Fish Forensics: Environmental DNA Detection of Juvenile Coho Salmon and Resident Salmonids in Pacific Coastal Streams

by

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BSc, University of Victoria, 2013

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of the Requirements for the Degree of

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Abstract

Conventional fish monitoring requires considerable investments of equipment and labour, and often harmful and potentially fatal techniques. Emerging methods allow detection of aquatic animals by collecting water and extracting DNA that has been shed to the environment (eDNA). Present knowledge gaps in the field include minimum densities necessary for consistent detection, and persistence of eDNA after a target species has left a site.

I conducted three experiments at a salmon hatchery in British Columbia to address these knowledge gaps. Water samples were taken from flow-through tanks with juvenile Coho Salmon densities ranging from 38.0g/1000L to 0.6g/1000L. To simulate field surveys in recently abandoned habitats, I sampled water from tanks after removing fish, at flow-through volumes ranging from 20,000L to 1,000,000L. Post removal sampling occurred starting at one hour and ending after just over four days of flow-through time. Water samples from tanks containing one or more fish tested positive for Coho DNA at least 70% of the time, increasing at higher densities. Samples taken after removing the fish had detection probability of 75% at flow-through volume of 40,000L. Detection failed at flow-through volumes greater than 80,000L.

In stream samples, all sites with Coho or salmonid presence confirmed by conventional trapping also tested positive for target species’ eDNA. Two sites tested positive for Coho eDNA where conventional methods failed, indicating a possible higher sensitivity of eDNA sampling. I also mapped the distribution of juvenile Coho Salmon
through multiple tributaries of a productive salmon system with conventional and eDNA detections.

This study improves on an emerging method with a new species by addressing existing uncertainties regarding eDNA detection threshold, and signal persistence through dilution in a simulated stream pool habitat. It also demonstrates that eDNA methods can be used to assess coastal streams for presence of juvenile and resident salmonid fishes.
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I couldn’t have done it without you.
Chapter 1
General Introduction

Fisheries monitoring in concept and practice

Determining the distribution and abundance of species is a central focus in ecology and resource management. This knowledge is critical in assessing the health of endangered populations, community composition of biologically diverse habitats, and determining the range expansion of globally destructive invasive species. It is also necessary for setting harvest targets for resources with significant social, economic, and cultural value.

Conventional monitoring of fish populations typically involves techniques such as minnow traps, seine/gill nets, snorkel/dive surveys, and electrofishing, which can be limited in several ways. First, there are high labor and material costs for training, equipment acquisition, site permitting, and accessing remote field locations (Bohmann et al. 2014). Necessary expertise for field technicians includes specific taxonomic knowledge and experience in field identification, which is often inexact for closely related species or at early life stages. Animal handling permits for invasive field sampling add to the complex logistics of equipping and transporting field personnel, and conducting research and assessment in compliance with regulatory bodies. Second, there is considerable uncertainty in abundance estimates and even detection (i.e., false absences) that can vary between the methods, and between environments using the same method (Lyon et al. 2014). Finally, conventional monitoring methods impose stress and harm on fish that can cause immediate damage or long term energetic and fitness
consequences (Cho et al. 2002, Braun et al. 2010). Electrofishing, the standard monitoring method for freshwater fish, is known to lead to spinal injury and brain hemorrhaging (Reynolds 2012). Associated mortality varies among species, but is has been documented in research on salmonids at rates as high as 36% (Clément and Cunjak 2010). In general, salmonids are a family group considered to be most susceptible to injurious effects of electrofishing (Snyder 2004).

**Theory and emergence of environmental DNA**

Averting harm to animals caused by live capture methods led to experiment and implementation of a wide variety of non-invasive genetic sampling techniques. A comprehensive review of the subject (Beja-Pereira et al. 2009) lists dozens of studies in which hair and skin, mucous and saliva, and faeces and urine are the sources of genetic samples. A single case of freshwater samples providing target species DNA is mentioned, acknowledging the first successful macrofaunal detection with environmental DNA (eDNA) techniques (Ficetola et al. 2008). The methods entail collecting small volumes of water and filtering or precipitating the DNA that animals shed to the environment without ever contacting the organisms themselves. Sources of this DNA include dead skin and scales, mucous, feces, or any other secreted or sloughed material that contains genetic information. The molecular target is a short (80-250 base pairs) (Bohmann et al. 2014) gene region that is unique to the target species or shared only within a target group. The target gene region is selected to be unique to the species of interest, or shared among all species of a higher taxonomic designation.
Assessment by eDNA methods can significantly reduce the time and expense of sampling (Jerde et al. 2011, Evans and Lamberti 2017), and eliminates stress and mortality imposed on animals by conventional methods. The limitations and biases of conventional methods are minimized with a standard collection protocol that can be carried out by technicians with minimal training, evidenced by the success of a citizen science program for monitoring an endangered amphibian (Biggs et al. 2014). eDNA techniques in freshwater systems begin with collecting water samples in sterile bottles and filtering or precipitating the DNA suspended in the water. Moving to the lab, eDNA is isolated with extraction kits or by established isolation techniques. Small volumes of DNA isolate are run in polymerase chain reaction (PCR) with primers targeting a unique gene sequence. If present, that sequence will be copied and synthesized in reaction, increasing concentration by several orders of magnitude. Primer specificity tests on tissue of target and co-occurring species are carried out prior to sampling to ensure no cross-reactivity exists between primers and non-target species. Presence can be scored by running post-PCR DNA on agarose gel and comparing target fragment lengths from amplified DNA against positive controls of tissue extracted DNA. Quantitative PCR (qPCR) offers two additional results: First, assurances of specificity are provided by matching melting curves (the temperature at which double stranded DNA dissociates) of experimental samples and positive controls. Furthermore, an extra target gene sequence must be matched to a probe for amplification to be recorded by fluorescence. Second, from fluorescence measures taken at every stage of the reaction, a quantitative estimate of starting DNA concentration can be made.
Since its recent introduction, eDNA has proven its value in many applications, such as determining invasion fronts (Darling and Mahon 2011), locating rare and endangered species (Thomsen et al. 2012b), and assessing community composition at higher sensitivity than conventional methods (Thomsen et al. 2012a, Kelly et al. 2014b). It has been applied in diverse environments, across wide taxonomic range, and is capable of resolving time scales from days to thousands of years (Thomsen and Willerslev 2014). However, eDNA methods still face considerable challenges in addressing uncertainties that relate to both environmental and methodological variation. DNA production rates can be organism and system specific, and can also vary among individuals and life stages (Pilliod et al. 2014, Klymus et al. 2015). Collection, filtration/precipitation, and preservation methods are also inconsistent among studies (Rees et al. 2014a, Takahara et al. 2014), making their effects measurable only within limited context of single experiments. Effects of discharge, substrate, and environmental inhibition are focal points for active research (Jane et al. 2015, Jerde et al. 2016, Wang et al. 2017), which await synthesis into overarching principles for general application in the field.

Two other critical uncertainties remain in eDNA research: minimum required densities for detection, and the persistence of eDNA in moving water (Barnes et al. 2014, Strickler et al. 2014). To date, experimental determinations of low-density detection thresholds and eDNA persistence have been performed in small aquaria and artificial ponds (Dejean et al. 2011, Piaggio et al. 2013), but none have incorporated discharge or flow rate to model DNA degradation and dilution in lotic systems at the lowest detection densities. eDNA models of reach scale habitat use have been made based on flow
estimates and downstream detection (Deiner and Altermatt 2014), or with a coarse metric of flow rate (Jane et al. 2015). These studies represent the current leading edge in low density detection with eDNA for freshwater wildlife, incorporating environmental variability of lotic systems.

The pioneering work of Ficetola et al. (2008) set an experimental standard for methodological validation of eDNA, a basic model which still applies to many eDNA studies today: confirmation of results in controlled conditions, and testing of methods in natural environments. Sampling eDNA from habitats well-studied by conventional methods has become another commonly employed design. After establishing the method in tightly controlled systems (e.g. aquaria, tanks, and artificial ponds) sampling for eDNA from closely monitored sites allows a second level of validation for results obtained from field samples. For an emerging monitoring technique, corroboration with proven methods that are near impervious to false positives adds considerable confidence. This general design of eDNA research has been replicated and improved upon countless times (e.g., Dejean et al. 2011, Takahara et al. 2012, Thomsen et al. 2012b, Veldhoen et al. 2016).

**eDNA application in a new environment**

Environmental DNA is an encouraging development for wildlife managers, as a rapid, non-invasive, standardized method for mapping distribution and estimating abundance. As it is applied on new species and in new systems, it requires a reassessment of sensitivity and specificity before results can be accepted with confidence. This research intends to calibrate the method for eDNA monitoring of juvenile Coho Salmon (*Oncorhynchus kisutch*), and resident salmonids (*Oncorhynchus* spp.) in Pacific coastal
streams. Pacific salmon are a species group of tremendous conservation concern, stressed by compound effects of habitat degradation and loss, and increasing fishing pressure (Gresh et al. 2000, Sethi et al. 2012). Dozens of local extinctions and an overall decline in abundance have been well documented throughout their range (Lackey 2003, Gustafson et al. 2007). Pacific Salmon also constitute a $750 million annual recreational and commercial fishery in British Columbia (DFO 2014), and provide key ecosystem services (Holmlund and Hammer 1999, Helfield and Naiman 2001). In this context, effective and informed management should be a focal concern for the salmon fishery, yet monitoring efforts and capacity have fallen dramatically over five decades in Canada (PFRCC 2004, Price et al. 2008). Legislative protection of freshwater habitats has also deteriorated in recent years (Hutchings and Post 2013). These habitats are subject to wide environmental variation and disturbance (Strayer and Dudgeon 2010), and reliance on them during critical stages of their life history put juvenile salmon at high risk. Coho Salmon are particularly reliant on these habitats, spending up to eighteen months rearing in freshwater (Groot and Margolis 1991).

Limited research in focused applications exists on the efficacy of eDNA for monitoring Pacific salmon (Laramie et al. 2014, Strobel et al. 2017), and to my knowledge, none has been performed to date on juvenile Coho Salmon in freshwater habitat. The next chapter of this thesis describes my field validation of eDNA methods for detecting Coho Salmon and resident salmonids in five small streams on the Central Coast of British Columbia, Canada. This research was conducted with support from the Hakai Institute at their field station on Calvert Island, where study streams are part of
long term hydrologic and biogeochemical monitoring projects. Fish communities in these streams were inventoried with conventional methods to provide a reference to which eDNA detections could be compared.

On the British Columbia coast, hatcheries for raising and releasing juvenile salmon have been operating for decades as a strategy to augment the salmon fishery. At Howard English Fish Hatchery on the Goldstream River, near Victoria, BC, I conducted several experiments in eDNA detection, described in the third chapter of this thesis. Their purpose was to determine minimum density necessary for consistent eDNA detection, and persistence of eDNA in moving water after the target species have abandoned a site. I used hatchery tanks up to 10,000L to simulate juvenile Coho Salmon habitat. Water in the tanks is sourced directly from a natural salmon spawning river, from a site upstream of a natural barrier to wild salmon and the hatchery discharge. The tanks’ size approximates that of pools in typical Coho Salmon rearing streams. The system improves on eDNA experimental designs that often employ small aquaria and treated tap water, making my results more generalizable to natural conditions.

A final concluding chapter will summarize findings from original research in the second and third chapters. General recommendations based on these findings will be discussed, as they relate to the larger field of eDNA experimentation and research. Lastly, potential for expanded application of eDNA monitoring of wild salmon populations at regional scale will be explored.
Chapter 2
Up the creek without a net: Mapping distribution of juvenile Coho Salmon and resident salmonids in Pacific Coast streams with environmental DNA

Abstract
Conventional monitoring of freshwater fish often involves harmful capture methods like electrofishing, and imposes handling stress that can lead to long term fitness consequences. For juvenile salmon, conventional capture occurs at an early life stage when they are most vulnerable to these effects. In recent years, environmental DNA methods for detecting aquatic vertebrate animals have been advancing at a rapid pace, with methodological validations in new ecosystems and with new species occurring regularly.

