected *F. psychrophilum* shedding rate of live coho salmon (e.g., 7.39×10^7 cells/fish/hour) occurred on day five (Figure 4.1C).

Control fish did not shed *F. psychrophilum* nor did they experience any mortality (data not shown). Likewise, inhibition of gene target amplification was not observed among water samples originating from the negative control or *F. psychrophilum*-exposed fish, as evidenced by all IPC C_q values falling within 28 – 34.

4.4.3.2. Shedding rates of *Flavobacterium psychrophilum* from dead fish

Atlantic salmon, coho salmon, and rainbow trout shed *F. psychrophilum* into the water from one day post-death (PD) to the end of the 98-day experiment (Figure 4.2A-C). Throughout the experiment, fish underwent post-mortem decomposition (Figure 4.3A-C). Because of extensive decomposition after day 98, sampling ceased.

Two Atlantic salmon died during the experiment, one from each replicate aquarium (Figure 4.2A). Initially (i.e., one day PD), *F. psychrophilum* shedding rates differed by ~15-fold, whereby one fish shed 2.20×10^7 cells/fish/hour and the other shed 3.45×10^8 cells/fish/hour (mean of $1.8 \pm 1.6 \times 10^8$ cells/fish/hour; Figure 4.2A; Table 4.3); however, shedding rates became more consistent between 3 - 14 days PD (e.g., differed by 1 - 3-fold). Over the next 12 weeks, the shedding rate of one Atlantic salmon decreased to 1.63×10^4 cells/fish/hour and then *F. psychrophilum* became undetectable, whereas the shedding rate of the other Atlantic salmon decreased but was still detectable (e.g., 4.51×10^6 cells/fish/hour; Figure 4.2A) 98 days PD.

Nine of the ten rainbow trout died during the experiment; therefore, individual *F. psychrophilum* shedding rates of two rainbow trout per replicate aquarium were measured (Figure 4.2B). Initially (i.e., one day PD), most (i.e., 3/4) rainbow trout were shedding ~10^8 cells/fish/hour (range of $4.91 – 8.20 \times 10^8$ cells/fish/hour), whereas one rainbow trout was shedding 8.73×10^7 cells/fish/hour (overall mean of $5.0 \pm 2.7 \times 10^8$ cells/fish/hour; Figure 4.2B; Table 4.3). By 5 days PD, all rainbow trout shed at substantially higher rates, whereby individual rainbow trout were shedding $0.28 - 3.59 \times 10^{10}$ cells/fish/hour (mean of $1.6 \pm 1.2 \times 10^{10}$ cells/fish/hour; Figure 4.2B; Table 4.3). Between 7 - 14 days PD, rainbow trout shedding rates decreased ~1 – 12,000-fold, and the shedding rate of one rainbow trout continued to decrease through the end of the experiment (final shedding rate of 1.36×10^8 cells/fish/hour; Figure 4.2B).

For the other three rainbow trout, shedding rates increased ~4 – 1,200-fold (e.g., to 0.68 – 3.5 × 10^9 cells/fish/hour) by 63 days PD. At the end of the 98-day experiment, these three rainbow trout were shedding 1.77 × 10^6 to 6.09 × 10^9 cells/fish/hour (mean of 1.6 ± 1.2 × 10^9 cells/fish/hour; Figure 4.2B; Table 4.3).

All ten coho salmon died during the experiment; therefore, individual *F. psychrophilum* shedding rates of two coho salmon per replicate aquarium were measured (Figure 4.2C). Initially (i.e., one day PD), most (i.e., 3/4) coho salmon were shedding ~10^8 cells/fish/hour (range of 2.71 – 9.26 × 10^8 cells/fish/hour), whereas one coho salmon was shedding 3.24 × 10^7 cells/fish/hour (overall mean of 4.8 ± 3.5 × 10^8 cells/fish/hour; Figure 4.2C; Table 4.3). By 3 days PD, shedding increased by ~1 – 100-fold for most (i.e., 3/4) coho salmon, whereby individuals were shedding between 0.38 - 8.45 × 10^9 cells/fish/hour (Figure 4.2C). In contrast, *F. psychrophilum* shedding rate decreased by ~100-fold (e.g., to 5.59 × 10^6 cells/fish/hour) for the other coho salmon. By 5 days PD shedding rates had increased for all coho salmon (range of 0.21 × 10^{10} cells/fish/hour; mean of 5.0 ± 3.8 × 10^9 cells/fish/hour; Figure 4.2C; Table 4.3) and continued to increase for 2/4 coho salmon through 7 days PD (range of 2.12 – 6.94 × 10^9 cells/fish/hour). In the other two coho salmon, a decrease in shedding rates was appreciated (range of 1.34 – 9.28 × 10^9 cells/fish/hour; Figure 4.2C). Over the next 13 weeks (i.e., through the end of the 98-day experiment), shedding rates generally decreased, and most (i.e., 3/4) coho salmon were still shedding *F. psychrophilum* (range of 4.95 × 10^5 to 1.28 × 10^7 cells/fish/hour; mean of 1.6 ± 2.6 × 10^9 cells/fish/hour; Figure 4.2C; Table 4.3).

4.4.4. Infection status in salmonids challenged with *Flavobacterium psychrophilum*

Flavobacterium psychrophilum was recovered in a pure form and as perfuse lawns (i.e., colony forming units were too numerous to count) from external lesions (e.g., muscle

ulcerations) and the kidneys of all dead Atlantic salmon, coho salmon, and rainbow trout. *Flavobacterium psychrophilum* was not recovered from the kidneys of any surviving Atlantic salmon (n = 8 fish; 49 days post-inoculation) or rainbow trout (n = 1 fish; 63 days post-inoculation). However, *F. psychrophilum* was recovered in a pure form and as a perfuse lawn from the eye of the only surviving rainbow trout. Because all coho salmon died after seven days, no survivors could be cultured.

4.5. Discussion

For the first time, *F. psychrophilum* shedding dynamics (e.g., time to shedding, shedding rate, and duration in live and dead fish) have been elucidated in Atlantic salmon and coho salmon, two of the most BCWD-susceptible salmonid species (Holt, 1987; Barnes and Brown, 2011; Nilsen et al. 2011). Addressing this knowledge gap was essential, as several studies showed that BCWD epizootics in Atlantic salmon and coho salmon are instigated by phenotypically distinct *F. psychrophilum* variants (Madetoja et al. 2001; Sundell and Wiklund, 2015; Sundell et al. 2019). Moreover, these *F. psychrophilum* variants differ from those affecting rainbow trout (Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Knupp et al. 2019), the sole species our entire understanding of *F. psychrophilum* shedding dynamics is based upon. Although some commonalities in shedding dynamics were observed among Atlantic salmon, coho salmon, and rainbow trout, multiple differences were also apparent.

Dead Atlantic salmon, rainbow trout, and coho salmon were found to shed *F. psychrophilum* cells at rates up to 5.4-, 8.5-, and 171.8-fold greater than their living counterparts, and did so for extended periods (e.g., up to 77, 63, and 93 days longer). This the first time these comparisons have been made among Atlantic salmon and coho salmon, demonstrating these species, in addition to rainbow trout, are efficient *F. psychrophilum* shedders. Madetoja et al.

(2000) also found dead rainbow trout shed at higher (e.g., up to ~100-fold) rates and for a longer duration (e.g., 59 days longer) than living rainbow trout. The difference in shedding rate among live and dead rainbow trout observed by Madetoja et al. (2000) is substantially greater than the 8.5-fold difference found herein for rainbow trout, while differences in shedding duration were similar (59 vs. 63 days; Madetoja et al. 2000). Taken together with the findings in Atlantic salmon and coho salmon, results suggest that some *F. psychrophilum* variants may be more efficiently shed by dead fish than others. Nevertheless, dead *F. psychrophilum*-infected fish clearly pose a significant risk for disease perpetuation within fish farms and hatcheries, underscoring the importance of implementing management strategies that aim to remove dead fish from rearing units quickly. Like *F. psychrophilum*, higher shedding rates in dead salmonids versus live salmonids have been noted in at least one other fish pathogenic *Flavobacterium* species (e.g., *F. columnare*, a cause of columnaris disease; Kunttu et al. 2009; LaFrentz et al. 2022), as well as *Aeromonas salmonicida* subspecies *salmonicida* (e.g., the cause of furunculosis; Rose et al. 1989), and therefore dead fish shedding may be an important transmission strategy among some bacterial fish pathogens.

Despite these similarities in shedding, multiple shedding differences with likely implications for *F. psychrophilum* transmission and BCWD management were also observed. For example, on day 11, live rainbow trout were shedding 2,923-fold more *F. psychrophilum* cells/hour compared to live Atlantic salmon. This difference continued to increase through day 21, which was the last day of *F. psychrophilum* detection among Atlantic salmon. Meanwhile (i.e., on day 21), *F. psychrophilum* shedding rates remained high among rainbow trout (e.g., 5.98×10^7 cells/fish/hour) and this species continued to shed for another two weeks. Adding a further layer of complexity, *F. psychrophilum* shedding rates among live Atlantic salmon and rainbow

trout tended to peak on and/or near days with mortality, and could possibly suggest that risk of transmission may vary by *F. psychrophilum* variant. For example, Atlantic salmon infected with US62 (ST277, in CC-ST232) died over three days, whereas rainbow trout infected with US87 (ST275) mostly died over 11 days (i.e., a >3-fold longer period). Madetoja et al. (2000) infected rainbow trout with a different *F. psychrophilum* variant and observed that fish died over a four-day period. Therefore, it appears some *F. psychrophilum* variants pose a greater transmission risk among live fish compared to others.

Differences in *F. psychrophilum* infection status were also apparent among rainbow trout and Atlantic salmon survivors, as the lone surviving rainbow trout was still infected with *F. psychrophilum* 63 days post-inoculation, in contrast to the surviving Atlantic salmon. Indeed, a common finding among previous in vivo *F. psychrophilum* challenge (Madetoja et al. 2000; Knupp et al. 2021a; Macchia et al. 2022) and surveillance studies (Madsen et al. 2005; Marancik and Wiens, 2013; Van Vliet et al. 2016; Knupp et al. 2019) is the existence of *F. psychrophilum* carriers. Where coho salmon fall in this regard is less clear, as US19 proved highly virulent (i.e., caused rapid and fulminant mortality) to exposed fish in the current study, despite being inoculated with an identical dose. Nevertheless, our findings suggest rainbow trout may be a longer-term infection reservoir and pose greater *F. psychrophilum* transmission risk compared to at least Atlantic salmon. With these findings in mind, the results of Chapter 3 add an additional layer of complexity to our understanding of *F. psychrophilum* transmission dynamics. The coho salmon-associated isolate US19 and Atlantic salmon-associated isolate US62 proved capable of infecting and causing disease and mortality in Atlantic salmon, rainbow trout, and coho salmon, albeit to varying degrees. Within the context of a facility rearing multiple salmonid species, generalist *F. psychrophilum* variants may pose a higher risk of cross-species transmission. Thus,

such facilities may need to implement spatial and/or physical barriers to limit cross-species transmission. In this context, future studies comparing transmission capabilities of host specific and generalist *F. psychrophilum* variants are warranted.

The higher *F. psychrophilum* shedding rates in live Atlantic salmon and rainbow trout coupled with delayed death [e.g., onset at 7 (Atlantic salmon) or 9 (rainbow trout) days] could affect *F. psychrophilum* transmission dynamics and increase the number of subsequent infections (i.e., the basic reproduction number, R_0; Delamater et al. 2019) as higher shedding rates over a longer period may provide *F. psychrophilum* with more opportunities to infect new hosts. On the other hand, coho salmon exhibited a different shedding patten – dying rapidly (e.g., ≤ 7 days) and potentially before peak shedding, which could potentially limit *F. psychrophilum* transmission if death occurred prior to maximum pathogen release. However, the initial shedding rate in coho salmon was ~10-fold higher than that of Atlantic salmon and rainbow trout, possibly compensating for the shorter shedding period. In the context of a fish farm or hatchery rearing unit, this could potentially enhance transmission despite the quick death. Moreover, if high early shedding followed by rapid death is a strategy for *F. psychrophilum*, it could be an effective way to maximize transmission while limiting the time for host immune response. Ogut et al. (2005) found *Aeromonas salmonicida* subsp. *salmonicida* transmission was host density dependent, whereby transmission increased with fish density. However, Ogut et al. (2005) also found R_0 was similar (e.g., 1.17 – 1.45), even over a wide range of densities (e.g., 0.36 – 9.13 fish/L), possibly indicating fish behavior changed with density. Whether these findings are emulated by *F. psychrophilum* remains to be determined but warrants investigation as they may have implications for *F. psychrophilum* transmission.

The observed differences in shedding dynamics across *F. psychrophilum* variants is likely influenced by pathogen and/or host factors. In this study, US87, identified in Chapter 3 as molecular serotype 2 (nearly equivalent to conventional serotype Th; Lorenzen and Olesen, 1997; Rochat et al. 2017), showed a protracted shedding period in rainbow trout. This variant was also observed to persist systemically in one (e.g., the lone surviving rainbow trout in this study) or more (e.g., survivors in Chapter 3) individuals. Collectively, these findings suggest that the rainbow trout immune system struggles to eliminate this variant, which likely contributed to its prolonged shedding. Considering the heightened transmission risk of this variant (ST275), it should be incorporated into the development of BCWD prevention and control strategies. US62 also exhibited prolonged shedding herein, albeit to a lesser extent than US87, but appeared to be successfully cleared by Atlantic salmon at the end of the experiment. This *F. psychrophilum* variant belonged to molecular serotype 1 (nearly equivalent to conventional serotype Fd; Lorenzen and Olesen, 1997; Rochat et al. 2017; Chapter 3) and was less host specific (Chapter 3). Moreover, these serotypes are less common in Atlantic salmon (Avendaño-Herrera et al. 2020) and thus possibly less capable of circumventing this species immune response, possibly helping to explain the shorter duration of shedding and lack of recovery from survivors herein and Chapter 3. US19 was highly virulent herein and caused mortality before a rigorous adaptive immune response could likely be mounted. Whether the shedding dynamics of these variants are representative of other *F. psychrophilum* variants affecting Atlantic salmon, coho salmon, and rainbow trout should be further investigated.

