Comparing the Richness of Pathogens around Open-Net Atlantic Salmon Marine Aquaculture Farms to Fallow Farms and Non-Farm Areas in Passamaquoddy Bay, New Brunswick

An Analysis of Methods and Proposal of Future Sampling Considerations

Honours Thesis

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Abstract

The Committee on the Status of Endangered Wildlife in Canada considers Atlantic salmon (*Salmo salar*) in the outer Bay of Fundy to be endangered. Despite decreases in fishing pressure since wild Atlantic salmon were overfished in the 1960s and 1970s, Atlantic salmon abundances have continued to drop across Atlantic North America, including in the Bay of Fundy. Atlantic salmon have been important for fisheries and are important for nutrient transfer across aquatic environments. Understanding factors that perpetuate Atlantic salmon declines is crucial for wild salmon population recovery efforts and restoration of the historical and ecological roles of the species. Salmon aquaculture currently poses a threat to outer Bay of Fundy wild Atlantic salmon. Pathogen transfer from open-net aquaculture farms into the surrounding water column is a potential risk for wild salmon. However, agents of oceanic Atlantic salmon mortality, and specifically how Atlantic salmon populations are impacted by marine salmon farms, remain largely undocumented in the literature. This study aimed to address knowledge gaps surrounding the impacts of marine aquaculture on wild Atlantic salmon by answering the question: how does the richness of pathogens differ around open-net Atlantic salmon marine aquaculture farms compared to fallow farms and non-farm areas in Passamaquoddy Bay, NB? Ocean water was sampled adjacent to active and fallow open-net pen salmon aquaculture sites, as well as at non-farm sites, in Passamaquoddy Bay. Water was filtered to capture pathogen eDNA in the water column. Quantitative PCR analysis of filters showed no detection of RNA from Piscine orthoreovirus or Atlantic salmon cold-water vibriosis (Atlantic salmon pathogens) and no detection of Atlantic salmon DNA or RNA at any of the tested sites, with the exception of one active site (where a small amount of Atlantic salmon DNA was detected). These results reveal potential issues with experimental design, including possible challenges associated with sampling distances from the net pens and tidal effects, pre-filter clogging, and bleach contamination. Avenues to address these challenges have been presented and should be considered by future studies, as well as used to inform continued development of global eDNA sampling standards and protocols.

1. Introduction

Atlantic salmon (*Salmo salar*) in the outer Bay of Fundy are considered endangered through COSEWIC (the Committee on the Status of Endangered Wildlife in Canada) assessment (DFO 2019a). In the 1960s and 1970s, overfishing of wild Atlantic salmon resulted in extensive decreases in populations (Parrish et al. 1998). Despite subsequent reductions in fishing pressure (Parrish et al. 1998), decreased abundances of Atlantic salmon have continued across Atlantic North America, including in the Bay of Fundy (DFO 2019b). Salmon populations in the inner Bay of Fundy became so low in the late 1900s that they risked regional extinction (Lacroix 2014). In the outer Bay of Fundy, salmon populations experienced slower, but still significant, reductions, with a 64% decrease in population size between 2001 and 2016 (Lacroix 2014; DFO 2019a).

Historically, wild Atlantic salmon have been a food source targeted by commercial fisheries, and are also important to indigenous and recreational fishers (COSEWIC 2011). Atlantic salmon are anadromous (DFO 2019a), meaning that they exist in freshwater and saltwater at different life stages (NOAA 2017). As such, Atlantic salmon also play a role in nutrient transfer across different river and oceanic environments (COSEWIC 2011). Currently, no wild fisheries are permitted to operate in the outer Bay of Fundy due to low wild salmon populations (DFO 2019a). Understanding factors that may contribute to and perpetuate the decline of wild Atlantic salmon is important when considering recovery efforts for wild salmon populations and restoration of the historical and ecological roles of the species.

It is known that reduced Atlantic salmon abundances are likely a result of salmon deaths that occur while at sea. However, exact agents of Atlantic salmon losses in the marine environment that contribute to population level declines remain undocumented in the literature (Lacroix & Knox 2005; Lacroix 2014). As well, despite being identified as a key stage in Atlantic salmon survival, little is known about the migration of juvenile Atlantic salmon from their rivers of origin to the ocean and the causes of impeded salmon success during this journey (Lacroix & Knox 2005).

Salmon aquaculture has been listed as a current threat to wild Atlantic salmon in the outer Bay of Fundy (DFO 2014), with the release of pathogens from aquaculture farms into the surrounding water column identified as a threat posed by open-net farms to wild salmon (Naylor et al. 2005). Globally, aquaculture is an important industry. As of 2016, the amount of global marine fish aquaculture for food production was 28.7 million tonnes (FAO 2018). Worldwide, Canada is the fourth largest country for Atlantic salmon farming (Government of New Brunswick [date unknown]), with New Brunswick comprising much (40%) of Canadian salmon farming (Government of New Brunswick [date unknown]; Carr et al. 1997). Salmon aquaculture has, however, been identified as one of the biggest risks to wild Atlantic salmon in the outer Bay of Fundy (DFO 2014).

Most Canadian Atlantic salmon production, including salmon farming in Passamaquoddy Bay (NB), occurs with the use of marine open-net pens (Weston 2013; Mayer 2018). Generally, these open-net pens are clusters of between six and twenty-four cages with netting that extends down into the ocean. Salmon in these cages are hatchery raised (Weston 2013). Open-net pens permit ocean water to move through them, allowing nutrients and waste from the cages to move into the ocean water neighbouring the cages (Brager et al. 2015).

Aquaculture net pens contain high abundances of salmon in a confined space, allowing amplification of pathogens and diseases, which can then spread into the surrounding ocean and could possibly infect wild salmon migrating near the net pens (Naylor et al. 2005). A pathogen is a living or non-living entity that “causes disease” (Alberts et al. 2002). Because pathogens can build up in marine open-net pens, the Bay of Fundy in New Brunswick has been divided into six Bay Management Areas (BMAs) to limit disease transfers associated with salmon aquaculture. BMAs contain aquaculture farms that are operational for three-year periods (DFO 2010; Gardner Pinfold Consultants Inc. 2016). Salmon within a BMA are removed from aquaculture sites after their three-year active period (DFO 2010). Aquaculture sites that were used in past years but are not currently stocked are known as fallow sites (Gardner Pinfold Consultants Inc. 2016).

Environmental DNA (eDNA) has been used to detect the presence of pathogens (which contain DNA; Alberts et al. 2002) in freshwater (Schmidt et al. 2013), and can also be applied to the marine environment (Berry et al. 2019). eDNA has been defined by Thomsen and Willerslev as "genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material" (Thomsen & Willerslev 2015). eDNA was first developed in the 1980s to overcome constraints related to "traditional detection methods" (Díaz-Ferguson & Moyer 2014) and is used across many fields. For example, eDNA has allowed researchers to locate freshwater invasive species and "estimate marine fish biodiversity" (Díaz-Ferguson & Moyer 2014). eDNA also possibly can be used to determine pathogen prevalence around marine salmon aquaculture facilities (Peter et al. 2018).

Low salmon survival across the Atlantic Ocean has been found in correlation with the presence of salmon aquaculture facilities (Ford & Myers 2008; DFO 2014). Nonetheless, the magnitudes of marine salmon farm impacts on specific wild Atlantic salmon populations are still poorly researched (Ford & Myers 2008). Disease transfer to wild salmon populations from salmon farms has been identified as a risk aquaculture poses to salmon populations (Naylor et al. 2005). There is therefore a need for more extensive research regarding impacts of pathogens from farmed salmon on wild Atlantic salmon in the Bay of Fundy.

The goal of this study was to better understand a possible mechanism of wild Atlantic salmon mortality in the marine environment and address existent gaps in research pertaining to the impacts of marine aquaculture on wild salmon in Atlantic Canada by investigating the richness of known pathogens of Atlantic salmon surrounding aquaculture open-net pens in Passamaquoddy Bay, NB, located in the Outer Bay of Fundy (Lacroix & Knox 2005). This study specifically aimed to answer the question: how does the richness of pathogens differ around open-net Atlantic salmon marine aquaculture farms compared to fallow farms and non-farm areas in Passamaquoddy Bay, NB? It was hypothesized that a greater richness of pathogens would be present in water samples collected in close proximity to Atlantic salmon aquaculture pens when compared to reference sites located far from marine aquaculture pens. It was also hypothesised that water samples collected around fallow farms would have a lower richness of

pathogens compared to active farms, but a higher richness of pathogens compared to reference (non-farm) sites.

This study encompasses the Quoddy region of the Bay of Fundy, however, the study region is referred to as Passamaquoddy Bay for the purposes of this paper. This study considers marine Atlantic salmon open-net pen aquaculture in this region. A two-week sampling period in July of 2019 is the temporal span of the present study.

To answer the research question, ocean water samples were taken adjacent to active and fallow open-net pen Atlantic salmon marine aquaculture sites, as well as at reference (non-farm) sites, in Passamaquoddy Bay. Water was filtered to capture pathogens present in the water samples, and eDNA was used to identify pathogen richness. The aim was for results to be used to understand pathogen composition (specifically the presence or absence of known Atlantic salmon pathogens, with results expected to be qualitative in nature) around Atlantic salmon open-net pens in Passamaquoddy Bay compared to fallow and non-farm sites.

