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Environmental DNA Plumes: Linking Fish Farm eDNA to Microbial Communities and Novel Detection of Transgenic eDNA

By

Alex Kajtar

A Thesis
Submitted to the Faculty of Graduate Studies
through the Great Lakes Institute for Environmental Research
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2021

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Environmental DNA plumes: Linking Fish Farm eDNA to Microbial Communities and Novel Detection of Transgenic eDNA

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DECLARATION OF CO-AUTHORSHIP

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows: I am the sole author of Chapter 1 and Chapter 4, and I am the primary author of Chapters 2 and 3. Chapter 2 was coauthored with Dr. Robert Delvin, and Dr. Daniel Heath and Chapter 3 was coauthored by Dr. Daniel Heath. In all cases, the primary contributions, including experimental input, data analysis, interpretation and writing were performed by the main author, and co-authors contributed to this thesis solely in an advisory capacity and in securing funds to perform the research.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

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I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

Finfish aquaculture has been on a steady rise, and to match human consumption an increase of open water fish farming is inevitable; however, the impacts of rearing high densities of fish on the surrounding ecosystem remains unclear. Transgenic fish have begun to be implemented in aquaculture to improve traits such as growth rate and feeding efficiency. However, concerns about the potential ecological impact if escaped transgenic organisms are diverse and widespread. Here we characterize the eDNA "plume" from an open water *Oncorhynchus tshawystcha* farm, and from a transgenic *Oncorhynchus kistuch* rearing facility. We utilize eDNA as a biomarker of sloughed Chinook salmon DNA from the farm and test for farm effects on bacterial community changes. We found evidence of an overall seasonal effect on eDNA concentration and localized distance effects relative to the farm in the fall. Our BC analyses showed strong seasonal effects as well as evidence of a distance (from the farm) on BC diversity. Despite the well-mixed characteristics of the sampled bay our findings indicate a radial effect of the fish farm plume on the surrounding waters. We also designed a transgene-specific assay to detect transgenic Coho salmon without interference from the wild-type genome and establish the range of detection from an effluent pipe. Our transgene-specific assay detected the growth hormone construct from environmental samples to 10 m from the effluent pipe, as well as two samples 150 m away and 1300m away from the effluent pipe, detecting extremely low traces of transgene DNA copies. This spatial inconsistency in transgenic eDNA detection may be due to sloughed organic matter accumulating, rather then breaking down into a homogenous mixture in marine water. This work establishes how eDNA can be used as a valuable tool for marine surveillance, providing data on the distribution of finfish DNA from a point source and identifying ecological impacts on the surrounding aquatic environment.

DEDICATION

To my dog Annie, Thanks for all the support.

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CHAPTER 1

General Introduction

Environmental DNA

Over the past two decades, researchers have developed the study of environmental DNA, or eDNA, as a molecular genetic surveillance technique, with applications in terrestrial and aquatic ecosystems. Environmental DNA detection techniques were first used on soil and water samples to study whole microbial communities and their interactions with the environment (Roose-Amsaleg et al., 2001; Taberlet et al., 2012). While microbial communities can be directly sampled in eDNA, all multi-cellular organisms leave behind a molecular genetic footprint in the form of sloughed organic matter, such as skin cells, fecal matter, and body fluids in their environment (Ficetola *et al.*, 2008). Species-specific or community markers can be used to detect macro-organisms based on the sloughed genetic material they leave behind (Ficetola *et al.*, 2008; Taberlet, Coissac, Hajibabaei, *et al.*, 2012; Wilcox *et al.*, 2013; Thomsen and Willerslev, 2015). Detection of vertebrates through eDNA has been used to identify rare and invasive species within aquatic ecosystems, without them being visually identified (Dejean *et al.*, 2012; Thomsen *et al.*, 2012; Spear *et al.*, 2015). The combination of eDNA, PCR and high-throughput sequencing (HTS) can be used to assess the microbial, as well as, animal and plant diversity in an aquatic ecosystem (Creer et al., 2016; Deiner et al., 2017a; Taberlet et al., 2012). Utilizing these methods, fish can be detected with high precision and minimal environmental impact, compared to traditional field methods, such as net capture or electrofishing (Jerde et al., 2011; Schmelzle & Kinziger, 2016; Wilcox et al., 2013). Overall, eDNA has been shown to be a valuable tool with a wide range of applications to investigate complex ecosystems.

eDNA Detection Techniques

Researchers can choose between single species detection by quantitative real time PCR (qRT-PCR), or multi-species (community) detection using PCR and HTS (meta-barcoding). Single species detection uses primers designed to target one or few organisms in an environmental sample. For example, Minamoto et al., (2017) used species-specific primer sets with qRT-PCR to conduct a temporal and spatial survey of *Chrysaora Pacifica* (Japanese sea nettle), in Maizuru Bay, Kyoto. They were able to match visual surveys with the distribution of eDNA within the bay, as well as temporal patterns of eDNA concentration correlated with the number of individuals observed. Meta-barcoding, on the other hand, utilizes PCR primers designed to amplify amplicons from an entire communty of organisms from an environmental sample. Evans et al. (2016) used eDNA metabarcoding in mesocosm experiments and were able to detect all nine species present, as well as, correlated their abundance with sequence read abundance. Balasingham et al. (2018) designed group-specific primers for HTS to detect three at risk species, one invasive species, and 78 native species of fish in tributaries of the Great Lakes. They demonstrated that eDNA metabarcoding for fish communities provides better spatial distribution data than single PCR target methods when examining an entire ecosystem. Depending on experimental design and research questions, qRT-PCR and metabarcoding using eDNA extracted from environmental samples provides an accurate approach for detecting a target species or community.

Microbial eDNA Metabarcoding

Environmental DNA metabarcoding has an important role in ecological research, specifically for quantifying community diversity within and among environments (Taberlet et al., *2012).* Microbial community studies based on eDNA analyses have compared bacterial assemblage patterns and specific functional adaptations across various ocean ecosystems around

the world (Rusch *et al.*, 2007; Zinger *et al.*, 2011). Determining the specific microbial assemblages that reside within niche environments can provide important information about the biological functions and patterns over time (Caruso, 2014). Treusch et al., 2009 identified three distinct bacterial communities in the northwestern Sargasso Sea during seasonal stratification: a surface community responding to low nutrients, a deep chlorophyll-rich community, and a mesopelagic community. They concluded that these specialized communities reflected the relative success of microbial populations in oligotrophic oceans. Analyzing such data across temporal replicates can provide insight into the environmental factors that may be influencing fluctuation and shifts in microbial community composition and function (Nogales et al., 2010; Giovannoni & Vergin, 2012). We know that microbial communities have vital roles in ecosystem biogeochemical cycles, and that their global biomass represents a huge carbon sink (Gilbert and Neufeld, 2014). Thus characterizing microbial community composition and dynamics can provide important information on the complex microbe-microbe and microbe-environment interactions that can affect aquatic ecosystems (Falkowski et al., 2008; Kallmeyer et al., 2012).

Ecology of eDNA

eDNA analyses is a relatively new approach for aquatic surveillance, hence most research has investigated its ability to detect species, with relatively little focus on the "ecology" of eDNA (Barnes & Turner, 2016). The ecology of eDNA is the of interactions between extra-organismal genetic material (eDNA) and the environment – these interactions influence the detection, quantification, and analysis of eDNA (Barnes and Turner, 2016) . Those interactions can be classified as origin, state, transport and fate of eDNA (Barnes & Turner, 2016). Studies have investigated how seasonal temperatures and the density of the source organism(s) can affect

degradation and abundance of eDNA (Takahara *et al.*, 2012; De Souza *et al.*, 2016). Deiner & Altermatt, 2014 studied the transport distance of invertebrate eDNA (*Daphnia longispina* and *Unio tumidus*) in a river system, and detected eDNA up to 9.1 km away from the source, indicating that invertebrate eDNA can persist over a long distance. However, factors such as water flow, species abundance and behavior, water temperature, and PCR inhibition can affect the detectability of an eDNA signal (Pilliod *et al.*, 2014; Jane *et al.*, 2015). With all the factors that can influence the characteristics of eDNA, further research is needed to assess how niche environments can affect eDNA ecology.

Aquaculture

Since 1990, the global trend for open-water fish capture has plateaued at ~85million tonnes, and aquaculture production has been on a steady rise due to demand that capture fisheries cannot meet (Duarte et al., 2009b, FAO 2020). Global fish production (in 2016) from aquaculture facilities reached 171 million tonnes, 88% of which was directed towards human consumption (FAO 2018). With this increase of fish production comes a higher number of farms using open-water systems to rear these organisms (Duarte et al., 2009). Such systems have sustainability issues due to the sensitivity of costal and inland environments from the output of nutrient waste of high densities of organisms (Wang *et al.*, 2012; Zhang, Bleeker and Liu, 2015). An increase in fisheries can have a variety of effects on the surrounding ecosystems, including farmed organisms escaping and potentially becoming invasive, eutrophication of surrounding waters, and disease or parasite transfer from captive to wild stocks (Diana, 2009). As aquaculture continues to grow, accurate and timely characterization of their influence on their surrounding environment is key to sustainable fish culture (Cole et al., 2009; Marra, 2005).

Genetically Modified Organisms

With the growing demand for fish production, the potential for transgenic organisms to increase production is being actively explored, as the production benefits of transgenes can help to maximize aquaculture production (Wakchaure & Ganguly, 2015). Transgenics is the insertion of a foreign, but functional gene construct into an organism's genome; the construct facilitates specific desirable biological processes (Pinkert et al., 1997). Desired effects from the insertion of foreign genes can include increases in disease resistance, cold tolerance, growth, and feed conversion ratios (Rasmussen and Morrissey, 2007). The insertion of a growth hormone transgene into *Oncorhynchus kisutch* (Coho salmon) has been shown to produce an increase of growth rate of up to 600%, while also reducing feed by 25% (Devlin et al., 1994). Such growth acceleration can be extremely valuable for aquaculture facilities to commercially produce organisms faster and at a higher feed efficiency. However, a primary concern with open-water farming of transgenic organisms is the risk of escapement, and potential breeding with native populations (Muir & Howard, 2002). Although transgenic fish have been shown to be reproductively out-competed by non-transgenic fish (Fitzpatrick *et al.*, 2011), they are more active and aggressive when feeding (Wakchaure & Ganguly, 2015). If transgenic fish become common in aquaculture, there will be a need to closely monitor these organisms at all stages of production (Aerni, 2004; Muir & Howard, 2002, Devlin et al., 1994). Using eDNA as a fast and sensitive surveillance method to detect the transgene in the environment may facilitate the safe application of transgenics in aquaculture.

Aquaculture Ecological Impacts

With the increase of aquaculture development, there is concern for the risks associated with rearing high densities of organisms in open water ecosystems (Karakassis *et al.*, 2000; Duarte *et al.*, 2009a). The organic matter from faeces, skin cells and residual food has been

shown to influence the seafloor and benthic microorganism communities (Danovaro et al., 2003; Karakassis et al., 2000; Luna et al., 2013; Olsen, 2008; Taberlet et al., 2012; Vezzulli et al., 2002; Wang et al., 2012). Although there is a knowledge gap in our understanding of the influence organic matter from farm output on pelagic ecosystems, studies have investigated their impact on benthic regions. He et al., (2019) sampled sediment from six salmon farms in British Columbia, Canada to measure the changes in foraminifera composition resulting from farm organic output. They found that foraminifera alpha diversity increased with distance from the farm and concluded that species diversity and composition was impacted by fish farming activities. With the potential for anthropogenic factors driving change in microbial communities around net-pen farming sites, it is important to have efficient and cost effective means of monitoring such changes (Caruso, 2014; Pawlowski *et al.*, 2016; He *et al.*, 2019). Environmental DNA metabarcoding of bacterial communities provides quantitative indices to accurately establish impacts of organic output (Pawlowski *et al.*, 2016; He *et al.*, 2019)

Multi-level Analysis

Diverse eDNA analyses have the potential to be combined to provide a comprehensive overview of study sites at multiple ecosystem levels. Combing vertebrate and microbial eDNA analyses can allow researchers to establish interactions and connections between these two levels of surveillance. This is especially true if vertebrate eDNA is composed of organic matter that directly influences bacterial communities composition (Leonard et al., 2000; Olsen et al., 2017; Vezzulli et al., 2002). Examining eDNA using qRT-PCR with species-specific primers can provide information such as presence, relative abundance, and distribution of an organism (Dejean, 2011; Takahara *et al.*, 2012; Laramie, Pilliod and Goldberg, 2015; Evans *et al.*, 2016). Pairing this single species analysis with eDNA metabarcoding data on bacterial community

composition can help establish functional relationships between bacterial communities and the genetic material sloughed from high densities of organisms (Creer et al., 2016; Deiner et al., 2017b; Handelsman, 2005; Taberlet et al., 2012). Therefore, utilizing multiple techniques of eDNA analysis for a single sample can provide a diverse data set to assess complex interactions within an environment.

Thesis Objective

This thesis aims to assess the ecological impact of aquacultural practices through eDNA analyses. Specifically, we propose to use eDNA as a multi-level surveillance technique for assessing the distribution of farm effluent. Our goal was to develop sensitive eDNA extraction and amplification techniques, characterize three-dimensional plumes of fish farm effluent, assess microbial community composition, and explore transgene construct mapping within complex marine environments. Specifically, we address two critical issues associated with open-water aquaculture surveillance.

In chapter two we address the increase of transgenic organisms in aquaculture and the need to detect potential transgenic escapees in aquatic environments. We designed a nested PCR assay to detect the growth hormone transgene from transgenic Coho salmon, without interference from their wild-type counterparts. We then established the detection distance of the growth hormone transgene from an effluent outflow pipe, and the assay's sensitivity for monitoring the use of transgenic organisms. In chapter three, we address how the plume of eDNA from a marine fish farm is driving change in the surrounding bacterial community. We conducted a three-dimensional transect sampling of the bay surrounding a Chinook salmon farm to investigate the relationships between Chinook salmon eDNA and bacterial community diversity. These chapters

emphasize the range of applications eDNA can be used for aquatic surveillance as a bioindicator to facilitate management changes for sustainable rearing practices.

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CHAPTER 2

Detection of Growth Hormone Transgene Environmental DNA in Marine Waters Adjacent to A Transgenic Coho Salmon Rearing Facility

Introduction

Aquaculture production has been rapidly increasing in response to the demand for, and limited natural supply of, seafood products (Sapkota et al., 2008). To match this demand there is a desire to improve aquaculture production efficiency through enhancement of, among others, growth, food conversion efficiency, and disease resistance. Genetic technologies have been explored to improve aquaculture, including production of genetically modified organisms (GMOs), or transgenic organisms, where foreign DNA associated with desirable traits are inserted into the host genome (Rasmussen & Morrissey, 2007). Growth-related transgenes are of great interest as candidates to enhance production efficiency due to their potential to stimulate appetite, growth rate and feeding efficiency (Fletcher & Davies, 1991; Rasmussen & Morrissey, 2007; Singh et al., 2019). In fish, growth hormone (GH) genes are seasonally regulated by the central nervous system inducing synthesis of IFG-I and IGF-II (insulin-like growth factors), ultimately stimulating growth (McKay *et al.*, 2004; Robertson et al., 2017). Growth hormone transgenic fish have been shown to increase their growth rate by up to 37 fold, while reducing feeding requirements by up to 25%, ultimately improving food conversion ratios (Devlin et al., 1994; Raven et al., 2008; Higgs et al., 2009). The production improvements resulting from

transgenic technologies are of interest for commercial use due to their ability to increase aquaculture production efficiency; however, regulatory agencies have ecological and environmental concerns (Aerni, 2015; Devlin et al., 2006; 2015; Kapuscinsji et al., 2007).

Concerns about potential ecological impacts resulting from transgenic fish that have escaped confinement are diverse and wide-ranging (Devlin, Sundström & Leggatt, 2015). For example, it has been shown that cultured transgenic salmon have inferior reproductive ability relative to salmon from nature (Bessey et al., 2004; Fitzpatrick et al., 2011; Leggatt et al., 2017); however, it is possible that transgenic fish could hybridize with wild conspecifics and adapt over time, improving their ability to introgress into wild populations with potential ecological impacts. GH-transgenic Coho salmon (*Onchorhyncus kisutch*) also show more risk-taking behaviour compared to wild-type salmon thus increasing their competitive ability to acquire food (Sundstrom et al., 2004). Thus transgenic Coho salmon juveniles may proliferate in a environment with low predation pressure and a high abundance of food (Sundstrom et al., 2004), but be eliminated in environments with high predator load and low food supply. Given the potential for transgenic salmon to have ecological impacts, there is a need to detect escapees with high sensitivity and precision within aquatic environments (Naylor et al., 2005).