I undertook a field validation of eDNA methods for juvenile Coho Salmon and resident salmonids in small coastal streams on the Central Coast of British Columbia. Fish communities in five streams near the Hakai Institute on Calvert Island were assessed with minnow trap and seine prior to conducting eDNA sampling. In comparison of the methods, eDNA detections of Coho Salmon and salmonid fish were made at all sites where the target group were confirmed present with conventional methods. Two sites tested positive for Coho DNA, and one for salmonid DNA, where conventional methods failed, indicating a possible higher sensitivity of eDNA sampling. I also mapped the distribution of juvenile Coho Salmon through multiple tributaries of a productive salmon system with conventional and eDNA detections.
Several environmental and experimental variables were found to affect the probability of eDNA detection. Higher DNA concentration in PCR was correlated to higher detection probability, but appeared to interact with both filtered water volume and fish abundance. Sediment size and discharge variation also appeared to affect downstream transport of eDNA, with greater eDNA dispersion in cobble and boulder, and at higher flows.

Lastly, I evaluated the effect of altering the threshold of evidence needed to conclude site level occupancy, by changing the required proportion of positive detections out of total PCR replicates. In 10 of 13 sites, the highest threshold of evidence was met or exceeded. Three sites scored detections that met lower thresholds. Practical and theoretical consequences of these results are discussed in both research and management contexts.

**Introduction**

The monitoring of aquatic biodiversity often involves a significant investment in time and resources and can produce biological data that is challenging to interpret (Jerde et al. 2011, Adams et al. 2017). Environmental DNA (eDNA) detection is an emerging rapid assessment method for the monitoring of rare and cryptic aquatic species that may augment or even replace more conventional methods. These conventional monitoring techniques can be limited in two ways: First, there are high labour and material costs for training, equipment acquisition, site permitting, and accessing remote field locations (Bohmann et al. 2014). Second, there can be considerable uncertainty in abundance estimates and even detection (i.e., false absences) that often relate to equipment

These limitations are minimized with standardized collection protocols of eDNA, which entail collecting small volumes of water and filtering or precipitating the DNA that animals shed to the environment, without ever contacting the organisms themselves. Assessment by eDNA methods can reduce the time and expense of sampling (Jerde et al. 2011), and eliminates stress and mortality imposed on animals by conventional methods. Minimal demands on labour and expertise associated with eDNA field work are demonstrated by the success of citizen science monitoring of an endangered amphibian (Biggs et al. 2014). Wearing latex gloves and collecting water without entering the pond were the only requirements to be met by citizen collectors while ladling water from 20 locations around the pond into a sealable bag.

Though rapidly advancing, the field of eDNA still faces several obstacles and unknowns that must be overcome before it can be used in a regulatory context that would be accepted by legislators and management agencies (Kelly et al. 2014b). These include effects of stream flow on downstream transport of eDNA (Jane et al. 2015), and primer specificity against closely related sympatric species with ability to hybridize (Wilcox et al. 2013). Sample collection and preservation methods are highly variable among studies, and trade-offs between them (e.g., cost and logistical feasibility, susceptibility to contamination or degradation) are not well understood or widely recognized (Takahara et al. 2014, Turner et al. 2014, Piggott 2016). Abundance estimation from eDNA results remains accurate only in relative terms, and within a single studies (Takahara et al. 2012,
Nathan et al. 2014). Experimental and laboratory control measures must also follow strict guidelines to ensure eDNA detections of target species are the result of a true signal, not contamination (Jerde et al. 2011, Thomsen and Willerslev 2014).

Inhibition of PCR from environmental agents (e.g., tannins, humic acids) is another concern of eDNA research (Lakay et al. 2007, Green and Field 2012). The prospect of false negatives due to inhibition exists in all eDNA field samples. A common, practical step to prevent PCR inhibition is dilution of isolated DNA to a concentration at which inhibitors no longer have an effect (Cao et al. 2012). This method does have inherent risk: DNA extract can be diluted to a point where inhibition is eliminated, but target DNA is also diluted to the point of signal distortion (Wang et al. 2017), or complete signal loss. Differences in collection and DNA isolation protocols have also been shown to influence eDNA results (Ficetola et al. 2008, Piaggio et al. 2013).

Another important consideration of eDNA results as they apply to conservation and management goals is determining what threshold of evidence must be met before a site or system is regarded as occupied (Veldhoen et al. 2016). When invasive or endangered species are involved, the conclusion of species presence or absence can have implications for management action. Currently, there is no industry or research standard on necessary sampling effort, laboratory replication, or minimum proportion of positive detections before a site or system can be considered occupied by a target species.

As these uncertainties are addressed, they must be revisited whenever the technique is applied in a new system and on a new study species. eDNA research on Pacific Salmon has been successful in focused applications (Laramie et al. 2014, Strobel
et al. 2017), but none to date has been performed detecting juvenile populations in freshwater systems. These juvenile populations are particularly vulnerable to adverse effects from conventional monitoring methods. Handling fish imposes stress with energetic consequences that can have long term fitness effects, especially in early development. Electrofishing is the most common and reliable procedure, but also entails the most direct harm to fish (i.e., brain hemorrhaging and spinal injuries (Clément and Cunjak 2010), and reduced egg survival (Cho et al. 2002)). In an era of reduced monitoring and management capacity for Pacific Salmon (Price et al. 2017), the prospect of a rapid, non-invasive, standardized method for determining species presence and abundance is an encouraging development for fisheries managers.

I set out to test eDNA methods for monitoring juvenile Coho Salmon (*Oncorhynchus kisutch*) and resident salmonids in five remote coastal streams near to the Hakai Institute on Calvert Island, in the Great Bear Rainforest of British Columbia, Canada. To validate eDNA methodology and assess detectability of juvenile and resident salmonids, I compare detections made by eDNA and conventional monitoring methods. Adopting proven techniques and successful pilot testing suggest that field detections will be made at high density sites, but no previous experience or research indicates how the method will perform at lower densities and across the breadth of environmental variation. I also explore possible effects of environmental variables such as substrate, stream discharge, and target species abundance. Process level variables including two different filtration methods, varying DNA isolation protocols, and template dilution in PCR are also investigated. Finally, I employ a modified standard from Veldhoen et al. (2016) to
evaluate effects of altering the evidence threshold required for determining presence of target species in these streams. I have several hypotheses based on pilot testing and literature review: 1. eDNA detections will correlate well with conventional detections, and eDNA detectability will increase with higher observed fish densities. 2. Higher sample volumes also should increase detectability, as may larger substrates. 3. Different presence/absence conclusions will result from altering evidence thresholds.

Methods

Field work: Conventional fish capture and eDNA sampling

Field work was conducted near the Hakai Institute on Calvert and Hecate Islands on the Central Coast of BC (Figure 1). Several study streams are subject to long term hydrologic and bio-geochemical monitoring by the Hakai Institute. I selected four of these streams for study (numerical IDs 1015, 844, 703, and 708 (common name Big Spring Creek), and added one nearby (867) to incorporate more habitat and fish community variation.

I surveyed fish communities and habitat in four of five study streams (867 excluded) in July 2013 (Figure 2). In each stream, six minnow traps baited with ~ 30g of wet cat food were set overnight for 18-24 hours. Where present, one or two pools per stream were repeat seined to depletion with a 1m x 3m x 64mm net. These assessments were performed in stream reaches selected as coarsely representative of the best fish habitat accessible within 1000m of the outflow to sea. Low gradient (<1°) reaches with pools were selected where present. Relative abundance and catch per unit effort (CPUE) estimates for each stream were used to characterize fish habitat quality and productivity. CPUE was scored as number of fish caught per seine pass or cumulative trap hours.
Conventional fish sampling in all streams did not constitute exhaustive community surveys, but rather snapshot community assessments based on opportunistic sampling in high quality fish habitat. Reaches were subdivided into six transects, upon which I measured bank full (BF) width and depth, and substrate composition. Bank full measures are taken from high water marks that reflect a maximum depth experienced in the previous wet season. Pool and cover forming large woody debris (LWD) were counted on the entire reach length, and qualitative assessment of canopy cover, undercut banks, periphyton growth, and habitat type were made. Water quality measures were also made for dissolved organic carbon (DOC), and pH.

Fish community and habitat assessment in watershed 867 was conducted in July 2014. Fish sampling was conducted in two locations: a series of step-pools in a higher-gradient (~2.5°) reach, 30m in length; and a small beaver-dammed pond above the assessed reach. Three baited minnow traps were placed in both locations overnight. Qualitative observations on canopy, substrate, stream width and depth, and habitat type were also made. Repeat visits to 703, 708, and 1015 were made in summer 2014. A small pond on 703 was surveyed for fish with a column of three minnow traps, and with hook and line. Two tributaries above the lake in 708 were assessed for fish presence with 3 minnow traps each, for sampling times of 20 and 22 hours, respectively. A reach above the lake in 1015 was assessed with six minnow traps, and a shallow sandy shoreline near inactive cutthroat trout redds was seined. A column of three traps was also placed in the centre of the lake to a depth of ~10m. All fish collection was performed with permission from provincial, federal, and First Nations authorities (BC FLNRO: NA13-88401 and
NA14-95317; for 2013 and 2014, respectively; DFO: XR 131 2013 and XR 131 2014; Heiltsuk and Wuikinuxv permission granted through Hakai Institute’s Coastal Watersheds research program). Animal handling permits were granted through University of Victoria Office of Research Services Animal Care Committee protocol 2013-008, renewed each year through to 2016.
Figure 1. Location of Calvert Island on the Central Coast of British Columbia, Canada. Environmental DNA and conventional monitoring of juvenile Coho Salmon and resident salmonids was performed in five streams near the Hakai Institute on Calvert Island.

In all streams, eDNA sample sites were selected upstream of any tidal influence to ensure no DNA from non-target marine fish species or adult salmon would be collected, and with adequate depth and flow to partially submerge and fill a 2L collection bottle. Prior to field sampling, 2L bottles were rinsed three times in 10% bleach to degrade any
residual DNA, and three times in double distilled H$_2$O (ddH$_2$O) to remove the bleach.

Instead of bleaching bottles between repeated site sampling on subsequent days, each bottle was dedicated to a sampling site. Upon returning to a site, sample bottles were rinsed three times with stream water, immediately downstream of the collection site, then filled with a stream water sample. Care was always taken to ensure that 2L samples were collected at a site upstream of any activity (site access, equipment preparation, etc.) to prevent contamination of samples with exogenous DNA.

Preliminary eDNA sampling was performed from August 27-31, 2014. Two samples were taken from the mouth of each study stream, and one additional sample from an upper reach of 708 (Figure 3, site code LT2). These samples were processed using a vacuum filtration method. A new filtration system was implemented in 2015, and all samples from 2015 were processed with a peristaltic pump filtration. Sampling was conducted from June 10-12, 2015. Three samples were taken from the stream mouths of 703, 844, and 867; four samples were taken from the stream mouths of 708 and 1015 (Figure 2). The purpose of eDNA sampling was to characterize fish communities across watersheds with eDNA, and compare against community assessments performed with conventional methods.

Further eDNA sampling was performed through several lakes and tributaries of 708 to map the distribution of Coho Salmon in the watershed, and investigate possible habitat related features within a watershed that could influence Coho presence and eDNA detectability. Twenty-one additional samples were taken from seven sites in stream 708 (Table 3, Figure 3).
**Vacuum filtration process for preliminary 2014 sampling**

Repeat sampling occurred on separate days (1-3 days apart) at each study stream mouth, and one sample was taken from site LT2 (Figure 3, Table 4) in stream 708. Two litres were collected at each site, placed in a cooler for 1-6hrs to return to base for filtration. After refrigeration at 4°C for 2-3 hours, a subsample of collected water (200-500mL) underwent vacuum filtration in sterilized single-use vacuum filter flasks (VWR 47mm diameter, 0.2µm pore size, cellulose nitrate/polyethersulfone membrane (CN/PES)) to trap the waterborne eDNA on the filter membrane. Filtration was stopped when no water drops were observed to pass through the filter for three minutes.