2. Literature Review

This literature review will explore information related to Atlantic salmon and the decline of this species. In particular, Bay of Fundy and Quoddy region descriptions and the importance of these regions for Atlantic salmon aquaculture in Canada will be discussed. Comprehensive background on Atlantic salmon (particularly in Atlantic Canada) will be displayed, including characteristics, importance, and declines of Atlantic salmon. Impacts of open-net pen aquaculture on wild Atlantic salmon will be examined, with a focus on escaped salmon from net pens and pathogen movement to wild salmon populations from aquaculture sites. Gaps in understanding about causes of declines and in how wild Atlantic salmon populations are affected by open-net pens will be considered, and the ability of eDNA sampling to help address these gaps will be highlighted. This review will provide an overview of Atlantic salmon and how open-net pen aquaculture threatens Atlantic salmon, with a focus on Atlantic Canada where possible.

2.1 The Bay of Fundy

2.1.1 Properties of the Bay of Fundy

The Bay of Fundy is a distinct region of the Atlantic Ocean in Canada bordering Nova Scotia and New Brunswick (Buzeta et al. 2003; Webb et al. 2007). Tides in the Bay of Fundy are extensive, reaching up to 16m. However, there are notable differences between tides throughout the Bay of Fundy. Expansive tides mean that waters of the Bay of Fundy generally are “well mixed” (Trites & Garrett 1983; Greenberg 1984; Buzeta et al. 2003). The Bay of Fundy also contains a wide range of wildlife, and is home to more than 100 fish species, including Atlantic salmon, which are endangered (as per COSEWIC’s assessment) in the inner and outer Bay of Fundy (Buzeta et al. 2003; DFO 2019a; DFO 2019c). Thus, the Bay of Fundy is a dynamic and complex oceanic region of Atlantic Canada characterized by intense tides and high turnover of materials that facilitate a great deal of biological diversity, making the Bay of Fundy a distinctive study region.

2.1.2 The Quoddy Region

The Quoddy Region is an important cultural, commercial, and ecological region of the Bay of Fundy and is situated between Maine in the United States and southwest New Brunswick in Canada (Buzeta et al. 2003). This region is divided into Inner Quoddy, which is made up of “Passamaquoddy Bay and the St-Croix Estuary,” and Outer Quoddy, which is made up of passages that are located towards the outside of Passamaquoddy Bay, as well as “the West Isles, […], the Wolves Islands and Letang estuary” (Buzeta et al. 2003). This region is affected by the extensive flushing that is typical of the Bay of Fundy, with between 15 and 16 days needed for ocean water to turnover in Passamaquoddy Bay (Trites & Garrett 1983; Buzeta et al. 2003).

The Quoddy region is also an “ecologically sensitive” region of the Bay of Fundy (Buzeta et al. 2003), and is, for Atlantic Canada, the marine region that is most ecologically at-risk (Buzeta et al. 2003; Buzeta 2014). The Quoddy region houses more than 2000 marine species (Buzeta 2014). As well, the

Quoddy region has and continues to serve as an important region for fish farming. Currently, this region is one of the biggest marine salmon farming locations on Canada's East Coast (Buzeta et al. 2003; Chang et al. 2014a), containing more marine aquaculture that anywhere else in the Bay of Fundy (Buzeta 2014). The outer part of the Quoddy Region has also been valued historically by Indigenous groups for food acquisition and cultural practices (Buzeta et al. 2003). The Quoddy Region is thus an important region for Eastern Canada's marine aquaculture production, as well as being culturally and ecologically valuable.

2.2 Atlantic Salmon (*Salmo salar*) in Atlantic Canada

2.2.1 Ecology of Eastern Canadian Atlantic Salmon

Atlantic salmon are native to freshwater rivers in Iceland, Europe, the northeast parts of North America, and the northwest parts of Russia (NOAA [date unknown]a). While diversity exists in the life histories of Atlantic salmon populations (Webb et al. 2007), almost all Atlantic salmon spend part of their life in freshwater and part of their life in the ocean (are anadromous) (Webb et al. 2007; DFO 2013; DFO 2019a). Atlantic salmon generally reproduce in their "natal river" systems (Webb et al. 2007; DFO 2019a), where they tend to build nests called redds in oxygen rich regions atop cobble or gravel riverbeds (COSEWIC 2011). In the outer Bay of Fundy, juvenile Atlantic salmon typically stay in freshwater for up to four years (DFO 2019a, DFO 2013). They then undergo smoltification (modifications to their anatomies to prepare for life in the ocean) (DFO 2013; Fjelldal et al. 2018) and make their way to the northern part of Atlantic Ocean, where they remain for between one and three years (COSEWIC 2011; DFO 2013; DFO 2019a). While in the marine environment, crustaceans, as well as fish, are predominantly consumed by Atlantic salmon (Webb et al. 2007), contrasting the smaller larvae (mainly fly larvae) and zooplankton consumed in freshwater (Keenleyside 1962; DFO 2013). Atlantic salmon (including those found in the outer Bay of Fundy), through spending their life partially in freshwater and partially at sea, have intricate life histories that provide spatial and temporal diversity in terms of possible

causes of population decreases. As such, these life histories warrant consideration when addressing Atlantic salmon declines.

2.2.2 Historical Importance of Atlantic Salmon in Atlantic Canada

Wild Atlantic salmon have historically contributed to the livelihoods of many groups of people, as well as being a vital component of healthy ecosystems. Since the late 18th and early 19th centuries, Atlantic salmon have been one important source of food for Europeans settling in Atlantic Canada, as well as contributing to local economic wellbeing through exports. Maritime "salmon exports" amounted to over 4 million pounds each year prior to 1814 (Dunfield 1985). Commercial fishing practices have continued to be important in Passamaquoddy Bay. The special ocean properties in the region, including an influx of freshwater from surrounding regions alongside low temperature seawater and a well-mixed water column, have led to fishing in this area being more prosperous that any other region of the Bay of Fundy (Scott 1983).

Atlantic salmon have also had unique importance to many indigenous groups in Canada, being key for cultural and spiritual practices of more than 49 indigenous groups. Wild Atlantic salmon were once present in high numbers in eastern Canada, and provided an important food source to multiple indigenous groups that are native to the regions located on the Bay of Fundy (Harper & Ranco 2009; COSEWIC 2011; TFC 2016).

Atlantic salmon also have ecological importance by bringing important nutrients from one region to another as they travel from freshwater to the ocean (Jonsson & Jonsson 2003; COSEWIC 2011). Jonsson & Jonsson (2003) investigated the relocation of nutrients and energy from the River Imsa in Norway to the Atlantic Ocean and vice versa, and found that adult salmon coming back to their rivers of origin from the Atlantic Ocean brought in (on average) "3176 kg, 735 kg and 132 kg [of C, N and P], respectively" across 19 years (the temporal scope of the study; Jonsson & Jonsson 2003), validating the role of Atlantic salmon in regional translocation of nutrients (Jonsson & Jonsson 2003).

Despite their historical importance, there are currently no operational fisheries for recreation or economic purposes, as well as no indigenous fisheries, permitted in the outer Bay of Fundy due to population declines (DFO 2019a). Because Atlantic salmon in Atlantic Canada have been and are still important to fishers, as well as having cultural and ecological importance, the loss of this species and related fisheries is a major concern for the livelihoods of many people and for the wellbeing of the freshwater and marine environments that these fish occupy.

2.2.3 The Decline of Atlantic Salmon in Atlantic Canada

Atlantic salmon began to decline in Atlantic Canada in the 1960s (Parrish et al. 1998). Reduced catch abundances were prevalent in the mid 20th century after large catches occurred between 1920 and 1930. There was a rebound (based on catch data) from the early 1960s until 1980, after which Atlantic salmon population abundances began to decrease once again (Parrish et al. 1998; Friedland et al. 2003; Chaput 2012). Atlantic salmon population declines have continued despite reductions in commercial fishing of Atlantic salmon since the 1960s (Parrish et al. 1998).

In much of Atlantic North America, and particularly in the outer Bay of Fundy, wild salmon population abundances are decreasing, with inner and outer Bay of Fundy salmon population sizes currently being as small as they have ever been historically (Parrish et al. 1998; COSEWIC 2011). Carr et al. (1997) found that amounts of wild Atlantic salmon each year coming back to the Magaguadavic River (connected to Passamaquoddy Bay) to spawn decreased between 1992 and 1996 (Carr et al. 1997). Declines have continued, exemplified by a 2011 COSEWIC report, which found that across the three generations of Atlantic salmon leading up to 2010, there was more than a 50% reduction in population size observed in the outer Bay of Fundy for salmon that spend a single winter in the ocean and for those that spend more than one winter in the ocean (COSEWIC 2011). A 2019 report by the Atlantic salmon Federation provided further evidence of Atlantic salmon declines, with 1346 small Atlantic salmon being found to come back to their rivers of origin in the Scotia-Fundy area of Atlantic Canada, representing one of the smallest documented returns (ASF 2019). Decreases in Atlantic salmon likely are caused by agents

in the ocean that impact salmon survival (Mills et al. 2013), with marine aquaculture discussed as a potential reason for Atlantic salmon declines (Peyronnet et al. 2007). Even with lesser fishing pressure since the 1960s, wild Atlantic salmon in the Bay of Fundy (and specifically within Passamaquoddy Bay) are currently still declining, with oceanic mortality likely a major cause of this decline.