The prolonged duration and high rate of *F. psychrophilum* shedding from dead fish may be influenced by multiple factors. In the absence of a host immune response, the conditions may become ideal for *F. psychrophilum* proliferation, possibly owing to the fish carcass serving as an

abundant protein source (i.e., *F. psychrophilum* main energy source; Duchaud et al. 2007; Chapter 2), this species' inherent proteolytic capabilities (Barbier et al. 2020), and water temperatures that not only slow fish tissue decomposition (Nobre et al. 2019) but are conducive to *F. psychrophilum* growth. Another possible contributing factor is the formation of biofilm by *F. psychrophilum*, which can form on various surfaces (Levipan and Avendaño-Herrera, 2017; Vidal et al. 2020), and is often comprised of multiple species (Schoina et al. 2022). In this context, *F. psychrophilum* has been shown to lyse cells of multiple bacterial species and use them as a growth source (Pacha and Porter, 1968).

In addition to elucidating important aspects of *F. psychrophilum* shedding dynamics in three salmonid species, this study also built upon the previous work of Marancik and Wiens (2013) to optimize their qPCR assay for detection and quantification of *F. psychrophilum* in water. Originally, Marancik and Wiens (2013) described this assay for the detection/quantification of *F. psychrophilum* in spleen tissue, with a LOD of 3.1 genome units per reaction and LOQ of ~486 colony forming units. Herein, this assay was further optimized to quantify *F. psychrophilum* from filtered water, with a LOD/LOQ of 30.94 ± 6.84 cells/mL. The sensitivity of the assay could potentially be improved by filtering a larger water volume and/or by increasing sample volume per reaction. Indeed, even a 50-mL water volume containing 10^8 *F. psychrophilum* cells/mL filtered quickly (i.e., no indication of filter fouling). Strepparava et al. (2014) also designed a *F. psychrophilum*-specific qPCR to quantify *F. psychrophilum* from water samples, and although the LOD was similar (e.g., 66 cells/mL), the LOQ was >100-fold higher (e.g., 3,300 cells/mL). Moreover, and in the hands of the study authors and at least two other laboratories, specificity issues were observed with this qPCR assay (unpublished data) and so alternatives were sought. An additional improvement for the qPCR assay herein was the addition

of an internal positive control, which allowed for monitoring of PCR-inhibition, a known source of "false negatives" with molecular assays (Kavlick, 2018). Moving forward, this qPCR assay will be instrumental to future studies assessing *F. psychrophilum* shedding dynamics and could have application to *F. psychrophilum* detection in environmental field settings.

Prior to this study, knowledge of *F. psychrophilum* shedding dynamics were limited only to rainbow trout that were infected with a single *F. psychrophilum* variant. Given that *F. psychrophilum* is now well-known as a genetically diverse pathogen of multiple farm and hatchery-reared salmonid species with varying phenotypes, coupled with the differential shedding results by variant/host species herein, future studies assessing shedding dynamics and underlying pathogen/host mechanisms are warranted and may lead to improved BCWD prevention and control. Overall, study results demonstrated dead Atlantic salmon, coho salmon, and rainbow trout shed *F. psychrophilum* at higher rates than their liver counterparts and for at least several months, thereby potentially posing a greater transmission risk. Therefore, and with the insight provided by a recent *F. psychrophilum* transmission model (Brenden et al. 2023), it remains critical for personnel raising fish to remove dead, *F. psychrophilum*-infected fish from rearing units quickly and frequently.

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APPENDIX

Table 4.1. Repeatability of *Flavobacterium psychrophilum* Marancik and Wiens (2013) qPCR assay.

Gene copies	C_q Mean	C_q SD	C_q CV (%)
Intra-assay			
100000000	11.34	0.12	1.06
10000000	14.63	0.09	0.65
1000000	17.83	0.09	0.48
100000	21.78	0.11	0.52
10000	24.61	0.12	0.48
1000	27.60	0.11	0.40
100	30.76	0.16	0.51
10	34.05	0.36	1.06
1	36.96	0.86	2.32
Inter-assay			
100000000	11.22	0.12	1.09
10000000	14.65	0.17	1.17
1000000	17.79	0.10	0.57
100000	21.58	0.13	0.61
10000	24.49	0.20	0.83
1000	27.58	0.12	0.42
100	30.69	0.20	0.65
10	34.01	0.31	0.90
1	37.05	0.83	2.25

Note: Mean, standard deviation, and coefficient of variation (CV) of the cycle threshold (C_q) values are shown. To determine intra-assay variation, the target gene was serially diluted in ten-fold increments over nine orders of magnitude (i.e., 10^8 gene copies/reaction – 1 gene copy/reaction) and eight replicate reactions of each dilution were PCR amplified and quantified. Inter-assay variation was determined by repeating the qPCR run on a separate plate using an identical number of replicate reactions. The assay was repeatable within and between runs, as CV percentages were <2.5 across the linear range (Standish et al. 2018).

Table 4.2. Evaluation of *Flavobacterium psychrophilum* DNA extraction procedure from water using quantitative PCR (qPCR). Key to abbreviations: SD, standard deviation; CV, coefficient of variation; IPC, internal positive control; C_q, cycle threshold.

Theoretical concentration of *F. psychrophilum*	qPCR-derived concentration of *F. psychrophilum*						*F. psychrophilum* DNA extraction efficiency (%)[b]
Mean	Mean	SD	Signal ratio[a]	CV (%)	C_q IPC (SD)	IPC CV (%)	
1.00×10^8	9.97×10^7	5.21×10^6	3/3	5.23	-	-	99.70
1.00×10^7	7.85×10^6	7.08×10^4	3/3	0.90	35.29 (0.32)	0.91	78.49
1.00×10^6	3.19×10^5	2.82×10^4	3/3	8.83	31.08 (0.26)	0.82	31.91
1.00×10^5	8.87×10^3	4.35×10^2	3/3	4.90	30.12 (0.21)	0.70	8.87
1.00×10^4	6.73×10^2	3.30×10^1	3/3	4.90	31.17 (0.15)	0.49	6.73
1.00×10^3	3.63×10^1	6.07×10^0	3/3	16.74	32.31 (0.11)	0.36	3.63
1.00×10^2	4.34×10^0	9.63×10^{-1}	10/10	22.19	31.56 (0.37)	1.17	4.34
1.00×10^1	1.92×10^0	9.95×10^{-1}	4/10	51.81	31.04 (0.24)	0.76	19.20
1.00×10^0	-	-	0/10	-	32.12 (0.36)	1.11	-
Median							14.03

[a] Number of amplified replicate reactions/total number of replicate reactions.

[b] Calculated as the quotient of the mean qPCR-derived concentration of *F. psychrophilum* and the mean theoretical concentration of *F. psychrophilum* cells, multiplied by 100.

Table 4.3. Mean *Flavobacterium psychrophilum* shedding rates (cells/fish/hour) ± standard deviation of dead Atlantic salmon, coho salmon, and rainbow trout on each sampling day. Number of fish sampled (N) is to the left of the host species name.

	Host species					
Sampling Day	N	Atlantic salmon	N	Coho salmon	N	Rainbow trout
1	2	$1.8 \pm 1.6 \times 10^{8}$	4	$4.8 \pm 3.5 \times 10^{8}$	4	$5.0 \pm 2.7 \times 10^{8}$
3	2	$5.0 \pm 0.8 \times 10^{7}$	4	$3.1 \pm 3.4 \times 10^{9}$	4	$1.9 \pm 0.5 \times 10^{9}$
5	2	$6.7 \pm 2.1 \times 10^{7}$	4	$5.0 \pm 3.8 \times 10^{9}$	4	$1.6 \pm 1.2 \times 10^{10}$
7	2	$1.7 \pm 0.7 \times 10^{8}$	4	$4.9 \pm 3.3 \times 10^{9}$	4	$8.1 \pm 6.3 \times 10^{9}$
14	2	$1.2 \pm 0.6 \times 10^{8}$	4	$4.4 \pm 5.1 \times 10^{9}$	4	$1.6 \pm 2.6 \times 10^{9}$
63	2	$6.9 \pm 6.9 \times 10^{6}$	4	$9.1 \pm 8.9 \times 10^{8}$	4	$1.6 \pm 1.2 \times 10^{9}$
98	1	$4.5 \pm 0.0 \times 10^{6}$	3	$4.9 \pm 5.6 \times 10^{6}$	4	$1.6 \pm 2.6 \times 10^{9}$

Figure 4.1. Mean *Flavobacterium psychrophilum* shedding rates (black bars with standard deviation) from and cumulative percent mortality (gray lines with standard error) of live A) Atlantic salmon (*Salmo salar*), B) rainbow trout (*Oncorhynchus mykiss*), and C) coho salmon (*O. kisutch*). *, water sampling occasion, which continued four weeks past the last detection of *F. psychrophilum*. //, indicates 6-day gap in time.

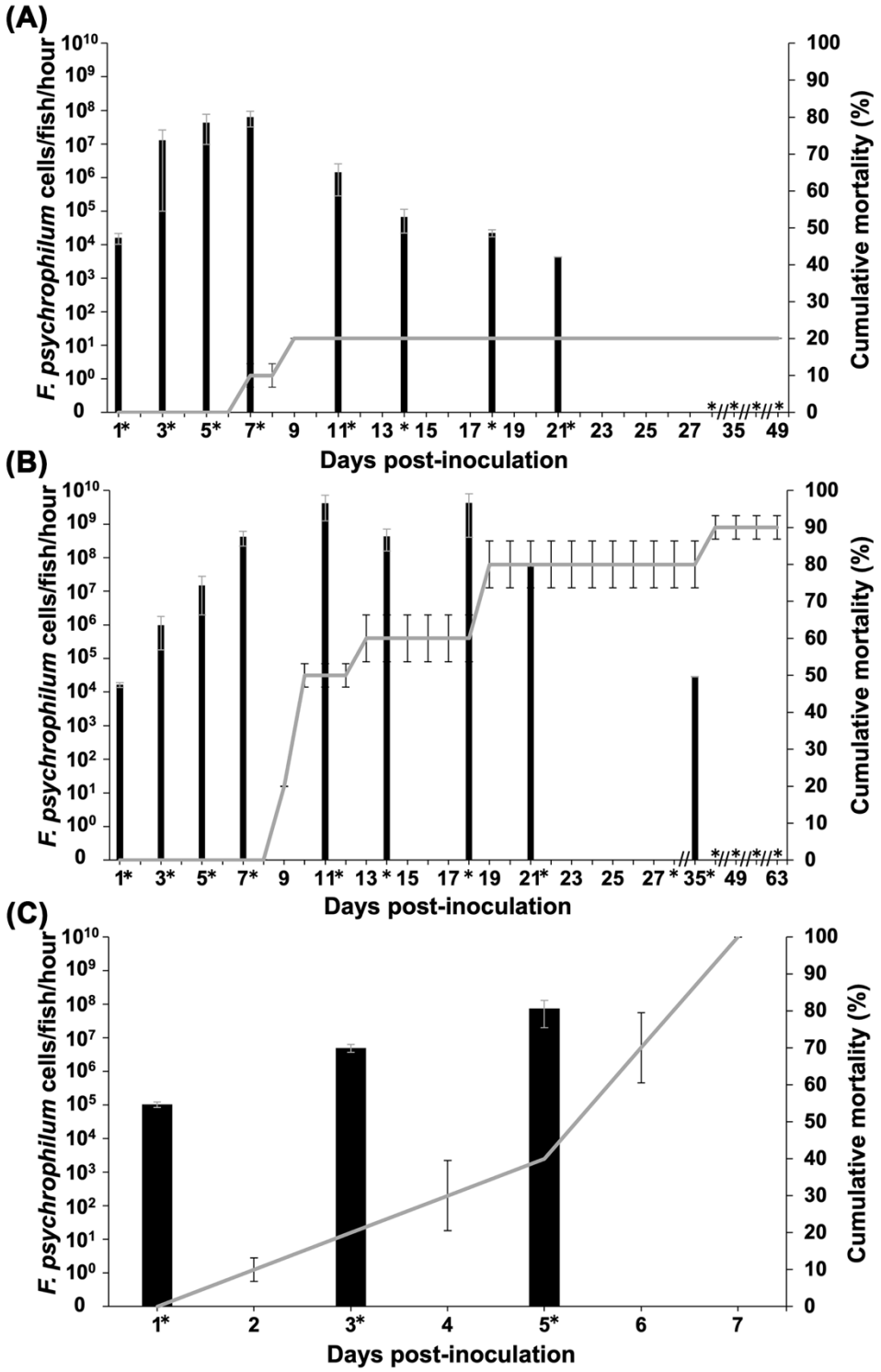


Figure 4.2. *Flavobacterium psychrophilum* shedding rates of dead individual (A) Atlantic salmon (*Salmo salar*), (B) rainbow trout (*Oncorhynchus mykiss*), and (C) coho salmon (*O. kisutch*). Legend explanation: ≤ 2 fish (F) per replicate (R) aquarium were maintained. Therefore, in the legend and as an example, R1.F1 corresponds to replicate one, fish one.