**Peristaltic pump filtration on high volume 2015 samples**

Subsamples of collected water (425-2000mL) were pumped through a 47mm diameter 0.45µm pore size mixed cellulose ester (MCE) filter membrane (Advantec MFS, Inc., Dublin, CA), with a Cole-Parmer Masterflex L/S 7553-80 peristaltic pump (Cole-Parmer, Montreal, QC) via a filtration unit consisting of ~1m of #16 silicone tubing (Baoding Signal Fluid, Baoding City, China) and a reusable 47mm in-line polypropylene filter holder (AMD Manufacturing, Mississauga, ON). Filtration was stopped when no water drops were observed to pass through the filter for one minute. Each filtration unit was decontaminated by pumping through 100mL of 10% bleach solution, 100mL of double distilled water (ddH2O), and then autoclaved at 121°C and 15psi for 20 minutes. Filtration units were re-used for replicate samples within streams and sites. MCE filter membranes were selected from several other competing types based on demonstrated higher eDNA
retention and extraction ability (Liang and Keeley 2013). Filtration protocol was adapted from Walsh et al. (2009).

With both filtration methods, membranes were removed from the filter holder and placed in sterile falcon tubes with forceps that were bleached, rinsed, and autoclaved as above. A single pair of decontaminated forceps was used to handle all within stream (or site) replicates. Filter membranes were frozen at -5ºC for 1-3 days, transported on ice for 12 hours, and then placed in -20°C freezer for periods ranging from 9-112 days.

**Laboratory processing: DNA isolation, PCR, and gel visualization**

Filter membranes were thawed and DNA extracted with MO-BIO PowerWater DNA isolation kits (MO-BIO Laboratories, Carlsbad, CA), following the manufacturer’s protocol for increased yield, which I modified to vary lysis buffer heat soak time from 0.5-1.75 hours (MO-BIO Laboratories, Carlsbad, CA). A subset of samples (all 2014 samples, and 2015 samples from 708 and 867) was quantified for DNA prior to amplification using a Qubit 2.0 fluorometer (Invitrogen Corp., Carlsbad, CA). Each sample was then run in triplicate PCR with template volumes of 4µl, 2µl, and 1µl in 20µl reactions, classified as high, medium, and low template concentrations. The range of template volumes was selected as a balance of three factors: true PCR replication at nearly equal template volumes, high template volume to increase likelihood of amplification at low target DNA concentration, and a modest dilution series as a check against PCR inhibition at high template concentrations. Reaction mixtures and conditions, and primer sequences are in Table 1.
Assay for Coho Salmon was taken from Rasmussen-Hellberg et al. (2010), targeting a highly conserved 95 base pair gene region of Cytochrome Oxidase I mitochondrial DNA. An NCBI Blast search returned no matches with any sympatric species in the forward primer. However, the reverse primer was a match with one deletion on Chinook Salmon. Salmonid family assay was taken from a project with Dr. Ben Koop at the Centre for Biomedical Research, University of Victoria, and designed using Primer3 software (Untergasser et al. 2012), targeting a 220 base pair SNP3 gene region on 16S ribosomal RNA that is shared among all Pacific Salmon, Rainbow Trout, and Cutthroat Trout, along with several other non-sympatric Salmoniformes. Of note was the finding through NCBI Blast that no *Cottus* or *Gasterosteus* sequences were found to match the salmonid primer, ensuring that detections made on the salmonid primer were not false positives from co-occurring Sculpin or Stickleback known to be present at these field sites.

PCR controls included a positive control test on template DNA extracted directly from Coho Salmon tissue, and triplicate no template controls (NTCs) to test the reaction components for contamination on each PCR plate. Gel electrophoresis was performed on five percent agarose gel, prepared with 1X sodium borate buffer and SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA). Two microliters of PCR product was run with 10µl Green GoTaq® Flexi Buffer (Promega, Madison, WI) aside 2µl 100 base pair gene ladder (Thermo Scientific, Waltham, MA) at 100V for 30-40 minutes. Imaging was done with EpiChemi® Darkroom Bioimager (UVP Bioimaging Systems, Upland, CA).
Samples with very low levels of target DNA can produce weak qualitative responses (i.e. faint band on agarose). Therefore, the system for scoring gel images was such that even very faint bands were classified as positive. Observer bias in classification of true positives and false positives from negative controls was tested using a blind re-scoring of 20 samples. The test resulted in a perfect correlation between initial and repeat scoring suggesting that positive classification bias was minimal.

Table 1. PCR reaction components and conditions, performed on Techne TC-412 thermal cycler (Bibby Scientific, Staffordshire, UK).

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1DNA free H₂O</td>
<td>7.9 – 10.9</td>
</tr>
<tr>
<td>2GoTaq buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>3MgCl₂</td>
<td>1.6</td>
</tr>
<tr>
<td>4dNTPs</td>
<td>0.4</td>
</tr>
<tr>
<td>5forward primer</td>
<td>1.0</td>
</tr>
<tr>
<td>6reverse primer</td>
<td>1.0</td>
</tr>
<tr>
<td>7GoTaq® hot start polymerase</td>
<td>0.1</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0– 4.0</td>
</tr>
</tbody>
</table>

1Invitrogen, Carlsbad, CA. 2,3,4,7Promega, Madison, WI. 5,6IDT, Coralville, IA.

Thermocycling profile: initial denaturation 95°C for 2.5mins, 35 cycles at (denaturation 95°C for 30s, annealing 62°C for 30s, elongation 72°C for 30s), final elongation 72°C for 5mins, final hold 4°C

Primer sequences: Coho forward CGCTCTTTCTAGGGATGATC; Coho reverse CTCCGATCATAATCGGCATG; salmonid forward GCCCATATGTCTTTGGTGG; salmonid reverse CTCGTCGATAGGGATTCTGG

eDNA CPUE and determination of site level presence or absence

At each sampling site, the proportion of total PCR positives out of total number of PCR runs (no. of samples x no. of replicates) was used as a relative CPUE measure for eDNA sampling. Site level presence/absence conclusions were made by adapting scoring standards and replication thresholds set out in (Veldhoen et al. 2016). At sites with total
technical replicates of 9 or 12, 3/9 or 4/12 cumulative positives were set as the standard threshold to confirm a site level positive. I also investigated the effect of raising and lowering the threshold level of positives by one on site level presence or absence determination. These thresholds were defined, respectively, as high certainty (4/9 or 5/12) and high sensitivity (2/9 or 3/12).

Results

eDNA fish detections from five streams near the Hakai Institute were similar to those made with conventional fish detection methods (Figure 2). eDNA detections were made at all streams and sites where fish were observed with conventional methods, both for salmonids and Coho Salmon. Additional eDNA detections were made where conventional sampling failed. This occurred at one stream for Coho Salmon, where only resident salmonids (*O. clarkii* and *Salvelinus malma*) were observed, and one for both salmonid and Coho Salmon, where no fish were observed.

Conventional fish detection methods determined Coho Salmon presence in two streams and resident salmonid presence in three streams. Coho Salmon were observed in streams 708 and 867, with higher CPUE and abundance in stream 708. Cutthroat Trout were observed in streams 703, 1015, and 708, with higher CPUE and abundance in stream 703 (Table 3). CPUE for conventional detection was positively associated with eDNA detectability for resident salmonids, but not for Coho Salmon. Physical variation among streams is defined in Table 2.
Table 2. Stream characteristics and fish species found using conventional detection methods. CCT: coastal cutthroat trout, CO: coho salmon, TSB: threespine stickleback, CS: coastrange sculpin, PS: prickly sculpin, DV: dolly varden. BF width, BF depth, include mean and (SD). Sediment size includes mean and (95% confidence interval).

<table>
<thead>
<tr>
<th>Stream</th>
<th>Catchment area (km²)</th>
<th>BF width (m)</th>
<th>BF depth (cm)</th>
<th>Sediment size (cm)</th>
<th>DOC (mg/L)</th>
<th>pH</th>
<th>Fish species observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>703</td>
<td>12.8</td>
<td>10.6 (2.8)</td>
<td>137.3 (70.6)</td>
<td>1.2 (1.1)</td>
<td>4.6</td>
<td>6.2</td>
<td>CCT</td>
</tr>
<tr>
<td>708</td>
<td>7.8</td>
<td>6.4 (0.5)</td>
<td>78.3 (23.6)</td>
<td>4.5 (1.9)</td>
<td>8.7</td>
<td>5.6</td>
<td>CCT, CO, TSB</td>
</tr>
<tr>
<td>844</td>
<td>5.7</td>
<td>4.2 (0.4)</td>
<td>113.9 (44.8)</td>
<td>20.7 (9.3)</td>
<td>14.0</td>
<td>5.0</td>
<td>none</td>
</tr>
<tr>
<td>867</td>
<td>1.2</td>
<td>1.5(est.)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.8</td>
<td>CO</td>
</tr>
<tr>
<td>1015</td>
<td>3.3</td>
<td>3.1 (0.8)</td>
<td>57.3 (19.6)</td>
<td>4.3 (0.8)</td>
<td>11.0</td>
<td>6.6</td>
<td>CCT, CS, PS, DV</td>
</tr>
</tbody>
</table>
Table 3. Conventional and eDNA catch per unit effort for target species in five streams on Calvert and Hecate Islands, British Columbia. Conventional data include total number of fish caught in parenthesis. eDNA CPUE results from 2015 (high volume samples) are recorded on the left, and combined 2014-15 results on the right of eDNA columns. Each waterbody has a single eDNA sample location that does not correspond directly to conventional capture method or location.

<table>
<thead>
<tr>
<th>Waterbody</th>
<th>Location</th>
<th>Method</th>
<th>Coho conventional</th>
<th>Coho eDNA</th>
<th>CCT conventional</th>
<th>salmonid eDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>708</td>
<td>assessed reach</td>
<td>seine</td>
<td>2.27 (29)</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>assessed reach</td>
<td>minnow trap</td>
<td>0.04 (4)</td>
<td>8/12 : 14/18</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>upper tributary</td>
<td>minnow trap</td>
<td>0.1 (8)</td>
<td>0</td>
<td>0.01 (1)</td>
<td>n/a</td>
</tr>
<tr>
<td>867</td>
<td>assessed reach</td>
<td>minnow trap</td>
<td>0.03 (2)</td>
<td>8/9 : 11/15</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>1015</td>
<td>assessed reach</td>
<td>seine</td>
<td>0</td>
<td>0</td>
<td>4/12 : 5/18</td>
<td>7/12 : 12/18</td>
</tr>
<tr>
<td></td>
<td>assessed reach</td>
<td>minnow trap</td>
<td>0</td>
<td>0</td>
<td>1.67 (5)</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>large lake</td>
<td>seine</td>
<td>0</td>
<td>0</td>
<td>0.03 (2)</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>upper tributary</td>
<td>minnow trap</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>703</td>
<td>assessed reach</td>
<td>seine</td>
<td>0</td>
<td>0</td>
<td>0.11 (12)</td>
<td>8/9 : 10/15</td>
</tr>
<tr>
<td></td>
<td>assessed reach</td>
<td>minnow trap</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4/9 : 4/15</td>
</tr>
<tr>
<td>844</td>
<td>assessed reach</td>
<td>seine</td>
<td>0</td>
<td>2/9 : 2/15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>assessed reach</td>
<td>minnow trap</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4/9 : 4/15</td>
</tr>
</tbody>
</table>
Figure 2. Comparison of conventional (conv.) and environmental DNA (eDNA) detection of Coho Salmon (coho) and resident salmonids (salmonid) from five watersheds on Calvert and Hecate Islands, British Columbia. 'Y' indicates positive detection, and 'N' indicates no detection at the standard threshold of replication, adapted from (Veldhoen et al. 2016).

eDNA detectability varied by stream and was influenced by target species density, field conditions, and sampling and process variation. Stream level variation in detectability influenced determination of presence at a site. All salmonid eDNA detections met the high certainty threshold, scoring ≥ 4/9 or 5/12 positive technical
replicates in all three streams where salmonid assay was tested. Of eleven sites with Coho Salmon eDNA detections, nine had positive replication exceeding the high certainty threshold, occurring in the two streams where Coho presence was confirmed by conventional methods (streams 708 and 867). Reducing the threshold to standard level resulted in addition of stream 1015 as Coho Salmon occupied. Further reduction to high sensitivity threshold resulted in site level Coho presence determinations in stream 844, and site HT2 (Table 4, Figure 3) in stream 708. No eDNA detections of Coho were made in stream 703, the field negative control system, where Coho are excluded by an impassable barrier.

eDNA detectability was also greater in peristaltic pump samples in all streams, with the exception of stream 708, which had the highest abundance of target species. Across all streams, filtrate volumes of peristaltic pump samples were 3-5 times greater than in vacuum filtration samples.