2.3 Open-Net Pen Salmon Aquaculture

2.3.1 Overview of Open-Net Pen Salmon Aquaculture

The use of open cages for salmon aquaculture in the marine environment first started in the late 20th century (Chang 1998). Aquaculture arose as a way to lower pressures on wild fish while satisfying rising global food demands (GAA [date unknown]; NOAA [date unknown]b; Chang 1998). The first occurrence of marine salmon aquaculture in open-net pens was in 1969 in Norway, and later New Brunswick in eastern Canada adopted these cages in 1978 (Chang 1998). As of 2012 in Southwestern New Brunswick, 45 operational salmon farms existed, exemplifying the industry's advancement (Chang 1998; Chang et al. 2014b). Open-net pens are cages with netting around them that sit at the ocean's surface and extend downward with the use of a weight to prevent movement of the cage with tides and water currents (Weston 2013). Ocean water and other small particles that it contains move freely between the open-net pens and surrounding oceanic ecosystems (Brager et al. 2015). Usually with open-net pen salmon aquaculture, salmon are hatchery-raised and then moved after one or more years to the sea. They are removed to go to market about another two years after entering the ocean (Weston 2013; Nguyen & Williams 2013). Globally, as well as in Canada, the most common practice for Atlantic salmon aquaculture is the use of oceanic open-net pens (Weston 2013; Nguyen & Williams 2013). Thus, since its origin, open-net pen aquaculture of Atlantic salmon has become the most prevalent form of aquaculture in Canada and worldwide.

2.3.2 Impacts of Open-Net Pen Salmon Aquaculture on Wild Atlantic Salmon

There is evidence that open-net pen marine aquaculture can negatively affect wild Atlantic salmon populations. Ford & Myers (2008) show that in the Bay of Fundy, the amount salmon returning to their rivers of origin to spawn was lower for those that would be in close proximity to salmon farms on their way to sea compared to non-farm areas in the Bay of Fundy. It was also found that the magnitude of this decline was inversely linked with the amount of salmon aquaculture in that region (Ford & Myers 2008). Further DFO and COSEWIC reports have also identified marine salmon farming as a possible agent of decreases in wild Atlantic salmon (COSEWIC 2011; DFO 2019a). Marine open-net pen aquaculture of Atlantic salmon is thus correlated with lesser wild Atlantic salmon survival.

The possibility of encounters between Atlantic salmon escapees from aquaculture pens and wild Atlantic salmon is one major concern associated with open-net pen marine aquaculture (COSEWIC 2011). There have been increases found in non-wild salmon infiltrating marine and freshwater environments. Carr et al. found that in the Magaguadavic River in New Brunswick, between 1992 and 1996, there were fewer wild Atlantic salmon coming back to the river each year (numbers of returnees being 294, 237, 131, 79, and 69 for each year consecutively), and a net increase in the amount of non-wild (farmed) salmon in this river (Carr et al. 1997). This is concerning because wild salmon and salmon raised in aquaculture facilities are able to reproduce with one another and have viable offspring (McGinnity et al. 2003), which may lessen the fitness of wild salmon populations. Fleming et al. (1996) demonstrated, through an experiment that recreated conditions needed for salmon reproduction, that overall, cultured Atlantic salmon had "11-19% the reproductive success of the wild [Norwegian salmon] […] when in competition" (Flemming et al. 1996). McGinnity et al. (2003) further showed that fewer farmed Atlantic salmon in Ireland that spent a single winter in the ocean came back to their rivers of origin compared to wild salmon when deposited in the ocean by the experimenters. The same was also true for all salmon groups that were a cross between wild and cultured salmon. However, salmon that were in the ocean over the span of two winters had more farmed salmon and crossbred salmon returning

to their rivers of origin compared to wild salmon (McGinnity et al. 2003). Thus, escapees from open-net pen marine aquaculture may impede reproductive success and survival of wild Atlantic salmon (although the impediment to salmon survival may depend on the time scale that is considered). However, relevant studies have focused on European countries rather than North America.

Pathogens being transferred from fish in aquaculture net pens into the surrounding water column has also been identified as a concern of open-net pen aquaculture, with particular concern for wild salmon populations (Naylor et al. 2005; Terlizzi et al. 2012). Rearing of large quantities of Atlantic salmon in close quarters in marine aquaculture cages often allow disease-causing agents to build up, which can be passed to wild fish travelling by the open-net pens because of unimpeded water movement between the pens and surrounding oceanic water column (Naylor et al. 2005; Johansen et al. 2011; Morton & Routledge 2016). Gustafson et al. (2007) found that in the Quoddy region (Bay of Fundy), there was a connection, on a short time scale, between oceanic water movement and the spread of a pathogen (infectious salmon anemia), between different Atlantic salmon farms nearby to each other (Gustafson et al. 2007). No field-based studies were found in the literature review pertaining specifically to disease or pathogen spread from marine aquaculture salmon farms to wild Atlantic salmon populations. Thus, while pathogens being spread from cultured to wild Atlantic salmon is a risk associated with open-net pen aquaculture, few studies validate how specific wild Atlantic salmon populations are impacted by this or the severity of such an impact.

2.4 Knowledge Gaps

The literature indicates oceanic mortality, and the uncertainty associated with it, is amongst the greatest reason for the declining size of Atlantic salmon populations, yet agents of at-sea salmon deaths are poorly understood and researched. Webb et al. (2007) and Simms (2017) have identified that information is lacking regarding entities that affect Atlantic salmon while at sea, and Lacroix & Knox (2005) further assert the lack of information pertaining to entities that effect Atlantic salmon on the way to sea. It is hard to quantify how many salmon die at sea of natural causes, as salmon may come back to

their home rivers after differing amounts of time in the North Atlantic Ocean, and fisheries also cause salmon deaths, which are hard to separate from natural deaths at sea (Chaput 2012). In addition, the way that environmental shifts in the ocean impact the mortality of Atlantic salmon while at sea is not well known (COSEWIC 2011), which is validated by Simms (2017), who calls for more attention and research into the role that changes to oceanic biotic systems resulting from climate shifts play in Atlantic salmon declines. Because survival as Atlantic salmon head out to and begin their life stage at sea is critical to overall population health, understanding the entities that affect Atlantic salmon while at sea is key to resolving population declines. As such, more research is needed in this realm.

As well, while aquaculture, and specifically pathogens released from aquaculture sites, has been found to possibly harm to wild salmon populations (Naylor et al. 2005), specific information regarding Atlantic salmon populations in North America and the impacts of pathogen releases from aquaculture on these populations is lacking. Most studies tend to focus on escapees from aquaculture facilities rather than pathogen spread when investigating aquaculture effects on wild Atlantic salmon, and little information is present in the literature about pathogens from aquaculture causing disease in wild fish (Terlizzi et al. 2012). No pathogen-related field studies pertaining to wild salmon in Atlantic Canada were found through this literature search. Research pertaining to diseases and disease prevalence in salmon, which exists for salmon raised via aquaculture, is largely not available for wild salmon populations (AAC 2014). As well, while some in-lab pathogen experiments have occurred, which can aid in understanding how likely a salmon population is to get a certain disease and what might happen if they do, these do not properly represent the real world and how living and non-living environmental components might alter the way a disease advances (AAC 2014). As such, there is a current need to provide more research regarding whether or not pathogen transfer to wild Atlantic salmon from marine aquaculture facilities is a cause of Atlantic salmon declines in the Bay of Fundy (and globally).

2.5 Environmental DNA (eDNA)

2.5.1 Overview of eDNA

Environmental DNA (eDNA) sampling can be used to gain an understanding of whether pathogens are present around marine open-net salmon pens, and thus whether pathogen spread can be a possible cause of wild Atlantic salmon declines. While there are many existent definitions of eDNA, eDNA is an organism's "genetic material" (separate from the organism itself) that has been sampled from the environment (Díaz-Ferguson & Moyer 2014; Thomsen & Willerslev 2015). eDNA sampling originated in the 1980s to characterize bacteria found in oceanic sediments (Díaz-Ferguson & Moyer 2014). As such, it is known that microorganisms can be targeted by eDNA sampling. eDNA has since been useful in conservation-related contexts, as at-risk or invasive organisms can be effectively identified using this tool (Ficetola et al. 2008). eDNA has notably been used to confirm that Asian Carp, an invasive fish, had infiltrated the great lakes (Jerde et al. 2013; Díaz-Ferguson & Moyer 2014; Huver et al. 2015).

2.5.2 eDNA as a Tool to Investigate Pathogen Spread in Marine Ecosystems

eDNA has been expanded from its initial use in freshwater to encompass marine ecosystems. Thomsen and Foote initially led the field in terms of applying eDNA techniques to oceanic environments (Díaz-Ferguson & Moyer 2014). Thomsen et al. (2012) and Foote et al. (2012) showed that fish and mammals (respectively) in the ocean in Denmark could be detected using eDNA methodologies. These studies validate that eDNA sampling can possibly work in marine environments. However, Foote et al. (2012) noted that the "greater dispersal and dilution of eDNA in marine ecosystems compared to lakes and ponds" poses a challenge for oceanic eDNA studies.

Researchers have also recently used eDNA to explore the presence of pathogens in aquatic environments. Huver et al. (2015) affirmed that *Ribeiroia ondatrae*, a pathogen that infects frogs, was located in North American wetlands using eDNA sampling. Peters et al. (2018) further showed that in ocean water treated with known pathogens of "farmed Atlantic salmon," eDNA techniques allowed for

identification of some of these pathogen species. Studies thus affirm that eDNA can be a useful tool in "determining pathogen presence" (Huver et al. 2015) in aquatic environments, and further that eDNA methodologies can possibly be applied to the marine environment to determine if pathogens are present around marine aquaculture facilities.