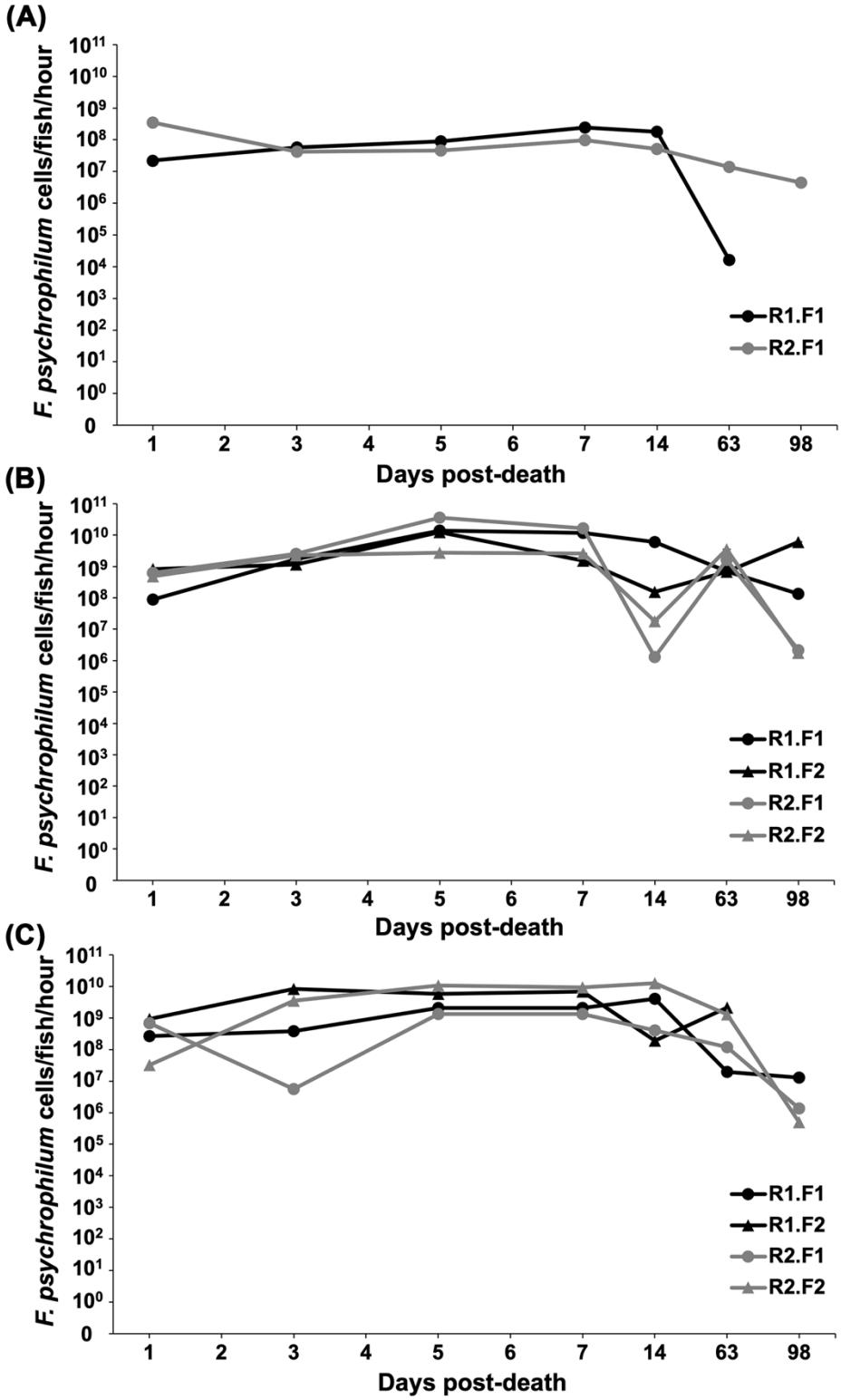
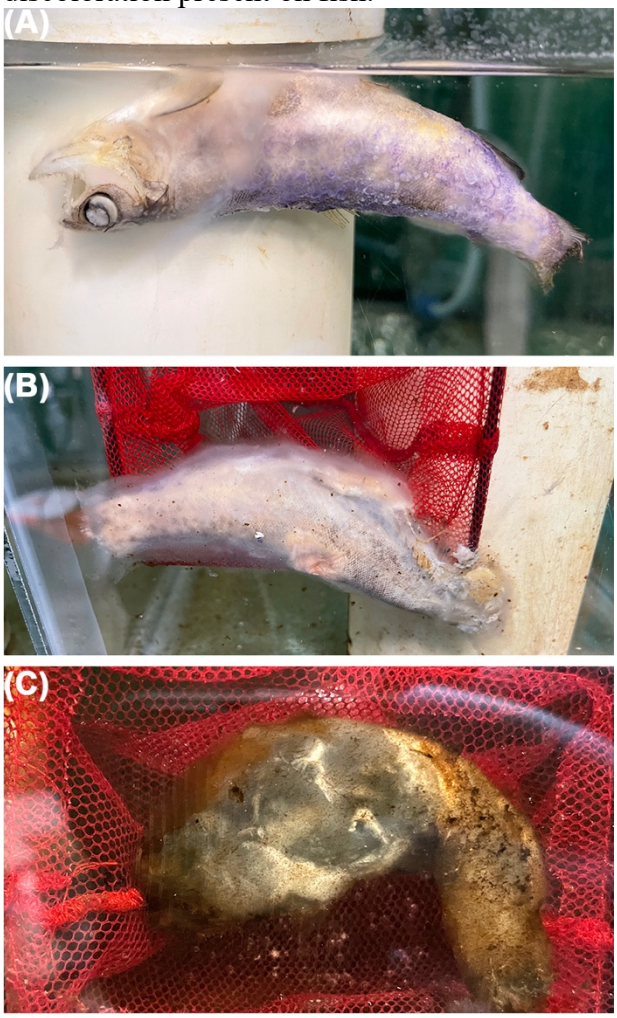


Figure 4.3. Representative images of dead fish used in *Flavobacterium psychrophilum* shedding experiment. (A) Coho salmon (*Oncorhynchus kisutch*) 12 days post death. (B) Rainbow trout (*O. mykiss*) 25 days post death. (C) Coho salmon 98 days post death. Note the yellowish discoloration present on fish.



Chapter 5:

Culturability of heterologous *Flavobacterium psychrophilum* multilocus sequence typing variants in three microcosms that simulate common fish farm and hatchery environments

5.1. Abstract

Flavobacterium psychrophilum, causative agent of bacterial coldwater disease (BCWD), is a widespread threat to the success of salmonid farms and hatcheries, where it causes substantial economic losses. In these environments, fish are fed frequently and reared at elevated densities, leading to the accumulation of uneaten food and/or raceway detritus. Such substrates are potential nutrient sources for *F. psychrophilum* when outside its host, possibly contributing to BCWD epizootic risk. Concurrently, hundreds of *F. psychrophilum* sequence types (STs; i.e., genetic variants) have been described using multilocus sequence typing (MLST) and findings show that some variants are more frequently associated with fish farms and hatcheries. Whether persistence strategies in these environments differ amongst *F. psychrophilum* variants remains unknown. To this end, the culturability of ten distinct *F. psychrophilum* MLST variants was evaluated for 13 weeks in three microcosms comprised of sterilized well water, sterilized well water with commercial trout feed, or sterilized well water with raceway detritus. These variants belonged to globally distributed clonal complexes (CCs) and/or are repeatedly recovered from fish farms and hatcheries. All ten *F. psychrophilum* variants remained culturable in each of the three microcosms for at least eight weeks, with bacterial concentrations often highest in the presence of raceway detritus. In addition, most (e.g., 90%) *F. psychrophilum* variants remained culturable for at least 13 weeks. Significant differences in culturability were observed both within and between microcosms, suggesting potential variability in environmental persistence strategies among specific variants. Collectively, results highlight the remarkable ability of *F. psychrophilum* to not only persist for weeks under nutrient limited conditions, but also thrive in the presence of organic substrates common in fish farm and hatchery rearing units.

5.2. Introduction

Flavobacterium psychrophilum, causative agent of bacterial coldwater disease (BCWD), poses a significant economic challenge by affecting multiple salmonid (Family *Salmonidae*) species reared in farms and hatcheries around the world (Loch and Faisal, 2017). The widespread success of this pathogen is multifactorial, in part relating to vertical transmission, circumvention of egg-surface disinfectants such as iodophor (Brown et al. 1997; Cipriano, 2005; Starliper 2011), and efficient horizontal transmission (Madetoja et al. 2000). Moreover, there are reports of reduced susceptibility to the few antibiotics that are approved to treat *F. psychrophilum* infections in food fish (Bruun et al. 2000; Van Vliet et al. 2017), and development of efficacious, licensed BCWD vaccines in the USA remain elusive to date (Gomez et al. 2014).

Another factor likely perpetuating losses caused by *F. psychrophilum* is its ability to survive outside its host for extended periods. Vatsos et al. (2003) found *F. psychrophilum* survived for 133 days in sterile stream water, while Madetoja et al. (2003) showed *F. psychrophilum* remained culturable in sterile lake water for 300 days. Although some fish farms and hatcheries use surface water sources (e.g., stream or river water; Strepparava et al. 2014), others use groundwater sources (e.g., well or spring water; Van Vliet et al. 2015), which differ in quality (e.g., hardness; Summerfelt, 2000) and may affect *F. psychrophilum* survival. Although not demonstrated for *F. psychrophilum*, water hardness has been shown to affect the biofilm formation of at least one other flavobacterial fish pathogen, *F. columnare* (Cai et al. 2013). Madetoja et al. (2003) found that *F. psychrophilum* could survive longer and proliferate more extensively in lake water microcosms that also contained natural beach sand. Other substrates, such as detritus and uneaten food (Schumann, 2021), may also affect *F. psychrophilum* survival but have yet to be examined. Given the common presence of detritus and uneaten food in fish

farms and hatcheries, investigating their role in *F. psychrophilum* survival could be pivotal for informing improved BCWD management strategies.

The considerable intraspecific diversity within *F. psychrophilum* may also affect its survival within fish farm and hatchery environments. In this context, multilocus sequence typing (MLST) has been applied to >1500 *F. psychrophilum* isolates worldwide, revealing over 260 distinct sequence types (STs; https://pubmlst.org/organisms/flavobacterium-psychrophilum). Importantly, the wealth of diversity within this species has been linked to phenotypic variation with respect to host associations, virulence, ultraviolet light susceptibility, and antimicrobial susceptibility (Van Vliet et al. 2017; Knupp et al. 2019; Sundell et al. 2019; Knupp et al. 2021a; Knupp et al. 2021b; Knupp et al. 2023, in press). However, it remains unclear if such intraspecific diversity also affects *F. psychrophilum* environmental persistence strategies. Indeed, previous studies examining *F. psychrophilum* environmental persistence strategies only used one to two isolates yet represent the totality of our knowledge (Vatsos et al. 2003; Madetoja et al. 2003). Several MLST-based studies suggested that certain *F. psychrophilum* variants (e.g., ST10, ST13, ST78, ST267, ST275, and ST253) are common in fish farms and hatcheries, as evidenced by their repeated detections in these environments (Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Van Vliet et al. 2016; Knupp et al. 2019; Li et al. 2021). In contrast to these reports, other variants, such as ST252 and ST256 (Van Vliet et al. 2016; Knupp et al. 2019), appear to be more common in wild and/or feral fish populations. Whether these disparities in recovery environment reflect differences in environmental persistence strategies or abilities to survive outside a host has yet to be thoroughly investigated.

To improve our understanding of *F. psychrophilum* environmental persistence strategies in fish farms and hatcheries, the culturability of ten *F. psychrophilum* variants, representing eight

MLST clonal complexes and two singletons, was measured for 13 weeks in three microcosms containing well water only, well water with detritus, and well water with commercial trout feed, to simulate environments commonly found in salmon and trout rearing facilities.

5.3. Materials and Methods

5.3.1. *Flavobacterium psychrophilum* isolate selection

A total of ten *F. psychrophilum* isolates, recovered from 2010 – 2021 from two gamete collection locations and six hatcheries in two U.S. states (e.g., Michigan and Pennsylvania) were selected for this study (Table 5.1). The isolates were recovered from three salmonid (Family *Salmonidae*) genera and five species, including Atlantic salmon (*Salmo salar*, *n* = 1), brown trout (*S. trutta*, *n* = 2), brook trout (*Salvelinus fontinalis*, *n* = 1), coho salmon (*Oncorhynchus kisutch*, *n* = 1), and steelhead trout (*O. mykiss*, *n* = 5; Table 5.1). The isolates were genetically diverse according to MLST, whereby most (*n* = 6) had been previously genotyped (Van Vliet et al. 2016; Knupp et al. 2019; https://pubmlst.org/fpsychrophilum/) and four were newly genotyped in this study following published protocols (Knupp et al. 2019). In total, eight isolates belonged to eight STs within five CCs, two isolates belonged to two distinct singleton STs, and all ten comprised globally relevant and/or widespread STs (Fujiwara-Nagata et al. 2013; Nilsen et al. 2014; Avendaño-Herrera et al. 2014; Knupp et al. 2019; Li et al. 2021; Table 5.1).