*Stream 703*

The highest rate of salmonid eDNA detection and Cutthroat Trout CPUE were observed here. A single PCR failure in the peristaltic pump samples occurred at high template concentration. In the vacuum filter samples, detections were made only on the second day of sampling. Total extracted DNA concentrations and stream water filtrate volumes in these samples were <1ng/mL in 300mL, and 4ng/mL in 500mL, respectively. Twenty-five millimetres of rain fell in the region between the sampling days (Environment Canada 2014).
*Stream 1015*

Coho Salmon DNA was detected here in samples using both filtration methods, despite failing to observe Coho with conventional techniques performed in multiple locations. Coho detections were made in two peristaltic pump samples: one at all template concentrations and another only at low template concentration. One Coho detection was made in a vacuum filter sample at high template concentration.

At least one salmonid detection was made in all eDNA samples. Salmonid eDNA detectability and Cutthroat Trout conventional CPUE were both lower than in stream 703. In low volume vacuum filter samples, the lone failure to detect salmonid eDNA occurred at low template concentration. In high volume peristaltic pump samples, detectability decreased with higher template concentrations. One sample had significant material loss in processing and was effectively diluted by a factor of four. This was the only sample to detect salmonid DNA at all three template concentrations. The other samples made detections only at medium and low template concentrations.

*Stream 844*

eDNA detections were made here only in the higher volume peristaltic pump samples. One sample detected Coho at high and medium template concentrations. Salmonid DNA was detected in the other two samples at the same concentrations.

*Stream 867*

Despite low density of target species, Coho eDNA detections were made in all vacuum and peristaltic pump samples. All peristaltic pump samples had perfect Coho detection
except for one failure at high template concentration. This occurred in the sample with much higher total DNA concentration (131ng/mL versus 6ng/mL and 23ng/mL). Coho DNA detection in the vacuum filter samples were made twice at high, and once at medium template concentrations.

Stream 708

Coho eDNA detections were made in all stream mouth samples, but with lower probability than in the lower density stream 867. In contrast with all other streams, failures occurred only in the high volume peristaltic pump samples and always at high template concentration. Vacuum samples from the stream mouth and site LT2 (Figure 3, Table 4) had perfect detection at all template concentrations.

Throughout the watershed, eDNA detections were made at multiple locations with the standard replication threshold (Figure 3). At site HT2, where multiple juvenile Coho were observed, only the high sensitivity threshold for determining site level positive was met. Considerable variation in eDNA collection and processing factors occurred across both years and through sample sites (Table 4). Within the nine sample sites, where heat treatment time differed between samples by at least a factor of two (range of 30-105 mins), the longer duration heat treatment produced greater total DNA concentrations in all but one of fifteen samples meeting these criteria (data not shown). At site HT2, both detections came from a single sample, which underwent long heat treatment and produced high total DNA concentration.
Figure 3. Environmental DNA detections of Coho Salmon throughout Big Spring Creek (watershed 708), near the Hakai Institute, Calvert Island, BC. Site level positives are based on the standard threshold of replication, adapted from (Veldhoen et al. 2016). Solid fill squares indicate positive detection, white fill indicates no detection.
Table 4. Environmental DNA processing and Coho Salmon detection results from nine sites in watershed 708, Calvert Island, British Columbia. At the stream mouth site, DNA concentration and eDNA detections from 2015 are recorded on the left, and combined 2014-15 results on the right. [DNA] = pre-amplification DNA concentration in ng/mL, [temp] = template DNA concentration in PCR.

<table>
<thead>
<tr>
<th>Site</th>
<th>Site ID</th>
<th>Mean filtrate volume (L)</th>
<th>Mean [DNA] ng/mL</th>
<th>Aggregated positives/technical replicates</th>
<th>Coho observed</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stream mouth</td>
<td>SM</td>
<td>1.4 : 0.3</td>
<td>30.4 : 264.0</td>
<td>8/12 : 14/18</td>
<td>Y</td>
<td>all failures at high [temp]</td>
</tr>
<tr>
<td>Trib 1</td>
<td>T1</td>
<td>1.0</td>
<td>3.3</td>
<td>9/9</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Trib 1A</td>
<td>T1A</td>
<td>1.1</td>
<td>10.8</td>
<td>9/9</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Mainstem &gt;Trib 1A</td>
<td>&gt;T1A</td>
<td>0.9</td>
<td>6.5</td>
<td>11/12</td>
<td>Y</td>
<td>failure at high [temp]</td>
</tr>
<tr>
<td>Trib 1 &gt; 1st Falls</td>
<td>T1&gt;F</td>
<td>0.8</td>
<td>30.1</td>
<td>6/9</td>
<td>N</td>
<td>all failures in highest [DNA] sample</td>
</tr>
<tr>
<td>Trib 1 &gt; 1st Lake</td>
<td>T1&gt;L</td>
<td>0.8</td>
<td>11.2</td>
<td>0/9</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>High Trib 2</td>
<td>HT2</td>
<td>0.6</td>
<td>10.9</td>
<td>2/9</td>
<td>Y</td>
<td>detect in high [DNA] at med and lo [temp]</td>
</tr>
<tr>
<td>Low Trib 2</td>
<td>LT2</td>
<td>0.3</td>
<td>71.9</td>
<td>6/6</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Trib 3</td>
<td>T3</td>
<td>1.0</td>
<td>15.1</td>
<td>3/6</td>
<td>N</td>
<td>no high [temp] detections</td>
</tr>
</tbody>
</table>
Discussion

Detection of Coho Salmon and salmonid family eDNA matched or exceeded detections using conventional methods at five remote streams on the Central Coast of British Columbia. This is consistent with observations of higher sensitivity of eDNA over conventional methods in varied ecosystem types (Pilliod et al. 2013, Janosik and Johnston 2015, Penârrubia et al. 2016, Wilcox et al. 2016). eDNA detectability and conventional method CPUE had a positive association for resident salmonids, but not for Coho Salmon. This may be due to interactions of target species abundance, sampled water volume, and template dilution, as they relate to environmental inhibition and eDNA detectability. Stream level characteristics of substrate size and relative discharge or flow rate also affect eDNA detectability in some cases. Further variation in stream by stream results reflects the unpredictability of complex systems with multiple interacting factors, as seen with modeling and testing eDNA detection in lotic systems (Schultz and Lance 2015, Furlan et al. 2016).

Altering the threshold level of positive technical replicates for determining site level positive had no effect at 10 of 13 sampling sites. Confidence of target species presence at these sites is high, and the collection and processing method appears robust for resident salmonid and Coho Salmon detection at densities found at these sites. However, lowering the stringency of the threshold changed the site level conclusions at three sites. Two streams where no Coho Salmon were observed with conventional methods (1015 and 844) would be reported as positive for Coho under standard and high sensitivity thresholds, respectively. An upper tributary site of stream 708, where Coho
juveniles were observed adjacent to the sampling site, would only be classified as Coho positive at the high sensitivity threshold. Two important conclusions emerge from these results. First, a different interpretation of the same raw data will lead to different conclusions about site level occupancy. eDNA researchers currently practice a wide variety of data interpretations, requiring different thresholds of evidence to draw site level conclusions (Biggs et al. 2014, Rees et al. 2014a, 2017, Peñarrubia et al. 2016, Piggott 2016). In the case of Rees et al. (2017), their threshold is considerably less stringent than my low certainty threshold, requiring only 1/12 positive replicates to conclude a site positive. This lack of research standard complicates implementation of eDNA as a valid assessment method for monitoring and management (Rees et al. 2014b), particularly in cases when a single species detection will instigate costly management actions. Second, the reality of false negatives with eDNA is illustrated in the case of very low positive replication from a sample taken adjacent to multiple individuals of the target species. Diminished signal at this site may have been due to increased settling and retention in nearly still, shallow water at very low flow on fine, porous substrate (Tréguier et al. 2014, Doi et al. 2017). Rees et al. (2017) found similar results, with 1/12 positive replicates from a site where target species had been observed. The question is raised of what threshold to set. The answer will incorporate management priorities, risk tolerance for type I and II error, and assay performance for each new species-environment combination, as performed in Veldhoen et al. (2016).

There appears to be an interaction between target species abundance, sampled water volume, and template concentration in PCR that affects eDNA detectability.
Inhibition of PCR due to environmental compounds is a likely cause of these failures (Thomsen and Willerslev 2014). The mechanism hypothesized to produce this interaction is dilution of inhibitors, based both on evidence from this thesis, and previously demonstrated inhibitor release at 5-10x dilutions (Cao et al. 2012, McKee et al. 2015). In low volume vacuum filter samples, and/or where target species density is low, detections tended to occur at higher template concentrations. Conversely, in high volume peristaltic pump samples, and/or where target species density is high, detections were more common at lower template concentrations. Low volume samples from low density streams may already fall below the threshold concentration of inhibitors’ effect, and further dilution only reduces target DNA concentration below the limit of detection (Schrader et al. 2012). In stream 844, where density is assumed to be lowest due to zero conventional capture, detections were only made in high volume samples at higher concentrations. High volume samples, inhibited at high template concentration (e.g. streams 708, 1015, 703, and 867), retain sufficient target DNA to amplify -and are released from inhibition- after template dilution. A similar result was documented in Takahara et al. (2014). Accidental dilution and subsequent detection of salmonid DNA at all template concentrations in one high volume sample from stream 1015 reinforces this hypothesis. In the cases of streams 703, 1015, and 867, target DNA appears to exist at densities such that low filtrate volume requires high template concentration, and vice versa. In stream mouth samples from stream 708 however, where highest target species abundance was observed, target DNA concentration was sufficiently high for perfect detection in the low volume vacuum filter samples. All high volume samples from the
same location experienced inhibition at high template concentration. This leads to suggesting a link between target species abundance and presence of environmental inhibitors. To my knowledge, this link is as yet undocumented in eDNA research on aquatic vertebrates. I propose that greater volume of animal waste products in high density streams are sources of this inhibition. Kreader (1996) demonstrated animal feces as an inhibitory agent, and inhibitor release with dilution, on experimental samples of bacteria. Several studies have demonstrated PCR inhibition due to other animal waste and blood products (Khan et al. 1991, Lantz et al. 1997, Mahony et al. 1998, Al-Soud and Rådström 2001).

This relationship between target species abundance, sample volume, and template concentration suggests a variable optimum concentration of template in PCR to maximize detection probability. Although dilution series at factors of 5 to 10 are more common (Nathan et al. 2014, Strobel et al. 2017), this finding suggests that a dilution series by successive halves might be necessary to find such an optimum concentration between inhibition and limit of detection. Variable detection through template dilutions of this magnitude have been documented elsewhere (Takahara et al. 2014), and corroborate my increased detection rate at lower template concentration.