2.6 Conclusion

This literature review showed that Atlantic salmon populations worldwide and in the Bay of Fundy are dwindling, and that this species is important, especially within the Bay of Fundy, to both people and to the health of the environment. Open-net pen aquaculture, which has become widespread globally and in Canada since its first occurrence in 1978, has possible involvement in Atlantic salmon declines. While a connection has been found between aquaculture that occurs in open-net pens and lesser wild salmon abundances, studies investigating specific reasons for this connection require more attention by scientists and experimenters. As well, literature regarding aquaculture impacts on Atlantic salmon mostly came from European-based studies, and more North American studies are needed. Through this review of relevant literature, important knowledge gaps regarding Atlantic salmon oceanic mortality and possible agents that have played a role in Atlantic salmon declines have become evident, affirming the need for further research into marine aquaculture impacts (particularly related to pathogens) on wild Atlantic salmon in Atlantic Canada. eDNA, a recently developed sampling approach, can be used to explore the potential role of pathogens in Atlantic salmon declines.

3. Methods

3.1 Sampling Location and Overview

Passamaquoddy Bay in New Brunswick, Canada is situated in the outer Bay of Fundy, and is part of the "Quoddy Region," which borders Maine and New Brunswick (Buzeta et al. 2003; Lacroix & Knox 2005). This region shares the high flushing rates that the Bay of Fundy is known for. Passamaquoddy Bay

was selected as the sampling location due to the prominence of aquaculture in this region (Buzeta et al. 2003).

Ocean water was collected between June 24th and July 5th, 2019 in Passamaquoddy Bay. Ten active aquaculture net pen sites were sampled, as well as five fallow (previously active) net pens and five reference (control) sites located away from active or fallowed sites (see Figure 1). The active net pens contain hatchery-raised Atlantic salmon and are operated by two main aquaculture companies (Cooke Aquaculture Inc. and Mowi Canada East). In regions with multiple active sites located within ~500 m of each other, one site was sampled. Active and fallow sites were sampled at tidal transitions to minimize water movement at the time of seawater collection. Reference sites did not require sampling at tidal transitions because there was not a need to target water moving through a net pen (as was the case with active and fallow farms).

3.2 Water Sampling and Filtration

At each site, samples consisted of ocean water collected with a submersible pump across a transect adjacent and parallel to the lease site boundary of the net pens (for active and fallow sites) or at reference sites (where point sampling occurred; locations shown in Figure 1). Due to variations in aquaculture site sizes, transects ranged from ~29.5 m to ~1037.8 m in length. However, a constant volume of 20 L was collected for all sites, and pumping speed remained constant. A DAVIS Drifter was deployed at each site to measure surface current speed and direction (CODE/DAVIS Drifter…2019). The boat used for sampling was sprayed with Virkon (a viral and bacterial disinfectant; Virkon S…2019) followed by water before new Bay Management Areas (BMAs) or farms operated by different aquaculture companies were sampled. DAVIS Drifters were sprayed with Virkon followed by water before being deployed at a new site.

Prior to water sample collection at a site, the submersible pump was primed for 300 seconds by cycling ocean water through the pump. This was done to ensure that there was no contamination from water collection at previous sites, as well as no Virkon residue, left in the pump. After priming, 20 L of

ocean water was collected at a depth of approximately 1 m along a transect of the lease site boundary of the active or fallow aquaculture farm. GPS waypoints and times were taken at the start and end of collection. Before sampling at a new site, the submersible pump was cycled in Virkon, followed by two cycles in distilled water. The submersible pump was primed with seawater again at the new site before sample collection.

Following water collection, samples were processed at the Fisheries and Oceans Canada Saint Andrews Biological Station in Saint Andrews, NB. For each site, the 20 L water sample was divided into four 2 L Nalgene bottles and pumped using a peristaltic pump through Masterflex tubing into a 1.6 µm GF/A Microfiber pre-filter to remove unwanted algae and sediment from the sample. Water was then filtered through a 0.22 µm Sterivex filter, followed by a "positively charged" Zeta Plus™ cellulose-based depth filter (2019 personal communication with C Rycroft, unreferenced; 2019 personal communication with K Miller, unreferenced; Zeta Plus [date unknown]). Tubing, bottles, and filter housings were cleaned using a 6% hypochlorite bleach solution, followed by distilled water (Figure 2).

Water sampling and filtration protocol were modeled after similar methods used by Dr. Kristi Miller, who conducted sampling to determine pathogens present around marine aquaculture sites in Quatsino, BC (2019 personal communication with K Miller, unreferenced). Figure A1 of Appendix A outlines procedures used for the present study. Dr. Miller has published extensively on salmon pathogens, co-authoring numerous recent publications on the topic, including Mordecai et al. (2019), Nekouei et al. (2019), and Thakur et al. (2019). Her research has also made use of qPCR techniques for pathogen detection (Bass et al. 2019).

3.3 DNA and RNA Extraction

Following filtration, filters were stored at 193.15 K (-80 °C) and sent to the Molecular Genetics Lab at the Department of Fisheries and Oceans Pacific Biological Station in Nanaimo, BC for extraction of DNA and RNA using a DNeasy kit, followed by quantitative PCR (qPCR) using the Fluidigm Biomark™ system (Miller et al. 2016). qPCR provides information about the number of PCR cycles

needed for sample DNA detection to be above "background levels" (this is called the threshold cycle, or Ct, value). Fewer cycles to reach the threshold ("lower Ct values") equate to more DNA present in the sample (Miller et al. 2016; Kralik & Ricchi 2017).

Potential pathogens selected for analysis can be found in Table A1 of Appendix A. A total of 47 pathogens and other agents (Miller et al. 2016) were chosen based on literature searches, guided by those selected by Lennox et al. (2019) in a study (currently in-review) on pathogens that infect brown trout in Norway, which share many possible disease-causing agents with Atlantic salmon.

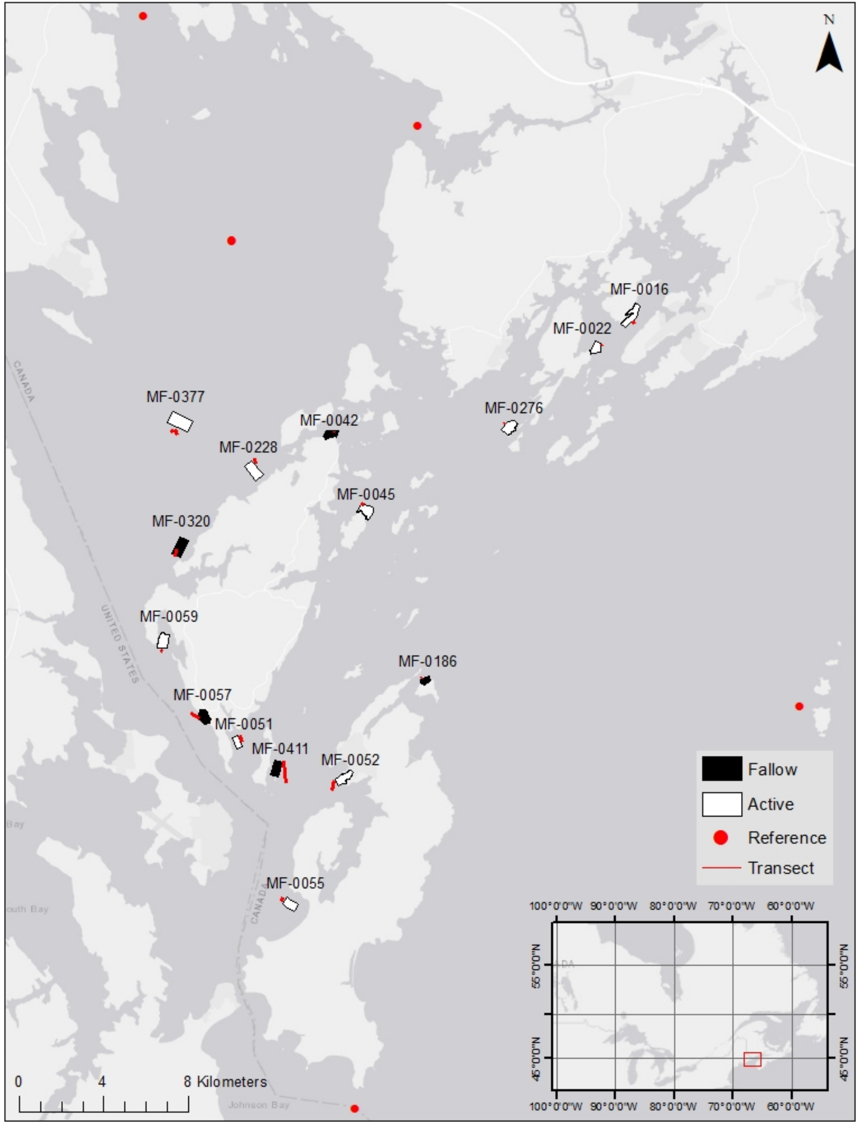

Figure 1 Locations of active and fallow marine aquaculture open-net pen sites, alongside reference sites, sampled in Passamaquoddy Bay, NB between June 24th and July 5th, 2019. Transects were not taken at reference sites (instead, point samples were collected at reference sites).