5.3.2. Bacterial inoculum preparation

The ten cryogenically preserved (maintained at -80 ºC) *F. psychrophilum* variants were revived on *F. psychrophilum* medium-A (FPM-A; Chapter 2), incubated for 48 hours at 15 ºC, and then visually inspected for purity. Each variant was inoculated into 150-mL of FPM-A broth and incubated with constant shaking (180 rpm) for 48 hours at 15 ºC. Bacteria were harvested via centrifugation (2,571 × *g*, 15 minutes), washed once using 0.22 µm filtered and autoclaved well

water (hereafter referred to as well water) that originated from a salmonid-rearing quarantine facility, and then resuspended in 15-mL of analogous water.

5.3.3. Microcosms

Three microcosms (i.e., treatment groups) that simulate environments common to fish-farms and hatchery facilities were prepared using 125-mL glass Erlenmeyer flasks with partially loosened screw caps. Each flask (n = 2 per isolate per treatment) contained 67.5-mL of either well water alone (treatment 1) or well water with 12.5 g (dry weight) raceway detritus (treatment 2) or commercial trout feed (treatment 3; see microcosm preparations below). A 2.5-mL aliquot of bacterial suspension was added to each flask and gently swirled ten times. To quantify starting bacterial concentrations (i.e., week 0), a 1-mL aliquot was collected from each flask, serially diluted 100,000,000-fold in ten-fold increments, plated on FPM-A in duplicate, and then incubated for seven days, after which final colony counts were performed. This sampling procedure was performed once per week for eight consecutive weeks, as well as on week 13. Prior to removing a sample for colony enumeration and on weeks 9 – 12 (i.e., when no sampling occurred), flasks were gently swirled ten times. Between samplings, flasks were incubated statically in the dark at 10 ºC (i.e., common temperature for BCWD epizootics; Barnes and Brown, 2011).

5.3.3.1. Fish-rearing facility well water

Ultraviolet light-treated, sand-filtered well water (pH 7.3) was obtained from the aquatics section of the Michigan State University – University Research Containment Facility (MSU-URCF), which is routinely used to rear salmonids. For this experiment, well water was filtered using 0.22-µm filter flasks with a polyethersulfone filter membrane (Santa Cruz Biotechnology, Dallas, TX, USA) and then aliquoted into 60 flasks (67.5 mL/flask); a subset of which (n = 20)

were immediately autoclaved at 121 ºC for 15 min. The remaining flasks were autoclaved after raceway detritus or commercial trout feed was added (see sections 5.3.3.2 and 5.3.3.3).

5.3.3.2. Raceway detritus

Detritus was selected for inclusion as a component of an experimental microcosm, as it represents an accumulation of uneaten food and fish byproducts (e.g., mucus and feces) commonly found in fish rearing units, thereby representing a potential nutrient source for *F. psychrophilum* while outside its host. To simulate this environment, detritus was siphoned from a single raceway of rainbow trout hatched and reared under quarantine conditions at MSU-URCF and collected on a mesh screen. Rainbow trout had been screened for *F. psychrophilum* via culture on FPM-A and verified free of bacterial infection (see Chapter 3). Amassed detritus was air-dried for 24 hours, finely crushed via mortar and pestle, and then stored in the dark at 4 ºC in a sterile glass bottle until use. Dried detritus was weighed (12.5 g/flask), added to flasks (n = 20) containing 67.5-mL filtered well water, and then autoclaved at 121 ºC for 15 min.

5.3.3.3. Commercial trout feed

One potential environmental reservoir for *F. psychrophilum* is uneaten fish feed, which can act as an organic substrate supporting bacterial proliferation. Furthermore, when fish are infected with *F. psychrophilum*, one of the first observable disease signs is often a decrease in feeding. This could result in an accumulation of uneaten feed in the rearing units, potentially promoting the persistence and proliferation of *F. psychrophilum*. For this reason, commercial trout feed Skretting #2 (Skretting USA, Tooele, Utah) was included as a treatment. This feed is specifically formulated for coldwater fish species, including rainbow trout, which are particularly vulnerable to BCWD during their early life-stages (Holt, 1987). Fresh feed was weighed (12.5

g/flask), added to flasks (n = 20) containing 67.5-mL filtered well water, and then autoclaved at 121 ºC for 15 min.

5.3.4. Data analysis

A linear mixed model was used to quantify the effect ($\log_{10}$ concentration in cfu/mL) of each microcosm (i.e., treatment) on the ten *F. psychrophilum* variants. The model included treatment, week, variant, the interaction between treatment and week, and the interaction between treatment and variant as fixed effects. Degrees of freedom for fixed effects were calculated using the Kenward-Roger method. Custom hypothesis tests as to differences in collective (i.e., all weeks combined) mean concentration among variants within the same treatment group and between treatment groups for the same variant were evaluated through pairwise comparisons of least-square means and adjusted for multiple comparisons using the Tukey-Kramer method ($\alpha = 0.05$). Analyses were performed using PROC MIXED in SAS® Version 9.4; custom hypothesis testing was performed using the LSMEANS statement and pdiff option.

5.4. Results

5.4.1. General model analyses

Based on the fitted linear mixed-effects model, treatment group (e.g., raceway detritus, commercial trout feed, and water only) effects differed significantly depending on week (df = 18, 543; F = 14.21; P-value < 0.0001) and *F. psychrophilum* variant (df = 27, 543; F = 17.70; P-value < 0.0001). Averaging observations across all ten variants and nine sampling weeks, mean *F. psychrophilum* concentrations were significantly higher in the raceway detritus treatment group compared to both the commercial trout feed (LSmeans estimate 1.64 ± 0.08; df = 543; t-value = 20.72; P-value < 0.0001) and well water only treatment groups (LSmeans estimate 1.22

± 0.08; *df* = 543; *t*-value = 15.46; *P*-value <0.0001). Likewise, mean *F. psychrophilum* concentrations were significantly higher in well water only compared to commercial trout feed (LSmeans estimate 0.42 ± 0.08; *df* = 543; *t*-value = 5.26; *P*-value < 0.0001).

5.4.2. *Flavobacterium psychrophilum* culturability in well water only

Initial *F. psychrophilum* concentrations within each well water only microcosm flask ranged from 2.14×10^7 cfu/mL to 1.78×10^8 cfu/mL (Figure 5.1A; Table 5.2) compared to 1.00×10^5 cfu/mL – 9.33×10^5 cfu/mL at week 13 (Figure 5.1A; Table 5.2). Averaging observations across all ten variants and nine sampling weeks, mean *F. psychrophilum* concentrations decreased between weeks zero and seven but increased on week eight before decreasing through the end of the study (e.g., week 13; Figure 5.1A; Table 5.3). However, mean concentrations of ST78 and ST267 increased between weeks zero and one (Figure 5.1A; Table 5.2). Also of note, the mean concentration of ST277 initially declined more rapidly than all other variants over the first two weeks, but by week five, its mean concentration aligned more closely with the other variants (Figure 5.1A; Table 5.2). Averaging observations for each variant across all nine sampling weeks, ST342 maintained the highest concentration, whereas ST277 had the lowest concentration, with the mean concentration difference between these two variants being significant (*df* = 543; *t*-value = 4.55; *P*-value = 0.0025; Table 5.4). Likewise, collective mean concentrations of ST286 and ST275 were significantly higher than ST277 (*df* = 543; *t*-values = 4.11 and 4.25; *P*-values = 0.0085 and 0.0148, respectively). For the remaining variant comparisons, collective mean bacterial concentrations were not significantly different from one another (*df* = 30; *t*-values = 0.01 – 3.97; *P*-values = 0.0707 – 1.0000; Table 5.4).

5.4.3. *Flavobacterium psychrophilum* culturability in well water with detritus

Initial bacterial concentrations within each well water with detritus microcosm flask ranged from 2.14×10^7 cfu/mL to 1.78×10^8 cfu/mL (Figure 5.1B; Table 5.5) compared to 0.00 cfu/ml – 5.25×10^7 cfu/mL at week 13 (Figure 5.1B; Table 5.5). Averaging observations across all ten variants and nine sampling weeks, mean *F. psychrophilum* concentrations increased between weeks zero and two, decreased on week three, increased on week four, and then decreased over the remainder of the study (Figure 5.1B; Table 5.3). Of note, larger differences in culturability were observed beginning week seven, whereby mean concentrations of ST353 (2.24×10^4 cfu/mL) and ST286 (6.76×10^5 cfu/mL) were lower than the other variants (9.77×10^6 – 3.72×10^8 cfu/mL; Figure 5.1B; Table 5.5). Averaging observations for each variant across all nine sampling weeks, ST253 maintained the highest mean concentration, whereas ST353 had the lowest mean concentration. In this context, most (e.g., $8/9 \approx 88.9\%$) *F. psychrophilum* variants had a significantly higher collective mean concentration compared to ST353 ($df = 543$; t-values = 4.17 – 7.77; P-values < 0.0001 – 0.0003), and ST253 had a significantly higher collective mean concentration compared to ST286 (ST286; $df = 543$; t-value = 4.74; P-value = 0.0011; Table 5.4). Similarly, ST277 had a significantly higher collective mean concentration compared to ST286 ($df = 543$; t-value = 4.17; P-value = 0.0119). In examining the remaining variant comparisons, collective mean concentrations were not significantly different ($df = 543$; t-values = 0.11 – 3.69; P-values = 0.0654 – 1.0000; Table 5.4).

5.4.4. *Flavobacterium psychrophilum* culturability in well water with commercial trout feed

Initial bacterial concentrations within each well water with commercial trout feed microcosm flask ranged from 2.14×10^7 cfu/mL to 1.78×10^8 cfu/mL (Figure 5.1C; Table 5.6) compared to 10 cfu/mL – 3.89×10^6 cfu/mL at week 13 (Figure 5.1B; Table 5.6). Averaging

observations across all ten variants and nine sampling weeks, mean *F. psychrophilum* concentrations decreased between weeks zero and two, increased between weeks three and five, and then steadily decreased over the remaining eight weeks (Figure 1C; Table 5.3). However, the mean concentration of ST267 increased between weeks zero and one before decreasing sharply over the next two weeks (Figure 5.1C; Table 5.6). Averaging observations for each variant across all nine sampling weeks, ST275 maintained the highest mean concentration, whereas ST267 had the lowest mean concentration and the mean concentration difference between these two variants was significant ($df = 543$; t-value = 14.67; P-value < 0.0001; Table 5.4). Likewise, collective mean concentrations of all other *F. psychrophilum* variants were significantly higher than ST267 ($df = 30$; t-values = 8.19 – 14.06; P-values < 0.0001; Table 5.4). Similarly, 66.7% (e.g., 6/9 variants) and 55.6% (e.g., 5/9 variants) of *F. psychrophilum* variants had collective mean concentrations that were significantly higher than ST13 or ST10, respectively (Table 5.4). Overall, collective mean *F. psychrophilum* concentrations were most variable in this microcosm, whereby each variant differed significantly from one to nine other variants (Table 5.4).

5.4.5. *Flavobacterium psychrophilum* culturability comparisons across microcosms

Overall, most *F. psychrophilum* variants (e.g., 8/10 = 80.0%) maintained higher collective (i.e., all weeks combined) mean concentrations in raceway detritus compared to commercial trout feed ($df = 543$; t-values = 3.93 – 18.84; P-values < 0.0001 – 0.0284; Table 5.7). Likewise, most *F. psychrophilum* variants (e.g., 8/10 = 80.0%) maintained higher collective mean concentrations in raceway detritus compared to well water only ($df = 543$; t-values = 3.89 – 9.63; P-values < 0.0001 – 0.0337; Table 5.7).

When comparing collective mean concentrations in commercial trout feed to well water only, no significant differences ($df = 543$; t-values = 0.18 – 3.16; P-values = 0.2758 – 1.0000)

were observed for most *F. psychrophilum* variants (e.g., 7/10 = 70.0%; Table 5.7). However, one variant (ST277) had a significantly higher collective mean concentration in the presence of commercial trout feed (df = 543; t-value = 3.82; P-value = 0.0422; Table 5.7), whereas the two other variants (ST13 and ST267) each had a significantly higher collective mean concentration in well water only (df = 543; t-values = 4.26 and 12.11; P-values = 0.0081 and < 0.0001, respectively; Table 5.7). Of note, one *F. psychrophilum* variant (ST353) had no significant differences in collective mean concentrations amongst all three microcosms (df = 543; t-values = 0.09 – 1.83; P-values = 0.9937 – 1.0000; Table 5.7).

5.5. Discussion

Although *F. psychrophilum* has been recovered from wild/feral fish (Van Vliet et al. 2016; Knupp et al. 2019; Harrison et al. 2021) and has caused mortalities in these populations (Davis, 1946), most BCWD epizootics occur in fish farms and hatcheries (Kum et al. 2008; Nilsen et al. 2011; Avendaño-Herrera et al. 2014; Knupp et al. 2019; Li et al. 2021). Indeed, the artificial rearing environment is ideal for *F. psychrophilum*, as fish are reared at elevated densities, providing enhanced opportunity for horizontal transmission (Kennedy et al. 2015). In this context, *F. psychrophilum* is efficiently shed from live and dead fish into the water column during BCWD epizootics (Madetoja et al. 2000; Chapter 4). Although some *F. psychrophilum* cells may be transmitted directly to a new host, others may encounter suspended solids that will eventually settle in the rearing unit. Two settable solids commonly found in fish farms and hatcheries are uneaten feed and detritus (Schumann, 2021). Despite being natural and common organic sources in fish rearing facilities, where they represent substrates for microbial proliferation (Dauda et al. 2019), controlled experiments evaluating *F. psychrophilum* survival in these microenvironments has not been reported. Herein, the 13-week culturability of ten

genetically diverse *F. psychrophilum* variants was evaluated in laboratory microcosms containing sterile well water, sterile well water with commercial trout feed, and sterile well water with raceway detritus. Overall, cfu yields across weeks and in well water only were similar among *F. psychrophilum* variants. Importantly, however, well water with raceway detritus led to significant increases in cfu yields amongst most *F. psychrophilum* variants. Moreover, well water with commercial trout feed also improved yields among some *F. psychrophilum* variants. These findings provide evidence that these common and natural settable solids have the potential to increase *F. psychrophilum* loads in fish farms and hatcheries, thereby potentially increasing the risk of BCWD. In addition to these findings, it was also apparent that some *F. psychrophilum* variants persisted well in all tested microcosms, whereas some were better suited to specific microcosms. Collectively, these findings not only improve our understanding of BCWD ecology and *F. psychrophilum* persistence outside its host but also have potential to improve BCWD management strategies.