Distinguishing transgenic from wild-type coho salmon based on morphological characteristics is not reliable (Sundström, Lõhmus & Devlin, 2015). Traditional capture-based survey methods would be ineffective to differentiate between wildtype and transgenic salmon, thus a molecular genetic approach over large geographic areas is required for transgene surveillance (Singh et al., 2019). Environmental DNA (eDNA), which is DNA extracted from environmental samples without direct sampling of the target species (Taberlet, Coissac, Hajibabaei, et al., 2012), has proven to be effective in detecting rare and early-stage invasive

species with great accuracy (e.g., Jerde et al., 2011; Goldberg et al., 2016). However, most published applications of eDNA for fish have employed mitochondrial DNA markers due to the high copy number and relative stability of mitochondrial DNA (Thomsen & Willerslev, 2015). Many factors influence the detection of eDNA, including abiotic environmental factors, target organism abundance and eDNA collection and extraction protocols, among others (Pilliod et al., 2014; Eichmiller, Miller and Sorensen, 2016; Goldberg et al., 2016). Therefore, applications of eDNA detection to nuclear transgenic genes in aquatic environments may not be technically straightforward, but the ability to detect GMOs in aquatic ecosystems is an important goal.

Here we design and optimize nested eDNA PCR assays and estimate their sensitivity to detect the nuclear growth hormone construct (pOnMTGH1) in transgenic Coho salmon (Devlin *et al.*, 1994). We apply this novel protocol to establish the transgene detection range from an outflow effluent pipe outside a research facility that rears transgenic Coho salmon. A reliable protocol to detect transgene constructs in aquatic environments will have important applications to environmental risk assessments, as well as for tracking during shipping and marketing.

Materials and Methods

Sample Collection

Sampling was conducted at the Fisheries and Oceans Canada facility in West Vancouver, British Columbia on November 19th, 2018. The facility has a single outflow pipe that discharges treated effluent from the fish rearing tanks into English Bay (Figure 1). Details on the housing and rearing of the transgenic Coho salmon at the West Vancouver facility are described in Alzaid *et al.*, (2018). At the time of sampling, there was a total of 188 transgenic Coho salmon (average weight of 4.29 kg, total 807.9 kg of biomass) reared in two mesocosm tanks receiving and discharging 900 L of seawater per minute.

Open-water samples were collected in 90 mL narrow mouth bio-tile™ (48 mm) specimen screw top plastic containers (Thermo Scientific) starting from the outflow pipe and progressing in three transects (Figure 1a). From the outflow pipe, water samples were collected at 0 m, 1 m, 3 m, 10 m, 30 m, 100 m, 300 m, and 1000 m from the outfall, as well as at the CAER dock (49.34033°N, -123.23259°E) and wharf (49.34012°N, -123.23303°E) outside the facility (Figure 1a). Additionally, water was collected at four negative control sites in English Bay ranging from 2.8 km to 6.4 km away from the facility (49.32958°N, -123.26285°E; 49.31273°N, -123.24963°E; 49.28492°N, -123.21499°E; 49.32691°N, -123.18607°E; 49.33524°N, -123.20764°E) (Figure 1b). Collection of water samples at each site was done at the surface and at 0.5 m to 10 m depth (depending on available depth) as site replicates. Water samples were also collected from holding tanks for transgenic and non-transgenic Coho salmon as positive and negative controls. All samples were stored on ice then frozen at -20°C within three hours of collection, and later shipped, inside a cooler filled with dry ice, to the University of Windsor for storage at -20°C until eDNA extraction and analyses.

Filtration and Extraction

Frozen water samples were thawed at room temperature and filtered with Cole-Parmer 0.8 µm pore size, 47 mm nylon filters (# RK-15945-28). A single filtration control consisting of 100 mL of ddH_2O through a blank filter was included after all field sample filtrations were completed. After filtration, each filter was cut in half (including the lab blank) with sterilized scissors and forceps, and one half was manually cut into thin strips (the other half was preserved for future use) before being placed into 2 mL sterile tubes that contained 0.5 mL of 1.0 mm glass beads (Bio-Spec Cat. No. 11079110). Each tube was filled with 400 µL of digestion buffer (5.844g NaCl, 50mL 1M tris-HCl pH 8.0, 20mL 0.5M EDTA, 50mL 10% SDS, diluted to 1.0L

ddH$_2$O) plus 200 µL of ddH$_2$O and homogenized for 1 min at 3,000 strokes per minute three times using a Mini-Beadbeater-24 (Fisher Scientific LTD, Bio-Spec.). Samples were centrifuged for 1 min at 13 000 g, before adding 2 µL of Proteinase K (20 mg/mL) and incubated with gentle rocking at 60°C for 1.5 hours. After incubation, the samples were held at 95°C for 10 min to deactivate the Proteinase K. Samples were then centrifuged for 5 min at 13 000 g, after which 150 µL of the supernatant was transferred to an extraction plate for robot-mediated bead extraction (Shahraki et al., 2019). Purified eDNA was eluted in 50 µL TE and stored at -20°C until further use.

Primer Development

Two sets of nested PCR primers were designed to amplify the growth hormone transgene construct (pOnMTGH1) in Coho salmon (Table 1). One primer set was designed to anneal to the metallothionein-B promoter and the other to the growth hormone-1 coding region in the transgene construct (Devlin et al. 1994; 2004). This arrangement of DNA sequence does not occur in the wild-type coho salmon genome and thus our primers specifically amplify only the transgene. The pre-amplification primer set (GH-115F & R; Table 1) was designed to amplify a 115 bp amplicon, while the second, nested, primer set (GH-107F & R; Table 1) amplified a 107 bp region within the initial 115 bp amplicon. Primers were designed using Primer3Plus and tested in the lab using DNA extracted from transgenic and non-transgenic Coho salmon fin clips to ensure the nested protocols were transgene-specific and to estimate sensitivity of transgene detection. Fin clip extracted DNA concentration was measured on a NanoVue 35 spectrophotometer (General Electric Company) producing a concentration of 1457 ng/µL. Nested primer sensitivity was determined using 1:4 dilutions series for 16 dilutions (one part ddH$_2$O and three parts extracted DNA), starting with the pre-amplification PCR (with 2µL of

template DNA) using the GH 115F & R primers followed by the nested triplicate quantitative real-time polymerase chain reaction (qRT-PCR) reactions (GH107F & R) using the dilution series of the fin clip transgenic Coho salmon DNA (details below). For all PCR assays, transgenic and non-transgenic Coho salmon DNA positive and negative controls (respectively) were used to ensure the nested primers only amplified the transgene construct. Sensitivity analysis was conducted for the dilution series standard curve to establish sensitivity threshold of the transgene construct as well as PCR efficiency. The sensitivity threshold was determined by the sensitivity limit of our nested PCR assay.

Table 1: Transgenic Coho salmon nested PCR primers

Primer Name	Fragment size	Primer	Annealing Temperature (°C)
GH-107F	107 bp (nested)	AAGAAGCGCGATCGAAAAG	60
GH-107R	107 bp (nested)	ACACTGACTTCCCACTGAAAA	60
GH-115F	115 bp	ACTAAAGAAGCGCGATCGAA	55
GH-115R	115 bp	TGGTACACTGACTTCCCACTG	55

PCR Protocols

Our PCR assay consisted of a 20-cycle pre-amplification with primer set GH-115F & R, and a second (nested) qRT-PCR using the pre-amplification PCR product and primer set GH-107F & R. The pre-amplification PCR consisted of 2.5 µL of 10x Taq reaction buffer (Bio Basic Canada Inc., Markham, On, Canada), 2.5 µL 20mM $MgCl_2$, 0.5 µM of forward and reverse GH-115F & R primers, 0.2 mM of each dNTP, 0.1 units of *Taq* polymerase (Bio Basic Canada Inc., Markham, On, Canada), 2.0 µL template DNA and ddH_2O for a total 25 µL reaction volume.

PCR conditions were an initial denaturation of 95°C for 2 min, then 20-cycles of 1 min 95°C, 30 s annealing at 55°C, 30 s extension at 72°C, with a single final extension at 72°C for 5 min and a final hold at 4°C. The second, nested, qRT-PCR consisted of 7.5 µL SYBR® green powerup (Thermofisher Scientific), 0.5 µM of forward and reverse GH-107F & R primers, 2.5 µL of the pre-amplification PCR product and ddH_20 for a total of 15 µL. qRT-PCR conditions were: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C denaturation for 15 s and 60°C annealing for 1 min. This protocol was used for our nested PCR standard curve dilution series DNA as well as our field eDNA samples. The initial pre-amplification PCR (GH115F & R) was run as a single assay with 2 µL of positive (transgenic Coho salmon DNA) and negative (non-transgenic Coho salmon DNA) controls, followed by the nested qRT-PCR run in triplicate for each PCR product and a second negative control, for a total of four assays per first round PCR. For every PCR plate we included a negative control of just PCR master mix (no template DNA). Therefore, the pre-amplification PCR included a negative control (as well as a positive control), and the nested qRT-PCR included a new negative control (as well as the initial negative control) run in triplicate. This was done to allow detection of contamination at each step of the nested PCR assay, should it occur.

Analysis

We employed conservative criteria for a positive transgene detection for the nested qRT-PCR assay. Individual nested qRT-PCR C_T values must be within the detection sensitivity range to be scored as a positive detection (C_T=4.7 to 27.5), and two out of the three triplicates must be positive detections for the sample to be deemed a positive detection. We also used the quantitative estimate of target transgene copy number possible with qRT-PCR and our standard curve: transgene copy number was calculated based on the serial dilutions standard curve and

known stock DNA concentration. Starting DNA concentration (from fin clip extraction) was converted into number of transgene copies present. To calculate the number of transgenes constructs we used an estimated Coho genome size of 1,600,000,000 bp multiplied by 650 Daltons (1 Dalton = 1.67×10^{-24}g) to establish that in 1 ng there are 578 Coho salmon genomes present. The transgene construct pOnMTGH1 is present five times within a Coho salmon genome and is hemizygous (Devlin, Sundström & Muir, 2006; Uh, Khattra & Devlin, 2006), therefore we multiply by five and divide by two, resulting in 1445 transgene constructs in 1 ng of DNA (a single construct weighs 6.9×10^{-4}). Ultimately, our stock DNA used to generate our dilution curve contained 840,990 transgene copies in 2 µL of DNA.

Results and Discussion

Our nested qRT-PCR had a PCR efficiency of 103% (slope= -2.87) (Figure 2) and a detection limit range of 0.003 to 0.05 copies (Figure 2). Our assay is based on the second PCR in a nested design, resulting in a high PCR efficiency and low C_T due to the preliminary 20-cycle PCR. In all PCR assays, none of our negative controls amplified at either stage of the nested PCR amplification, these included our filtration control, PCR blanks and the field negative sites; indicating no contamination due to lab or field procedures. This is remarkable due to nested PCR assays frequently generating type I error (false positive), and may be due to our stringent detection requirements which is critical to minimize bias in eDNA detection (Ficetola *et al.*, 2015). Furthermore, our nested PCR assay showed positive detection in the transgenic Coho salmon tank water and no positive detection for the non-transgenic tank water samples. Therefore, no wild-type Coho DNA would be identified as a positive detection with our nested assay.

With the extremely low copy number detection limit (0.003 to 0.05) our nested PCR assay is highly sensitive to very low concentrations of the target transgene in the environment, making our field sample analyses powerful, despite the low total water volume used for eDNA isolation (90 mL). In comparison, Lacoursière-Roussel *et al.*, 2016, filtered 1.0 L water samples to detect Lake trout (*Salvelinus namaycush)* eDNA and estimated concentration through qPCR (mitochondrial COI gene markers) and standard curves (constructed from fish tissue DNA dilutions). Their reported eDNA concentrations varied from 2.6 to 4278.7 pg/L (2.6 x 10^{-3} to 4.27 ng/µL). Another study from Lacoursière-Roussel et al (2016), tested different eDNA filter capture methods with Brook Charr *(Salvelinus fontinalis*) eDNA tanks samples, and quantified the concentration through qPCR (cytochrome b gene markers). They found that 1.0 L of water filtered through glass fiber 0.7-µm filters captured on average 81.12 ng/L (0.081 ng/µL) eDNA concentration, with a median of 4.67 ng/L (4.67 x 10^{-3} ng/µL). Our eDNA extraction method and qRT-PCR protocol for a nuclear transgene thus produced a comparable detection limit, despite the nuclear target transgene and small sample volume.

Only seven of our 62 field eDNA samples generated a positive detection for the growth hormone transgene (Table 1s). All three triplicates of these samples produced a positive signal with C_T values ranged from 25 to 27 (0.003 to 0.025 transgene copies) and C_T standard errors ranging from 0.032 to 0.135 (Supplementary Material Table S1). Among the seven positive field samples, five were within 10 m of the outflow pipe; however, we did not observe consistency across the depth replicates, nor was there consistency across the distances on the three directional transcripts (Figure 3). Two opportunistically sampled sites (30th Street and Wharf) that produced a positive signal were unexpected, since they are >1000m and >100m, respectively, from the outflow pipe. Other such sites, such as the CAER dock (~100m) and the very distant negative

control sites (2.8 km to 6.4 km), did not produce any amplification. Our detection profile shows a gradual decline in detection as distance increased from the outflow pipe with a loss of signal at 10 m (other than the 30th Street and Wharf locations; Figure 1). The lack of spatial consistency among sites within 10 m of the outflow pipe (and between at depth and surface samples) may be the result of environmental factors such as marine current patterns, tidal flow, or upland runoff effects, or perhaps variation in the persistence of the target transgene construct itself. The observed very localized transgene signal detection is surprising, given that this facility has been rearing transgenic Coho salmon and discharging tank water into English Bay for three decades. The positive detections of the growth hormone transgene at the Wharf and 30th Street sites may be due to organic genetic material (e.g., feces, mucus, gametes etc.) aggregating, resulting in non-uninform distribution based on stochastic tidal patterns ultimately contributing to the spatial inconsistency of transgenic eDNA found from the effluent pipe. Ultimately, the low copy numbers detected is a major contributor to the lack of consistency among positive detections. Furthermore, the hemizygous nature of the construct within the Coho genome may also be influencing the spotty detection from environmental samples.

Of the 15 samples collected within three meters of the outflow pipe, only four produced a positive signal for the transgene (Figure 3), reinforcing that significant variation in transgene detection is observed at low concentrations in natural marine ecosystems. The small volume of water we filtered for eDNA extraction (90 mL) may be a contributing factor to our limited detection distance; however, logistics prevented the collection, proper preservation, and shipping of larger volumes. Filtering a larger volume of water, such as the common one-liter sample volume, would increase our retention of organic material, and would thus increase our detection capacity by approximately ten-fold. However many factors can influence the quantity of DNA

retained after filtration and extraction, such as filter type extraction protocol, and PCR inhibitors (Eichmiller, Miller and Sorensen, 2016; Hinlo *et al.*, 2017).

The use of eDNA detection methods to detect transgenic organisms may be restricted to rearing water at farms and rearing facilities to assess whether transgenic fish are present. Detection in natural aquatic environments using our nested PCR approach is inconsistent, and thus only positive detections are reliable, since it is likely our study had a high level of type II error (false negatives). However, this shortcoming may be overcome with larger filtration volumes, although the challenges associated with nuclear eDNA markers remain. The nested PCR assay described here provides a cost-effective and non-intrusive method of determining whether farm effluent does contain traces of the transgene. The detection distance from the effluent pipe of 10 m is relatively short; however, a limited spatial detection range may be advantageous when locating potential escapees due to the reduced interference from target eDNA carried from the captive fish. With open-water farms containing high densities of organisms, transgenic farm eDNA plumes may produce false positive detection for molecular assays trying to verify escapee presence. Our work also highlights the lack of long-term retention of the transgene construct within a natural marine ecosystem, even though transgene eDNA has been continuously drained into the bay for three decades. Therefore, we speculate transgenic eDNA does not persist in aquatic environments for extended periods, despite the long-term exposure from the rearing facility. Alternative applications for eDNA surveillance can include estimating the biomass of transgenic individuals in natural environments for ecological risk assessments (Li et al., 2015), as well as for testing shipments of live fish (or frozen seafood) to determine the presence, or absence of regulated transgenic organisms (Collins *et al.*, 2013).

The benefits of transgenic organisms to the aquaculture industry are substantial; however, a reliable means of surveillance is crucial for the widespread adoption of such technologies. We provide a sensitive transgene eDNA protocol to detect one candidate transgene construct in a complex aquatic environment. Our qRT-PCR assay effectively differentiates between transgenic and non-transgenic salmon; however, the nature of the nuclear gene target (transgene construct) may be contributing to our limited detection distance, especially when compared to traditional mitochondrial molecular markers for fin fish (Deiner *et al.*, 2017b). Ultimately, this approach provides a means of detecting transgenic organisms from environmental samples without cross-amplification from the wild-type counterparts, perhaps improving the environmental security of rearing transgenic organisms.