3.4 Limitations

There are a few limitations associated with these methods. The length of transects varied based on farm sizes and based on the water current speed and direction at each site. The boat used for sampling could only operate on the lowest gear when the submersible pump was in the water, and as such was subjected to movement by ocean currents, which impacted transect length and prevented the use of a consistent transect length at each site. A constant pumping speed was maintained, as well as a constant volume of water taken (20 L) at each site, to mitigate unequal transect lengths. As well, sampling directly adjacent to the net pens was not approved by the aquaculture companies in Passamaquoddy Bay. Instead, samples could only be taken as close as the lease site boundary of the farm, which could impact the richness of pathogens found at each site. The long processing time for each water sample (about 20 minutes for filtration, not including equipment sterilization), as well as a lack of filters, limited the number of sites that could be sampled in Passamaquoddy Bay (not all active or fallow farms could be sampled). This was mitigated by not sampling active sites in close proximity (within ~500 m) of one another. As well, sampling occurred over a two-week period from late June to early July of 2019, and as such, this study does not consider possible pathogens that may be present at other times of the year or changes in pathogen richness across different years.

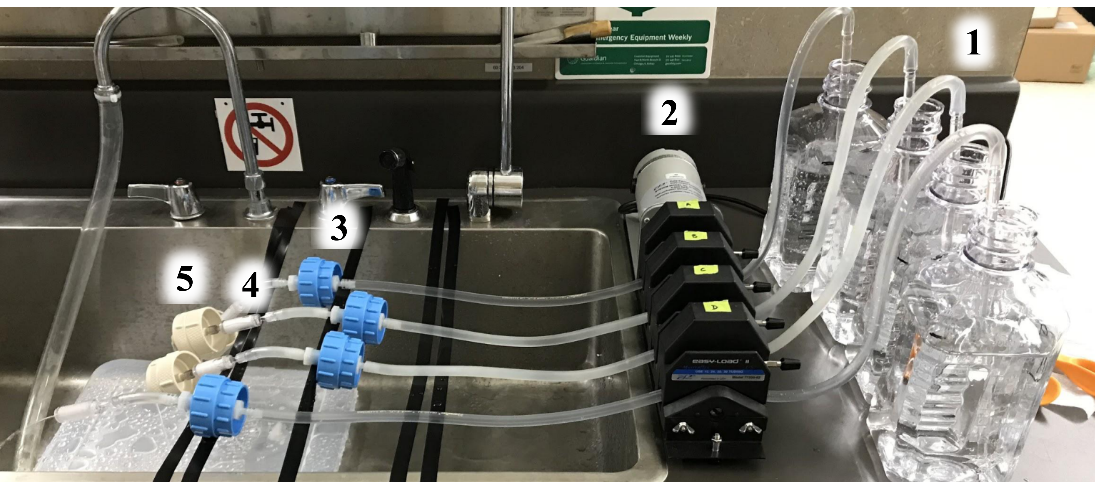

Figure 2 Filtration set-up at the St. Andrews Biological Station (Fisheries and Oceans Canada) in Saint Andrews, NB. Water was pumped from four 2 L Nalgene bottles (1) using a peristaltic pump (2) through a 1.6 µm GF/A Microfiber pre-filter (3), followed by a 0.22 µm Sterivex filter (4) and a charged filter (5).

4. Results

qPCR analysis of Streivex filters from three active (MF-0051, MF-0045, MF-0052) and two fallow (MF-0042 and MF-0320) sites revealed undetermined results (no detection) for the presence of Piscine orthoreovirus (PRV) and Atlantic salmon cold-water vibriosis (ASCV) (pathogens of Atlantic salmon). For all sites tested, with the exception of MF-0045, Atlantic salmon DNA and RNA were not found in detectable amounts on the Sterivex filters. A high cycle threshold (Ct) value of 39.58 was obtained for MF-0045, revealing a small amount of Atlantic salmon DNA detected at this site (Miller et al. 2016) (Table 1). It should be noted that not all filters were analyzed due to the lack of detections on the tested filters.

Table 1 Results from qPCR analysis of DNA and RNA extracted from Sterivex filters following filtration of seawater from active and fallow open-net Atlantic salmon marine aquaculture farms in Passamaquoddy Bay, NB. Ct values for Atlantic salmon, Piscine orthoreovirus (PRV) and Atlantic salmon cold-water vibriosis (ASCV) are shown. Undetermined results indicate a non-detection.

Site Code	DNA/RNA	Site Type	Atlantic Salmon Ct	PRV Ct	ASCV Ct
MF-0057	DNA	Fallow	Undetermined	-	-
MF-0042	DNA	Fallow	Undetermined	-	-
MF-0320	DNA	Fallow	Undetermined	-	-
MF-0411	DNA	Fallow	Undetermined	-	-
MF-186	DNA	Fallow	Undetermined	-	-
MF-0320	RNA	Fallow	Undetermined	Undetermined	Undetermined
MF-0042	RNA	Fallow	Undetermined	Undetermined	Undetermined
MF-0052	DNA	Active	Undetermined	-	-
MF-0051	DNA	Active	Undetermined	-	-
MF-0276	DNA	Active	Undetermined	-	-
MF-0228	DNA	Active	Undetermined	-	-
MF-0045	DNA	Active	Undetermined	-	-
MF-0377	DNA	Active	Undetermined	-	-
MF-0055	DNA	Active	Undetermined	-	-
MF-0059	DNA	Active	Undetermined	-	-
MF-0016	DNA	Active	Undetermined	-	-
MF-0022	DNA	Active	Undetermined	-	-
MF-0052	RNA	Active	Undetermined	Undetermined	Undetermined
MF-0045	RNA	Active	39.58	Undetermined	Undetermined
MF-0051	RNA	Active	Undetermined	Undetermined	Undetermined

5. Discussion

The aim of the present study was to address knowledge gaps related to factors that may contribute to wild Atlantic salmon mortality during their early marine life history, and specifically to determine if the production and release of pathogens from marine open-net pen salmon aquaculture could pose a risk to juvenile salmon during their migration to sea (Lacroix & Knox 2005; Naylor et al. 2005; Lacroix 2014) and thus be a contributing factor to the decline of salmon populations. However, qPCR analysis of Sterivex filters used to collect pathogens at active and fallow aquaculture sites in Passamaquoddy Bay, NB, where open-net pen aquaculture is prevalent, did not reveal detection of RNA for two pathogens common to aquaculture sites (PRV and ASCV; Table 1). Detection of Atlantic salmon DNA or RNA was also not found, with the exception of at the active site MF-0045, where a low level of Atlantic salmon RNA was detected (Table 1).

Several possible reasons for the lack of detection of pathogen and Atlantic salmon DNA and RNA will be discussed below. It is important to note that the lack of detection of pathogens does not mean that the sampled aquaculture sites are free of pathogens and that these sites do not pose a risk to wild salmon. The most likely explanation is that there were issues with the sampling design. In the remainder of this paper, potential challenges related to the collection of eDNA in the field will be reviewed, and next steps to address these challenges will be proposed. Specifically, three aspects of sampling design that potentially prevented the detection of pathogen and Atlantic salmon DNA and RNA will be discussed: 1) sampling distances from net pens and tidal effects; 2) pre-filter effects and phytoplankton blooms; and 3) bleach contamination.

5.1 Sample Collection in Relation to Net Pen Locations and Tidal Effects

Passamaquoddy Bay is a diverse region that poses a unique challenge for water sampling. The extensive tides in Passamaquoddy Bay (the tidal range is 6 m on average) and quick turnover of the water column (between about 15 and 16 days; Buzeta et al. 2003) contribute to a rapid movement and

replacement of seawater, and to the likely swift dilution of pathogens present in the water column surrounding marine salmon farms. As such, sample collection in the present study may not have occurred at the appropriate minimum distance from net pen sites to obtain detectable pathogen signals. Determining this minimum sampling distance is challenging in Passamaquoddy Bay, and at present no study that I am aware of has attempted to define this. For the present study, the minimum sampling distance from active net pens permitted by aquaculture companies in discussions prior to field work was 100 m. Distances from the net pens ranged from 100 m to 389 m across sites depending on the lease site boundaries of each sampled aquaculture farm. Given the hydrologic complexities and considerable flushing of Passamaquoddy Bay, it is possible that the 100 m minimum distance was too far from the net pens to capture detectable amounts of pathogen and salmon eDNA coming off of the net pens.

In addition to farm proximity, sampling farms in the midst of tidal changes in Passamaquoddy Bay poses a unique challenge. Past research has described the complexity and variability of tides and currents in Passamaquoddy Bay (Greenberg et al. 1998). If water was moving quickly through the net pens in the opposite direction of sampling equipment, it is possible that pathogen DNA would not be present in samples in detectable amounts. While the present study attempted to sample during tidal transitions to minimize water movement and dilution of pathogens surrounding active and fallow farm sites, Passamaquoddy Bay has been shown to be a dynamic and hard to predict oceanic region in terms of water movement (Greenberg et al. 1998). Winds, alongside the prevalence of bays and islands within the sampling region, could have altered water circulation during sampling, making the direction of water flow surrounding sampling sites difficult to determine. As such, regional current and tidal anomalies may have carried microbial matter away from the sampling equipment during water sampling, which, in conjunction with the minimum 100 m sampling distance from the aquaculture farms, could have affected pathogen and Atlantic salmon detection amounts in our samples.