All *F. psychrophilum* variants remained culturable in filtered well water for 13 weeks, and at similar concentrations over most weeks, possibly suggesting metabolic responses among most variants are comparable in this nutrient-limited environment. Vatsos et al. (2003) reported one *F. psychrophilum* isolate (MLST variant unknown) remained culturable for 19 weeks in filtered stream water. Likewise, Madetoja et al. (2003) found a different *F. psychrophilum* isolate (MLST variant unknown) remained culturable for 300 days (~43 weeks) in filtered lake water. In contrast to the slow decrease in bacterial concentrations observed amongst most *F. psychrophilum* variants herein and in previous studies (Vatsos et al. 2003; Madetoja et al. 2003), concentrations of *F. psychrophilum* variant ST277 decreased rapidly over the first two weeks but then consistently increased over the following three weeks. These results possibly suggest that *F.*

psychrophilum variant ST277 may be less equipped to transition from a nutrient-rich environment to a nutrient-limited one. Despite this, *F. psychrophilum* variant ST277 demonstrated a capacity to recover, possibly by exploiting the substantial number of cells that initially died as a growth source. Indeed, *F. psychrophilum* has been demonstrated to lyse and utilize bacteria for its proliferation (Pacha and Porter, 1968). However, these findings may also mean under natural conditions, where a significant mass of bacterial cells is less likely to be available, the survival potential of this specific variant might be compromised. Nonetheless, results provide further evidence *F. psychrophilum* is capable of persisting for weeks under nutrient-limited conditions.

Most of the analyzed *F. psychrophilum* variants persisted at high concentrations (e.g., ~10^7 – 10^8 cfu/mL) in the presence of raceway detritus for at least seven weeks. The apparent success of *F. psychrophilum* in the presence of this common fish farm and hatchery substrate may be related to the composition of raceway detritus, which is primarily composed of fish feces and uneaten food, both of which are rich in nutrients, including protein (Hardy and Gatlin, 2022; Schumann, 2021; Skretting USA). Genomic studies have indeed indicated that proteins are the primary energy source for *F. psychrophilum* (Duchaud et al. 2007; Castillo et al. 2021), findings further corroborated by in vitro growth studies (Holt, 1987; Alvarez and Guijarro, 2007; Oplinger and Wagner, 2012; Chapter 2). Interestingly, these detrital compounds only appear to better support the persistence of most studied *F. psychrophilum* variants in the first eight weeks in comparison to raw trout feed. Indeed, by week 13, concentrations of most *F. psychrophilum* variants were higher in the presence of commercial trout feed compared to detritus. Cautiously extrapolating these in vitro findings to field conditions suggests that raceway detritus may serve as a greater "short-term" substrate for *F. psychrophilum* proliferation, whereas commercial trout

feed may be a better "long-term" substrate, a matter highlighting the importance of removing detritus and feed from fish farm and hatchery rearing units.

Although similar trends in culturability were apparent in each microcosm, potential differences in environmental persistence strategies and survival responses among *F. psychrophilum* variants within a specific microcosm were also apparent. For instance, the culturability of *F. psychrophilum* variant ST267 was significantly less than all other variants in the presence of commercial trout feed. Interestingly, ST267 was the only variant that grew between weeks zero and one in the presence of commercial trout feed, while the concentrations of all other variants decreased by ≥10-fold. A similar growth pattern for ST267 was observed in the other microcosms. Thus, results may suggest that certain *F. psychrophilum* variants, when faced with sub-optimal nutrient conditions, initially respond by mobilizing energy reserves rather than adapting to a new nutrient source. Lending support to this theory, one genomic study (Duchaud et al. 2007) identified genes in one *F. psychrophilum* variant (ST20 in CC-ST10) that encode cyanophycinase and cyanophycin synthetase – enzymes necessary for cyanophycin production. Although the biological function of cyanophycin has not been explored in *F. psychrophilum*, it is known that other bacterial species use cyanophycin as a carbon/nitrogen (i.e., energy) storage mechanism, employing it for energy in sub-optimal nutrient conditions (Krehenbrink et al. 2002). Whether cyanophycin is common amongst *F. psychrophilum* variants and/or is used in this capacity remains to be investigated. In contrast to ST267, overall culturability of *F. psychrophilum* variant ST353 in the presence of commercial trout feed was like most other variants; however, this variant's culturability in the presence of detritus was significantly worse than most other variants, and was the only non-culturable variant at 13-

weeks. Thus, some *F. psychrophilum* variants may be better suited to different hatchery microenvironments.

Findings may lend support to previous molecular epidemiology research studies that indicated certain *F. psychrophilum* MLST variants, such as ST253, are more adapted to the fish hatchery environment, a claim based on the frequent recovery of ST253 from the same facility over several years (e.g., 2010, 2013, 2017, and 2020; Knupp et al. 2019). For instance, *F. psychrophilum* variant ST253 had the highest collective mean concentration in the presence of raceway detritus and was the only variant to persist a high concentration ($\geq 10^7$ cfu/mL) up to the 13th week, indicating a possible superior adaptation to this environment compared to other variants. Moreover, ST253 was culturable for 13 weeks in both other microcosms, and at moderately high concentrations (e.g., ~10^5 – 10^6 cfu/mL). Recognizing that some *F. psychrophilum* variants may thrive in common fish farm and hatchery environments may lead to improved BCWD management strategies, including altering feeding to minimize waste accumulation, adjusting rearing unit disinfection protocols, or even considering selective breeding programs for fish resistant to these highly successful *F. psychrophilum* variants.

Although this research provides evidence that *F. psychrophilum* can survive for extended periods in microenvironments commonly found in fish farms and hatcheries, further studies are needed to assess the virulence of variants originating from these settings, particularly under conditions where the bacterium has not been nutrient-deprived (e.g., in the presence of commercial trout feed and raceway detritus). Madetoja et al. (2003) exposed rainbow trout via immersion to one *F. psychrophilum* isolate that had been in sterile lake water for 14 days, finding it was moderately virulent (e.g., approximately 50% cumulative mortality). In addition, this same *F. psychrophilum* isolate retained most (e.g., 50% reduction in cumulative mortality) of its

virulence after subcutaneous injection into rainbow trout after being in sterile lake water for 49 days. In this context, if *F. psychrophilum* can remain virulent after extended periods of starvation, then perhaps under conditions when nutrients are available, *F. psychrophilum* will retain its virulence or potentially become more virulent. Indeed, Kinnula et al. (2017) found *F. columnare* (a causative agent of columnaris disease; LaFrentz et al. 2022) virulence increased proportionally with nutrient availability and possibly promoted virulence factor activation.

In conclusion, it is well-recognized that *F. psychrophilum* is a substantial threat to both aquaculture farms raising fish for consumption and hatcheries dedicated to conservation and stock enhancement efforts. Results herein provide evidence for the first time that multiple *F. psychrophilum* variants can persist for weeks in well water, a common groundwater source for fish farms and hatcheries, and at moderate concentrations. Moreover, it is apparent that raceway detritus is a growth source for most *F. psychrophilum* variants, elevating bacterial concentrations for weeks. Thus, efforts to remove raceway detritus fish farm and hatchery rearing units may be very beneficial to reducing overall *F. psychrophilum* loads, and possibly a source of BCWD outbreaks. Notably, however, some variants demonstrated an ability to persist and thrive using not only raceway detritus but commercial trout feed as well, which may potentially confer a fitness advantage in the fish farm and hatchery environment. In contrast, other variants appear to be only suited to one type of nutrient environment. This distinction underscores the complexity of BCWD ecology, and highlights the need for management strategies that consider the varying survival strategies and growth preferences of different *F. psychrophilum* variants. Nevertheless and in the interim, it is recommended that personnel raising fish enhance rearing unit hygiene practices, particularly focusing on removing detritus and especially during BCWD outbreaks as

removing this environmental reservoir could pay dividends in reducing bacterial loads and infection risk.

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APPENDIX

Table 5.1. *Flavobacterium psychrophilum* isolates selected for this study. The multilocus sequence typing clonal complex (CC), sequence type (ST; i.e., genetic variant), recovery location, host of origin, year of isolation, and starting concentration (i.e., at week 0) in the microcosm experiment are presented for each isolate.

Isolate	CC	ST	Recovery location[a]	Host species[b]	Year	Reference[c]
US019	9	13	MI-W-1	COS	2010	Van Vliet et al. (2016)
US075	10	10	PA-H-1	STT	2016	Knupp et al. (2019)
US053	10	78	MI-H-2	STT	2011	Van Vliet et al. (2016)
US464	10	275	MI-H-3	STT	2019	This study
US524	10	342	MI-H-2	STT	2021	This study
US531	191	267	MI-H-2	STT	2021	This study
US062	232	277	MI-W-2	ATS	2012	Knupp et al. (2019)
US461	286	286	MI-H-4	BNT	2019	This study
US503		253	MI-H-5	BNT	2020	This study
US487		353	MI-H-6	BKT	2020	This study

[a] Key to recovery location: U.S. state (MI, Michigan; PA, Pennsylvania) – Facility (H, hatchery; W, weir) – Site code (1 – 6).

[b] Key to host species: ATS = Atlantic salmon (*Salmo salar*); BKT = Brook trout (*Salvelinus fontinalis*); BNT = Brown trout (*S. trutta*); COS = Coho salmon (*Oncorhynchus kisutch*); STT = Steelhead trout (*O. mykiss*).

[c] Link to pubMLST database for *F. psychrophilum*: https://pubmlst.org/fpsychrophilum/.

Table 5.2. Weekly mean concentrations (in $\log_{10}$ colony forming units) ± (standard error) of the ten *Flavobacterium psychrophilum* sequence types (i.e., genetic variants) used in this study, as measured in the well water only microcosm.

	Week									
ST	0	1	2	3	4	5	6	7	8	13
13	8.10 (0.00)	7.37 (0.37)	6.30 (0.30)	6.50 (0.20)	6.09 (0.09)	6.20 (0.20)	5.97 (0.57)	5.68 (0.50)	5.89 (0.41)	5.74 (0.56)
10	8.15 (0.00)	7.84 (0.06)	7.00 (0.00)	6.76 (0.11)	6.59 (0.29)	6.09 (0.09)	5.48 (0.00)	4.97 (0.57)	5.39 (0.21)	5.27 (0.13)
78	8.25 (0.00)	8.29 (0.11)	7.24 (0.06)	6.39 (0.39)	6.20 (0.20)	6.27 (0.27)	5.63 (0.03)	5.54 (0.06)	6.00 (0.00)	5.09 (0.39)
275	8.15 (0.00)	8.09 (0.09)	7.63 (0.15)	6.72 (0.02)	6.59 (0.11)	6.30 (0.00)	6.09 (0.09)	6.00 (0.00)	6.18 (0.00)	5.74 (0.44)
342	7.85 (0.00)	7.79 (0.25)	7.18 (0.00)	7.18 (0.00)	6.94 (0.01)	6.85 (0.00)	6.58 (0.40)	6.13 (0.35)	6.12 (0.19)	5.97 (0.07)
267	7.33 (0.00)	7.39 (0.09)	7.72 (0.12)	6.24 (0.24)	6.14 (0.40)	6.00 (0.00)	5.50 (0.20)	5.35 (0.350	5.79 (0.39)	5.50 (0.24)
277	8.15 (0.00)	6.30 (0.00)	4.50 (0.20)	5.00 (0.00)	5.35 (0.05)	6.09 (0.09)	5.50 (0.10)	5.57 (0.09)	5.57 (0.09)	5.18 (0.00)
286	8.15 (0.00)	7.67 (0.07)	7.48 (0.00)	7.39 (0.09)	7.15 (0.15)	6.54 (0.00)	5.64 (0.10)	5.77 (0.23)	6.15 (0.15)	5.89 (0.41)
253	8.15 (0.00)	6.91 (0.07)	7.06 (0.00)	6.53 (0.35)	6.35 (0.35)	6.40 (0.00)	6.24 (0.24)	5.89 (0.41)	6.00 (0.30)	5.44 (0.44)
353	8.15 (0.00)	7.62 (0.08)	6.65 (0.00)	6.45 (0.15)	6.00 (0.00)	6.00 (0.00)	6.00 (0.00)	5.64 (0.10)	6.00 (0.00)	5.00 (0.00)

Table 5.3. Weekly mean concentrations (in $\log_{10}$ colony forming units) ± (standard error) of the three experimental microcosms (e.g., well water, well water with raceway detritus or commercial trout feed).