Substrate and discharge variation also correlates to variation in eDNA detectability, with a possible interaction of effects. In stream 703, failure to detect salmonid DNA on the first day of vacuum filter sampling could be due to combined effect of retention of eDNA in the fine sediments, and rainfall between the sampling days. In spite of modest dilution of signal that occurs with higher flows, heavy rain
between the sampling days may have stirred up DNA that settles at the bottom of the deeper pools, allowing it to be transported downstream to the sampling location.

Persistent eDNA signal extracted from sediments has been demonstrated to last months after it is no longer detectable in surface waters (Turner et al. 2015). Also, a behavioral response of salmonids to increased flows, experimentally demonstrated by Taguchi and Liao (2011), leading to higher metabolic rates and greater eDNA production, is also possible (Lacoursière-Roussel et al. 2016). A test of eDNA re-suspension in experimental streams (Shogren et al. 2017) illustrates the complexity of variable responses and multiple interacting factors (i.e. transport, retention, re-suspension, degradation), but also posits delayed re-suspension in relatively finer substrates, like those measured in stream 703, unique in this study. In contrast, stream 867 has high Coho Salmon eDNA detectability in spite of low target species abundance (Table 3). I identify three likely contributing factors: short transport distance (<200m) from occupied sites to sampling location (Jane et al. 2015), high flow velocity and mixing in a high gradient stream (Pilliod et al. 2013), and higher rate of downstream eDNA transport in cobble/boulder substrate (Jerde et al. 2016).

Further variation in results characterizes other unknowns and stochastic elements of eDNA research. Increasing filtrate volume should improve detectability, both on first principles and in models (Schultz and Lance 2015, Mächler et al. 2016), but these results do not universally support this. eDNA transport has been demonstrated at a scale exceeding that of this study (Deiner and Altermatt 2014), but dispersion/cohesion of eDNA along that transport distance can affect not only site level detectability, but also
within site sample variability (Barnes and Turner 2016, Furlan et al. 2016), as observed for Coho eDNA in stream 1015. Complex interactions of excretion, absorption, transport, collection, extraction, inhibition and dilution confound results of eDNA research (Roussel et al. 2015, Evans and Lamberti 2017). With limited ability to control environmental variation across studies, every effort should be made to standardize sample processing and data interpretation. A heightened standard of rigor for eDNA processing that includes a general amplification test for inhibition, experimental determination of assay performance and error rates, and blind scoring of samples and controls is described in Veldhoen et al. (2016). The general amplification test targets chloroplast DNA that is ubiquitous in freshwater systems, and a failure to detect this target is a strong indicator of inhibition. Probe-based assay design, quantitative PCR, and high cycle number are other options for improving specificity and sensitivity in eDNA research. More widely applied, these improvements would serve to increase confidence and generalizability in eDNA results.

In this study, I demonstrate effectiveness of an improved high volume collection protocol for environmental DNA monitoring of resident salmonids and juvenile Coho Salmon in small coastal streams. At multiple sites and streams, eDNA appears to be more sensitive than conventional monitoring methods, and with analysis protocols that consider methodological error rates, I have high confidence in site-level conclusions for target species presence or absence. Sensitivity and confidence in future studies, and generalizability among studies, could be increased by incorporating several processing, analysis, and design improvements. As methods are improved and refined, and findings
are replicated across systems, environmental DNA can be used to augment and replace conventional monitoring on species of high commercial and conservation value.
Chapter 3
Raising the bar with a lower detection threshold: Sensitive eDNA detection of Coho Salmon in experimental pools

Abstract

Environmental DNA (eDNA) is an emerging method for non-invasive detection of aquatic animals. Its rapid advance has seen successful application with a multitude of species across a wide range of environments, verified by conventional monitoring methods. However, several uncertainties remain as obstacles to implementation of eDNA as a viable alternative monitoring tool, and experimental benchmarks are necessary for interpreting eDNA data collected from the field. Present knowledge gaps include minimum densities necessary for consistent detection, and persistence of eDNA after a target species has left a site.

I conducted three experiments at a salmon hatchery in British Columbia to address these knowledge gaps. Water samples were taken from flow-through tanks with juvenile Coho Salmon densities ranging from 38.0g/1000L to 0.6g/1000L. To simulate field surveys in recently abandoned habitats, I sampled water from tanks after removing fish, at flow-through volumes ranging from 20,000L to 1,000,000L. Post removal sampling occurred starting at one hour and ending after just over four days of flow-through time. Water samples from tanks containing one or more fish tested positive for Coho DNA at least 70% of the time, increasing at higher densities. Samples taken after removing the fish had detection probability of 75% at flow-through volume of 40,000L. Detection failed at flow-through volumes greater than 80,000L.
This study sets a new experimental benchmark for eDNA detection at low animal density and models loss of signal through dilution after removing fish from a simulated stream pool habitat. It also demonstrates the utility and future potential of using hatchery infrastructure for the study of eDNA in controlled experiments.

**Introduction**

Advances in non-invasive sampling for wildlife monitoring have broadened the scope of research possibilities and increased capabilities of management agencies (Beja-Pereira et al. 2009). In the past decade, environmental DNA (eDNA) has emerged on the forefront of these non-invasive monitoring techniques for rare and cryptic aquatic species (Ficetola et al. 2008, Thomsen et al. 2012b, Piaggio et al. 2013). eDNA methods in freshwater systems entail collecting small volumes of water and filtering or precipitating the DNA that animals shed to the environment without ever contacting the organisms themselves.

The method has been applied successfully in diverse environments across wide animal taxonomy, at higher sensitivity than with conventional methods (Minamoto et al. 2011, Thomsen et al. 2012a). However, eDNA methods still face considerable challenges in addressing uncertainties regarding minimum densities necessary for reliable and consistent detection, and the persistence of eDNA in moving water (Barnes et al. 2014, Strickler et al. 2014). To date, experimental determinations of low-density detection thresholds and eDNA persistence have been performed in small aquaria and artificial ponds (Dejean et al. 2011, Piaggio et al. 2013). With no circulation or discharge of water, these study systems and laboratory setups tend to be poor approximations of natural environments. Moreover, these calibration studies seldom actually approach the low end
of naturally observed densities in the wild. The open-sea tank experiment at Monterey Bay Aquarium (Kelly et al. 2014a) sets the current standard of low density eDNA testing, having detected a marine fish species to genus level at a density of 1.7g biomass/1000L.

Beyond shortcomings of generalizability from laboratory and microcosm experiments to natural environments, standardization of eDNA results across studies is complicated by the wide range of processing and analysis protocols currently in use (Thomsen and Willerslev 2014, Barnes and Turner 2016). Approaches for testing samples for PCR inhibition (i.e., dilution, purification, general target amplification) are inconsistently applied. Effect of dilution on inhibition for PCR is generally accepted (Green and Field 2012), but its effect on missed detection rate is less well known. Diluting template for inhibitor release also reduces the likelihood that target DNA enters the reaction in sufficient quantity for amplification. As this contributes to detection failures, many studies treat some frequency of missed detections as inevitable. Consequently, different studies will carry out different levels of laboratory replication, opting to take even single PCR positive replicates as sufficient evidence to conclude site level detection (McKelvey et al. 2016, Rees et al. 2017). Similarly, determination of experimental false positive rates is often overlooked. This is even more troubling from a management perspective: low detection rate from field sampling needs to be distinguishable from some background level of false positive error. Experimental determination of this background level is critical for confidence in eDNA detections from sites where target species presence is unknown.
I set about addressing these shortcomings and knowledge gaps with three controlled experiments designed to detect Coho Salmon (*O. kisutch*) in simulated stream environments. Coho Salmon are a species both of conservation and commercial interest, and their fishery is augmented in British Columbia by many hatcheries operating on rivers near the coast. Using hatchery rearing tanks with controlled densities of juvenile Coho Salmon, I sought to determine a minimum fish density for detection with eDNA methods, and model the loss of eDNA signal via degradation and dispersion after fish have left small and large pool habitats. I also tested processing and analysis protocols, and different data interpretation methods to determine effects on detection probability, and site level conclusions for presence or absence of target species.

**Methods**

**Study System**

Experiments in this study were conducted at the Howard English Fish Hatchery along the Goldstream River (hereafter “Goldstream Hatchery”), near Victoria, B.C. The Goldstream River is home to a sizeable annual run of Chum (>20,000), and modest Coho and Chinook runs (~1000 and <50, respectively). There is a waterfall barrier to anadromous fish several kilometers from the river mouth. Goldstream Hatchery is located several kilometers more above this waterfall barrier, meaning any evidence of salmon in the reach above the falls is attributable to hatchery fish. A second manmade fish barrier immediately above the hatchery completely isolates the upper reach from either wild or hatchery-reared anadromous salmon. This model reproduces classic exclusion experiments in salmon research (*e.g.*, Hocking et al. 2013) and therefore provides an
excellent setup for controlled experimentation with juvenile salmon eDNA. Fish DNA present in the upper reach belongs exclusively to isolated freshwater fish populations, and is assumed to contain no DNA from anadromous salmon. Water samples taken above the hatchery represent no-salmon controls, and samples from hatchery tanks receive treatment of salmon DNA at experimentally manipulated densities.

In the hatchery, rearing populations of Coho, Chum, and Chinook Salmon number between 150,000 and 300,000. Coho are present at all times, and Chinook and Chum are present for two to four months in spring. Two sizes of flow-through rearing tanks were available for density manipulation experiments at increasing scale: 370L and 10,000L. All tanks are plumbed directly from the river and independent of one another. Water is pumped into a tank untreated and unfiltered, and drains straight out again to the river. River water flows through the tanks, as opposed to being recirculated. Thus, each tank is an independent replicate, unaffected by changes in density or abundance in the other tanks. Water is never mixed between tanks. Fish in experimental tanks do not require supplemental feeding because input of invertebrates and particulate plant matter from the river is adequate feed for fish at these densities. This eliminated the potential of fish DNA from feed pellets contributing to total DNA measures, as observed in Kelly et al. 2014. Pilot study experiments with 25 juvenile Coho in a 370L tank had zero mortality for three months duration (Peter McCully, personal communication, 2014).

Experimental Design

Prior to designing three separate experiments, an initial pilot study was conducted. This pilot experiment constituted four 370L tanks with a range of fish densities (0, 1, 5, 25
individuals; 0.02g/L - 0.4g/L). Perfect detection at low replication (one 2L sample/tank and duplicate PCR) suggested further experiments should target densities below 0.02g/L. Mass of each fish in all four tanks was recorded (\( \bar{x} = 5.7g, \ SD = 1.2g \)).

The first of three principal experiments was designed to determine the probability of eDNA detection through the range of naturally occurring fish densities, to a minimum density where detection failed. Four 10,000L tanks, with flow through rates of 140 – 155L/minute served as the experimental units. Five 2L water samples were collected from each tank at densities of 1, 2, 4, 8, 16, 32, and 65 fish (0.6g – 38.0g/1000L). Samples were collected 24 hours after additions of fish to allow eDNA shedding and degradation/dispersion to equilibrate. This range of densities was expected to include the threshold at which eDNA detections cease to be effective. The highest experimental density slightly exceeds a maximum estimated density for juvenile Coho Salmon in coastal BC, approximated from Rosenfeld et al. (2000), who estimated juvenile Coho densities to a maximum of 3 individuals per linear or square metre of stream. Because this experiment measures density per volume, I assigned an arbitrary stream depth of 1m to Rosenfeld’s observations. This conversion likely biases their natural density estimates downward, because streams of bank full width <5m (from which a large majority of their observations occur) typically have average depths below 1m (Rosenfeld et al. 2000). As a result, the densities at which I sampled are likely to be much lower than natural densities of productive salmon streams.