The constraints associated with water sampling as described here will continue to pose unique challenges to future researchers addressing similar questions in the Quoddy region, and careful consideration of sampling distances from aquaculture farms and flow regimes will be required in the

design of future studies. While both farm proximity and tidal effects may have played a role in minimizing pathogen and Atlantic salmon detection from water samples in this study, due to the abundance of Atlantic salmon (from aquaculture) in the Quoddy region, detectable amounts of Atlantic salmon genetic material would be expected regardless of these factors. Because no Atlantic salmon DNA or RNA was detected through qPCR analysis of Sterivex filters (with the exception of one site, where low detection was found; Table 1), likely other mechanisms contributed to the non-result.

5.2 Pre-filter Effects

As of 1987, phytoplankton population dynamics have been documented in the Bay of Fundy bordering southwestern New Brunswick, with Passamaquoddy Bay having an active phytoplankton monitoring station since 1999 (Martin et al. 2014). Data collected in 2001 show that phytoplankton in Passamaquoddy Bay (specifically picophytoplankton, such as *Synechococcus*, nanophytoplankton and bacteriophytoplankton) were present in the highest abundances in the end of summer and early fall months (August to the end of September; Martin et al. 2014). Additional data show that, from 1999 to 2009, total phytoplankton abundance is generally highest in July (NOAA 2018), affirming the occurrence of seasonal phytoplankton blooms in Passamaquoddy Bay.

In the present study, a 1.6 µm GF/A Microfiber prefilter was used to remove unwanted algae and sediment from water samples collected at all 20 sites. Because sampling occurred from late June to early July of 2019, it is possible that the prefilters became clogged by the larger phytoplankton abundances present in Passamaquoddy Bay during this time. Pathogens should, in theory, pass through the pre-filter, and be captured by subsequent filters. However, if the GF/A pre-filter was clogged with phytoplankton, the microorganisms (and DNA) of interest may have been prevented from passing through the prefilter into the Sterivex and charged filters, thus accounting for the lack of detection of pathogens on the Sterivex filters.

Padilla et al. (2015) filtered differing volumes (between 0.05 and 5 L) of water through a 1.6 µm GF/A prefilter followed by a 0.22 µm Sterivex filter with a peristaltic pump. These are consistent with the

filter types and sizes that were used in the present study. The authors found that as filtration volume increased, a greater richness of bacteria was captured by the prefilter while a lesser richness of bacteria was captured by the Sterivex filter. The authors propose that changes to the make-up of microbes found within the filters at differing filtration volumes may be explained by the prefilters clogging with increasing sample volumes. This could cause smaller cells that typically would have passed through the 1.6 µm prefilter pores (to the Sterivex filter) to remain on the prefilter, thus increasing prefilter microbial diversity. The 2 L filtration volume used in the present study aligns with the upper limits of volumes tested by Padilla et al. (2015). Based on results from Padilla et al. (2015), it is possible that prefilter clogging may have inhibited pathogen and Atlantic salmon detection on the Sterivex filters in the present study, as pathogens and salmon DNA and RNA may have remain trapped on the clogged prefilter rather than passing through to the Sterivex and charged filters. However, it should be noted that no extreme clogging was observed across water samples during water filtration.

The GF/A pre-filters were not intended to be analyzed for pathogen or Atlantic salmon eDNA. However, because of the lack of detection of DNA or RNA on the Sterivex filters, the GF/A prefilters will be analyzed. GF/A prefilter analysis was underway at the Molecular Genetics Lab at the Department of Fisheries and Oceans Pacific Biological Station during the writing of this thesis; however, results were not yet available.

5.3 Bleach Contamination

It is well documented in the literature pertaining to eDNA collection and analysis that the use of bleach can prevent sample detection by PCR (Rodgers 2017). While there is no globally accepted standard for strength of bleach solution to be used for decontamination and preparation of eDNA sampling gear for use in the field, it has been found that a 2-3% sodium hypochlorite concentration is essential for a high level of sterilization, although a 0.55% sodium hypochlorite concentration is largely acceptable (this is equal to a 10% bleach solution; Dickie et al. 2018). For the present study, cleaning of equipment was done with a 6% sodium hypochlorite bleach concentration based on suggestion from other

researchers in the field. Although all equipment was rinsed extensively with distilled water following bleaching, it is possible that the high (6%) hypochlorite concentration used left trace bleach residues within the filtration system. If this was the case, bleach contamination could have prevented detection of pathogen RNA and Atlantic salmon DNA and RNA in our samples.

5.4 Discussion of Positive Result

One positive detection of Atlantic salmon RNA was found surrounding active net pen site MF-0045 (Table 1). The lessening of any of the potential issues with sampling design discussed above may explain this anomalous successful detection. For example, it is possible that ocean current speeds were lower at MF-0045 compared to other active sites. This could potentially reduce water movement at the time of sampling around the net pen site, allowing Atlantic salmon cells to be captured in the water sample collected. Alternately, there may have been lesser phytoplankton present in the water column surrounding site MF-0045 compared to other sites, which would have reduced the effects of possible filter clogging. It is also possible that the sampling equipment used to collect water surrounding active farm MF-0045 and filter housings used for filtration were more thoroughly rinsed with distilled water than for other samples, reducing bleach contamination and allowing detection of Atlantic salmon RNA. However, the Ct value of 39.58 (Table 1) represents a large number of qPCR cycles needed for detection to pass a threshold amount, which indicates a small amount of Atlantic salmon RNA detected surrounding MF-0045 (Miller et al. 2016). Given the prevalence of Atlantic salmon in active farm sites, a higher amount of Atlantic RNA detection would be expected. The lower than expected detection amount could be as a result of the aforementioned sampling design challenges.

6. Conclusions

In summary, ocean water samples were collected surrounding active, fallow, and reference open-net pen marine aquaculture sites in Passamaquoddy Bay, NB. Samples were filtered and underwent qPCR to determine the richness of Atlantic salmon pathogens (based on the presence or absence of pathogen DNA

and RNA) surrounding each sampling site. No RNA of PRV or ASCV (known pathogens of Atlantic salmon) was detected around tested active and fallow sites. This lack of detection likely resulted from sampling challenges such as the collection of samples at large distances from net pen sites, tidal uncertainties, possible pre-filter clogging and possible bleach contamination of filtration equipment. The non-result obtained does not negate the need for future research to address the impacts of marine salmon aquaculture on wild Atlantic salmon.

6.1 Next Steps and Avenues for Future Research

Commercial Atlantic salmon aquaculture production is increasing in parts of Atlantic Canada, and the farming of salmonids more generally is predicted to triple in Nova Scotia alone in the near future (DFO 2019d). An audit conducted by the Commissioner on the Environment and Sustainable Development "concluded that Fisheries and Oceans Canada did not adequately manage the risks associated with salmon aquaculture consistent with its mandate to protect wild fish. […] It had not made sufficient progress in completing the risk assessments for key diseases that were required to understand the effects of salmon aquaculture on wild fish. It also had not defined how it would manage aquaculture in a precautionary manner in the face of scientific uncertainty" (Office of the Auditor General of Canada 2018). This report highlights that, while results from the present study were inconclusive, it is still important that researchers move forward with trying to address existent knowledge gaps regarding the impacts of pathogens from marine open-net pen aquaculture farms on wild Atlantic salmon populations and the possible contribution of marine salmon aquaculture to declines of wild salmon populations. It is important to address logical next steps and avenues for future research. Specifically, this paper proposes alternate sampling strategies that can be adopted by future researchers who aim to quantify pathogen richness in dynamic oceanic regions such as Passamaquoddy Bay that pose a unique challenge for eDNA sampling.

Point samples have been most commonly used in past research when conducting eDNA sampling (Thomas et al. 2019). Transects, while holding value in principle in that they could possibly capture a

more representative sample of the water column, proved difficult in practice due to the dynamic water currents and tides in Passamaquoddy Bay. As such, when conducting sampling in the future, point samples would be a more feasible water sampling approach. Multiple point samples could be taken at each site and combined to ensure a representative water sample. Point samples should, if possible, be taken directly adjacent to active and fallow farm sites, which will require strengthening relations and promoting collaboration with aquaculture companies in sampling regions. As well, having a better understanding of current and tidal flows surrounding aquaculture farms can help better inform where water samples should be taken in relation to the farms.

Additionally, it would be useful for future eDNA studies in Passamaquoddy Bay to attempt water sampling at varied distances from the aquaculture net pens (including a sample being taken within the net pen as a control), with samples being tested for the presence of Atlantic salmon and pathogen eDNA to understand the distance at which detection of Atlantic salmon and pathogens becomes limited. This type of study would help begin to define a minimum distance for successful eDNA testing around marine aquaculture sites, as well as provide information regarding the risk of pathogen transfer to wild migrating Atlantic salmon with increasing distances from the farm sites.

As well, as discussed, the use of a 1.6 µm GF/A prefilter in conjunction with a larger filtration volume of 2 L in the present study might have impeded pathogen and Atlantic salmon detection in subsequent filters (this is validated by Padilla et al. 2015). Moving forward, the best option for water filtration would be to avoid the use of a prefilter altogether. This could be achieved through altering sampling times to avoid the mid to late summer period in which algal blooms typically occur in Passamaquoddy Bay (Martin et al. 2014; NOAA 2018), and instead conduct sampling in the spring or early summer. However, if sampling is being conducted in a region with a perpetually turbid water column or where algal blooms are unavoidable, selecting a prefilter with a larger pore size than 1.6 µm may be favorable to reduce the chance of prefilter clogging and increase the ability of microorganisms, alongside DNA and RNA fragments, to pass through the prefilter. Additionally, reducing filtration

volume to 0.1 L or less when using a prefilter would reduce the likelihood of filters clogging (Padilla et al. 2015).