	Week									
Microcosm	0	1	2	3	4	5	6	7	8	13
Well water	8.04 (0.06)	7.53 (0.13)	6.88 (0.21)	6.52 (0.15)	6.34 (0.12)	6.27 (0.06)	5.86 (0.10)	5.65 (0.10)	5.91 (0.08)	5.48 (0.11)
Raceway detritus	8.04 (0.06)	8.47 (0.04)	8.54 (0.04)	8.43 (0.08)	8.52 (0.05)	8.33 (0.08)	7.91 (0.16)	7.06 (0.28)	6.84 (0.25)	4.56 (0.47)
Commercial trout feed	8.04 (0.06)	6.54 (0.13)	5.66 (0.22)	5.72 (0.42)	5.98 (0.41)	6.18 (0.33)	5.82 (0.36)	5.51 (0.41)	5.44 (0.37)	5.41 (0.42)

Table 5.4. Differences of least square mean estimates ± standard error (SE) for the interaction between treatment group (e.g., raceway "detritus", commercial trout "feed", or "water" only) and ten *Flavobacterium psychrophilum* variants (e.g., ST13, ST78, ST277, ST10, ST286, ST275, ST353, ST253, ST342, ST267). Pairwise comparisons between variants within the same treatment are provided. Tukey-Kramer adjusted *P*-values for multiple comparisons are shown (α = 0.05). Table is ordered by treatment.

Treatment 1	Isolate 1	Treatment 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
Detritus	ST13	Detritus	ST78	0.2894	0.2502	543	1.16	1.0000
Detritus	ST13	Detritus	ST277	-0.1201	0.2502	543	-0.48	1.0000
Detritus	ST13	Detritus	ST10	0.4208	0.2502	543	1.68	0.9983
Detritus	ST13	Detritus	ST286	0.9224	0.2502	543	3.69	0.0654
Detritus	ST13	Detritus	ST275	0.2385	0.2502	543	0.95	1.0000
Detritus	ST13	Detritus	ST353	1.6799	0.2502	543	6.71	<.0001
Detritus	ST13	Detritus	ST253	-0.2631	0.2502	543	-1.05	1.0000
Detritus	ST13	Detritus	ST342	0.1787	0.2502	543	0.71	1.0000
Detritus	ST13	Detritus	ST267	0.02690	0.2502	543	0.11	1.0000
Detritus	ST78	Detritus	ST277	-0.4095	0.2502	543	-1.64	0.9989
Detritus	ST78	Detritus	ST10	0.1315	0.2502	543	0.53	1.0000
Detritus	ST78	Detritus	ST286	0.6330	0.2502	543	2.53	0.7582
Detritus	ST78	Detritus	ST275	-0.05086	0.2502	543	-0.20	1.0000
Detritus	ST78	Detritus	ST353	1.3906	0.2502	543	5.56	<.0001
Detritus	ST78	Detritus	ST253	-0.5525	0.2502	543	-2.21	0.9288
Detritus	ST78	Detritus	ST342	-0.1107	0.2502	543	-0.44	1.0000
Detritus	ST78	Detritus	ST267	-0.2625	0.2502	543	-1.05	1.0000
Detritus	ST277	Detritus	ST10	0.5410	0.2502	543	2.16	0.9433
Detritus	ST277	Detritus	ST286	1.0425	0.2502	543	4.17	0.0119
Detritus	ST277	Detritus	ST275	0.3587	0.2502	543	1.43	0.9999
Detritus	ST277	Detritus	ST353	1.8001	0.2502	543	7.20	<.0001
Detritus	ST277	Detritus	ST253	-0.1430	0.2502	543	-0.57	1.0000
Detritus	ST277	Detritus	ST342	0.2988	0.2502	543	1.19	1.0000
Detritus	ST277	Detritus	ST267	0.1471	0.2502	543	0.59	1.0000
Detritus	ST10	Detritus	ST286	0.5015	0.2502	543	2.00	0.9771
Detritus	ST10	Detritus	ST275	-0.1823	0.2502	543	-0.73	1.0000
Detritus	ST10	Detritus	ST353	1.2591	0.2502	543	5.03	0.0003
Detritus	ST10	Detritus	ST253	-0.6840	0.2502	543	-2.73	0.6009
Detritus	ST10	Detritus	ST342	-0.2422	0.2502	543	-0.97	1.0000
Detritus	ST10	Detritus	ST267	-0.3939	0.2502	543	-1.57	0.9995
Detritus	ST286	Detritus	ST275	-0.6839	0.2502	543	-2.73	0.6013
Detritus	ST286	Detritus	ST353	0.7576	0.2502	543	3.03	0.3673
Detritus	ST286	Detritus	ST342	-0.7437	0.2502	543	-2.97	0.4086
Detritus	ST286	Detritus	ST267	-0.8955	0.2502	543	-3.58	0.0914
Detritus	ST275	Detritus	ST353	1.4414	0.2502	543	5.76	<.0001
Detritus	ST275	Detritus	ST253	-0.5016	0.2502	543	-2.01	0.9771
Detritus	ST275	Detritus	ST342	-0.05985	0.2502	543	-0.24	1.0000
Detritus	ST275	Detritus	ST267	-0.2116	0.2502	543	-0.85	1.0000
Detritus	ST353	Detritus	ST253	-1.9431	0.2502	543	-7.77	<.0001
Detritus	ST353	Detritus	ST342	-1.5013	0.2502	543	-6.00	<.0001
Detritus	ST353	Detritus	ST267	-1.6530	0.2502	543	-6.61	<.0001
Detritus	ST253	Detritus	ST342	0.4418	0.2502	543	1.77	0.9963
Detritus	ST253	Detritus	ST267	0.2900	0.2502	543	1.16	1.0000
Detritus	ST342	Detritus	ST267	-0.1518	0.2502	543	-0.61	1.0000
Feed	ST13	Feed	ST78	-1.1281	0.2502	543	-4.51	0.0029
Feed	ST13	Feed	ST277	-1.3593	0.2502	543	-5.43	<.0001

Table 5.4. (cont'd)

Treatment 1	Isolate 1	Treatment 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
Feed	ST13	Feed	ST10	-0.2469	0.2502	543	-0.99	1.0000
Feed	ST13	Feed	ST286	-0.7860	0.2502	543	-3.14	0.2891
Feed	ST13	Feed	ST275	-1.6210	0.2502	543	-6.48	<.0001
Feed	ST13	Feed	ST353	-1.4694	0.2502	543	-5.87	<.0001
Feed	ST13	Feed	ST253	-1.2197	0.2502	543	-4.88	0.0006
Feed	ST13	Feed	ST342	-1.3749	0.2502	543	-5.50	<.0001
Feed	ST13	Feed	ST267	2.0485	0.2502	543	8.19	<.0001
Feed	ST78	Feed	ST277	-0.2312	0.2502	543	-0.92	1.0000
Feed	ST78	Feed	ST10	0.8812	0.2502	543	3.52	0.1083
Feed	ST78	Feed	ST286	0.3421	0.2502	543	1.37	1.0000
Feed	ST78	Feed	ST275	-0.4929	0.2502	543	-1.97	0.9818
Feed	ST78	Feed	ST353	-0.3412	0.2502	543	-1.36	1.0000
Feed	ST78	Feed	ST253	-0.09153	0.2502	543	-0.37	1.0000
Feed	ST78	Feed	ST342	-0.2468	0.2502	543	-0.99	1.0000
Feed	ST78	Feed	ST267	3.1766	0.2502	543	12.70	<.0001
Feed	ST277	Feed	ST10	1.1124	0.2502	543	4.45	0.0038
Feed	ST277	Feed	ST286	0.5733	0.2502	543	2.29	0.8963
Feed	ST277	Feed	ST275	-0.2617	0.2502	543	-1.05	1.0000
Feed	ST277	Feed	ST353	-0.1101	0.2502	543	-0.44	1.0000
Feed	ST277	Feed	ST253	0.1396	0.2502	543	0.56	1.0000
Feed	ST277	Feed	ST342	-0.01564	0.2502	543	-0.06	1.0000
Feed	ST277	Feed	ST267	3.4078	0.2502	543	13.62	<.0001
Feed	ST10	Feed	ST286	-0.5391	0.2502	543	-2.15	0.9455
Feed	ST10	Feed	ST275	-1.3741	0.2502	543	-5.49	<.0001
Feed	ST10	Feed	ST353	-1.2224	0.2502	543	-4.89	0.0005
Feed	ST10	Feed	ST253	-0.9727	0.2502	543	-3.89	0.0333
Feed	ST10	Feed	ST342	-1.1280	0.2502	543	-4.51	0.0029
Feed	ST10	Feed	ST267	2.2954	0.2502	543	9.18	<.0001
Feed	ST286	Feed	ST275	-0.8350	0.2502	543	-3.34	0.1804
Feed	ST286	Feed	ST353	-0.6834	0.2502	543	-2.73	0.6030
Feed	ST286	Feed	ST253	-0.4336	0.2502	543	-1.73	0.9972
Feed	ST286	Feed	ST342	-0.5889	0.2502	543	-2.35	0.8664
Feed	ST286	Feed	ST267	2.8345	0.2502	543	11.33	<.0001
Feed	ST275	Feed	ST353	0.1516	0.2502	543	0.61	1.0000
Feed	ST275	Feed	ST253	0.4013	0.2502	543	1.60	0.9992
Feed	ST275	Feed	ST342	0.2461	0.2502	543	0.98	1.0000
Feed	ST275	Feed	ST267	3.6695	0.2502	543	14.67	<.0001
Feed	ST353	Feed	ST253	0.2497	0.2502	543	1.00	1.0000
Feed	ST353	Feed	ST342	0.09443	0.2502	543	0.38	1.0000
Feed	ST353	Feed	ST267	3.5179	0.2502	543	14.06	<.0001
Feed	ST253	Feed	ST342	-0.1553	0.2502	543	-0.62	1.0000
Feed	ST253	Feed	ST267	3.2682	0.2502	543	13.06	<.0001
Feed	ST342	Feed	ST267	3.4234	0.2502	543	13.68	<.0001
Water	ST13	Water	ST78	-0.1061	0.2502	543	-0.42	1.0000
Water	ST13	Water	ST277	0.6633	0.2502	543	2.65	0.6676
Water	ST13	Water	ST10	0.02875	0.2502	543	0.11	1.0000
Water	ST13	Water	ST286	-0.4008	0.2502	543	-1.60	0.9993
Water	ST13	Water	ST275	-0.3650	0.2502	543	-1.46	0.9999
Water	ST13	Water	ST353	0.03091	0.2502	543	0.12	1.0000
Water	ST13	Water	ST253	-0.1133	0.2502	543	-0.45	1.0000
Water	ST13	Water	ST342	-0.4748	0.2502	543	-1.90	0.9891
Water	ST13	Water	ST267	0.08658	0.2502	543	0.35	1.0000
Water	ST78	Water	ST277	0.7694	0.2502	543	3.08	0.3335

Table 5.4. (cont'd)

Treatment 1	Isolate 1	Treatment 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
Water	ST78	Water	ST10	0.1349	0.2502	543	0.54	1.0000
Water	ST78	Water	ST286	-0.2947	0.2502	543	-1.18	1.0000
Water	ST78	Water	ST275	-0.2588	0.2502	543	-1.03	1.0000
Water	ST78	Water	ST353	0.1371	0.2502	543	0.55	1.0000
Water	ST78	Water	ST253	-0.00712	0.2502	543	-0.03	1.0000
Water	ST78	Water	ST342	-0.3686	0.2502	543	-1.47	0.9998
Water	ST78	Water	ST267	0.1927	0.2502	543	0.77	1.0000
Water	ST277	Water	ST10	-0.6345	0.2502	543	-2.54	0.7540
Water	ST277	Water	ST286	-1.0641	0.2502	543	-4.25	0.0085
Water	ST277	Water	ST275	-1.0282	0.2502	543	-4.11	0.0148
Water	ST277	Water	ST353	-0.6323	0.2502	543	-2.53	0.7601
Water	ST277	Water	ST253	-0.7765	0.2502	543	-3.10	0.3140
Water	ST277	Water	ST342	-1.1380	0.2502	543	-4.55	0.0025
Water	ST277	Water	ST267	-0.5767	0.2502	543	-2.31	0.8902
Water	ST10	Water	ST286	-0.4296	0.2502	543	-1.72	0.9976
Water	ST10	Water	ST275	-0.3937	0.2502	543	-1.57	0.9995
Water	ST10	Water	ST353	0.002160	0.2502	543	0.01	1.0000
Water	ST10	Water	ST253	-0.1420	0.2502	543	-0.57	1.0000
Water	ST10	Water	ST342	-0.5035	0.2502	543	-2.01	0.9760
Water	ST10	Water	ST267	0.05783	0.2502	543	0.23	1.0000
Water	ST286	Water	ST275	0.03587	0.2502	543	0.14	1.0000
Water	ST286	Water	ST353	0.4317	0.2502	543	1.73	0.9974
Water	ST286	Water	ST253	0.2876	0.2502	543	1.15	1.0000
Water	ST286	Water	ST342	-0.07394	0.2502	543	-0.30	1.0000
Water	ST286	Water	ST267	0.4874	0.2502	543	1.95	0.9843
Water	ST275	Water	ST353	0.3959	0.2502	543	1.58	0.9994
Water	ST275	Water	ST253	0.2517	0.2502	543	1.01	1.0000
Water	ST275	Water	ST342	-0.1098	0.2502	543	-0.44	1.0000
Water	ST275	Water	ST267	0.4515	0.2502	543	1.80	0.9948
Water	ST353	Water	ST253	-0.1442	0.2502	543	-0.58	1.0000
Water	ST353	Water	ST342	-0.5057	0.2502	543	-2.02	0.9746
Water	ST353	Water	ST267	0.05567	0.2502	543	0.22	1.0000
Water	ST253	Water	ST342	-0.3615	0.2502	543	-1.44	0.9999
Water	ST253	Water	ST267	0.1998	0.2502	543	0.80	1.0000
Water	ST342	Water	ST267	0.5613	0.2502	543	2.24	0.9160

Table 5.5. Weekly mean concentrations (in $\log_{10}$ colony forming units) ± (standard error) of the ten *Flavobacterium psychrophilum* sequence types (i.e., genetic variants) used in this study, as measured in the well water with raceway detritus microcosm.