Figures and Tables

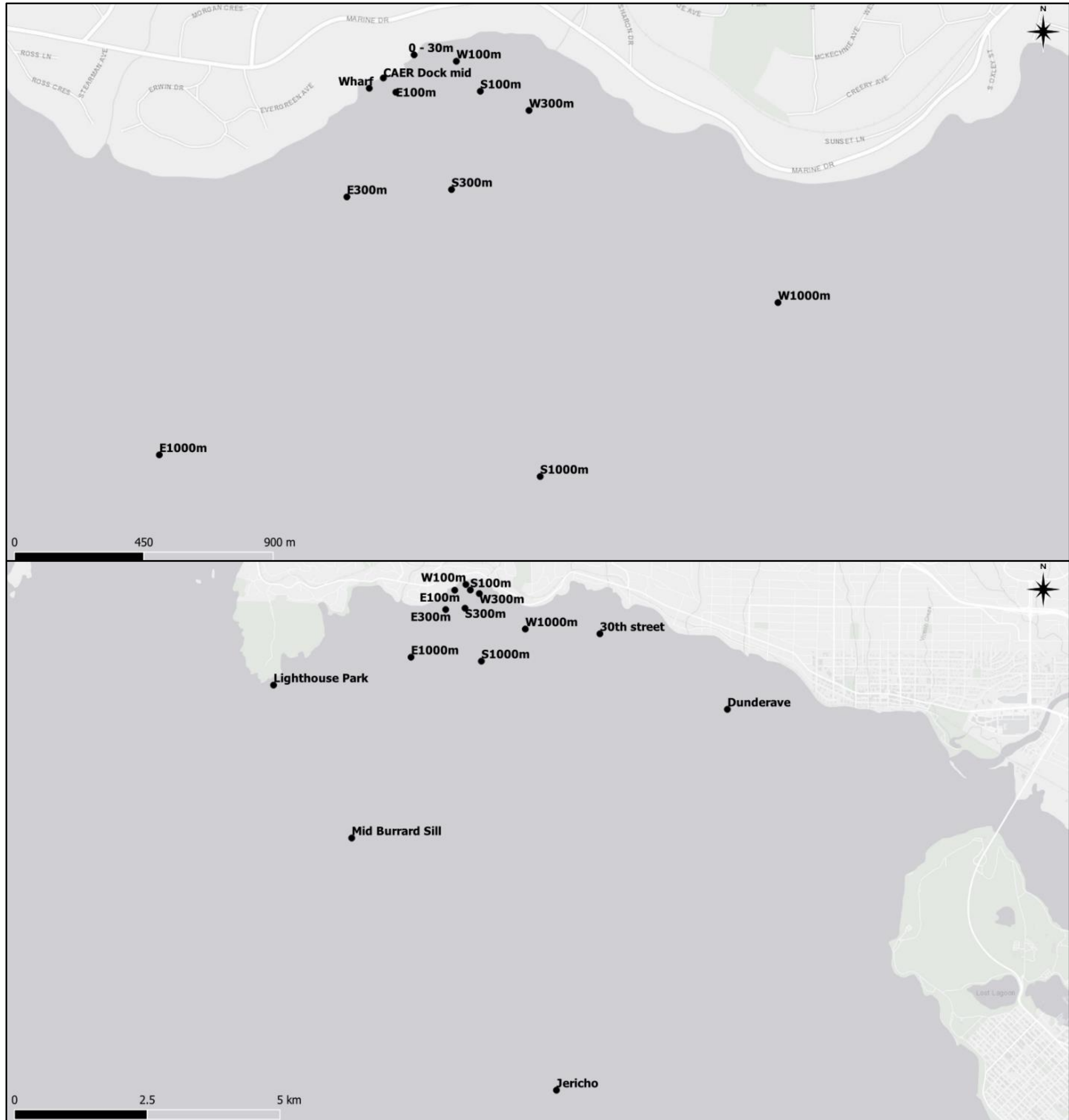

Figure 1: eDNA water sample locations within English Bay, Vancouver, British Columbia. Sample sites are identified with dots, and the sample design consists of transects from the DFO facility effluent pipe ("W" = west; "C" = center; "E" = east) plus opportunistically sampled sites (Wharf, CAER Dock) and far sites (Jericho, Lighthouse Park, Mid Burrand Sill, Dunderave and 30th Street). Figure 1a shows sample transect locations 0 –

1000m (Samples 0 – 30m are represented as one point). Figure 1b shows all additional far site locations taken within English Bay.

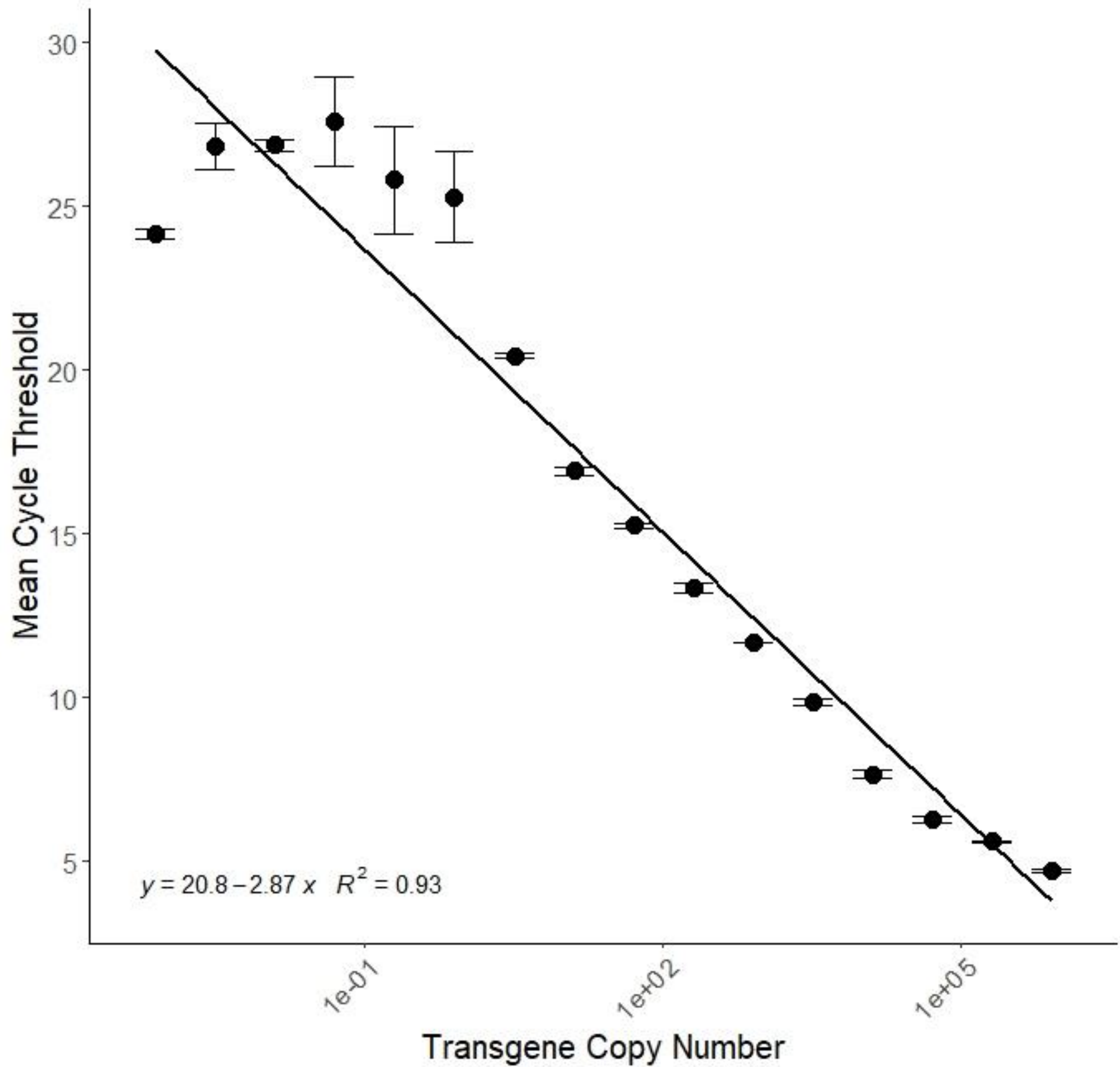

Figure 1: Serial dilution of Transgenic Coho Salmon DNA nested PCR protocol with primer GH-107 nested within GH-115 after a pre-amplification of 20 cycles. X-axis represented in a log10-scale of transgene copy number; Y-axis represent the mean cycle threshold values from qRT-PCR.

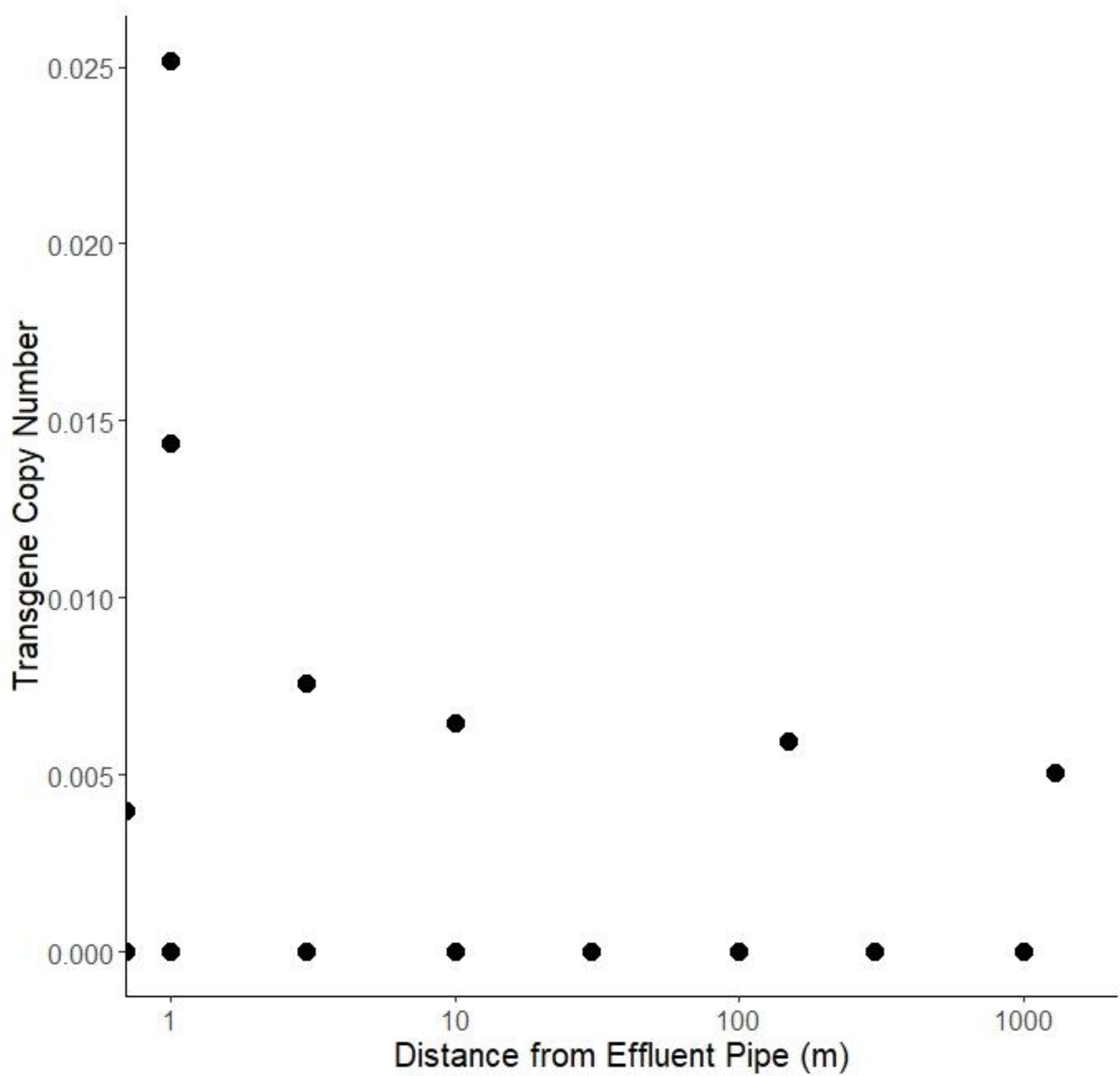

Figure 3: Transgene copy number plotted against distance (m) of water samples from the effluent pipe (represented in a log10-scale). The three transects and depth/surface samples were combined, as well as the opportunistic samples, to represent the concentration of DNA by distance in a two-dimensional figure.

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Supplementary Materials

Supplementary Materials Table 1s: Sample site information and transgene eDNA detection results for each field sample (including transgenic tank control). Included are; GPS coordinates (latitude and longitude), depth (Surface and At Depth), Mean cycle threshold (C_T) with standard error, Mean concentration (ng/µL), and number of triplicates positive. Subheadings used to separate samples but Field site, Opportunistic samples, Positive Controls, Negative Controls, and far sites

Sample	Latitude	Longitude	Depth	Mean C_T	Mean Copy Number	Triplicates Positive
Field Sites						
E0m	49.3408	-123.232	Surface	27.7 ± 0.0110	0.0039	3/3
S0m	49.3408	-123.232	Surface	Undermined	N/A	0/3
W0m	49.3408	-123.232	Surface	Undermined	N/A	0/3
E1m	49.3408	-123.232	Surface	26.1 ± 0.135	0.014	3/3
	49.3408	-123.232	At Depth	Undermined	N/A	0/3
S1m	49.3408	-123.232	Surface	Undermined	N/A	0/3
	49.3408	-123.232	At Depth	Undermined	N/A	0/3
W1m	49.3408	-123.232	Surface	Undermined	N/A	0/3
	49.3408	-123.232	At Depth	25.4 ± 0.0316	0.025	3/3
E3m	49.3408	-123.232	Surface	Undermined	N/A	0/3
	49.3408	-123.232	At Depth	Undermined	N/A	0/3
S3m	49.3408	-123.232	Surface	Undermined	N/A	0/3
	49.3408	-123.232	At Depth	26.9 ± 0.127	0.0075	3/3
W3m	49.3408	-123.232	Surface	Undermined	N/A	0/3
	49.3408	-123.232	At Depth	Undermined	N/A	0/3
E10m	49.34069	-123.232	Surface	27.1 ± 0.0798	0.0064	3/3
	49.34069	-123.232	At Depth	Undermined	N/A	0/3
S10m	49.34072	-123.232	Surface	Undermined	N/A	0/3
	49.34072	-123.232	At Depth	Undermined	N/A	0/3
W10m	49.34082	-123.232	Surface	Undermined	N/A	0/3
	49.34082	-123.232	At Depth	Undermined	N/A	0/3
E30m	49.34054	-123.232	Surface	Undermined	N/A	0/3
	49.34054	-123.232	At Depth	Undermined	N/A	0/3
S30m	49.34063	-123.231	Surface	Undermined	N/A	0/3

	49.34063	-123.231	At Depth	Undermined	N/A	0/3
W30m	49.34087	-123.231	Surface	Undermined	N/A	0/3
	49.34087	-123.231	At Depth	Undermined	N/A	0/3
E100m	49.34004	-123.232	Surface	Undermined	N/A	0/3
	49.34004	-123.232	At Depth	Undermined	N/A	0/3
S100m	49.34006	-123.23	Surface	Undermined	N/A	0/3
	49.34006	-123.23	At Depth	Undermined	N/A	0/3
W100m	49.34067	-123.23	Surface	Undermined	N/A	0/3
	49.34067	-123.23	At Depth	Undermined	N/A	0/3
E300m	49.33791	-123.234	Surface	43.3	1.46E-8	1/3
	49.33791	-123.234	At Depth	Undermined	N/A	0/3
S300m	49.33806	-123.23	Surface	Undermined	N/A	0/3
	49.33806	-123.23	At Depth	Undermined	N/A	0/3
W300m	49.33967	-123.228	Surface	42.6 ± 1.24	2.57E-8	3/3
	49.33967	-123.228	At Depth	Undermined	N/A	0/3
E1000m	49.33266	-123.24	Surface	Undermined	N/A	0/3
	49.33266	-123.24	At Depth	Undermined	N/A	0/3
S1000m	49.33222	-123.228	Surface	Undermined	N/A	0/3
	49.33222	-123.228	At Depth	Undermined	N/A	0/3
W1000m	49.33576	-123.22	Surface	42.3 ± 1.01	3.27E-8	3/3
	49.33576	-123.22	At Depth	Undermined	N/A	0/3
Opportunistic sites						
Wharf	49.34012	-123.233	Surface	27.2 ± 0.0274	0.0059	3/3
	49.34012	-123.233	At Depth	Undermined	N/A	0/3
CAER Dock	49.34033	-123.233	Surface	Undermined	N/A	0/3
	49.34033	-123.233	At Depth	Undermined	N/A	0/3
30th Street	49.33524	-123.208	Surface	27.4 ± 0.0854	0.0051	3/3
	49.33524	-123.208	At Depth	Undermined	N/A	0/3
Positive Controls						
Transgenic Tank (1)	-	-	-	25.9 ± 0.169	0.016	3/3
Transgenic Tank (2)	-	-	-	26.1 ± 0.281	0.014	2/3
Transgenic Tank (3)	-	-	-	24.5 ± 0.210	0.05	2/3
Transgenic Fin Clip	-	-	-	12.6 ± 0.089	722	3/3
Negative Controls						
Non-Transgenic Tank (1)	-	-	-	Undermined	N/A	0/3
Non-Transgenic Tank (2)	-	-	-	Undermined	N/A	0/3
Non-Transgenic Tank (3)	-	-	-	Undermined	N/A	0/3