To reduce possible bleach contamination of filter housings and other equipment, sodium thiosulfate, a “bleach neutralizer” (UBC [date unknown]), could be used to rinse equipment following bleach treatment. This has been observed in previous eDNA studies. D’Auriac et al. (2019) used sodium thiosulfate to remove bleach residue during eDNA sampling of an invasive Norwegian plant species. Agersnap et al. (2017) and Rusch et al. (2018) also used sodium thiosulfate for neutralization of bleach when cleaning equipment during eDNA water sampling and filtration. Moving forward, a lower bleach concentration (closer to a 10% bleach solution, as has been observed in much of the eDNA literature) should also be used when sterilizing equipment to further prevent the possibility of bleach contamination.

To reiterate what has been discussed, the non-result observed in the present study does not necessarily mean an absence of pathogens surrounding marine open-net pen aquaculture sites in Passamaquoddy Bay, NB, especially since Atlantic salmon eDNA was also not detected (with the exception of one positive detection) around these sites, and thus does not negate the threat that these facilities may pose to wild Atlantic salmon populations, nor does it negate the need for this potential threat to be further investigated. Given that inadequacies in sampling design are the most likely explanation for the non-result observed, the alternative sampling approaches discussed above can provide insight into how to best conduct eDNA sampling in unique marine environments such as Passamaquoddy Bay, NB. The proposed alternate sampling techniques may prove particularly useful in future studies that aim to quantify pathogen amounts and diversity surrounding marine aquaculture farms and address the knowledge gaps that the present study set out to address. These alternative sampling approaches also affirm the need for a global set of eDNA sampling standards and protocols. Best practices when moving forward in the field of eDNA sampling can be informed by the shortcomings the present study, which may prove valuable in informing continued advances in creating widespread eDNA standards.

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Appendix A

eDNA protocol complete_2019-12-02 V.3

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ABSTRACT

The following document is a technician manual for field collection and filtration of environmental DNA samples.

This procotol has been adapted from:

1) "eDNA collection and filtering; Molecular Genetics Lab"
by Dr. Kristi Miller and Amy Tabata, from the Department of Fisheries and Oceans Canada Molecular Genetics Lab.

2) "Beach Seining and eDNA water sample collection GRDI project team, May 2019" by Dr. Anais Lacoursiere. Adapted from Colleen Kelloff's protocol and originally from Small volume (1-3L) filtration of Coastal Seawater Samples by David Walsh, Elena Zaikova and Steve Hallam DOI: 10.3791/1163. Further adapted from Hakai Institute's protocol by GRDI eDNA team.

GUIDELINES

Environmental DNA Sensitivity:

"Environmental DNA (eDNA) analysis is highly sensitive to contaminant DNA including microbial DNA in the lab environment. Follow the protocols carefully with special attention to the measures to maintain cleanliness including:
-Frequent changing of nitrile gloves.
-2L sample bottle and pump tubing cleaning with diluted bleach followed by water rinsing and drying after each sampling & processing event. "

Excerpt from: "Protocol: eDNA collection and filtering" by Dr. Kristi Miller and Amy Tabata, from the Department of Fisheries and Oceans Canada Molecular Genetics Lab.

Please pay close attention to notes throughout the protocol as they contain important information.

Note: The following document has been edited to address field obstacles technicians encountered. Please understand that this document comes with no guarantees regarding sterility or viability for samples collected. Protocol was developed specifically for authors' purposes.

MATERIALS TEXT

Field site kits will contain the following:
1) 20L collection carboy
2) Tygon collection tubing (sterile)
3) Submersible pump
4) Milli-Q water spray bottle
3) 10% Bleach solution ([1.5%] hypochlorite) spray bottle
4) Nitrile gloves
5) Bucket with lid (8L) x 3
6) Plastic collection bin for used equipment
7) Clipboard
8) 16L of dH_2O
9) Volume concentrated bleach to prepare [1.5%] cleaning solution for pump

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10) Cable ties
11) Shop towels
NOTE: Many of these materials will be common between sites and must be wiped down (water, bleach, water) while in transit between sites.

Lab site kits will contain the following:
1) 2L Nalgene sample bottles
2) MasterFlex tubing (1X 106cm, 2X 3cm lentghs)
3) Luer locks
4) Sterivex filters
5) Swinnex filters
6) Parafilm
7) Double dead-ended, non-vented, Sterivex caps
8) 60mL luer lock syringe
9) Pre-labelled whirl-pak
10) Bench coat
11) Datasheet
12) Milli-Q water spray bottle
13) 10% Bleach solution ([1.5%] hypochlorite) spray bottle
14) Shop towels
15) Mounted MasterFlex motor and pump heads
16) MasterFlex controller
17) Waste water collection bucket

BEFORE STARTING

Before reaching the collection-cycle site:

1) Connect pump to marine battery. **Make sure the battery is turned off, else the pump will be damaged.**
2) Prep the carboy collection line. When this apparatus is engaged it will allow the carboy to hang securely over the side of the vessel effectively reducing the power required to pump water from depth.

eDNA Water Sample Collection

1 Collect a sealed site kit.

With new gloves remove one end of collection tubing from autoclave bag and attach it to the submersible pump. Make sure to keep at least 60cm of tubing in the bag and uncontaminated.

2 Clip the pump safety line to the boat before lowering the pump into the water, uncoiling the tubing from the autoclave bag as it is lowered. With the safety line fully extended, the submersible pump should be 1-1.5m below the surface.

Submersible pump depth will vary slightly due to wave action.

3 Remove the remainder of the collection tubing from the autoclave bag, making sure to avoid touching the last 30cm of tubing as it will be inserted into the collection carboy. This end of the tubing will be held over the side while the system is flushed.

4 Turn on the pump and allow water to cycle through for 5 minutes.

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5 During pump cycling:

1) Record the following information on eDNA datasheet:
-Date and vessel.
-Site code and transect number.
-Sea and weather conditions.
-Technicians on board (collection tech (CT) initials and hydrography technician (HT) initials).
2) Deploy CTD.
3) Deploy Davis drifters.
4) Record CTD data on data sheet.

NOTE: The CTD and Davis drifters should be deployed on the lee side of the vessel, otherwise samplers run the risk of contaminating the submersible pump, tubing and collection carboy. CTD will be rinsed liberally with distilled water and Davis drifters treated with between sites. Make sure they are deployed on the lee side of the vessel.

6 Holding the collection tubing in one hand, remove the cap of the 20L collection carboy and insert the collection tubing. Add approximately 1L of site sea water to the carboy, remove the tubing, replace cap and shake vigourously for 30 seconds. Discard water in carboy and repeat another five times more to adequately rinse collection carboy.

NOTE: It is best to have two technicians available for this job to decrease the liklihood of accidental contamination.

7 After the pump has been cycled and the carboy has been properly rinsed (6X total), place approximately 15cm of the tubing in the carboy. Make sure to watch the water level; the tubing should not become submerged in the water sample and should make as little contact with the inside of the carboy as possible. The tubing will need to be slowly retracted as the water level rises.

8 Wrap the carboy opening in aluminium foil to minimize accidental contamination from the air. Ensure the collection tubing is secure and will not slip during transit.

9 Indicate the equipment is ready and collection can begin to the Pilot/Captain.

10 Turn on pump to begin water collection.

11 Record sampling information on datasheet:
1) Start lat and long
2) Start time
3) Additional comments (changes in sea state, situations that arise etc)

12 During collection, watch the angle of the pump line. If it deviates more than 20 degrees from the vertical, make a note of approx angle and how long it persisted.

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13 Once transect and water collection is complete, turn off the pump, remove tubing from the carboy and secure the cap. Store the 20L water sample securely for transit and cover with tarp to keep carboy as cool as possible.

NOTE: If temperature is particularly high, cover the carboy caps with the aluminum foil (the same used to secure the collection tubing), wet a towel with sea water and cover the carboys with the towel before laying tarp on top. Icepacks can also be laid on the wet towel to help keep samples cool.

14 Retrieve the pump from the water and record the following information on the eDNA datasheet:
1) End lat and long
2) End time
3) Additional comments (changes in sea state, situations that arise etc)

15 Remove the collection tube from the pump and set tubing in used equipment bin.

16 Gently place the pump in the bleach bath bucket and turn it on. Allow the pump to cycle in the bleach for 3 minutes.

NOTE: Make sure to wipe down the pump safety line and power cord with fresh water, then bleach, followed by dH2O.

[Bleaching may be replaced by cycling in multiple distilled water baths followed by cycling at site before attachment of collection tubing. Updates on this to come.]

17 Turn off the pump and allow it to drain as much bleach as possible before transferring it to the dH2O bath. Turn it on and allow it to cycle for 6 minutes. Once the water bath is complete, turn off the pump and move it the dH2O storage bath while in transit.

Return to step 1 at the next site.

Lab processing

18 Perform bench and equipment sterilization by treating all surfaces (bench and equipment) with standard water, bleach, dH2O, ethanol (70%)

19 Select the first 20L sample for processing. Invert the carboy 10X to ensure the sample is well mixed.

20 Change gloves.

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21 Using 2L Nalgene bottles, fill to the 2000mL mark (measured previously with graduated cylinder) with sample water. Repeat for each of the 4 x 2L sample bottles.

> NOTE: 2 of the 2L bottles will be used to flush and prime the peristaltic pump system.