	Week									
ST	0	1	2	3	4	5	6	7	8	13
13	8.10 (0.00)	8.60 (0.05)	8.74 (0.04)	8.87 (0.06)	8.70 (0.04)	8.74 (0.14)	8.57 (0.09)	7.89 (0.41)	7.24 (0.24)	4.65 (0.25)
10	8.15 (0.00)	8.39 (0.21)	8.35 (0.05)	8.20 (0.20)	8.44 (0.26)	8.00 (0.00)	7.70 (0.04)	7.22 (0.74)	6.70 (0.00)	4.74 (0.19)
78	8.25 (0.00)	8.65 (0.05)	8.69 (0.09)	8.65 (0.05)	8.70 (0.04)	8.51 (0.03)	8.18 (0.00)	7.18 (0.00)	6.50 (0.50)	3.89 (0.41)
275	8.15 (0.00)	8.57 (0.03)	8.72 (0.02)	8.54 (0.06)	8.53 (0.13)	7.90 (0.00)	7.54 (0.06)	6.99 (0.18)	6.91 (0.09)	5.85 (0.00)
342	7.85 (0.00)	8.57 (0.09)	8.70 (0.04)	8.30 (0.00)	8.60 (0.00)	8.48 (0.00)	8.33 (0.15)	7.24 (0.06)	7.30 (0.00)	4.94 (0.19)
267	7.33 (0.00)	8.33 (0.15)	8.54 (0.06)	8.46 (0.28)	8.69 (0.09)	8.60 (0.00)	8.41 (0.24)	7.59 (0.59)	7.87 (0.03)	5.99 (0.21)
277	8.15 (0.00)	8.60 (0.05)	8.55 (0.15)	8.63 (0.03)	8.63 (0.03)	8.68 (0.28)	8.42 (0.042)	8.57 (0.09)	7.70 (0.04)	5.37 (0.37)
286	8.15 (0.00)	8.44 (0.04)	8.42 (0.12)	8.39 (0.09)	8.30 (0.00)	8.09 (0.09)	7.30 (0.00)	5.83 (0.83)	5.50 (0.50)	2.44 (0.73)
253	8.15 (0.00)	8.45 (0.15)	8.54 (0.00)	8.60 (0.30)	8.59 (0.11)	8.48 (0.00)	8.35 (0.05)	7.73 (0.08)	8.10 (0.20)	7.72 (0.41)
353	8.15 (0.00)	8.15 (0.15)	8.20 (0.20)	7.70 (0.00)	8.00 (0.00)	7.85 (0.00)	6.30 (0.00)	4.35 (0.95)	4.59 (0.59)	0.00 (0.00)

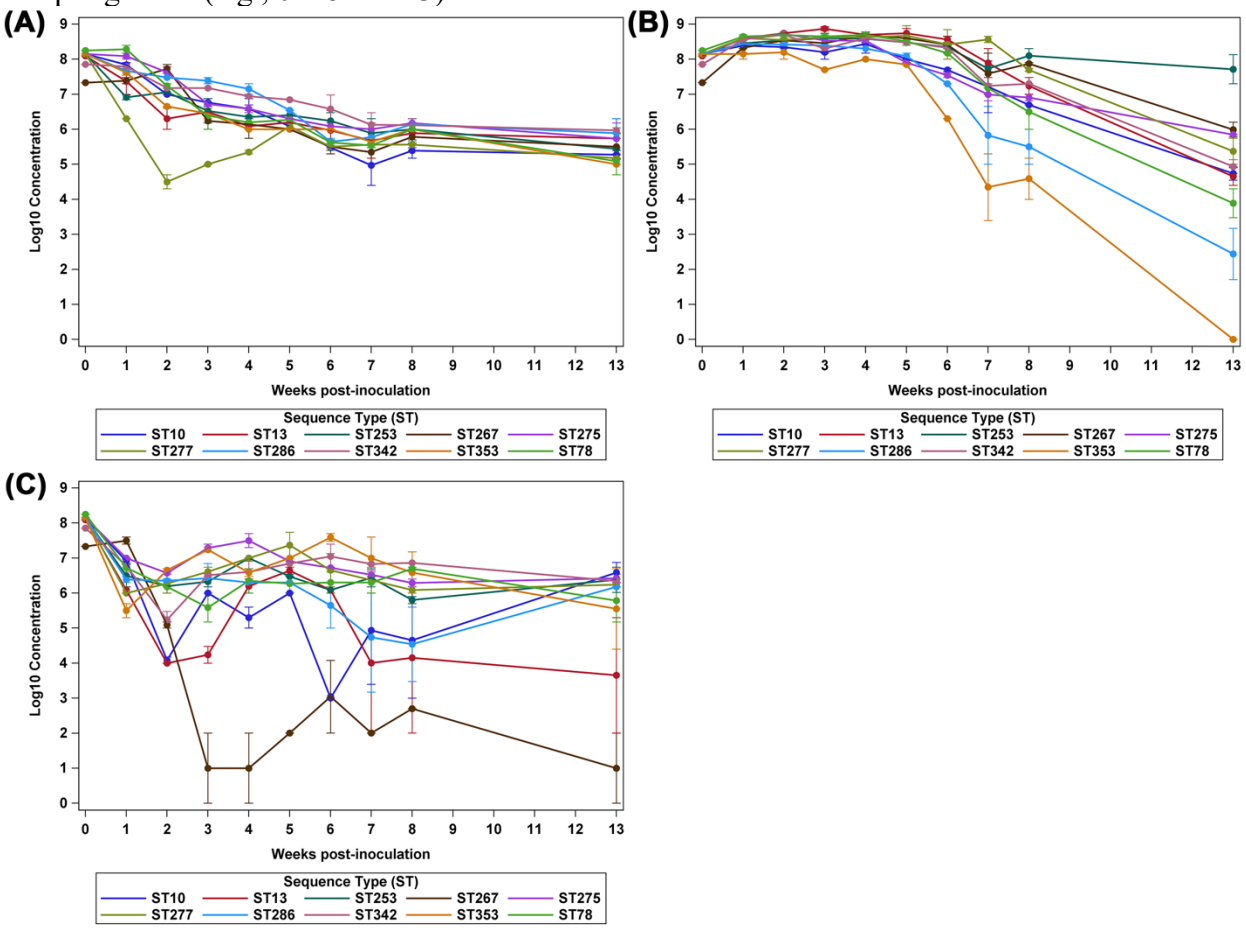
Table 5.6. Weekly mean concentrations (in $\log_{10}$ colony forming units) ± (standard error) of the ten *Flavobacterium psychrophilum* sequence types (i.e., genetic variants) used in this study, as measured in the well water with commercial trout feed microcosm.

	Week									
ST	0	1	2	3	4	5	6	7	8	13
13	8.10 (0.00)	6.09 (0.09)	4.00 (0.00)	4.24 (0.24)	6.20 (0.20)	6.64 (0.10)	6.09 (0.09)	4.00 (2.00)	4.15 (2.15)	3.65 (1.65)
10	8.15 (0.00)	6.91 (0.07)	4.09 (0.09)	6.00 (0.00)	5.30 (0.30)	6.00 (0.00)	3.00 (0.00)	4.94 (1.54)	4.65 (1.65)	6.59 (0.29)
78	8.25 (0.00)	6.72 (0.02)	6.18 (0.00)	5.59 (0.41)	6.35 (0.35)	6.27 (0.27)	6.30 (0.30)	6.30 (0.30)	6.70 (0.04)	5.79 (0.61)
275	8.15 (0.00)	7.00 (0.00)	6.57 (0.03)	7.29 (0.11)	7.50 (0.20)	6.90 (0.00)	6.72 (0.02)	6.53 (0.13)	6.29 (0.11)	6.42 (0.12)
342	7.85 (0.00)	6.78 (0.18)	5.24 (0.24)	6.51 (0.03)	6.60 (0.3)	6.85 (0.00)	7.05 (0.35)	6.83 (0.02)	6.86 (0.01)	6.34 (0.05)
267	7.33 (0.00)	7.50 (0.10)	5.09 (0.09)	1.00 (1.00)	1.00 (1.00)	2.00 (0.00)	3.04 (1.04)	2.00 (0.00)	2.70 (0.00)	1.00 (1.00)
277	8.15 (0.00)	6.00 (0.00)	6.27 (0.27)	6.61 (0.13)	7.00 (0.00)	7.37 (0.37)	6.65 (0.48)	6.37 (0.37)	6.09 (0.09)	6.24 (0.06)
286	8.15 (0.00)	6.39 (0.09)	6.35 (0.05)	6.42 (0.42)	6.30 (0.30)	6.30 (0.00)	5.65 (0.65)	4.74 (1.56)	4.54 (1.06)	6.18 (0.00)
253	8.15 (0.00)	6.49 (0.32)	6.20 (0.20)	6.33 (0.15)	7.00 (0.00)	6.48 (0.00)	6.09 (0.09)	6.44 (0.26)	5.80 (0.10)	6.38 (0.36)
353	8.15 (0.00)	5.50 (0.20)	6.65 (0.05)	7.24 (0.06)	6.59 (0.11)	7.00 (0.00)	7.59 (0.11)	7.00 (0.60)	6.59 (0.59)	5.55 (1.15)

Table 5.7. Differences of least square mean estimates ± standard error (SE) for the interaction between treatment group (e.g., raceway "detritus", commercial trout "feed", or "water" only) and ten *Flavobacterium psychrophilum* variants (e.g., ST13, ST78, ST277, ST10, ST286, ST275, ST353, ST253, ST342, ST267). Pairwise comparisons between treatments for each variant are provided. Tukey-Kramer adjusted *P*-values for multiple comparisons are shown ($\alpha = 0.05$).

Treatment 1	Isolate 1	Treatment 2	Isolate 2	Estimate	SE	DF	*t*-value	*P*-value
Detritus	ST13	Feed	ST13	2.6924	0.2502	543	10.76	<.0001
Detritus	ST13	Water	ST13	1.6254	0.2502	543	6.50	<.0001
Feed	ST13	Water	ST13	-1.0670	0.2502	543	-4.26	0.0081
Detritus	ST78	Feed	ST78	1.2749	0.2502	543	5.10	0.0002
Detritus	ST78	Water	ST78	1.2299	0.2502	543	4.92	0.0005
Feed	ST78	Water	ST78	-0.04497	0.2502	543	-0.18	1.0000
Detritus	ST277	Feed	ST277	1.4532	0.2502	543	5.81	<.0001
Detritus	ST277	Water	ST277	2.4088	0.2502	543	9.63	<.0001
Feed	ST277	Water	ST277	0.9556	0.2502	543	3.82	0.0422
Detritus	ST10	Feed	ST10	2.0246	0.2502	543	8.09	<.0001
Detritus	ST10	Water	ST10	1.2333	0.2502	543	4.93	0.0004
Feed	ST10	Water	ST10	-0.7913	0.2502	543	-3.16	0.2758
Detritus	ST286	Feed	ST286	0.9840	0.2502	543	3.93	0.0284
Detritus	ST286	Water	ST286	0.3022	0.2502	543	1.21	1.0000
Feed	ST286	Water	ST286	-0.6818	0.2502	543	-2.73	0.6082
Detritus	ST275	Feed	ST275	0.8329	0.2502	543	3.33	0.1844
Detritus	ST275	Water	ST275	1.0220	0.2502	543	4.08	0.0163
Feed	ST275	Water	ST275	0.1891	0.2502	543	0.76	1.0000
Detritus	ST353	Feed	ST353	-0.4569	0.2502	543	-1.83	0.9937
Detritus	ST353	Water	ST353	-0.02363	0.2502	543	-0.09	1.0000
Feed	ST353	Water	ST353	0.4333	0.2502	543	1.73	0.9972
Detritus	ST253	Feed	ST253	1.7359	0.2502	543	6.94	<.0001
Detritus	ST253	Water	ST253	1.7753	0.2502	543	7.10	<.0001
Feed	ST253	Water	ST253	0.03943	0.2502	543	0.16	1.0000
Detritus	ST342	Feed	ST342	1.1388	0.2502	543	4.55	0.0024
Detritus	ST342	Water	ST342	0.9720	0.2502	543	3.89	0.0337
Feed	ST342	Water	ST342	-0.1668	0.2502	543	-0.67	1.0000
Detritus	ST267	Feed	ST267	4.7140	0.2502	543	18.84	<.0001
Detritus	ST267	Water	ST267	1.6851	0.2502	543	6.74	<.0001
Feed	ST267	Water	ST267	-3.0289	0.2502	543	-12.11	<.0001

Figure 5.1. 13-week culturability of *Flavobacterium psychrophilum* sequence type (ST) 10, ST13, ST78, ST253, ST267, ST275, ST277, ST286, ST342, and ST353 in microcosms containing (A) sterilized well water only, (B) sterilized well water with raceway detritus, or (C) sterilized well water with commercial trout feed. Standard error between replicate flasks on each sampling week (e.g., 0 – 8 and 13) are shown.