Non-Transgenic Fin Clip	-	-	-	Undermined	N/A	0/3
Filter Control	-	-	-	Undermined	N/A	0/3
PCR 1st negative	-	-	-	Undermined	N/A	0/3
PCR 2nd negative (1)	-	-	-	Undermined	N/A	0/3
PCR 2nd negative (2)	-	-	-	Undermined	N/A	0/3
PCR 2nd negative (3)	-	-	-	Undermined	N/A	0/3
Far sites						
Lighthouse Park	49.32958	-123.263	Surface	Undermined	N/A	0/3
	49.32958	-123.263	At Depth	Undermined	N/A	0/3
Mid Burrard Sill	49.31273	-123.25	Surface	Undermined	N/A	0/3
	49.31273	-123.25	At Depth	Undermined	N/A	0/3
Jericho	49.28492	-123.215	Surface	Undermined	N/A	0/3
	49.28492	-123.215	At Depth	Undermined	N/A	0/3
Dundarave	49.32691	-123.186	Surface	Undermined	N/A	0/3
	49.32691	-123.186	At Depth	Undermined	N/A	0/3

CHAPTER 3

Marine Environmental DNA plumes: Linking Salmon Farm eDNA and Bacterial Communities

Introduction

With the rapid increase of the human population in the 20th and 21st century, the demand for seafood has increased dramatically (Duarte *et al.*, 2009b). Capture rates of wild fish could not match the growing demand, leading to the growth of aquaculture, specifically, rearing aquatic organisms in open water systems (Diana, 2009). This brings urgency to the need for environmentally sustainable practices to keep aquaculture within the carrying capacity of inland and costal bodies of water (Edwards, 2015). Modern aquaculture relies on agro-industrial pelleted feed, antibiotics, large areas of open water for production, and high rearing densities (Edwards, 2009; Sapkota et al., 2008). These factors increase the organic output from farms, and influence the surrounding microbial communities, potentially leading to eutrophication and reduced bacterial community (BC) diversity and changes in community composition (Heisler *et al.*, 2008; Caruso, 2014). It is critical to maintain aquaculture rearing within ecological limits to minimize environmental degradation, as the environment provides vital ecosystem services for the health of not only the farmed fish, but also wild organisms and, ultimately, human well-being (Millennium Ecosystem Assessment, 2003). These considerations are important for open water cage-base fish production, as these systems generate organic effluent that is dispersed into the surrounding environment (Edwards, 2015). Therefore, quantitative monitoring of the effects of aquaculture systems on the surrounding ecosystem to establish guidelines suited for environmental protection is overdue (Cole *et al.*, 2009).

Methods for aquatic ecosystem assessment are usually based on direct sampling approaches (e.g., visual surveys, vertebrate and invertebrate capture-based assessments, water quality measurements, candidate bacterial species culture) that are expensive and time consuming, especially in remote areas (Lacoursière-Roussel *et al.*, 2016a). Monitoring aquatic ecosystems with molecular genetic approaches is a more efficient, cost effective, and non-destructive option (Creer *et al.*, 2016). Environmental DNA (eDNA) is extracted from material filtered from water samples and used to assess species presence or community composition based on sloughed material, or whole microorganisms present in the water sample (Taberlet et al., 2012; Thomsen & Willerslev, 2015). Using specific polymerase chain reaction (PCR) primers to amplify and quantify template eDNA from one or more species in an environmental sample can be a powerful approach to environmental assessment (Deiner et al., 2017; Ficetola et al., 2008). Aside from determining the presence/absence of a target species, the relative abundance of the target DNA can be estimated using quantitative real-time PCR (qRT-PCR) assays (Kelly *et al.*, 2014; Lacoursière-Roussel *et al.*, 2016a). However, to effectively monitor aquatic systems and diagnose specific changes that may result from fish farming, more than just the presence of the farmed fish species eDNA must be considered: specifically, the bacterial community composition (BCC) can provide valuable ecosystem health information (Rosa *et al.*, 2001; Luna *et al.*, 2013). High-throughput sequencing (HTS) of eDNA samples can be used to characterize the BCC and thus indirectly, potential resulting cascade effects (Bentzon-tilia & Sonnenschein, 2016; Abdelfattah et al., 20z18). Combining the detection and quantification of farmed fish eDNA with BC characterization can provide insights into the potential scope for fish farm effects on the surrounding aquatic ecosystem.

Previous studies have used eDNA analyses to address diverse issues concerning the distribution of fishes and the composition of complex communities in costal and inland bodies of water. For example, Takahara et al., (2012) demonstrated that common carp (*Cyprinus Carpio* L.) biomass and eDNA concentration were positively correlated, and that eDNA could be used to estimate the species distribution in a natural environment. O'Donnell et al., (2017) found evidence of a decrease in fish community similarity based on eDNA with distance between sampling sites in a marine offshore environment. Those two studies demonstrate eDNA applications for single fish species and fish species communities, highlighting the diverse potential for eDNA-based monitoring of vertebrates in aquatic ecosystems. One such application is to monitor commercial aquaculture facilities using organic waste generated from fish in caged aquaculture systems dispersed into the surrounding water. The effluent from such facilities can potentially affect BCC (Olsen & Olsen, 2008). For example, Olsen et al., (2017) reported that the nutrient-loading from a Chilean salmon farm had a significant impact on the BCC in the surrounding waters; however, they did not test for spatial or temporal effects. Little is known about the potential interactions between salmon farm eDNA and aquatic microbial composition (e.g., how microbes effect the degradation of eDNA or how eDNA affect the composition of the BC). Using eDNA to measure potential fish farm effects on the surrounding ecosystem may provide a cost effective and accurate methodology for quantifying aquaculture impacts on the immediate environment.

Our study was designed to characterize the spatial and temporal patterns of fish eDNA and BC diversity around a commercial marine salmon farm. The study was conducted off Quadra Island, British Columbia, Canada, where an organic Chinook salmon farm rears its fish at low densities. The farm is situated in the Discovery Passage, a narrow channel that connects

two larger bodies of water, where the marine water is thoroughly mixed through tidal flow. We hypothesize that the nature and position of the net-pens will affect the landscape of eDNA within the well-mixed marine system (Lin *et al.*, 2012), and that eDNA can be used as a measure of farm effluent to estimate its influence on the surrounding microbial diversity. We predict that the distance from the net-pens will directly affect the eDNA landscape (decrease with distance), but that sampling depth will not have an effect due to the well-mixed characteristics of the bay. We also predict a seasonal effect on eDNA concentration due to changing tidal patterns and salmon spawning in the fall. To test these predictions, we sampled water in a three-dimensional grid-based survey, and measured Chinook eDNA concentration and BC alpha and beta diversity over three days in the spring and fall. We combined nested quantitative real-time PCR (qRT-PCR) and HTS to quantitatively characterize the effects of the fish farm on the distribution of the eDNA signal (the farm "eDNA plume") and the BC. The application of a combination of eDNA analyses and HTS metabarcoding of BCC to quantify the plume of a commercial salmon farm may help regulatory agencies to set evidence-based environmental protection protocols.

Materials and Methods

Study System

Yellow Island Aquaculture (YIAL) is a Chinook salmon farm and hatchery that employs organic rearing practices and is located in the Discovery Passage between Vancouver Island and Quadra Island (Figure 1). This 25 km marine channel has strong currents (up to 30 km/hr), which provide mixing of nutrients and particulates throughout the ecosystem. The YIAL net-pen site receives particularly strong currents from the Seymour Narrows (Discovery Passage), providing exceptional tidal mixing of the marine water year-round. The YIAL Chinook salmon are reared at low densities (5 kg/m^3) and are fed at $< 2\%$ body weight per day.

Sampling

Marine water samples were collected by boat at ~50 sites in radiating transects around the YIAL net-pens (Figure 1) each day. Depending on weather conditions, the total number of sample sites varied from 48 to 53 per day. Each site was sampled at three depths (1 m, 5 m, and 10 m) using one litre Van Dorne bottles. Immediately after collection, water samples were transferred into sterile 500 mL screw top Nalgene Bottles and kept in a cooler on ice until filtration. Sampling was conducted in the spring (n = 436) and fall (n = 424) of 2018 over three consecutive days, between 9:00 am and 12:00 pm to account for tide and current changes (June 4th to 6th 2018, November 3rd to 5th 2018). Tide and current data for Seymour Narrows was collected from Fisheries and Oceans Canada (tides.gc.ca). Our sampling was conducted at the end of slack tide of each morning; our spring sampling was on an ebb tide while the fall sampling was on a flood tide. Nalgene bottles were sprayed with 10% bleach solution and rinsed with fresh water prior to leaving land each day before sampling. After 1.5 to 2 hours of sampling, samples were brought to shore and filtered through Whatman® glass microfiber filters (47 mm diameter; 1.2 µm pore size; Whatman, Maidstone, UK). To minimize DNA degradation, all samples were filtered within 4 hours of collection using a vacuum pump. Filters were cut in half with sterilized scissors and forceps and stored in separate 2 mL tubes filled with a high salt buffer (40 mL 0.5 M EDTA disodium dehydrate (18.61 g/100 mL, pH to 8.0 with NaOH), 25mL 1 M sodium citrate (trisodium salt dehydrate 29.4 g/100 mL), 700 g ammonium sulfate (powdered), 935 mL ddH_2O, 1 M H_2SO_4) and stored at -20°C. Each day after field samples were processed, two field control samples were prepared with distilled water filtered and processed in the same manner as field samples, a total of 12 controls were created across the six days of sampling (three days in spring and three in fall).

eDNA Extraction

Field samples were thawed at room temperature in the lab and centrifuged at 13000 g for 10 min to compact the filter paper within the 2 mL tube. The high-salt buffer was removed from the tube and we added 1.5 mL ddH_2O to rinse the salt from the filter. Samples were centrifuged again at 13000 g for 5 min and the water was removed. The ddH_2O rinse and removal was repeated, after which 200 µL of 1.0-mm packed dry glass beads (BioSpec) and 400 µL of digestion buffer (5.844 g NaCl, 50 mL 1 M tris-HCl pH 8.0, 20 mL 0.5M EDTA, 50 mL 10% SDS, diluted in 1L ddH_2O) was added to the sample and homogenized for 3 min at 3,000 strokes per minute using a Mini-Beadbeater-24 (Fisher Scientific LTD, BioSpec.). Samples were then centrifuged at 13000 g for 5 min and 2 µL of Proteinase K (20 mg/mL) was added to each sample followed by incubation overnight at 37°C with gentle mixing. Following incubation, the Proteinase K was deactivated at 95°C for 10 min and the tubes centrifuged at 5000 g for 5 min. We removed 150 µL of the supernatant for robot bead extraction to extract eDNA (Tecan Freedom Evo150 Liquid Handling Platform, Perkin Elmer) following the protocol described in Shahraki et al., (2019). We eluted the eDNA in 100 µL of TE and stored it at -20°C until further use.

Chinook eDNA Analysis

Primer Development

Two sets of primers were designed to amplify a fragment of the *Oncorhynchus tshawystcha* (Chinook salmon) cytochrome *c* oxidase subunit 1 (CO1), using a two-step nested PCR protocol. This protocol consists of an initial pre-amplification PCR with one set of primers, and a second (nested) PCR to amplify a region within the first PCR's amplicon. Sequence data

for the Chinook salmon CO1 gene was collected from NCBI GenBank (JX960926.1) and primers were designed using Primer3Plus. We aligned CO1 sequences of related *Oncorhynchus* species (*O. keta, O. gorbushcha, O. mykiss, O. nerka, O. kisutch*) and positioned primers for *O. tshawystcha* in a region with the highest sequence divergence from the other aligned sequences (Figure 2). Nested primers were designed to exclude most congeners; however, they can cross amplify *O. kisutch* DNA due to the close genetic relationship with *O. tshawystcha*. Target specificity of designed primers was checked *in silico* using NCBI BLAST (Altschul *et al.*, 1997) with the GenBank database. The initial primer set (CO1-132F & R; Table1) was designed to pre-amplify a 132 bp amplicon, while the second nested primer set (CO1-93F & R; Table1) amplified a 93 bp region within the initial 132 bp amplicon. Primer sensitivity was tested in the laboratory using Chinook salmon fin clip extracted DNA, and Chinook salmon mesocosm water filtered eDNA. Nested primer sensitivity was determined using a 1:4 dilution series (16 dilution rounds) of known concentration fin clip extracted Chinook salmon DNA, using the nested PCR protocol: pre-amplification using CO-132F & R primers followed by triplicate nested qRT-PCR (CO1-93F & R) (details below).

Nested eDNA Amplification

Nested eDNA PCR amplification consisted of a 15-cycle pre-amplification PCR stage with primer set CO1-132F & R and a second, 45 cycle, qRT-PCR stage using the pre-amplification PCR product and primer set CO1-93F & R. Our nested PCRs included all samples collected from the field, including blank field controls, and lab-based PCR (negative) controls. Every pre-amplification PCR plate included a negative control consisting of a no template DNA reaction; these were included as templates in the second (triplicate) qRT-PCR run. We also included new negative (blank) controls in the second (nested) qRT-PCR to test for contamination

in the second, longer cycle PCR. The pre-amplification PCR stage consisted of 2.5 µL of 10x *Taq* reaction buffer, 2.5 µL of 20 mM $MgCl_2$, 0.5 µM of forward and reverse primer (CO1-132F & R), 0.2 mM of each dNTP, 0.1 unit of *Taq* polymerase (Bio Basic Canada Inc., Markham, On, Canada), 2.0 µL extracted eDNA and ddH_20 for a total 25 µL reaction volume. Pre-amplification PCR conditions were an initial denaturation of 95°C for 2 min, then 15 cycles of 1 min 95°C, 30 s annealing at 55°C, 30 s for 72°C extension, final extension at 72°C for 5 min and final hold at 4°C.

The second qRT-PCRs (CO1-93F & R) consisted of 6 µL SYBR® green PowerUp, 3.15 µL ddH_2O, 0.5 µM of forward and reverse primer CO1-93 and 2.05 µL of pre-amplification PCR product for a total 12.05 µL volume. 10 µL of this was then transferred to a 384 well plate for thermo-cycling to minimize volume variation during transfer to the reaction plates. The qRT-PCR consisted of an initial hold stage of 50°C for 2 min, then 95°C for 10 min, followed by 45 cycles, of 95°C denaturation for 15 s, and 60°C annealing temperature for 1 min. The initial pre-amplification PCRs were run as single assays, followed by nested qRT-PCRs (CO1-93) run in triplicate for each pre-amplification PCR product. All qRT-PCR reactions were 384 well plate reactions on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems).

eDNA Concentration Calculation

To determine the concentration of Chinook salmon eDNA in our samples, we created a standard curve (see section 2.4) using our nested PCR assay and serially diluted known-concentration Chinook salmon DNA. The serially diluted samples were amplified in triplicate with the nested PCR assay (as above) to produce relative cycle threshold (C_T) values for each dilution. We fit a log-linear curve to the Chinook salmon DNA concentration and relative C_T

data to allow the conversion of C_T values into initial template eDNA concentrations (Ellison *et al.*, 2006). eDNA concentration value data were used for all analyses (i.e., we did not use raw C_T value data for any subsequent analyses)

Bacterial Community Analysis

Next-Generation Sequencing Library Preparation

We used 16S meta-barcoding to characterize the bacterial community at each of the sample sites. To optimize our use of available sample barcode sequences (n = 384) for high throughput sequencing (HTS) we used primer sequence variants (at the 5' end) of the 16S V5-V6 primers (He, Chaganti & Heath, 2018) (Table 1). Amplification of the V5-V6 region of the 16S rRNA gene (~350 bp) consisted of a first round PCR with 2.5 µL of 10x *Taq* buffer, 3.5 µL of 20mM $MgCl_2$, 0.2mM of each dNTP, 0.5µM of each forward and reverse primer, 0.1 unit of *Taq* polymerase (Bio Basic Canada Inc., Markham, On, Canada), 2 µL of eDNA sample and ddH_2O to 25 µL total volume. PCR conditions were set to an initial denaturation of 95°C for 2min, then 40 cycles of 1 min 95°C, 30 s annealing at 55°C, 30 s for 72°C extension, final extension at 72°C for 5 min and final hold at 4°C. First round PCR products were cleaned to remove primer dimer and fragments less then 100 bp using Agencourt AMPure XP beads (Beckman Coulter Genomics GmbH, Mississauga, ON, Canada).