Prior to sampling, all experimental tanks were drained, pressure washed, and scrubbed with 10% bleach solution to degrade any residual DNA (Prince and Andrus
1992). Upon refilling, four pre-fish negative control water samples were taken from each tank to determine effectiveness of decontamination procedures. Experimental samples, those taken from tanks containing juvenile Coho Salmon at manipulated densities (N = 140 in experiment 1), each provided a presence/absence response. Twenty-one concurrent negative control water samples (three per sampling day) were also taken from the tank inflow, sourced upstream of any possible exposure to hatchery salmon DNA.

The second experiment measured signal decay due to the combined effects of DNA degradation and dispersion via stream discharge, after removal of fish. Four 370L tanks, with inflow rates of approximately 2L/min, underwent identical decontamination procedures. Pre-fish negative control sampling was reduced from four to three. A single juvenile Coho Salmon was placed in each tank, and given a three-day period for DNA production and degradation/dispersion to equilibrate. Three baseline samples were taken from each tank prior to removal of the fish. Triplicate sampling was conducted at times calculated to have 50%, 25%, 12.5%, and 1% of the original baseline water remaining: equivalent to dilutions of 50%, 75%, 87.5%, and 99%. Tank fill rates were estimated by averaging three filling times of a 20L bucket in each tank, and used to calculate sampling times for desired dilutions with this equation:

\[ t = -\frac{V}{\lambda} \ln(1 - \phi) \] (1)

Where \( t \) is the sampling time in minutes after fish removal, \( V \) is the volume of the tank in litres, \( \lambda \) is the flow-through rate in litres per minute, and \( \phi \) is the desired dilution level (i.e. 50%, 75%, 87.5%, 99%). Water samples taken at the specified dilutions (N = 48) each provided a presence/absence response. Fifteen concurrent negative control samples
were taken from the inflow source at times spaced evenly through the duration of the experiment. Mass of each fish was recorded ($\bar{x} = 5.4g$, SD = 0.6g).

The third experiment repeated the sampling post-removal to measure signal decay, but scaled up in the 10,000L tanks, with flow-through rates of 140-155L/minute. This estimate was made by averaging three complete filling times for each tank. Decontamination and pre-fish negative control sampling (three per tank) was performed identically as above. Three juvenile Coho Salmon, contained in minnow traps (biomass $\bar{x} = 26.1g$/tank, SD = 1.0g), were placed in the tanks just below the surface near the inflow, and the system was allowed to equilibrate for one week prior to baseline sample collection (4 per tank). Traps were removed and triplicate 2L samples collected from each tank at times representing cumulative flow-through volumes of 20,000L, 40,000L, 80,000L, 160,000L, and 1,000,000L. During the course of the four and a half day experiment, five concurrent negative control samples were taken from the inflow source above the hatchery. A total of 17 negative control samples were taken from the third experiment and through all experiments, a total of 83 negative control samples were taken.

Sample collection and filtration

In sampling from the tanks, a 2L bottle was partially submerged at arm’s reach (~0.5m) from the edge of the tank and filled with water from the surface. Concurrent negative control sample bottles were filled directly from the tank inflow source, or from a head-pond upstream of the hatchery. Water samples were filtered through 47mm diameter 0.45µm mixed-cellulose ester (MCE) filter membranes (Advantec MFS, Inc., Dublin,
CA, ON), with a Cole-Parmer Masterflex L/S 7553-80 peristaltic pump (Cole-Parmer, Montreal, QC), and a filtration unit consisting of ~1m of #16 silicone tubing (Baoding Signal Fluid, Baoding City, China) and a reusable 47mm in-line polypropylene filter holder (AMD Manufacturing, Mississauga, ON). Filtration protocol was adapted from Walsh et al. (2009) to allow for filtration of four 2L samples simultaneously. MCE filter membranes were selected from several other competing types based on demonstrated higher eDNA retention and extraction ability (Liang and Keeley 2013). In experiment 3, 24 out of 70 samples were processed with filter membranes from a different manufacturer (PALL Corp., New York, NY), although specifications on material and pore size were the same. Equal proportions of each filter manufacturer were employed among tanks and treatment levels, and filter type was included in detection models. After filtration, filter membranes were removed from the filter holder and placed in falcon tubes with sterile forceps. Filter membranes were then frozen at -20ºC within 30 minutes of filtering, remaining there for periods ranging from 27 to 164 days.

Prior to sample collection, all sampling equipment underwent a decontamination procedure: 2L sample bottles were rinsed three times in 10% bleach to degrade any residual DNA, and three times in double distilled H$_2$O (ddH$_2$O) to remove the bleach. Each filtration unit was decontaminated by pumping through 100mL of 10% bleach solution, 100mL of ddH$_2$O, and then autoclaved at 121ºC and 15psi for 20 minutes. Filtration units were re-used for replicate samples within tank and treatment level. Forceps for handling filter membranes were rinsed three times in 10% bleach and three times with ddH$_2$O, then autoclaved as above.
For increased assurance against contamination in the field, a “cooler blank” was also used (Jerde et al. 2011). This entailed filling a 2L bottle with ddH$_2$O, bringing it to the experimental site, and subjecting it to the same handling and processing as the experimental samples. The frozen filter membrane of this cooler blank returned to the lab and became a “lab process blank” whereby ddH$_2$O underwent the same isolation protocol as experimental samples. Two such process blanks were run: one subject to potential hatchery contamination (filtered alongside experimental site samples), and another laboratory blank, subject only to potential contamination from DNA extraction and PCR procedures. This was done to ensure the equipment decontamination process was effective, contamination at the experimental site did not occur, and lab sourced ddH$_2$O was free of potentially contaminating DNA.

Assay selection and validation

A Coho Salmon assay from Rasmussen-Hellberg et al. (2010), targeting a highly conserved 95bp region of Cytochrome Oxidase I (COI) gene of mitochondrial DNA, was selected. A genbank (Geer et al. 2009) search of these primer sequences was done to identify potential issues of non-specific binding and amplification of DNA from species most likely to contaminate samples, either from field or lab sources (ie. Oncorhynchus spp.; Esox spp. and Lepeophtheirus salmonis, respectively). An NCBI Blast search returned no matches with any sympatric species in the forward primer. However, the reverse primer was a match with one deletion on Chinook Salmon.
Coho Salmon assay was optimized for annealing temperature and validated for specificity against naturally co-occurring *Oncorhynchus* spp. Annealing temperature optimization was done by running 12 pre-fish tank negative control samples and 12 experimental samples (one fish/10kL) in PCR at annealing temperatures of 58, 60, and 62ºC. The optimum annealing temperature was determined by prioritizing specificity (non-reactivity with non-target DNA) over sensitivity (target DNA detection at low density), whereby a marginal loss of specificity at a given temperature would not justify even significant increases of sensitivity. The primer was tested for Coho specificity on tissue-extracted DNA from Chum (*O. keta*), Pink (*O. gorbuscha*), Sockeye (*O. nerka*), and Chinook salmon (*O. tshawytscha*), as well as Coastal Cutthroat Trout (*O. clarkii clarkii*) and Rainbow Trout (*O. mykiss*). At least two genomic DNA samples from all sympatric *Oncorhynchus* spp. were run in PCR with the Coho primer to rule out cross-reactivity. The Coho assay did cross-amplify on Chinook tissue, but practical experimental controls were deemed sufficient to exclude the possibility that Chinook DNA was entering the system and influencing results.

Extra measures were taken to rule out primer cross-reactivity with Cutthroat Trout, as that species was known to inhabit Goldstream River above the hatchery (the negative control sampling location) and was considered a possible contributor to false positives from the experimental site. Template DNA extractions were made on fin clips from three individuals and run in triplicate PCR, in a dilution series crossing four orders of magnitude (N=36).
Lab processing

Filter membranes were thawed and DNA extracted with MO-BIO PowerWater DNA isolation kits (MO-BIO Laboratories, Carlsbad, CA), following a modified manufacturer’s protocol for increased yield by applying heat treatments for a range of 1-24 hours instead of the standard 1 hour. Each sample was then run in PCR, with reagents and under conditions described in Table 5. To test for any effect of template dilution – indicating possible correlation between target DNA concentration and band intensity, or, conversely, environmental inhibitors – a modest range of template DNA dilutions was performed through the experiments. Dilutions were selected on an ad hoc basis, as was number of PCR replicates, and therefore varied among the experiments as follows: Pilot experiment: Duplicate PCR at 1µL of template DNA; Experiment 1: Single PCR at 1-7µL of template per reaction, 17 samples that scored negative were re-run in triplicate at 1µL per reaction; Experiment 2: Single PCR at 2µL per reaction, one treatment re-run at 4µl and 1µl template volumes; Experiment 3: All samples run in triplicate PCR with template DNA volumes of 4µl, 2µl, and 1µl, subset of samples had additional triplicate PCRs at 1µL template volume (N = 26 at 6 technical replicates, N = 3 at 9 technical replicates). Replication hierarchy and numbers for all experiments is described in Table 6. PCR controls included a positive control test on template DNA extracted directly from Coho Salmon tissue and triplicate no template controls (NTCs), to test the reaction components for contamination, with every PCR plate.

Reaction products were then visualized on agarose gel to determine presence or absence of amplified target DNA. Presence scores were determined by a visually
distinguishable band at target fragment length corresponding to the positive control.

Because samples with very low levels of target DNA can produce weak qualitative responses (i.e. faint band on agarose), the system for scoring gel images was such that even very faint bands were classified as positive. Observer bias in classification of true positives and false positives from negative controls was tested using a blind re-scoring of 20 samples. The test resulted in a perfect correlation between initial and repeat scoring suggesting that positive classification bias was minimal. Sequencing of PCR products was performed in the pilot stages to confirm Coho Salmon DNA in positive control samples. Subsequent samples collected through the experiments were not sequenced.

Table 5. PCR reaction components and conditions, performed on Techne TC-412 thermal cycler (Bibby Scientific, Staffordshire, UK).

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1DNA free H₂O</td>
<td>7.9 – 10.9</td>
</tr>
<tr>
<td>2GoTaq buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>3MgCl₂</td>
<td>1.6</td>
</tr>
<tr>
<td>4dNTPs</td>
<td>0.4</td>
</tr>
<tr>
<td>5forward primer</td>
<td>1.0</td>
</tr>
<tr>
<td>6reverse primer</td>
<td>1.0</td>
</tr>
<tr>
<td>7GoTaq® hot start polymerase</td>
<td>0.1</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0– 4.0</td>
</tr>
</tbody>
</table>

1Invitrogen, Carlsbad, CA. 2,3,4,7Promega, Madison, WI. 5,6IDT, Coralville, IA.