Chapter 6:

Conclusions and future research

6.1. Conclusions

Despite nearly a century of research, effective prevention and control of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS) remains elusive. Recent studies have identified multiple predominating *Flavobacterium psychrophilum* multilocus sequence typing (MLST) clonal complexes (CCs) and sequence types (STs; i.e., genetic variants) causing BCWD epizootics globally, including in the United States of America (USA). Studies have also begun to advance our understanding of how *F. psychrophilum* diversity relates to virulence, susceptibility to antimicrobials, host species association, and geographical distribution. Resulting from these advancements, multiple unanswered questions emerged regarding the potential factors associated with the apparent success of some of these variants, such as their affinity for certain host species, the influence of the artificial rearing environment, differences in shedding dynamics among variants and salmonid species, and the adequacy of current gold-standard research/diagnostic culture media. These knowledge gaps are likely contributing to productivity losses for salmonid farms and challenges in hatchery-based conservation efforts by impeding BCWD diagnosis, and the development of effective BCWD management, prevention, and control strategies. Towards filling these knowledge gaps, I conceptualized, designed, and executed a series of in vitro and in vivo experiments.

In Chapter 2, I developed two new culture media, *F. psychrophilum* medium-A and -B (FPM-A and FPM-B), that significantly improved the recovery of a wide diversity of *F. psychrophilum* variants in the laboratory. Considering most BCWD research projects have a culture component, these new media will be instrumental in helping researchers achieve their study goals, and ultimately will contribute to the mitigation of BCWD-associated losses. Indeed, FPM-A could be considered the backbone of Chapters 3 – 5, as findings of those studies may

have been obscured by a less sensitive culture medium. Apart from demonstrating the utility of FPM-A and FPM-B under controlled laboratory conditions, both media were highly successful under field conditions, as evidenced by their recovery of *F. psychrophilum* from naturally infected Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), and steelhead trout (*O. mykiss*) broodstock when compared to the current gold-standard medium for *F. psychrophilum* detection, tryptone yeast extract salts agar. Since the development of FPM-A, it has also been used to recover *F. psychrophilum* from multiple salmonid species experiencing BCWD in Michigan state fish hatcheries, and thus has been crucial for not only BCWD diagnosis but guiding timely treatment recommendations. Similarly, FPM-A is now also being used by other laboratories and with reportedly good success. Thus far, these new media have shown tremendous promise for not only BCWD research efforts but the widespread diagnosis of BCWD.

In Chapter 3, I investigated the host specificity of *F. psychrophilum* variants US19-COS, US62-ATS, and US87-RBT, which belong to MLST CCs (e.g., CC-ST9, CC-ST232, and CC-ST10) commonly associated with a single salmonid species - coho salmon, Atlantic salmon, and rainbow trout, respectively. This was the first study to simultaneously cross-challenge multiple salmonid species with multiple putatively host specific *F. psychrophilum* variants via immersion. Such an experimental design was necessary to appropriately assess the host specificity of each variant as it not only controlled for differences in inoculum preparation, age-related susceptibility, and environmental factors but also avoided bypassing external immune defenses. Study results supported MLST-based observations that some *F. psychrophilum* variants (e.g., US87-RBT) are host specific, whereas others (e.g., US19-COS and US62-ATS) have a wider host range. The underlying pathogen/host mechanisms contributing to these findings require

further investigation, but given that each variant belonged to a different serogroup, which may affect how the host "views" the pathogen, it is plausible sero-variation played a role. Nonetheless, assessing the host specificity of *F. psychrophilum* was crucial as it can guide the selection of variants for inclusion in BCWD vaccines, support the evaluation of vaccine efficacy, provide insight into BCWD transmission dynamics, and aid in the further development of BCWD-resistant salmonids.

In Chapter 4, I assessed *F. psychrophilum* shedding dynamics (e.g., time to shedding, shedding rate and duration) in rainbow trout and, for the first time, in coho salmon and Atlantic salmon. Live fish from all examined species were capable of shedding *F. psychrophilum*, with notable differences in shedding patterns and durations being observed across host species and *F. psychrophilum* variants. These differences may imply the existence of diverse transmission strategies, potentially varying based on the specific interaction between the host species and the infecting *F. psychrophilum* variant. Bridging the findings from Chapters 3 and 4, it seems that host specificity of *F. psychrophilum* variants may provide distinct advantages. In Chapter 3, US87-RBT was specific to rainbow trout and found to persist in survivors, whereas the other two, more generalist variants, were not. In Chapter 4, US87-RBT displayed the longest shedding duration in live fish and, once again, was the only variant recovered from survivors. Although survivor data for coho salmon was inconclusive due to total mortality, these findings suggest a possible correlation between host specificity, shedding duration, and survival in hosts. Therefore, host specificity may not only influence survival but also play a role in shaping transmission dynamics by affecting the shedding behavior of *F. psychrophilum*. Dead fish from all examined species were also found capable of shedding *F. psychrophilum*, and did so at higher rates and for a longer duration compared to live fish. This finding underscores the necessity of efficient

management strategies aimed at prompt removal of dead fish from rearing units as dead fish shedding also appears to be an important transmission strategy for *F. psychrophilum*. Whether the shedding dynamics observed herein are emulated by other *F. psychrophilum* variants remains to be determined but warrants further investigation as it will lead to a more comprehensive understanding of *F. psychrophilum* transmission dynamics, and thus potentially inform more widely adoptable BCWD management strategies.

In Chapter 5, I assessed the culturability of ten diverse *F. psychrophilum* variants in microcosms that simulate fish farm/hatchery rearing unit environments. All *F. psychrophilum* variants demonstrated remarkable adaptability by persisting under nutrient-limited (e.g., well water only) and nutrient-rich (e.g., raceway detritus and commercial trout feed) conditions, suggesting potential environmental reservoirs for this bacterium. However, and for the first time, it was apparent that most *F. psychrophilum* variants proliferated best in the presence of raceway detritus compared to the other microcosms, as evidenced by maintaining significantly higher concentrations (e.g., ~10^7 – 10^8 colony forming units/mL) for several weeks. This finding is particularly significant, as it suggests *F. psychrophilum* could not only survive but thrive in the presence of raceway detritus, possibly posing a greater transmission risk, thereby underscoring the importance of regularly removing detritus from rearing units. Besides these similarities, it was also evident that some *F. psychrophilum* variants may be better suited to these environments, which could partially explain their repeated recovery from hatcheries over multiple years. Overall, examining this important aspect of BCWD ecology offered valuable insight into additional factors potentially affecting transmission dynamics. Applying these findings to fish farm and hatchery facilities, it may be beneficial to invest more time and resources into thoroughly cleaning rearing units during disease outbreaks. At the same time,

avoiding overfeeding by observing feeding behavior and adjusting as needed may lower *F. psychrophilum* loads in diseased rearing units and therefore disease spread. These measures could significantly aid in controlling BCWD-associated losses, and enhance the overall health and productivity of fish farms and hatcheries.

In total, the findings of my dissertation support the hypothesis that *F. psychrophilum* intraspecific diversity plays an important role in shaping our understanding of BCWD ecology. In this context, I found *F. psychrophilum* diversity influences our ability to detect and diagnose BCWD, and affects its interaction with its host and environment. Thus, it is crucial that future studies also consider intraspecific diversity when selecting study isolates to ensure a comprehensive understanding of BCWD ecology, which shapes how we develop and implement BCWD management, prevention, and control strategies. Indeed, this approach holds significant promise for mitigating losses of multiple salmonid species, thereby improving fish farm productivity and hatchery conservation and stock enhancement efforts.

6.2. Future research

Although the findings of my dissertation made significant strides in optimizing BCWD research and diagnostic tools, elucidating host-pathogen interactions, unraveling *F. psychrophilum* transmission dynamics, and exploring its persistence in fish farm and hatchery environments (i.e,. BCWD ecology), they have also brought to light numerous new and unexplored questions. These knowledge gaps, if pursued, hold the potential to further deepen our understanding in each of these facets and, ultimately, enhance our ability to diagnose, manage, and control BCWD more effectively.

Building upon Chapter 2, a critical avenue for future research lies in the evaluation of the diagnostic sensitivity and specificity of the newly developed FPM-A and FPM-B culture media

via controlled laboratory experiments. The observed limited to non-existent non-target bacterial growth during my field experiments provides promising preliminary evidence of high specificity. However, to validate this, more extensive testing is necessary. Furthermore, refining the specificity of these media by incorporating antibiotics, such as the neomycin sulfate used during field testing, could further suppress non-target bacterial growth. However, the potential impact of this antibiotic (or others) on the recovery of *F. psychrophilum* variants needs to be assessed to ensure any enhancement in specificity doesn't compromise the sensitivity of the medium. In the context of sensitivity, the new media successfully detected *F. psychrophilum* from multiple naturally infected salmonids under field conditions and from more fish than TYES. However, controlled laboratory experiments are critical for definitively assessing diagnostic sensitivity, as the true infection prevalence among the fish examined herein was unknown. Another avenue of future research is testing the ability of the new media to recover *F. psychrophilum* from other tissues, such as reproductive fluids, or environmental samples. Considering disease dynamics are complex in natural and aquaculture settings, the ability to detect and isolate the pathogen from various sources could greatly aid in early detection and effective disease control measures. Finally, comparing the sensitivity of the new culture media to molecular assays (e.g., conventional PCR, qPCR, and LAMP) could offer a more comprehensive evaluation of their performance. This comparison would provide valuable insights into the capability of the media to detect low-level infections and contribute to a broader understanding of the BCWD prevalence and distribution.

Chapter 3 marked a significant step forward in the understanding of *F. psychrophilum* disease ecology by cross-challenging multiple salmonid species via immersion with putatively host specific variants. Yet, to gain a more comprehensive understanding, it would be beneficial

to evaluate additional isolates that belong to the same variants tested in this Chapter, as well as variants that have not yet been tested. It could also be insightful to investigate the ability of these variants to cause disease in closely related salmonid species that appear less susceptible to *F. psychrophilum* infection, such as Chinook salmon. Such an experiment coupled with transcriptomics may help to identify markers that make Chinook salmon less susceptible to BCWD. These findings could then possibly be leveraged to selectively breed other salmonid species to have similar disease-resistance traits. Our study also highlighted that *F. psychrophilum* variant US62-ATS, belonging to molecular serogroup 1 (seemingly equivalent to serogroup Fd), was highly virulent to rainbow trout and Atlantic salmon. Although serogroup Fd is known to affect rainbow trout, it is not often that this serogroup has been reported in Atlantic salmon. In this context, knowledge regarding the serogroups and serotypes affecting salmonids in the USA is scant but is of utmost importance considering this information will likely be useful in developing a cross-protective BCWD vaccine. Thus, additional studies addressing the serodiversity of salmonids in the USA are warranted.

In Chapter 4, I found that both live and dead Atlantic salmon, coho salmon, and rainbow trout shed *F. psychrophilum*, but dead fish did so at higher rates and for a longer duration. However, the implications of these findings for disease transmission are unclear partly due to the contrasting behaviors of live and dead fish. Live fish, despite shedding less bacteria, might pose a higher transmission risk due to their mobility. In contrast, although dead fish shed higher bacterial loads, they are stationary, which might limit transmission to a localized area. Together, this highlights the need to understand host behavior in conjunction with shedding dynamics. In a similar context, cohabitation studies have proven valuable for understanding disease transmission in rainbow trout, but similar research has yet to be conducted with Atlantic salmon

and coho salmon. Hence, future studies should focus on confirming the importance of horizontal transmission in these species. Moreover, comparing the infectivity of *F. psychrophilum* shed from live and dead fish could significantly influence management strategies. Synthesizing findings from Chapters 3 and 4, the consequences of generalist variants like US62-ATS, which was highly virulent to both Atlantic salmon and rainbow trout should be examined. In this context, future research elucidating whether US62-ATS is more likely to be shed from and successfully infect the same-species (e.g., Atlantic salmon shedding and transmitting to Atlantic salmon) or different-species (e.g., Atlantic salmon shedding and transmitting to rainbow trout) should be investigated as it could have significant implications for transmission in facilities rearing multiple salmonid species. Also observed in Chapters 3 and 4 was the ability of US87-RBT (i.e., the rainbow trout specific variant) to persist in survivors, suggesting some fish become carriers. In this context, understanding the potential role of carriers in perpetuating BCWD epizootics is worth further investigation. Notably, investigating how long carriers harbor *F. psychrophilum*, whether they shed the bacterium and under what conditions (e.g., times of increased stress), could further contribute to our understanding of BCWD transmission dynamics.

Expanding on the insights from Chapter 5, the role of water chemistry in *F. psychrophilum* proliferation and survival should be considered for future studies. Like the known effect of water hardness on biofilm formation in *F. columnare*, alterations in water chemistry could potentially influence *F. psychrophilum* biofilm formation and consequently, this bacterium's persistence in fish farms and hatcheries. By manipulating water conditions, it may be possible to create an environment less conducive for *F. psychrophilum*, thereby reducing bacterial loads in fish farms and hatcheries. My findings indicated that the presence of

commercial trout feed and raceway detritus enhanced the proliferation of certain *F. psychrophilum* variants. However, the virulence of *F. psychrophilum* after being exposed to these conditions for various time periods, and the likelihood of transmission from these sources to fish, remains to be elucidated. Consequently, future studies should focus on these areas to improve our understanding of the complex disease dynamics in fish farms and hatcheries. Furthermore, understanding the mechanisms that facilitate *F. psychrophilum* persistence outside its host is crucial. Genomic studies focusing on variants exhibiting differential survival capacities could provide key insights into these mechanisms. For instance, exploring the role or existence of cyanophycin, a putative energy storing polymer, in *F. psychrophilum* variants may shed light on potential strategies this bacterium employs for persistence outside its host. Understanding these aspects would significantly enhance our capacity to mitigate BCWD outbreaks and improve fish farm and hatchery disease management strategies.