We used a second short-cycle ligation PCR with the first round PCR amplicons as template to add the HTS adaptor sequences and sample barcodes for library preparation. The ligation 6-cycle PCR consisted of 7.5 µL ddH_20, 2.5 µL of 10x *Taq* buffer, 3.5 µL of 20 mM $MgCl_2$, 0.2mM of each dNTP, 0.5 µL Uni-B adaptor (Table 1), 0.5 µL Uni-A Barcode, 10 µL of cleaned PCR product, and 0.1 unit of *Taq* polymerase (Bio Basic Canada Inc., Markham, On,

Canada). The second short cycle PCR consisted of a 2 min denaturation at 95°C for 2 min, followed by 6 cycles of 95°C denaturation for 30 s, 55°C annealing for 30 s, 72°C elongation temperature for 30 s, and a final extension at 72°C for 5 min with a final hold at 4°C. Final barcoded PCR products were run on agarose gel to determine band intensity, and then pooled, based on band intensity. Finally, samples were purified using an Epoch Life Sciences, GenCatch™ PCR purification kit and quantified using a High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga ON, Canada) to determine DNA concentration. Library pools were diluted to 60pm/L before loading on to Ion Gene Studio S5 (Thermo Fisher Scientific) 530 chip for sequencing.

HTS Data Analysis

Demultiplexing and quality filtering of raw sequence data was performed in QIIME 1.9.0 (Caporaso *et al.,* 2011). The 16S rRNA split library was filtered with minimum quality score = 25, minimum/maximum length = 200/1000, no ambiguous bases, no mismatches allowed in the primer sequence and variable barcode length; however, primer sequences were not removed from the sequences (to differentiate between 16S PCR primer variants). Operational taxonomic units (OTUs) were defined with 97% sequence similarity using the default UCLUST algorithm with all sequence data from the split library. The resulting OTU table was filtered to remove OTUs with fewer than 10 reads as well as all taxonomically unassigned or non-bacterial OTUs.

Statistical analyses

eDNA Concentration Analyses

Data Transformation: We found many samples amplified at extremely low C_T values, generally indicative of high template concentrations; however, these were determined to be due to false amplification resulting from the first-round PCR products dimerizing with the second PCR primers. To confirm this, we selected 16 random PCR samples with C_T values below 20 and characterized them on an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany); all samples showed no evidence of the expected DNA amplicon. Instead, very small fragments were identified, consistent with amplification of dimers in our nested PCR assay. Therefore, samples with C_T values < 20 were identified as false positives and set to a template DNA concentration of zero for our analyses. We also set all qRT-PCR assays with "undetermined" C_T values, or C_T values > 38, to a template concentration of zero. We included all qRT-PCR triplicate data from each sample (codes as replicates) in our analyses, this resulted in a skewed towards zero distribution. To compensate for this, we transformed the data using arcsine square root (Sokal and Rohlf, 2012).

Concentration contours were computed using Surfer® 16.6.484 (Golden Software, LLC) for each day and depth of sampling to visually represent the spatial distribution of Chinook eDNA concentration within the bay. The mean eDNA concentration ($ng \cdot \mu L^{-1}$) from the qRT-PCR triplicates was used for each sample site. Data for each day was then plotted using a radial basis gridding function to generate the concentration contour maps. Each map was developed with the same contour concentration scale for consistency and to allow interpretation of relative color among figures.

All statistical analyses for eDNA concentration were done in R Studio (RStudio Team 2015). We tested for the effect of five categorical independent factors: **season** (spring, fall), **day** (1 – 3), **distance** (in the net-pens (50m x 150m), close (375m x 625m), medium (500m x 875m), far (750m x 1250m)), **polar direction** (North, North West, West, South, South West, in the net-pens), and **depth** (1m, 5m, 10m), with general linear models (GLM).

We analyzed our eDNA concentration data starting with a temporal model that included all factors and possible two-way interactions (besides day interactions) to determine the role of two temporal effects: 1) replicate sampling "day" effects and 2) season effects.

We then separated our data by season to test for spatial effects (depth, polar direction and distance) and their interactions on Chinook salmon eDNA distribution, as well as day effects (without interactions). We used Tukey pairwise comparisons to test for subfactor differences for all significant main effects. All GLM model outputs were generated from a Type II analysis of deviance (R package "car", version 3.0-7) for GLM model objects to produce F-test statistics.

BCC: Alpha and Beta Diversity

The alpha diversity measure Chao1 was estimated for the BC at each sampled site using QIIME 1.9.0 (Caporaso *et al.,* 2011). The final filtered OTU tables were rarefied to 1200 reads, and Chao1 was calculated for each sample based on the rarefied data. Beta diversity estimates were generated in PAST 4.03. (Hammer, Harper and Ryan, 2001) where the final filtered OTU table (not rarefied) was input into a multivariate ordination principle component analysis (PCA) to generate principle components (PCs) via a correlation matrix, for each sample. Two principle components (PC 1 and 2) were chosen as independent variables for our analyses; PC 1 with an eigenvalue of 327 and explaining 22% of the variance, and PC 2 with an eigen value of 199 and

explaining 13% of the variance. Chao 1 and the two PCs were analyzed in the same manner as our eDNA analyses (see above); however, with linear models.

Chinook eDNA and Microbial Diversity Spatial Correlation

We tested for correlations between Chinook eDNA concentration (arcsine square root transformed) and measures of BC diversity (Chao1, PC 1 and 2) using R Studio (RStudio Team 2015). The averages of the triplicate estimates of the qRT-PCR eDNA concentration estimates were used to correlate with BC metrics from the same sample. We performed the Pearson correlation analyses separately for the two sampling seasons, but across all sample days, sites, and depths within season combined. We also performed a Mantel test using PAST 4.03. (Hammer, Harper and Ryan, 2001) for matrix co2rrelations between a Bray-Curtis Similarity Matrix generated from our filtered OTU table, and an eDNA concentration (not transformed) pairwise Euclidean distance matrix with 9999 permutations.

Results

Primer Specificity

We aligned mitochondrial CO1 sequences of *O. keta*, *O. gorbushcha*, *O. mykiss*, *O. nerka, and O. kisutch* against O. *tshawystcha* to establish the sequence similarity of our nested primer for cross amplification (Figure 2). Our Chinook salmon pre-amplification primers (CO1-132F & R) and nested primers (CO1-93F & R) most closely matched *O. kisutch* CO1 sequence (CO1-132F = 100%, CO1-132R = 95%, CO1-93F = 100%, CO1-93R= 100%) due to their close genetic relationship. Other Oncorhynchus species sequence similarities to our Chinook nested primers ranged from 70% to 95% and can be seen in Figure 2.

eDNA Concentration

Our field controls included 12 blank samples, and thus our triplicate nested qRT-PCR assay had a total of 36 field control reactions. Of the 36 controls, 13 produced C_T values ≥ 38 and the remaining 23 produced C_T values ≤ 9, all indicative of no detectable Chinook salmon DNA. For the nine 384-well plate qRT-PCRs runs, we included 27 pre-amplification PCR blank controls, and 27 nested 2nd round qRT-PCR blank controls. Among the 27 pre-amplification PCR blank controls, five produced C_T values ≥ 38, and the remaining 22 produced C_T values ≤ 10, all indicative of no detectable Chinook salmon DNA. Of the 27 nested 2nd round qRT-PCR blank controls, all produced undetermined C_T values (as these reactions had no pre-amplification, they did not produce primer dimer amplification C_T values). All field samples with $C_T \geq 38$ were set to a template concentration of zero, consistent with the limit of detection generated in our standard curve (see below).

Our nested qRT-PCR has a PCR efficiency of 95% (slope=-3.44; Figure 3) and had a limit of detection of 2.31×10^{-7} ng·µL^{-1} and the triplicates generally had low standard error. At very low concentrations of eDNA (1.0×10^{-8} ng·µL^{-1}), our assay produces high standard error among the triplicates (Figure 3). Therefore, we set our detection limit to be consistent with our PCR negative controls ($C_T = 38$). In total, we assayed Chinook salmon eDNA samples using 2,425 qRT-PCRs (including triplicate assays), of those, 911 qRT-PCRs were either below the detection threshold or were identified as showing primer dimer amplification (see above) and were thus set to zero template concentrations. The 1513 positive Chinook salmon eDNA assay concentrations ranged from 5.18×10^{-6} ng·µL^{-1} to 9.5×10^{-2} ng·µL^{-1}. The mean (± SEM) concentration of all the 2,425 reactions (including zeros) was 2.90×10^{-4} (± 1.55×10^{-5} ng·µL^{-1}). The mean Chinook salmon eDNA concentration for the spring sample (including zeros) was 2.50×10^{-4} (± 1.75×10^{-5} ng·µL^{-}

^{1}). The mean concentration for fall sample (including zeros) was 3.37 x 10^{-4} ng·μL^{-1} (± 2.59 x 10^{-5}). To visualize the spatial distribution of eDNA within the bay, we computed contour concentration maps for each day and depth within both seasons (Figure 4).

eDNA Concentration GLM models

We used a GLM model to test for temporal effects (i.e., day and season) on eDNA concentration; this produced significant effects for season (p = 1.9 x 10^{-2}), day (p = 9.4 x 10^{-3}), polar direction (p = 1.9 x 10^{-7}), and multiple interactions effects (Supplementary Table S1). We then used separate season models to analyze for spatial effects on eDNA concentration in the spring and fall. Our spring model produced significant effects for day (p = 5.7 x 10^{-6}), depth (p = 3.9 x 10^{-3}), polar direction (p = 0.01), and interaction effects for depth x polar direction (p = 1.0 x 10^{-3}), distance x polar direction (p = 0.05Table 2). We ran a Tukey *post hoc* analysis and found significant differences between depth 1m versus 5m (p = 0.01), 5m versus 10m (p=0.03), and northwest versus west (p=0.01; Supplementary Table S3). Our fall model produced significant effects for distance (p = 0.02), depth (p = 0.02) polar direction (1.2 x 10^{-7}), and interactions effects for depth x polar direction (p = 4.1 x 10^{-3}) and distance x polar direction (p = 4.4 x 10^{-4}; Table 2). We ran a Tukey *post hoc* analysis and found highly significant differences for distance localized to only the net-pens (p = < 0.0001), depth between 1m and 5 m (p = 0.05), and several polar directions (Supplementary Table S3).

Bacterial communities

Approximately 24 million raw sequence reads were obtained from HTS of the PCR amplified V5 and V6 regions of the 16S rRNA gene. Once singletons, doubletons, and unassigned and non-bacterial sequences were removed, approximately 11.5 million reads

remained across 5398 OTUs. When we removed all OTUs with read numbers <0.001% of the total sequence read number, 10,394,800 sequences across 1498 OTUs remained.

Alpha Diversity

To calculate alpha diversity (Chao1), samples were rarefied to 1,200 sequence reads and 65 out of 770 samples that did not reach the 1,200 read depth threshold and were dropped from the alpha diversity analysis. We then analyzed the Chao1 index with our initial model to test for temporal effects. Our model generated a significant effect for season x depth ($p = 0.01$; Supplementary Table S3). We then separated the models into spring and fall to test for spatial effects within each season. Our fall model produced no significant effects, and our spring model produced a distance effect ($p = 0.05$; Table 3). We then ran a Tukey *post hoc* analysis and only found a marginally significant difference between distances far and medium ($p=0.09$; Supplementary Table S5)

Beta Diversity

PC 1 analyzed with the temporal model generated a significant, effect for season ($p = 2.2 \times 10^{-16}$; Supplementary Table S2). We then separated our analysis into seasonal models. Our fall model produced no significant effects (Table 3), and our spring model only produced significant effects for day ($p = 1.2 \times 10^{-4}$; Table 3).

PC 2 analyzed with the temporal model produced significant effects for day ($p = 2.3 \times 10^{-3}$), season ($p = 2.2 \times 10^{-16}$), distance ($p = 0.01$) and interaction effects for season x distance ($p = 2.2 \times 10^{-3}$) and season x polar direction ($p = 0.01$; Supplementary Table S2). We then separated our models into season-specific analyses. Our fall model produced significant effects for day (p

= 1.3×10^{-8}), distance ($p = 1.1 \times 10^{-3}$), and depth ($p = 5.2 \times 10^{-3}$; Table 3). We then ran a Tukey *post hoc* test for depth and distance, and found that the differences between 1m and 10m were significant ($p = 3.8 \times 10^{-3}$), and differences between far and close distances were also significant ($p = 7.0 \times 10^{-4}$; Supplementary Table S3). Our spring model produced significant effects for day ($p = 9.4 \times 10^{-3}$), distance ($p = 5.6 \times 10^{-3}$), and polar direction ($p = 0.04$; Table 3) We then ran a Tukey *post hoc* test for distance and polar direction and the driving factor for the distance effect was the difference between the far and medium ($p = 8.6 \times 10^{-3}$) and far and net-pens distances ($p = 0.02$), and polar direction had no individual factor driving significance (Supplementary Table S3).

Microbial Diversity and eDNA Spatial Correlation

We performed a correlation analysis comparing our BC metrics (Chao1, PC 1 and PC 2) with eDNA concentration (arcsine square root transformed) for the two sampling seasons independently (Figure 5). In the spring season, Chao1 produced a significant positive correlation with eDNA concentration; however, it explained very little of the variance ($p = < 0.001$, $R = 0.3$; Table 7), and neither of the principle component axes (PC 1 and 2) generated significant correlations. In the fall season, Chao1 ($p = < 0.001$, $R = 0.2$) and PC 1 ($p = 0.02$, $R = 0.1$) produced significant positive correlations with eDNA concentration but no correlation for PC 2 ($p = 0.77$, $R = -0.01$); however, again little of the variance was explained. Our Mantel test comparing the Bray-Curtis similarity matrix and eDNA concentration (not transformed) Euclidean distance matrix produced no significant correlation in either season (spring: $p = 0.8$, fall: $p = 0.9$).

Discussion

As the demand for seafood continues to expand, more aquaculture facilities will rear high densities of fish in open water cage systems, leading to increased organic output from farm effluent that can drive changes in the surrounding ecosystem (Wu, 1995; Karakassis *et al.*, 2000). Many factors can influence the dispersal of nutrients and the intensity of environmental interactions driven by farm sites, including rearing density, feeding practices, water mixing, proximity to other farm sites, and the nature of the microbial ecosystem (Olsen, 2008; Edwards, 2015; Ottinger et al., 2016). Establishing how sloughed genetic material from net cage aquaculture systems disperses in a natural and complex environment and how it may interact with the surrounding BCs could help to establish effective and evidence-based regulations for environmental protection. Here we used Chinook salmon eDNA and bacterial community metabarcoding as markers to map the area of effect of a salmon farm site and to quantify how the plume of the farm varies spatially and temporally to gain insight on how farm effluent diffuses in a complex marine environment.

To quantitatively map the salmon farm eDNA plume, we assessed Chinook eDNA concentration variation and BC diversity around a commercial salmon farm in British Columbia, Canada. We examined spatial and temporal factors that we predicted would be important for eDNA concentration and microbial diversity variation. Previous research has focused on the persistence of eDNA under various environmental conditions and on quantifying how far the eDNA signal can be detected away from the source organism(s) (e.g., Lance et al., 2017; Pilliod et al., 2014; Balasingham et al., 2018). Such studies are integral to understanding how abiotic and biotic factors influence the detectability of eDNA in complex natural ecosystems. However, most such studies quantify eDNA at a limited number of discrete points within a one or two-dimensional sampling design. Depending on the specific study system, the eDNA plume from

the source organisms needs to be assessed in three-dimensions, to provide a complete picture of eDNA-ecosystem interactions. Our results show evidence of eDNA heterogeneity within the three-dimensional sample space, despite the well-mixed nature of the sampled marine system (Lin *et al.*, 2012). The stochastic nature of the eDNA concentration signal is highlighted in our eDNA concentration contour maps, which show chaotic temporal and spatial variation across the bay, at different depths and different times.