*Thermocycling profile:* initial denaturation 95°C for 2.5mins, 35 cycles at (denaturation 95°C for 30s, annealing 62°C for 30s, elongation 72°C for 30s), final elongation 72°C for 5mins, final hold 4°C

*Primer sequences:* Coho forward CGCTCTCTAGGGGATGATC; Coho reverse CTCCGATCATAATCGGCATG
Table 6. Type and numbers of replicates taken for all experiments. Two litre samples were taken directly from tanks (pre-fish negatives, baseline positives, and treatment samples), or from inflow source (concurrent negative controls). PCR replicates were run at varied template DNA volumes.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pre-fish Tank Negatives</th>
<th>Concurrent Negative Control Samples</th>
<th>Baseline Positive Control Samples</th>
<th>Samples Per Treatment</th>
<th>PCR Replicates Per Sample</th>
<th>Template DNA Volume (µL) in PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilot</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1 per tank</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1: 10kL 1-65 fish</td>
<td>4 per tank</td>
<td>21</td>
<td>NA</td>
<td>5 per tank</td>
<td>1 - 3</td>
<td>1, 4, or 7</td>
</tr>
<tr>
<td>2: 370L dilution</td>
<td>3 per tank</td>
<td>15</td>
<td>3 per tank</td>
<td>3 per tank</td>
<td>1 - 3</td>
<td>1, 2, and 4</td>
</tr>
<tr>
<td>3: 10kL dilution</td>
<td>3 per tank</td>
<td>5</td>
<td>4 per tank</td>
<td>3 per tank</td>
<td>3 - 9</td>
<td>1, 2, and 4</td>
</tr>
</tbody>
</table>
Statistical analysis

In the first and second experiments, high overall detection rates limited analysis to comparison of detection probability against the experimentally determined false positive rate, below. For the third experiment, binary response data were analyzed in R (RStudio Team 2015) using generalized linear mixed models (GLMMs) in the R package “lme4” and its “glmer” function (Bates et al. 2015). Repeated measures on individual sample bottles required inclusion of a sample bottle random effect. I also included a random effect of tank by dilution. Selecting sample bottle nested within tank by dilution random effect structure was based on several criteria: repeated measures on sample bottles had no crossover between tank and dilution, so nesting was appropriate. Tank alone had only 4 levels, which has inadequate replication to set as a random effect. However, tank by dilution (i.e., Tank 1-10kL,…Tank 2-20kL,…Tank 3-40kL,…Tank 4-80kL,…Tank 4-160kL) are also sample clusters, but with twenty levels. This allowed a model with a tank based random effect, while maintaining the primary fixed effect of dilution. Fixed effects of dilution level, filter type, and template DNA concentration in PCR were included in the candidate model set, with an interaction between dilution and template concentration. Suitability of other co-variate predictors (i.e. Wait time pre-filtering, freezer time post-filtering, heat treatment time in DNA isolation, process batch, and PCR plate) was explored, with respect to even blocking among treatments, prior to inclusion in any models. Co-linearity in several of these factors precluded their analysis. Model comparison and selection was performed with AIC based on steps outlined in Zuur et al.
The candidate model set was selected based on results from the field chapter, and intentionally applied treatments in the experiment.

A comparison of tank-level detection rate at different thresholds of evidence was also made by adapting a standard from Veldhoen et al. (2016). A high evidence threshold was set at a minimum of two positive detections among all bottles and replicates, increasing to three if 9 PCR replicates were performed (i.e., 2/3, 2/6, 3/9). If this threshold level of positive replication was met, detection would be scored in that tank. The low threshold was set at a single positive PCR replicate to score detection in the tank.

All pre-fish and concurrent negative controls (N = 83) were analyzed together to determine a false-positive detection rate for the method. Initially, all positive experimental samples were regarded as true positives, and a best model determined. Then, positive detection rate at each treatment level was tested for significance above the false detection rate by chi-squared test for significance, at $\alpha = 0.05$.

**Results**

In the first experiment, Coho Salmon eDNA was detected across all experimental densities from 0.6 - 38.0g/1000L. Detection probability was high through all treatment levels: 70-100%. Triplicate PCR increased detectability at all densities to at least 85%, an increase from the lowest detection rate of 70% observed at 0.6g - 2.2g/1000L with one PCR replicate (Table 7a, b). In the second experiment, all samples were positive for Coho Salmon DNA through the first three sampling periods, corresponding to tank volume dilutions of 50%, 75%, and 87.5%. At the final sampling period (99% dilution), detection
probability was 75% (Figure 4). At the 50% dilution sampling period, amplification failures occurred in three samples when 2µl template was used, and one sample when 4µl of template was used. Perfect detection occurred using 1µl of DNA template in PCR.

Table 7. eDNA detections of increasing number of juvenile Coho Salmon in 10,000L flow-through tanks with a) one PCR replicate per sample and b) triplicate PCRs. With triplicate PCR, one or more positive replicates count as sample level detection.

<table>
<thead>
<tr>
<th>No. of fish</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positives /20</td>
<td>14</td>
<td>15</td>
<td>14</td>
<td>18</td>
<td>15</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Positives (%)</td>
<td>70</td>
<td>75</td>
<td>70</td>
<td>90</td>
<td>75</td>
<td>100</td>
<td>85</td>
</tr>
</tbody>
</table>

b)

| Positives /20 | 17 | 17 | 17 | 19 | 18 | 20 | 20 |
| Positives (%) | 85 | 85 | 85 | 95 | 90 | 100 | 100 |
Figure 4. Agarose gel image of amplified Coho Salmon eDNA from experiment 2. Batches of 12 (a-d) represent 3 sample replicates from 4 experimental tanks at dilutions of 50% (a1-3), 75% (b), 87.5% (c), and 99% (d), after removal of fish. The 50% dilution samples were run again at lower (a2) and higher (a3) template DNA concentrations in PCR. The subsequent gel images are superimposed and a 1kb ladder is included with a3.

In the third experiment, Coho Salmon eDNA was detected in at least 75% of samples from densities of 0.6g – 2.4g/1000L. Detection rates in the third experiment were consistent with predictions of reduced detectability with increasing dilution level. At baseline density (2.4g/1000L), all samples scored positive. Detection probability fell incrementally through two dilutions by half (down to 0.6g/1000L), and a single PCR replicate scored positive at 0.3g/1000L (Figure 5). The top model included dilution, filter
type, and template concentration, with a model weight of 0.64. Detection probability was greater at higher fish densities (P<0.001) and at lower template concentrations (P<0.0001). Advantec filter membranes (Advantec MFS, Inc., Dublin, CA) were also associated with higher detection probability (P<0.05). Model weights and parameter estimates for the top model are in Tables 8 and 9.
Figure 5. Rate of Coho Salmon eDNA detection through experimentally manipulated fish densities (data points include number of fish per tank) in 10,000L rearing tanks at Goldstream Hatchery, near Victoria, British Columbia. Crosses denote proportion out of 20 samples taken while fish were in the tank; Xs denote proportion out of 12 samples taken after removing fish and diluting the signal in the tank. Logistic curve derived from base generalized linear model of detection \( \sim \) log fish density (g/m\(^3\)).
Table 8. Top model set for eDNA detection of Coho Salmon, with dilution (D), filter type (F), and template concentration (T) parameters.

<table>
<thead>
<tr>
<th>Model</th>
<th>Residual Df</th>
<th>Residual Deviance</th>
<th>ΔAIC</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>D + F + T</td>
<td>267</td>
<td>103.4</td>
<td>0.0</td>
<td>0.64</td>
</tr>
<tr>
<td>D + F + T + D*T</td>
<td>266</td>
<td>103.4</td>
<td>2.0</td>
<td>0.24</td>
</tr>
<tr>
<td>D + T</td>
<td>268</td>
<td>102.2</td>
<td>4.0</td>
<td>0.09</td>
</tr>
<tr>
<td>D + T + D*T</td>
<td>267</td>
<td>102.2</td>
<td>5.9</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 9. Parameter estimates for top model from Table 8.

| Estimate         | Standard Error | Z Value | Pr (>|Z|)   |
|------------------|----------------|---------|-------------|
| (intercept)      | 4.9            | 1.4     | 3.7         | 0.000253   |
| dilution         | -1.0           | 0.3     | -3.8        | 0.000162   |
| template         | -2.1           | 0.5     | -4.1        | 0.0000363  |
| filter (Advantec)| 2.1            | 1.0     | 2.2         | 0.0308     |

Detection probability is sensitive to the evidence threshold selected to determine tank level positive (Figure 6). Detections at the tank level in the first and second experiment were made 100% of the time at all densities and at both high and low thresholds. Experiment 3 densities of 2.4g and 1.2g per 1000L also had detections in all tanks at both high and low thresholds. At the high evidence threshold, detection rate fell to one tank out of four at 0.6g/1000L, and zero detection at lower densities. At the low evidence threshold, all tanks had detections at 0.6g/1000L and one tank had detection at 0.3g/1000L. No detections were made at 0.2g/1000L, and therefore samples taken at 0.02g/1000L were not processed and analyzed.
Figure 6. Rate of Coho Salmon eDNA detection through experimentally manipulated fish densities in four 10,000L rearing tanks at Goldstream Hatchery, near Victoria, British Columbia. Green represents a higher weight of evidence threshold to score a tank level detection, currently in place in industry in BC. Red represents a minimum weight of evidence threshold acceptable in research.
Coho Salmon assay specificity test against Cutthroat Trout resulted in zero amplification in all samples, dilutions, and replicates. Temperature optimization in PCR determined 62°C annealing temperature gave equal sensitivity (15-17 detections out of 20 samples), while limiting false positives to 1/20, versus up to 12/20 at 58 °C. In experimental and laboratory controls, neither the cooler blank nor process blank produced false detections (N = 2 samples x 2 PCRs each), nor did any PCR negative controls, indicating that equipment sterilization procedures were effective, and cross contamination in processing did not occur. Concurrent and pre-fish experimental negative controls produced six false detections out of 83 samples, making a 7% global false positive rate for the technique. A statistically significant difference (P < 0.0001) between this false detection rate and experimental detection rates was found at all treatment levels down to a density of 0.6g/1000L.

Discussion

In these experiments I demonstrate high detectability of juvenile Coho Salmon eDNA in hatchery rearing tanks, and loss of detection at a low density threshold achieved by dilution after removal of fish. In occupied tanks, perfect detection at the tank level and high detection at the sample level (85%) demonstrate that this technique is a powerful detection method for juvenile Coho Salmon, at densities below those typically observed in natural rearing streams (Grant and Kramer 1990, Rosenfeld et al. 2000). High tank level detection probability through extended dilutions after removing fish exceeded the predicted limit of detection. The threshold density below which detection became
unreliable was found at dilutions below 0.6g/1000L, equivalent to one five gram juvenile Coho Salmon in approximately 14.5m$^3$ of water.

Fish density emerged as a significant predictor of detection probability in all models. Inclusion of template concentration and filter type in the high-weighted models suggests processing variation remains an important consideration for collecting and analyzing eDNA samples. PCR inhibition from environmentally sourced compounds is commonplace in eDNA research (Schmidt et al. 2013, Deiner et al. 2015, Doi et al. 2015). Dilution of template as I performed is the simplest and most cost-effective method for reducing inhibition, though DNA purification kits can be more successful at inhibitor release, while maintaining qPCR accuracy and detection sensitivity (McKee et al. 2015). Increased detection probability at lower template concentrations indicates likely inhibition at the higher template concentrations in the third experiment. Similarly, template dilution on samples from the 50% dilution sampling period of experiment 2 scored perfect detection only at the lowest template concentration. This contradicts results from the Chapter 2 field study on eDNA detection of Coho Salmon (MacAdams, unpublished data), where template concentration interacted with environmental conditions (target species density and habitat characteristics), affecting detection probability. Based on these experiments, a guideline for future eDNA research would be to use a range of template concentrations in PCR. Also, filter membranes of different specifications should be tested for efficacy in different environments (Liang and Keeley 2013, Turner et al. 2014).
Zero detection in the no template controls (NTCs), as well as laboratory and cooler blanks, suggests that cross-contamination of samples and reagents was not a concern. However, a 7% false positive rate from experimental negative controls does raise a problematic issue in eDNA research (Darling and Mahon 2011, Kelly et al. 2014b). Although sound logical justifications can be made to include results with low-level contamination (Bylemans et al. 2017), confidence in detections is limited to those occurring at rates sufficiently higher than the false positive rate as to be distinguishable from background error. Robust statistical methods for incorporating known false-positive error have been performed (Ficetola et al. 2015, Lahoz-Monfort et al. 2016), but more rigorous primer design and validation process should be carried out before eDNA studies begin, to reduce or eliminate occurrence of false positives (Veldhoen et al. 2016).

Sequencing from PCR products for a subset of experimental samples would also serve to validate positive results, and should be carried out in future studies.