22 With one hand, remove one end of the sterilized tubing from the autoclave bag. Using your other hand expose the pointed end of a 10mL pipette, using aseptic technique.

23 Insert the tappered end of the 10 mL pipette into the MasterFlex tubing. Withdraw the pipette from packaging and carefully insert into the "priming" 2L filter bottle, making sure that the bulk of the MasterFlex tubing remains in the autoclave bag.

24 Remove the remainder of the MasterFlex tubing from the autoclave bag and load into associated pump head. Ensure the end of the tubing does not come into contact with any lab surfaces.

> NOTE: Secure the tube ends with clips if necessary.

25 Repeat for the remaining priming bottles.

26 Prime the tubes by turning the pump on and setting the controller to the FWD direction at max speed of 2. Adjust tubing to dislodge as many bubbles as possible. If any persist, make note on sampling sheet.

27 Allow the system to run and stop the pump before the the priming bottle is emptied (before air bubbles are drawn up into the pipettes).

28 Transfer each pipette from the priming bottle to a designated 2L sample bottle. Be careful to minimize contact between pipette and neck of the bottles.

29 Collect the blue Swinnex housing with pre-loaded GF/A pre-filter from sterile lab kit. Attach a barbed luer-lok adapter to the threaded inlet end and insert into the tubing coming from the MasterFlex pump head.

30 Using the appropriate adapter, collect a sterile 2-3cm piece of tubing from the sterile lab kit and connect it to the outlet end of the blue GF/A pre-filter Swinnex housing.

31 Collect a Sterivex and add a barbed luer-lock adapter using aspetic technique.

32 Attach the Sterivex inlet port to the piece of tubing coming from the blue pre-filter Swinnex .

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33 Using another barbed adapter, connect the outlet of the Sterivex to the third and final 2-3cm piece of tubing.

34 Taking care to avoid touching the in- and outlet, attach a barbed luer-lok adapter to the threaded inlet of the pre-loaded beige Swinnex housing.

35 Fit the barbed-end of the adapter (attached to the beige Swinnex inlet) into the 2-3cm piece of MasterFlex tubing coming from the Sterivex filter housing.

36 Direct the outlet of beige Swinnex into the waste container. Secure tubing and filters with clamps if necessary. Ensure nothing will dip into or become submerged in the waste bucket as the volume increases. Make sure the tubing is not pinched at the attachment point.

NOTE: At this point, the system should be ready to filter. Working from the water sample to the waste container the sequence of filters is as follows:
1) Blue Swinnex GF/A pre-filter
2) Sterivex
3) Beige Swinnex charged filter

37 Turn on the pump to the FWD position with max speed=2. Check for leaks and good flow.

NOTE: If flow in any of the tubes seems slow, check that the tubing is properly installed in the pump head. If problem persists, check the swinnex filters to ensure only a single filter paper was installed in the housing.

38 Filter the entire 2L volume.

NOTES:
If water flow becomes notably reduced, it is likely the filter is clogged.
a) Record the volume filtered.
b) Remove filter, place on clean kimiwipe and cover. Return to this once the new filter has been installed. Follow steps 41-42 for Swinnex and steps 44-46 for Sterivex.
c) Use a maximum of two filters per 2L collection bottle.
d) Record all information on datasheet including:
  1) Time the filter was changed and how long the process took (approx number of minutes).
  2) Volume processed by first and second filter.
  3) Any observations.

Sample Preservation and Storage

39 When filtering is complete, sterilize gloves with bleach followed by thorough rinse with distilled water.

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40 Remove the beige Swinnex housing from the pump system.

41 Ensure to have two sterile forceps and labelled epi tube on hand.

42 Carefully open Swinnex and locate the filter. Grasp the edges of the filter paper with the sterile forceps and roll the filter in on itself so the surface with the sample is on the inside of the tube you have created. Place the folded filter into the labelled epi.

NOTES:
Do your best to minimize contact between the portion of the filter containing the sample. Try to restrict forcep manipulation to the edges of the filter paper.

43 Carefully remove the Sterivex filter. Avoid contact with the in- and outlet.

44 Remove the luer-lock adapter from the inlet. If there is water in the filter, collect a 60mL syringe from sterile lab kit and draw in air before attaching to the Sterivex. Depress the syringe to purge the air from the filter housing.

NOTES:
DO NOT DRAW AIR INTO PLUNGER WHEN ATTACHED TO THE STERIVEX.

45 Remove the syringe from the Sterivex and wipe both ends with clean kimwipe before adding blue dead-end caps to in- and outlet ports. If the caps do not fit snuggly, secure caps with parafilm.

46 Place sealed Sterivex filter into labelled 2oz whirl-pak.

47 Store both Swinnex and Sterivex filters at -80 and ensure all the data has been recorded correctly.

48 Disassemble the blue pre-filter Swinnex and discard prefilter.

49 Disassemble the pump system and move all site-specific equipment to designated tote for cleaning. Perform bench and equipment sterilization by treating all surfaces (bench and equipment) with standard water, bleach, dH2O, ethanol (95%) wipe down sequence.

50 Repeat process (Sample processing and preservation section) for the next set of samples.

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Equipment clean-up and sterilization

51 Fill 2L Nalgene bottle with 1L of 10% bleach solution. Secure cap and shake vigourously for 2 minutes, making sure the bleach comes into contact with all interior surfaces of the bottle. Pour the bleach into the next bottle. Bleach needs to be replaced after every 4 sample bottles.

52 Once all filtering is complete, collect all MasterFlex tubing for steriliztion and load into pump heads. Using the 2L bottles just bleached, add 1L of fresh 10% bleach solution. Using the same pipettes used in sample filtering, pump the full 1L of bleach through the system. Collect the discharged bleach in another set of collection bottles.

Repeat this step with the next set of tubing. 4 sets of tubing can be processed before the bleach needs to be refreshed.

53 Repeat steps 51 and 52 3X with distilled water to thoroughly flush the system. Change the distilled water after each bottle and set of tubing. Set bottles and tubing on clean bench coat to dry.

54 Clean the collection tubing by attaching it to the MasterFlex tubing using a T-adapter while running through tubing sterilization steps.

NOTE: If collection tubing is daisy-chained to the MasterFlex tubing during sterilization, refresh solutions after 2 sets rather than every 4.

55 Soak all Swinnex housing and adapters in a 10% bleach solution for a minimum of 6 minutes. Follow up with three distilled water baths. Soak equipment in each distilled water bath for 5 minutes followed by aggitation. Lay equipment out to dry.

56 Place all adapters, tubing, and bottles in UV hood for treatment.

After UV sterilization is complete, replace cap on sample bottles and set off to the side for next days sampling.

57 Collect pieces of Swinnex housing, load new filters and place assembled housing in designated autoclave bags.

58 Place UVed tubing into bags with loaded Swinnex housing and autoclave at 121°C for 30 mins.

59 Collect all 20L carboys. Add 2L of 10% bleach solution, recap and shake vigourously for 2 minutes. Make sure bleach comes into contact with all surfaces. Transfer bleach into the next container and repeat process. The bleach can be reused for 4 carboys before it needs to be refreshed.

60 When the last bottle has been treated, remove the caps from each 20L carboy and pour bleach into each cap and allow to sit for 60 seconds. This will clean the threads of the caps.

While the caps incubate, wet a shop towel with bleach and wipe the necks of the 20L carboys.

61 Repeat steps 59 and 60 3X using distilled water. Change the distilled water after 2 carboys.

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62 Place submersible pump in 10% bleach solution and allow to cylcle for 3 minutes. Transfer to 3 subsequent water baths allowing to cycle for 3 minutes each.

63 Remove submersible pump from water bath, allow to drain and place on separate designated bench coat to dry overnight.

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Figure A1 Standard Operating Procedure (9 Pages) for water sampling and sample filtration, created by Claire Rycroft (Dalhousie University MSc student) and last modified on December 2nd, 2019 using protocols.io.

Table A1 List of possible pathogens (and other entities) to be looked for in the present study, created by Julia Fast in November of 2019 based on literature searches and guided by a paper by Lennox et al. (2019) that is currently in-review.

Pathogen/Other	Notes
aeromonas salmonicida	
Atlantic salmon calicivirus	limited information available in the literature
Atlantic salmon paramyxovirus	on farms, may not have been found in wild
dermocystidium salmonis	on farms, may not have been found in wild
flavobacterium psychrophilum	on farms, may not have been found in wild
gyrodactylus salaris	
ichthyobodo sp.	
infectious pancreatic necrosis virus	
infectious salmon anemia virus	
moritella viscosa	
neoparamoeba perurans	on farms, may not have been found in wild
orthomyxovirus	
paranucleospora theridion	on farms, may not have been found in wild
parvicapsula pseudobranchicola	on farms, may not have been found in wild
piscichlamydia salmonis	on farms, may not have been found in wild
piscine myocarditis virus	
piscine reovirus	
piscirickettsia salmonis	
renibacterium salmoninarum	
reovirus MGL	assuming this is similar to piscine reovirus
rotavirus	limited information available in the literature
salmon alphavirus	
salmon gill poxvirus	
sphaerothecum destruens	limited information available in the literature
spironucleus salmonicida	limited information available in the literature
tenacibaculum maritimum	on farms, may not have been found in wild
tetracapsuloides bryosalmonae	
totivirus	limited information available in the literature
vibrio anguillarum	
vibrio salmonicida	
viral hemorrhagic septicemia virus	
yersinia ruckeri	
herring assay	
mackerel assay (if available)	