Our analyses of variation in eDNA concentration across the two seasons supported our hypothesis that seasonal differences in farm and tidal activity would significantly affect the landscape of eDNA within the bay. Our significant season effect of eDNA concentration was expected due to the Chinook salmon sexual maturation in the fall season: the release of gametes can contribute substantially to eDNA signal (Erickson *et al.*, 2016). The life history stage of the target eDNA species has been shown to be an important factor for eDNA output, independent of gamete release, more specifically, the increased activity associated with mating behaviours (Laramie, Pilliod and Goldberg, 2015; O'Donnell *et al.*, 2017). Seasonal effects on eDNA detection and distribution have been reported in previous studies, generally showing that species mating seasons are linked to increased detection of eDNA (De Souza *et al.*, 2016). Interestingly, we found evidence of a day effect on eDNA concentration in the spring season sampling, but not the fall. The random day to day effects on the eDNA plume may be associated with tidal flow and/or variation in farm activity. However, the lack of day effects in the fall season may be due to stronger tidal flow (mean currents of 8.4 km/hr and fall 21.5 km/hr, spring and fall respectively) from the Seymour narrows producing greater mixing in the fall. Along with variations in tidal flow across seasons, the ebb and flood differences during samples times may also be contributing to the temporal effects and spatial effects detected. The ebb tide during the

spring sampling and flood tide during the fall sampling may influence the distribution of Chinook eDNA, as well as bacterial communities in the system.

Our spatial analysis of seasonal eDNA concentration was designed to test our hypothesis that the position of the fish farm should drive distance and polar direction effects on eDNA distribution in the bay, but that depth should not have an effect due to localized mixing. We found evidence of a consistently significant polar direction effect around the net-pens across both seasons. The bathymetry of the bay produces a localized gyre that drives water movement within the bay, we theorize the gyre flow influences the Chinook salmon DNA, ultimately producing directional effects. Surprisingly, we found significant depth effects in both seasons; we predicted depth effects would be minimal due to the well-mixed characteristics of the bay (Lin *et al.*, 2012). One possible explanation of these depth effects may be benthic organic material, originating from the farm, being re-suspended by tidal flow. Mean Chinook DNA concentrations for sampled collected at 1 m (Fall = 3.8×10^{-4}, Spring = 1.9×10^{-4}), 5 m (Fall = 9.2×10^{-3}, Spring = 3.4×10^{-4}) and 10 m (Fall = 3.3×10^{-4}, Spring = 2.2×10^{-4}) show higher concentrations for 5 m samplesUnsurprisingly, we also found a significant distance effect, where eDNA decreased with distance from the farm; however, the effect was limited to the fall season. The fact that distance effects were only found in the fall may be due to the higher point source eDNA at the net-pens resulting from the fish reaching sexual maturity and releasing gametes. The higher eDNA output may contribute to the diffusion and dilution patterns in the bay, despite the tidal mixing generated from Seymour Narrows and the bathymetry of the bay. YIAL is a relatively low-production salmon farm (~50 metric tonnes), hence greater distance effects on farm eDNA plumes may be expected for larger farms.

Our analysis of BC diversity (Chao 1, PC 1 and 2) generally resulted in significant

seasonal effects, likely due to bacterial community variation with season due to temperature, nutrient and light effects (Treusch *et al.*, 2009; Giovannoni and Vergin, 2012; Kaestli *et al.*, 2017). Curiously, we also found significant sample day effects, which we did not expect; however, short term temporal effects have been reported in other aquatic bacterial community studies (Yannarell *et al.*, 2003; Crump and Hobbie, 2005; Shahraki, Chaganti and Heath, 2020). The variation in the BCC over the three sampling days is not likely associated with changes in the nature of the community, but rather with highly dynamic tidal flow regimes that affect species composition through replacement, although diel cycles may also play a role (Shahraki, Chaganti and Heath, 2020). Interestingly, our spatial effects on beta diversity were exclusive to PC 2, which was primarily loaded by *Flavobacteriales* species (Supplementary Tables S6). This bacterial order has been reported as a dominant taxonomic group in marine ecosystems, likely due to high levels of organic substrates, where they play a role in degradation and decomposition of organic matter (Waśkiewicz and Irzykowska, 2014). We found significant distance effects relative to the salmon farm in both seasons for PC 2, suggesting that farm output had a direct effect on BC diversity. The seasonality of the farm's influence on the BCs extended across all measures of BC diversity (i.e. Chao 1, PC 1 and 2), highlighting the critical nature of environmental effects, including changes in tidal characteristics, that vary season to season. Intriguingly, we found significant depth effects in the fall season, despite the high tidal flows and expected water column mixing in the bay. We speculate that the very high tidal flows may stir up benthic microbes that are influenced by farm-related organic matter that settles at the bottom of the bay (Kawahara *et al.*, 2009; Turner, Uy and Everhart, 2015; He *et al.*, 2020).

To assess possible functional relationships between BC diversity and eDNA, we ran correlation analyses between various BC diversity metrics and Chinook salmon eDNA

concentration. The observed positive relationship between eDNA and Choa1 represents an increase in bacterial species diversity with higher levels of Chinook salmon eDNA concentration. Our correlation analyses of beta-diversity (PC 1 and 2) and eDNA concentration produced a significant correlation, but only for PC 1 within the fall season. The positive correlation between PC 1 and eDNA concentration indicates that differences in eDNA concentration are associated with increased bacterial divergence. Samples with higher PC scores indicate a higher variation of bacterial species from the other samples, while samples with lower PC scores are similar. This increase in bacterial diversity is evident in the top three bacteria driving PC 1: *Oceanospirillales, Rhodobacterales, and Flavobacteriales,* especially compared to PC 2 which is mainly driven by *Flavobacteriales.* These three orders of bacteria are known for their diverse abilities to metabolize organic and inorganic materials for energy. More specifically, *Flavobacterium* have been found to hydrolyze polysaccharides (Waśkiewicz and Irzykowska, 2014), and *Rhodobacterales* have been shown to produce secondary metabolites from organic and inorganic compounds (Pohlner *et al.*, 2019). We suspect the positive correlation between eDNA and BC diversity is the result of complex bacterial community interactions stemming from farm organic output, which is indirectly reflected in eDNA signal strength. Curiously, the BC beta-diversity - eDNA correlations were limited to the fall season, despite the consistent positive correlations between BC alpha diversity (Chao 1) and eDNA concentration in both seasons. We hypothesize this is due bacterial communities adjusting to the increase of temperature from the winter, allowing for BC growth and diversification into the summer. Our Mantel test did not show any correlation between Bray-Curtis and eDNA distance matrices, perhaps due in part to the low statistical power of the non-parametric Mantel test relative to our parametric correlation analyses (Legendre, Fortin and Borcard, 2015).

Type I (false positive) and type II (false negative) errors are both critical considerations for interpreting eDNA analyses (Darling and Mahon, 2011). Negative controls are essential to assess field contamination to allow critical assessment of error in later interpretation of eDNA generated detection data (Ficetola et al., 2015). Because our nested PCR eDNA assay is highly sensitive, it is prone to detecting low levels of contamination, potentially inflating false positive outcomes. Although such errors may be present in this study, we compensated by setting stringent detection thresholds and by including all zero concentration data in our analyses. Therefore, with these adjustments, combined with the lack of evidence for false positives based on our negative controls, type I errors likely do not have a large effect on our overall spatial or temporal outcomes. Another possible source of false detection is related to the detection of wild salmon species; our nested primers will cross amplify Coho salmon eDNA, and, of course, wild-source Chinook salmon eDNA. However, there is no expectation for substantial numbers of either species in the sampled area and the eDNA output from the farmed Chinook salmon should swamp possible wild source eDNA. Nevertheless, we cannot rule out the possibility of wild salmon eDNA affecting our results.

The aquaculture industry will continue to use open water cage systems to meet the demand for seafood, although there is increased regulatory pressure to limit such culture practices (Smart, 2020). As net cage aquaculture continues, the need to accurately measure the extent of impact of fish farms will become more urgent. We implemented a three-dimensional survey to assess if the nature and position of a salmon farm would influence the surrounding aquatic landscape using Chinook salmon eDNA and BC meta-barcoding data. We hypothesized that the net-pens would affect the landscape of eDNA, despite the well-mixed system, and that eDNA could be used as a measure of farm effluent to establish the "farm plume". We found both

significant spatial and temporal effects on Chinook salmon eDNA concentrations around the net-pens. Our results indicate that in complex and well-mixed system, eDNA can be still used as a reliable biological marker for salmon farm effluent, and thus possible environmental effects. Our BC analyses also showed spatial effects relative to the farm on BC diversity, plus we found significant correlations between BC diversity and Chinook salmon eDNA concentration. Thus, a combination of eDNA and BC analyses can provide insight into relationships between salmon farms and microbial community variation. The purpose of this study was to characterize the eDNA "plumes" from an open-water salmon farm and while both eDNA and BC diversity exhibited plume-like behaviour, more work needs to be done on the potential environmental impacts of such plumes. Therefore, using eDNA sampling to assess BCC and target vertebrate species can provide quantitative evidence of the spatial and temporal scope of influence for fin fish farms on the surrounding environment.

Figures and Tables

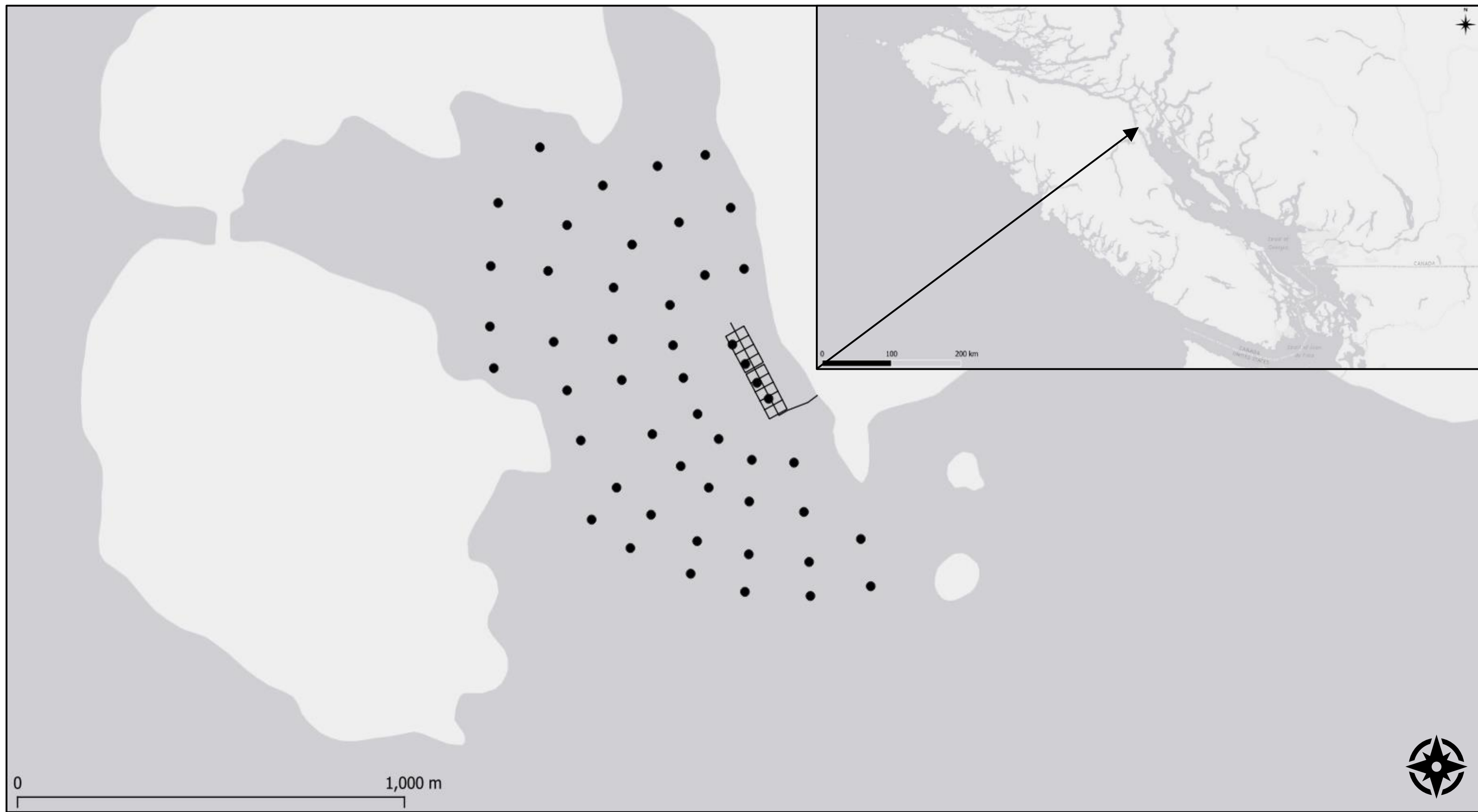

Figure 2*:* Area map of study site Yellow Island Aquaculture (YIAL) between Vancouver Island and Quadra Island, British Columbia, Canada. Inset map shows Vancouver Island indicating where the YIAL site is (arrow). Approximate locations of the sample sites around the salmon net-pens are represented as black dots.

CO1-132F + CO1-93F

keta 5′ – gcaggagcatctgtcgacttaaccatcttctccctccatttagctggaatctc

gorbushcha 5′ – gcagggcatccgtcgacttaactatcttctcccttcatttagctggaatctc

mykiss 5′ – gcaggagcctctgttgatttaactatcttctcccttcatttagctggaatctc

nerka 5′ – gcgggagcctctgttgacttaaccatcttctcccttcatttagctggaatttc

kisutch 5′ – gcaggagcctcagttgatctgactatcttctcccttcatttagccgggatctc

tshawytscha 5′ – **gcaggagcctcagttgatct**gacgatcttctcccttcatttagccgggatctc

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CO1-132R + CO1-93R

keta 5′ – ccattatcaacataaaacccccagctatttctcagtaccaaaccccgcttttt

gorbushcha 5′ – ccattatcaacataaaaccaccggcaatctctcagtaccaaaccccactttttt

mykiss 5′ – ccattattaacataaaacctccagccatctctcagtaccaaacccccctttc

nerka 5′ – ccattattaatatgaagcccccagccatctctcagtaccagaccccactttt

kisutch 5′ – ccattattaacataaagcccccagctatctctcagtaccaaaccccactttttt

tshawytscha 5′ – ccattattaacataaaacccccggcta**tctctcagtaccaaacccca**cttttt

Figure 2: Aligned mitochondrial CO1 sequences (5' to 3') of *O. keta*, *O. gorbushcha*, *O. mykiss*, *O. nerka, O. kisutch*, and *O. tshawytscha*. CO1-132 F & R primers are bolded, and CO1-93 F & R primers are highlighted in yellow. Red highlighted nucleotide base-pairs indicate mismatched sequences to *O. tshawystcha* within primer regions.