The hatchery sources its water from a natural salmon spawning river, making the experimental system of rearing tanks I employed a suitable analogue of natural Coho Salmon rearing habitat. Analyzing tank level detectability increased the sensitivity of my method to 100% at densities from 38.0 – 0.6g/1000L, and lowered the minimum fish density at which detections were made to 0.3g/1000L. Analyzing detectability at the tank/site level (instead of sample level) is more applicable in a wildlife management context, because site level presence or absence (e.g., stream, pool, lake) is the fundamental question of wildlife management and monitoring. Sample level detectability
and replication, though important as technical considerations, are less relevant in terms of establishing target species presence and distribution in a monitoring context.

Altering the threshold of evidence required for concluding site level presence is a consideration that will have management, and also legal implications, as endangered and invasive species are monitored with eDNA (Kelly et al. 2014b, Veldhoen et al. 2016). In this experiment, different interpretations of the same raw data led to true detections or false negatives. For example, applying the higher threshold of evidence led to detection failures in 3 of 4 tanks at 0.6g/1000L, and complete loss of signal at 0.3g/1000L. At the lower evidence threshold, tank level failures occurred at 0.3g/1000L, and signal loss at 0.2g/1000L. This trade-off between certainty and sensitivity by altering the evidence threshold poses a conundrum for regulatory and management agencies. Sampling for environmental assessment or endangered/invasive species monitoring often occurs at sites where managers are naïve to presence or absence of target species. Changing the evidence threshold, and hence the data interpretation, can lead to different conclusions about site presence. In this context, classifying sites as present or absent for target species could lead to regulatory designations for either protection or development. Setting appropriate thresholds of evidence will be dependent upon target species and assay performance at controlled sites, and must be determined by combining experimental trials and field testing with a priori knowledge of presence or absence. Methods for performing these trials from a statistically rigorous approach are described in Veldhoen et al. (2016), and should be undertaken before conducting eDNA monitoring at field sites for which no monitoring knowledge exists. With experimental tests as a guide, management priorities
and risk-tolerance for different types of error (i.e., false positives or missed detections) will dictate how wildlife resource managers will interpret results from this imperfect detection method.

This work establishes reliable eDNA detection of juvenile Coho Salmon at and below typically observed natural densities. I model a detection threshold through decreasing density, and compare detectability against an experimentally derived false positive error rate. With these experiments, I have performed necessary calibration of eDNA methods to demonstrate its efficacy in detection of juvenile Coho Salmon in natural environments. I made consistent detection at lower densities than previously shown in controlled experiments (Kelly et al. 2014a), with 85% reliability at density equivalent to one five gram juvenile Coho Salmon in 14.5m$^3$ of water. With more robust quantitative PCR methods -involving probe-based assays and up to 50 amplification cycles (Veldhoen et al. 2016), sensitivity of eDNA methods will increase further. Improved laboratory methods and quantitative experiments, with decreasing densities of target species and mixed species groups, will validate eDNA monitoring as a reliable method for determining distribution and estimating abundance of biologically sensitive species and commercially valuable resources.
Chapter 4
General Conclusion

Thesis Overview

Conducting this research was an exciting opportunity to apply a novel technique on a new study species and system. The exercise of methodological testing and refinement was a conceptual and practical challenge, making the successes all the more rewarding. The steps from sample collection to raw result are many with eDNA, and the time between them is long when the entire processing pipeline is undertaken by a single graduate student who is learning the molecular techniques of DNA isolation, PCR, and agarose imaging as the project proceeds. Many a quiet and private “huzzah!” was made with arms raised, sitting on a stool in front of a computer screen with several blurry white bars on it, contrasted to spackled black background: target species presence confirmed, matched to positive control! This was my experience in troubleshooting and proofing eDNA methods: technical work that is highly applied, and hugely satisfying for a tinkerer like myself. I admit to having said quite a number of shorter words as well.

The focus of the application brought me to an entirely different realm of research. It was not ‘target species’. It was Coho Salmon in coastal British Columbia, where their history of human use and stewardship extends back millennia, and their present status is both highly valued and threatened across their range (PFRCC 2004, DFO 2014, Price et al. 2017). I applied the technical process of eDNA in several streams near the Hakai Institute, where long term ecological monitoring projects seek to trace the relationships between ocean, land, and the living things that make both their home. Successfully
identifying species and family of fish in these streams with eDNA, verified by conventional capture methods, has opened the door to using eDNA for monitoring of wild Pacific salmon.

However, through the door of eDNA monitoring stands much variability and uncertainty. It is an emerging field of study that has made rapid advancement (Bohmann et al. 2014), but several limitations remain: quantifying limit of detection with respect to animal density (Roussel et al. 2015); modeling dispersion, settling, and degradation in moving water (Jerde et al. 2016, Shogren et al. 2017); and source and state of eDNA with respect to individual and population characteristics (Jerde et al. 2011, Barnes and Turner 2016). The latter might never be resolved with eDNA, and fish in hand will still be required to monitor population age and sex structure, and individual condition. The second is an area of active research that I address with observations from Chapter 2. The former is the focus of three quantitative experiments from my third thesis chapter. Four 10,000L rearing tanks and juvenile Coho Salmon at Howard English Fish Hatchery on Goldstream River near Victoria, British Columbia provided the experimental setup. Density manipulations and dilution series equivalent to a single 5 gram fish in 200m$^3$ of water were performed to answer these two questions: 1. What density of fish is necessary to make reliable eDNA detection for juvenile Coho Salmon? 2. How long does the eDNA signal persist after they have abandoned a localized habitat?

**Important Findings**

The field validation performed near the Hakai Institute demonstrated the efficacy of eDNA for detecting juvenile Coho Salmon and resident salmonids in small coastal
streams. Habitat variation and fish communities were characterized with standard assessment methods and conventional trapping in five streams. Despite variability in habitat, hydrology, and water chemistry, eDNA detections were consistent with known fish species in these streams. Crucially, no Coho Salmon DNA was detected in the negative control system, where sampling occurred above a waterfall barrier impassable by anadromous fish. Observed variation in detectability among streams was found to correlate with environmental characteristics such as substrate, discharge, and target species density, and environmental inhibition of PCR was hypothesized as a primary mechanism causing this variation. In multiple cases where no conventional fish detections were made, eDNA detections occurred at rates significantly higher than a false positive rate derived in experiments.

In addition to a low false positive rate based on extensive control sampling, results from the hatchery experiments indicate that eDNA detection of juvenile Coho Salmon is consistent and reliable at and below naturally observed densities in streams. The power of the method exceeded expectations expressed in the first two experimental designs: detection was 75-100% through all treatments from 38.0 – 0.6g biomass per 1000L. A long dilution series in the tank, after removing fish, pushed the minimum density for detection to less than one quarter of the previous experimental benchmark (Kelly et al. 2014a) – 0.3 grams of biomass in 1000L of water. This detection was made in a 10,000L tank after removing three caged fish (~28g of biomass) and flushing through an additional 70,000L. In this smooth walled fiberglass tank, juvenile Coho Salmon DNA remains in detectable quantities after the tank’s volume is diluted by a factor of 8. What
precisely this means about field detection after out-migration in natural habitats, with porous substrate, and variable mixing and discharge, is to be determined.

From both research sites, I found that optimum processing parameters (i.e., template dilution) can be dependent on sampling condition. From field samples, manipulating template DNA concentration in PCR by a comparatively small factor of four paid dividends in increased sensitivity, and in a novel result. Without that small dilution series, many detections would have been missed in the field study. I found a natural example to corroborate results from Takahara et al. (2014), where template dilution increases detectability with high volume samples. I also found a unique case where target species abundance correlates with inhibition of PCR. This result is, to my knowledge, as yet unreported in eDNA research, and supported with a plausible mechanism for inhibition via animal waste products (Kreader 1996, Al-Soud and Rådström 2001). Template dilution is a technical consideration that can be addressed with minor variations in processing and targeted replication, and based on my findings, should be employed as the minimum test of PCR inhibition. More robust inhibition tests and prevention are also common practice in eDNA research (McKee et al. 2015, Piggott 2016).

From a conceptual standpoint, I found that altering the threshold weight of evidence will change conclusions of site level presence or absence. In the experimental tank, these come out as false negatives occurring below the limit of detection. That limit varies with the threshold selected; if you want certainty, you lose sensitivity, and vice versa. In the field, however, the compromise of certainty and sensitivity must be made
based on informed interpretation and management priority, mindful of potential for legally required management action based on that interpretation.

**General Implications**

Before speaking to greater implications and future directions of this research, some weaknesses must be addressed. I did fall short in two important ways on the lab processing side. Firstly, my PCR assay design and testing were not up to research standards. I used an “off-the-shelf” assay for detecting Coho Salmon, taken from a study identifying salmon species in commercially available seafood (Rasmussen-Hellberg et al. 2010). My limited assay proofing efforts, which were inadequate for modern microbiological and eDNA study, determined that this primer had cross-reactivity with Chinook Salmon. Cross-amplification in PCR with a congeneric species and false positives in experiment would likely be avoided with greater efforts in this area. That is not to say that the original researchers used an invalid assay, because probe-based quantitative PCR was also employed by Rasmussen-Hellberg et al. (2010). The use of a probe in PCR adds extra assurance of specificity because it requires a third gene region near the centre of the target fragment to match, as well as both primers, for amplification to be successfully recorded (VanGuilder et al. 2008).

I also used conventional PCR and imaging on agarose gel, which are out of date and strictly qualitative compared to modern quantitative and droplet digital PCR (qPCR and ddPCR) (Doi et al. 2015), which allow estimates of starting DNA concentration. A quantitative measure of DNA concentration is essential for moving eDNA from a binary indicator of presence to a continuous measure of abundance (Lodge et al. 2012).
Quantitative PCR and probe-based assay design are standard practice today that I did not employ. Several other best practices of eDNA research are described in Veldhoen et al. (2016), and should be applied as standard in future studies.

In spite of these weaknesses, the methods performed far better than I imagined possible at the outset. True signal of target species detection greatly exceeds the noise of background variability at experimental densities below those observed in natural systems, and below those seen in all but one previous eDNA experiment. I adapted current filtration and processing techniques to increase sampled water volume, and number of samples that an individual can process in a work day. Many filtration setups are limited in the volume of water they can process, as well as the number of samples they can filter concurrently (Thomsen et al. 2012b, Wilcox et al. 2015). Though not typically reported, and lacking evidence to the contrary, I submit that my processing day at Goldstream Hatchery of sixty-three 2L samples in a 24 hour period is an eDNA filtration record for one technician. This was made possible by modifying the protocol of (Walsh et al. 2009) to filter four samples at once. Improvements on previous methodologies, combined with strong proofing in field trial and experimental test, have made eDNA detection of juvenile and resident salmonids in coastal streams a viable tool for local and regional monitoring of Pacific salmon.

**Future Directions**

The groundwork laid in this thesis presents environmental DNA as a ready-to-use tool for monitoring salmonid fish in freshwater. Approximately 2500 coastal streams line the British Columbia coast. Less than 1% of them are assessed for salmon returns annually
by Federal Agencies, while over 2000 are no longer or never have been (Price et al. 2008, 2017). Increasing costs and funding shortfalls are responsible. This thesis presents eDNA methods for Pacific salmon and British Columbia at a crucial time in the history of their management by humans. The largest part of that historical management, both geographically and temporally, has been carried out by coastal First Nations communities (Jones et al. 2010, Dale and Natcher 2015). The expansion of destructive colonial policies severed the mutually sustaining relationship between traditional stewards and natural resources. In recent years, great efforts have been made by many of these First Nations to reclaim their position as stewards and managers for sustainable resource use (Adams et al. 2014). eDNA can be used equally by these local First Nations managers as by regional-scale government managers to increase monitoring capacity. Cost and time savings of eDNA over conventional methods will enable managers to increase both volume of data collected, and local detail. Current and future collaborations with industry, and Federal, Provincial, and First Nations’ governments will put this new tool in the hands of the people who both need it most, and are best positioned to use it to greatest conservation effect.
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