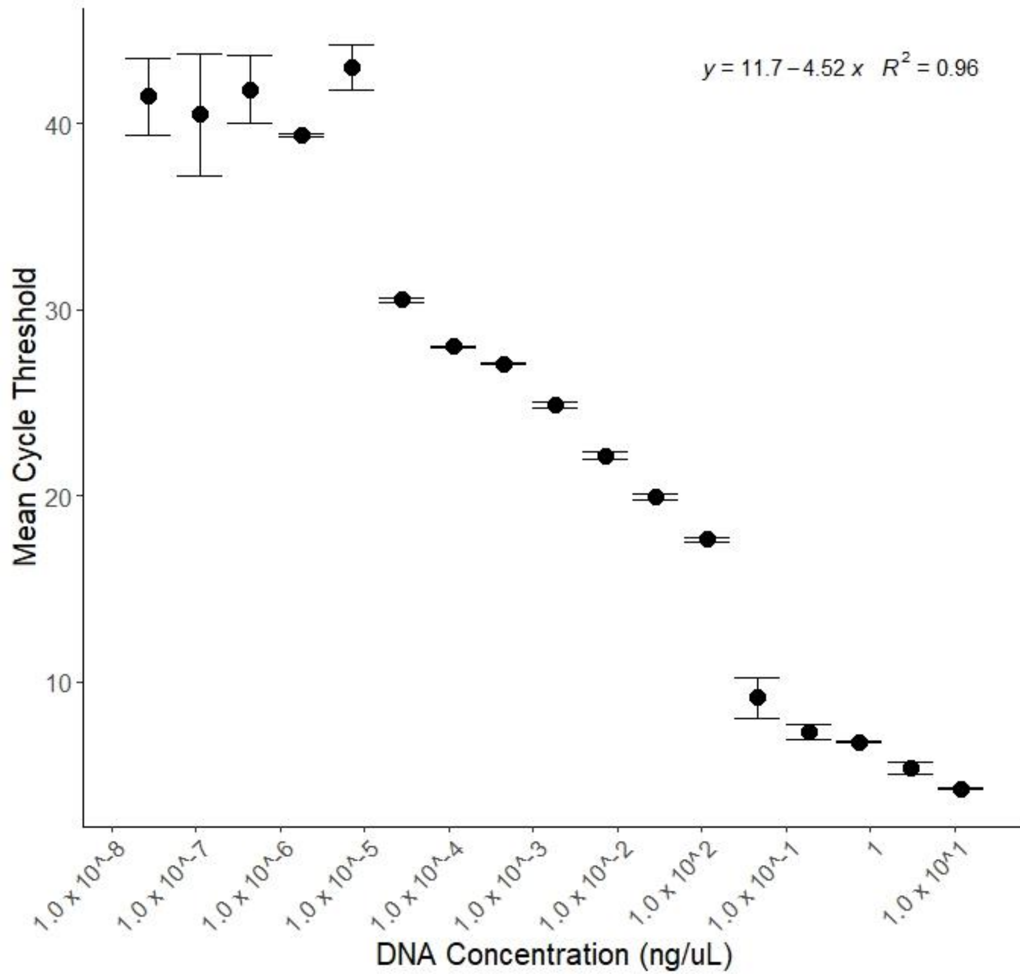
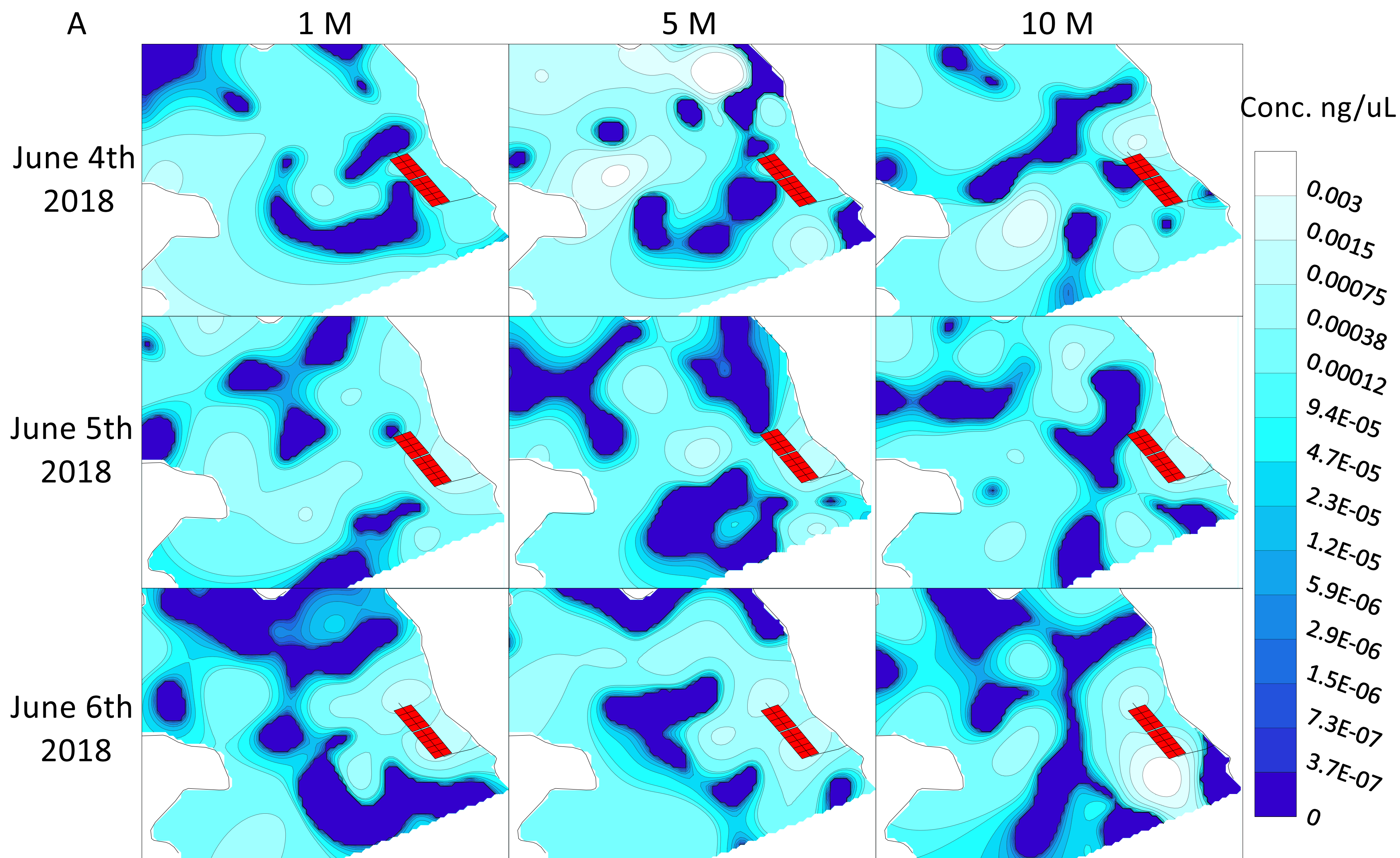


Figure 3: Nested PCR primer protocol (CO1-93F & R nested within C01-132 after 15-cycle pre-amplification PCR) of 1:4 serial dilution of fin clip extracted Chinook salmon DNA. X-axis represented in a log10-scale, showing DNA concentration (ng/µL), Y-axis showing the mean C_T values of the nested qRT-PCR


A
1 M
5 M
10 M
June 4th 2018
June 5th 2018
June 6th 2018
Conc. ng/uL
0.003
0.0015
0.00075
0.00038
0.00012
9.4E-05
4.7E-05
2.3E-05
1.2E-05
5.9E-06
2.9E-06
1.5E-06
7.3E-07
3.7E-07
0

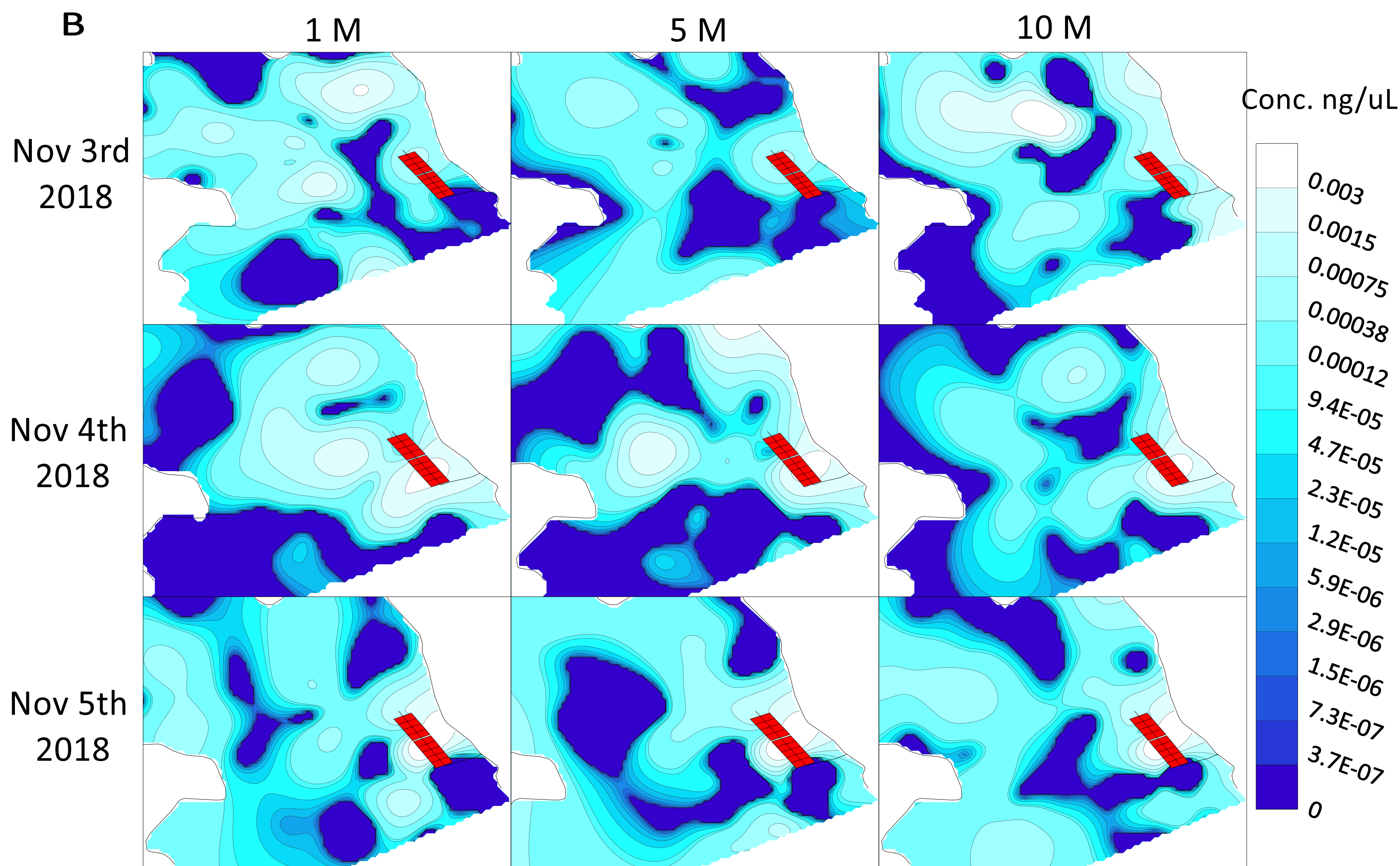


Figure 4: Chinook eDNA concentration contour maps for Spring (A) and Fall (B) seasons at each depth sampled. Yellow Island net-pens are represented in red and arcsine square root transformation DNA concentration (ng·µL^{-1}) contours are represent with the dark blue to white gradient, lighter shades indicating higher eDNA concentrations. The land and regions not covered by sample sites are not shown.

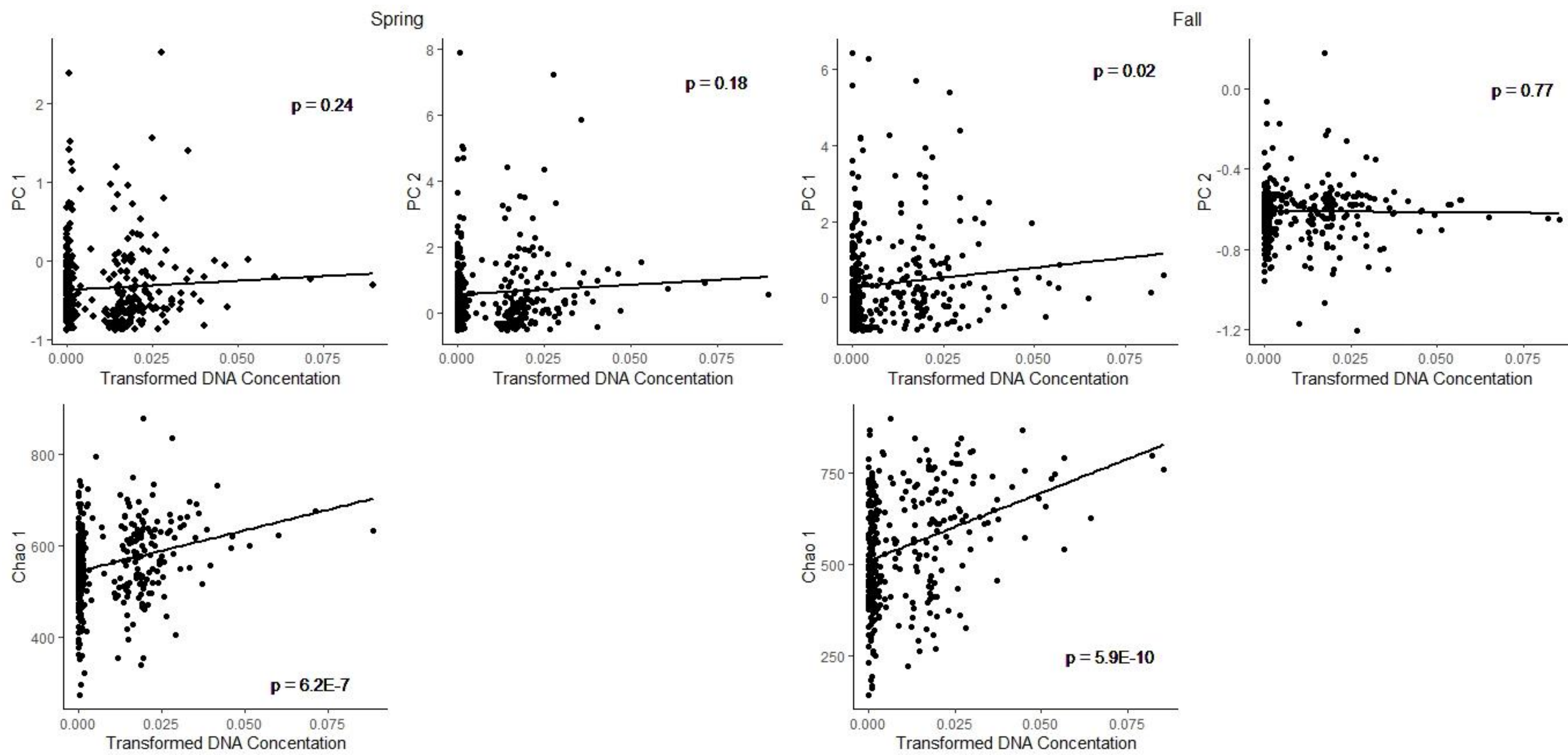


Figure 5: Scatter plots of bacterial community metrics (Chao 1, PC 1 and 2) versus average Chinook eDNA concentration (arcsine square root transformed), separated by season (fall and spring). Solid line shows Pearson correlation relationship.

Table 2: Primer Table: Chinook salmon nested PCR primers, Variations of 16S V5-V6 bacterial primers (Variable region underlined, Uni-A Forward (underlined sequence consisting of 10-12 base pair barcode sequence) and Uni-B adaptor Reverse primers.

Target	Primer Name	Forward Primer	Reverse Primer
O. tshawytscha (Chinook)	CO1-93 (nested)	CTC CCT TCA TTT AGC CGG G	TACT GAG AGA TAG CCG GGG G
O. tshawytscha (Chinook)	CO1-132	GCA GGA GCC TCA GTT GAT CT	TGG GGT TTG GTA CTG AGA GA
V5-V6 16S rRNA	16S_V5V6_A	ACC TGC CTG CCG *ATCG*[1] ATT AGA TAC CCN GGT AG	ACG CCA CCG AGC CGA CAG CCA TGC ANC ACC T
V5-V6 16S rRNA	16S_V5V6_C	ACC TGC CTG CCG *CTGA*[1] ATT AGA TAC CCN GGT AG	ACG CCA CCG AGC CGA CAG CCA TGC ANC ACC T
V5-V6 16S rRNA	16S_V5V6_D	ACC TGC CTG CCG *TAGC*[1] ATT AGA TAC CCN GGT AG	ACG CCA CCG AGC CGA CAG CCA TGC ANC ACC T
	Uni-A (Forward) Uni-B (Reverse)	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG XXX XXX XXX X[2] GAT ACC TGC CTG CCG	CCT CTCT ATG GGC AGT CGG TGA TAC GCC ACC GAG C

1. *Variable sequence (underlined) in V5-V6 primers used to maximize sample barcode sequences for multiplex sequencing.*
2. *Uni-A primer with 10-12 base sequences ("XXX…") represents variable barcode (sample identifier) sequences.*

Table 2: Summary output table for general linear model analysis of eDNA concentration, separated by season (spring and fall) to test for spatial effects. Includes main effect factors and selected interactions, sum of squares (Sum Sq), degrees of freedom (DF), F-value (F), and p-value (P).

Factor/Interaction			
Spring eDNA Concentration	DF	F-value	p-value
Day	2	12	5.74E-06
Distance	2	0.45	0.63
Depth	2	5.5	3.9E-03

Polar Direction	4	3.1	0.016
Distance x Depth	4	0.89	0.46
Depth x Polar Direction	8	3.2	1.0E-03
Distance x Polar Direction	8	1.8	0.058
Fall eDNA Concentration			
Day	2	2.7	0.065
Distance	2	3.8	0.021
Depth	2	3.5	0.027
Polar Direction	4	9.5	1.23E-07
Distance x Depth	4	1.7	0.14
Depth x Polar Direction	8	2.8	4.14E-03
Distance x Polar Direction	8	3.5	4.47E-04

Table 3: Summary output table for linear model analysis of BC metrics (Chao 1, PC 1 and PC 2), separated by season (spring and fall) to test for spatial effects. Includes main effect factors and selected interactions, degrees of freedom (DF), F-value (F), and p-value (P).

Factor/Interactions	**DF**	**Chao 1**		**PC 1**		**PC 2**	
Spring		F	P	F	P	F	P
Day	2	0.37	0.68	9.2	1.2E-04	4.7	9.4E-03
Distance	2	3.0	0.051	1.3	0.25	5.2	5.6E-03
Depth	2	1.6	0.19	1.4	0.24	0.12	0.88
Polar Direction	4	0.35	0.84	1.6	0.15	2.5	0.042
Distance x Depth	4	0.18	0.94	1.7	0.14	1.2	0.27
Depth x Polar Direction	8	0.39	0.92	0.34	0.94	0.31	0.96
Distance x Polar Direction	8	0.84	0.56	1.2	0.2	0.73	0.65
Fall							
Day	2	0.84	0.43	0.48	0.61	19	1.34E-08
Distance	2	0.48	0.61	1.5	0.20	6.	1.1E-03
Depth	2	2.5	0.081	1.7	0.18	5.3	5.2E-03
Polar Direction	4	1.74	0.13	1.2	0.27	2.1	0.070
Distance x Depth	4	0.74	0.56	1.0	0.38	1.0	0.39
Depth x Polar Direction	8	1.7	0.089	0.42	0.90	0.70	0.68
Distance x Polar Direction	8	0.66	0.72	0.59	0.78	0.84	0.56

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Supplementary Materials

Supplementary Table S1: Summary output table for general linear model analysis of eDNA concentration. Simplified model includes all factors and interactions, without sample day effect interactions, to test for season effects. Includes main effect factors and interactions, degrees of freedom (DF) F-value, and p-value.

Factor/Interaction	DF	F-value	p-value
Season	1	5.4	1.99E-02
Distance	2	0.77	4.62E-01
Depth	2	0.031	9.70E-01
Polar Direction	4	9.2	1.93E-07
Day	2	4.6	9.41E-03
Season x Depth	2	8.4	2.32E-04
Season x Distance	2	3.1	4.64E-02
Season x Polar Direction	4	4.2	2.14E-03
Distance x Depth	4	1.5	1.79E-01
Depth x Polar Direction	8	1.2	2.57E-01
Distance x Polar Direction	8	3.2	1.16E-03

Supplementary Table S2: Summary output tables for linear model analysis of BC metrics (Chao1, PC 1 and PC 2). Simplified model includes all factors and interactions to test for temporal effects. Includes main effect factors and interactions, F-value (F), and p-value (P).

Factor/Interaction	**Chao1**		**PC 1**		**PC 2**	
	F	P	F	P	F	P
Day	1.24	0.29	2.43	0.09	6.12	0.00
Season	2.10	0.15	104.15	< 2e-16	435.13	< 2.2e-16
Distance	2.34	0.10	2.53	0.08	4.14	0.02
Depth	0.50	0.61	0.68	0.51	0.24	0.79
Polar Direction	1.09	0.36	1.92	0.10	2.29	0.06
Season x Depth	3.98	0.02	2.43	0.09	0.17	0.84
Season x Distance	0.03	0.97	0.56	0.57	6.13	0.00
Season x Polar Direction	1.58	0.18	0.93	0.45	2.97	0.02
Distance x Depth	0.72	0.58	1.62	0.17	1.59	0.17
Depth x Polar Direction	1.89	0.06	0.56	0.81	0.41	0.91
Distance x Polar Direction	0.69	0.70	0.54	0.83	0.73	0.67

Supplementary Table S3: Tukey *post hoc* table for eDNA, Chao 1 and PC 2 analyses (separated by season). Includes subfactor interactions (of significant factors tested in season analyses), difference of means (Diff), and p-value (P).

Interactions	**Fall eDNA**		**Spring eDNA**		**Fall Chao 1**		**Spring Chao 1**		**Fall PC 2**		**Spring PC 2**	
	Diff	*P*	*Diff*	*P*	*Diff*	*P*	*Diff*	*P*	*Diff*	*P*	*Diff*	*P*
Depth												
1 m x 5 m	-2.22E-03	0.05	2.46E-03	0.01	-23	0.50			-0.028	0.18		
1 m x 10 m	-4.44E-04	0.88	3.17E-04	0.93	-44	0.08			-0.050	3.80E-03		
5 m x 10 m	1.78E-03	0.14	-2.14E-03	0.03	-21	0.56			-0.023	0.32		
Distance												
Netpens x Close	-2.48E-02	< 0.0001					3.7	0.99	1.2E-03	1.00	-0.63	0.20
Netpens x Fedium	-2.28E-02	< 0.0001					5.2	0.99	3.5E-02	0.80	-0.46	0.44
Netpens x Far	-2.41E-02	< 0.0001					-19.2	0.88	6.8E-02	0.29	-0.87	0.02
Close x Medium	2.02E-03	0.27					1.5	0.99	3.4E-02	0.23	0.17	0.69
Close x Far	7.09E-04	0.91					-22.8	0.28	6.7E-02	7.48E-04	-0.24	0.39
Medium x Far	-1.31E-03	0.47					-24.3	0.096	3.3E-02	0.12	-0.41	0.01
Polar Direction												
Netpens x N	2.58E-03	6.38E-01	2.16E-04	1.00							0.22	0.99
NW x N	-2.87E-04	1.00E+00	-1.17E-03	0.93							0.24	0.79
S x N	5.77E-03	1.61E-03	-7.93E-04	0.99							-0.02	1.00
SW x N	4.21E-03	1.34E-02	4.31E-04	1.00							0.24	0.76
W x N	4.38E-03	2.06E-02	2.74E-03	0.28							0.54	0.07
NW x Netpens	-2.87E-03	4.27E-01	-1.39E-03	0.90							0.02	1.00
S x Netpens	3.19E-03	4.35E-01	-1.01E-03	0.98							-0.23	0.98
SW x Netpens	1.63E-03	8.99E-01	2.15E-04	1.00							0.02	1.00
W x netpens	1.80E-03	8.82E-01	2.53E-03	0.48							0.33	0.92
S x NW	6.05E-03	9.89E-05	3.77E-04	1.00							-0.26	0.78
SW x NW	4.50E-03	6.75E-04	1.60E-03	0.61							-0.01	1.00
W x NW	4.67E-03	2.02E-03	3.91E-03	0.01							0.30	0.56

SW x S	-1.56E-03	8.58E-01	1.22E-03	0.91							0.25	0.76
W x S	-1.39E-03	9.31E-01	3.54E-03	0.10							0.56	0.08
W x SW	1.66E-04	1.00E+00	2.31E-03	0.30							0.31	0.48

Supplementary Table S4 **:** Principle component loading table with assigned taxonomic groups. Top 20 highest loading values are represented for PC 1 and PC 2 out of the 1500 OTUs used for beta diversity analysis.

PC 1 loading	**Kingdom**	**Phylum**	**Class**	**Order**	**Family**	**Genus**
0.051039	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Candidatus Portiera
0.052373	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Candidatus Portiera
0.048438	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Candidatus Portiera
0.052217	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
0.048651	Bacteria	Proteobacteria	Gammaproteobacteria	Thiohalorhabdales	Thiohalorhabdaceae	
0.048545	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae	
0.050026	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.048834	Bacteria	Bacteroidetes	Saprospirae	Saprospirales	Saprospiraceae	
0.04891	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9	
0.052458	Bacteria	Proteobacteria	Alphaproteobacteria			
0.049671	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	
0.05088	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9	
0.050631	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Nitrospinaceae	Nitrospina
0.048649	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	
0.049409	Bacteria	Planctomycetes	OM190	CL500-15		
0.048947	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
0.04922	Bacteria	Verrucomicrobia	[Pedosphaerae]			

0.052698	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales		
0.052505	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
0.048294	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	
PC 2 loading	**Kingdom**	**Phylum**	**Class**	**Order**	**Family**	**Genus**
0.064793	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Ulvibacter
0.063454	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	
0.062637	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.063585	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.066324	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.063317	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	
0.064107	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter
0.066194	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.06314	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales		
0.065407	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.063666	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	
0.066413	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.065566	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.064033	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	
0.062928	Bacteria	Cyanobacteria	Chloroplast	Stramenopiles		
0.064573	Bacteria	Bacteroidetes	Saprospirae	Saprospirales		
0.063871	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola
0.066685	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
0.063096	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavivirga

CHAPTER 4

General Conclusions

As the demand for seafood increases, a growing need for aquaculture facilities to increase production through GMO's or higher density rearing is predicted (Sapkota et al., 2008; Diana, 2009). As facilities adjust their rearing practices, either through adoption of GMO technology and/or higher rearing densities, the potential for impacts on the surrounding ecosystem increase: microbial communities in particular are highly sensitive to their surrounding environment (Bentzon-tilia & Sonnenschein, 2016; Lv et al., 2016). The effluent discharged from open-water farms has been shown to influence the community composition of microbes, through localized increases in inorganic and organic nutrients (fish by-products (wastes and mortalities) and pelleted feed) released into the system (Wu, 1995; Cole et al., 2009; Wang et al., 2012). Although the GMOs in aquaculture have been shown to have improved food conversion ratios (Devlin et al., 2001; Rasmussen & Morrissey, 2007), there is concern for the potential environmental impacts if they were to escape captivity (Naylor et al., 2005; Devlin, et al., 2006; 2015). Environmental DNA (eDNA), the sloughed material from multi-cellular organisms or whole microorganisms that are collected from a filtered water sample (Taberlet et al., 2012; Thomsen & Willerslev, 2015), can be a valuable approach to assess farm influences on the surrounding water quality, and detect fugitives that have escaped confinement. This thesis describes novel eDNA detection and surveillance methods with two related but distinct applications, highlighting the broad value of the approach. We demonstrate a variety of applications eDNA can be used for different environmental monitoring goals, from targeting single gene constructs, to assessing the interactions between salmon eDNA markers and bacterial community diversity.

Traditional methods used to monitor aquatic ecosystems (e.g., capture based identification, SCUBA surveys, microbial culturing, etc.) are intrusive, time consuming and costly (Thomsen & Willerslev, 2015; Schmelzle & Kinziger, 2016). On the other hand, eDNA surveys require the collection of water samples and can provide data on the biodiversity of an ecosystem, across taxonomic groups, making it a valuable tool for monitoring complex and niche environments (Taberlet, Coissac, Hajibabaei, *et al.*, 2012; Goldberg *et al.*, 2016). Moreover, monitoring of remote coastal and inland fish farms can be challenging due to harsh conditions and may require specialized equipment (e.g., water quality measurements, chemical analyses, bacterial culture). Therefore, the collection and filtration of water samples for eDNA extraction to be shipped for processing, provides an efficient and logistically straightforward methodology to monitor remote locations (Roussel et al., 2015).

Based on this thesis, our work addresses two main issues for aquaculture facilities: the impact of fish farm effluent on the surrounding environment and monitoring transgenic eDNA in aquatic systems. In chapter two, we developed highly sensitive PCR primers to detect the nuclear growth hormone transgene (OnMTGH1) of transgenic Coho salmon in environmental water samples, even in the presence of their non-transgenic counterpart. Despite this sensitive assay, there was no spatial consistency in detecting the transgene signal from the facility outflow pipe. We theorize this may be due to the nuclear GH gene having reduced persistence in aquatic environments compared to standard mitochondrial genes used for finfish eDNA detection. We also speculate that the lack of spatial consistency of the transgene may be due to detections resulting from accumulation of sloughed genetic material (feces, mucus) inside the outflow pipe. Therefore, facility effluent would not be a homogenous mixture of transgenic eDNA. For monitoring potential escapees, this assay provides localized detection without the concern of

wild-type eDNA interference. This assay can also be used to verify the presence of GMOs during trade of frozen or live seafood. In chapter three, our eDNA analysis of the farm "plume" showed evidence of farm activity effects on the surrounding aquatic environment. We speculate Chinook salmon spawning in the fall, along with increased tidal mixing in the fall, produced detectable differences of farm discharge across seasons. Furthermore, the BC analyses also showed farm effluent influences on the surrounding waters. Despite the well-mixed characteristics of the system, our spatial and temporal analysis of BC diversity generated significant distance from the farm effects in the spring season. Conducting our eDNA survey in transects, across two seasons provided a power measure of how the farm effluent was interacting with the BC as it dispersed in the bay. Using these methodologies for open water cage-based salmon farming can provide management direction on sustainability based on specific local environmental characteristics.

Establishing the potential correlations between fish farm eDNA plumes and BCs can be used to quantify fish farm impacts on the surrounding ecosystem and prevent long term effects. Characterizing bacterial species and identifying correlations with sloughed eDNA can provide indications of an overall influence of fish farm effects from organic output and verify if changes in rearing practices need to be made. In our YIAL study we found three orders of bacteria: *Oceanospirillales, Rhodobacterales,* and *Flavobacteriales,* driving our beta diversity analysis with a positive correlation with Chinook salmon eDNA. Other studies examining fish farm practices have found positive correlations between the increase of fish biomass and organic matter, and microbial community abundance/diversity (Caruso et al., 2003; Mirto et al., 2012; Quero et al., 2020). Evidence of the microbial impacts from fin fish farming, derived from bioindicators such as eDNA and bacterial community assessments, can be used to predict long lasting impacts on the surrounding ecosystem (Verhoeven et al., 2018). Therefore, utilizing

bacterial metabarcoding and eDNA detections is a valuable tool to support changes in rearing practices to sustain stable ecosystem biodiversity.

The main weakness with eDNA analyses is the potential of type I (false-positive) and type II errors (false-negative) for species detection (Darling & Mahon, 2011). False detections relating to site occupancy can result in biased analyses, potentially leading to improper assessment of surveillance data, for example in early invasive species monitoring (Bailey et al., 2014). However, appropriate replicated designs (sample sites and PCR assays) can help statistical models correct the effects of possible type I and type II errors (Ficetola et al., 2015; Lahoz-Monfort et al., 2016). In chapter three, to compensated for the possibility of high false positive detection resulting from our nested PCR assay, we set stringent detection limits and included all zero set values in our statistical analyses. By including all triplicate PCR assays (including zero set values) in our statistical analyses, the possibility for false-positive errors to generate bias in our outcomes was reduced. Therefore, when conducting eDNA surveys with the intent of management action or policy change, it is critical to assess for false detection errors (both positive and negative) within complex data sets (Mackenzie & Royle, 2005). Without properly replicated study designs, the outcome of eDNA-based assays can be misleading and drive improper management decisions (Roussel et al., 2015).

Future Work

Our chapter two study provides a novel assay for the presence/absence detection of transgenic Coho salmon with the presence of it is wild-type counterpart. Studies have begun to relate quantity of eDNA detected to species abundance in aquatic environments based on biomass data (Lodge *et al.*, 2012; Lacoursière-Roussel *et al.*, 2016b). Quantifying the abundance

of transgenic organisms through eDNA would provide the potential for long term quantitative monitoring of escapees that may reproduce with native populations. Therefore, predicting the potential increase of transgenic organisms in the wild, through estimating the abundance of individuals, would provide an early indicator similar to early detection of invasive species (Balasingham et al., 2018).

Our chapter three study provides methodologies to examine fish eDNA and BC across temporal and spatial scales in a well-mixed marine system. Curiously both eDNA and BC analyses produced sample day effects within our sampling seasons, suggesting fine scale diel variation between each sample day. Although studies have found that day/night cycles can influence bacterial activity (Shahraki, et al., 2020), eDNA variation may also be influenced by tidal impacts or organism activity. To study the factors that effect diel dynamics, we suggest mesocosm experiments control for day/night cycles and separate stagnant and high-mixed systems. Controlling for tidal, light, and mixing within these experiments may provide insight into how eDNA and BCs are influenced by each other and their environment. We also would suggest examining the effect of salinity on these parameters, specifically investigating how freshwater bacteria would interact with high outputs of eDNA without the well-mixed characteristics from a marine system such as Seymour Narrows. Identifying the eDNA and BC interactions among costal and inland caged facilities can provide insight into how each system responds to farm eDNA plumes.

Conclusion

The overall objective of this thesis was to assess the ecological impact of aquaculture facilities through eDNA analyses. We developed a transgene-specific assay to detect potential

escapees and established how transgenic eDNA is non-uniformly distributed from an effluent pipe. We assessed how Chinook salmon eDNA is distributed around a commercial salmon farm, and how spatial and temporal factors affected that distribution. We also assessed potential interactions between the salmon farm and BC diversity. Although more work needs to be done to establish standardized guidelines for the collection and processing of eDNA samples, there is more to explore for the application of eDNA for aquatic surveillance via the combination of BC characterization and vertebrate spatial and temporal detection. We have developed the groundwork for assessing not only the distribution of eDNA and detection of specific gene constructs but using eDNA as a biomarker for the “plume” of farm effluent. This thesis provides methodologies to assess the ecological influence of sloughed eDNA from aquacultural facilities on the surrounding BC diversity, and detect GMOs without interference from wild-type equivalents. Overall, we demonstrate the ability to assess the distribution of fin fish DNA in aquatic environments, as well as provide data-driven evidence to fish farm facilities of the ecological impact on their unique surrounding environment.

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