Optimizing eDNA methods to assess marine ecosystem health

By

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B.Sc., University of British Columbia, 2016

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We acknowledge and respect the Lək̓ʷəŋən (Songhees and Xʷsepsəm/Esquimalt) Peoples on whose territory the university stands, and the Lək̓ʷəŋən and W̱SÁNEĆ Peoples whose historical relationships with the land continue to this day. I thank the the Kitasoo/X'ais x'ais nation for their support and partnership.

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Abstract

The world is losing biodiversity at an unprecedented rate due to climate change and anthropogenic development. The global implications of these losses are catastrophic, and drastic improvements are needed in conservation and biomonitoring methods to track these changes. My PhD research focuses on optimizing environmental DNA (eDNA) methods as a scalable biomonitoring method. eDNA, which refers to genetic material that can be extracted from environmental samples, can provide insight into species occupancy, composition, and abundance. To perform this research, we developed many sensitive and robust qPCR assays using a whole mitogenome approach and a rigorous validation pipeline. The thesis explores the application of eDNA methods to address different conservation challenges in four marine ecosystems. First, we examined the distribution of the Olympia oyster (*Ostrea lurida*) in Barkley Sound, BC, employing a targeted eDNA assay and exploring how tidal and freshwater dynamics affect DNA signal strength. Second, we evaluated DNA extraction methods for detecting sea otter (*Enhydra lutris*) DNA in sediments, contributing to the understanding of species distribution and improving protocols for degraded marine mammal DNA detection. Third, we investigated the impacts of marine pollution in the Pacific Northwest by developing sensitive eDNA assays for indicator species, correlating their abundance with ecological responses to organic enrichment. Finally, we compared eDNA surveys to SCUBA diving for surveying rockfish species, demonstrating that eDNA can capture greater species richness while addressing challenges in species identification amongst a large closely related genus.

Overall, this research illustrates the potential of eDNA as an efficient tool for marine conservation, highlighting its application across different environments and conservation contexts. The findings provide critical insights for monitoring marine biodiversity and inform management strategies to protect vulnerable species and ecosystems.

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Thesis Format and Manuscript Claims

The thesis is presented in a manuscript format. Chapters 2, 3, 4 and 5 are written in a manuscript style containing an Abstract, Introduction, Methods, Results, Discussion and Conclusions. Chapter 1 provides background information and introduces the overall theme of the thesis. Chapter 6 summarizes and concludes the major findings of the work and provides suggestions for future directions. Both Chapter 1 and Chapter 6 include text from l previously published book chapter placing eDNA in the context of the rapidly growing number of genomics applications.

Chapter 1/6: **Neha Acharya-Patel,** Michael J. Allison, Caren C. Helbing. 2021. Environmental DNA: Revolutionizing ecological assessments with genomics. *Genomics and the Global Bioeconomy* (ed Adriana Suarez-Gonzales, Catalina Lopez-Correa) **Ch 5**, 103-117, Elsevier. Neha Acharya-Patel and Michael Allison wrote the chapter with the supervision of Caren Helbing.

Chapter 2: **Neha Acharya-Patel,** Sarah Dudas Emma T. Groenwold, Hajeong Lee, Michelle Bigg, and Caren C. Helbing. Using Environmental DNA (eDNA) to assess Olympia Oyster distribution in Barkley Sound, BC. This manuscript is in preparation for submission to the Journal of Shellfish Research. Neha Acharya-Patel, Sarah Dudas, and Michelle Bigg collected and processed samples from the field. Neha Acharya-Patel, Emma Groenwold and Hajeong Lee did all the lab sample processing. Neha Acharya-Patel ran all statistical analyses. Neha Acharya-Patel wrote the manuscript with the participation of the co-authors.

Chapter 3: **Neha Acharya-Patel,** Mark Louie D. Lopez, Matthew T. Bonderud, Hajeong Lee, Emma T. Groenwold, Erin U. Foster, Linda M. Nichol, and Caren C. Helbing. Comparison of DNA extraction methods for detecting the sea otter (Enhydra lutris) in marine sediment. This manuscript has been submitted to the eDNA Journal. Neha Acharya-Patel, Mark Louie D. Lopez, and Matthew T. Bonderud lead study design. Neha Acharya-Patel ran all statistical analyses. Neha Acharya-Patel wrote the manuscript with the participation of the other co-authors.

Chapter 4: **Neha Acharya-Patel,** Karen Cram, Emma T. Groenwold, Hajeong Lee, Abigail G. Keller, Breanna Bomback, Shirley Lyons, Rene L. Warren, Lauren Coombe, Chris Lowe, Lauren C. Bergman, Farida Bishay, Inanc Birol, Tara A. Macdonald and Caren C. Helbing. Monitoring marine pollution effects through targeted environmental DNA (eDNA) testing in the Pacific Northwest. This manuscript has been accepted for publication by thes Marine Pollution Bulletin. Neha Acharya-Patel, Emma Groenwold, and Hajeong Lee did all the lab sample processing. Neha Acharya-Patel, Breanna Bomback and Abigail G. Keller ran all the statistical analysis. Neha Acharya-Patel wrote the manuscript with the participation of the co-authors.

Chapter 5: **Neha Acharya-Patel,** Emma T. Groenwold, Matthew Lemay, Rute Clemente-Carvalho, Evan Morien, Sarah Dudas, Emily Rubidge, Cecilia Yang, Lauren Coombe, René Warren, Alejandro Frid, Inanc Birol, and Caren C. Helbing. 2024. Comparison of environmental DNA and SCUBA diving methods to survey keystone rockfish species on the Central Coast of British Columbia, Canada. *Ecological Indicators*. **160.** 111830.

10.1016/j.ecolind.2024.111830. Neha Acharya-Patel lead conceptualization and study design. Neha Acharya-Patel collected and processed all field samples. Neha Acharya-Patel Emma Groenwold, and Rute Carvalho did all the lab sample processing. Neha Acharya-Patel, Matt Lemay and Evan Morien did the Bioinformatic analysis for metabarcoding data. Neha Acharya-Patel wrote the manuscript with the participation of co-authors.

Chapter 1: Introduction

1.1 Biodiversity Loss and Global Biomonitoring Needs

It is well known that the world is losing biodiversity at an unprecedented rate due to climate change and anthropogenic development [1,2]. The global implications of these losses are catastrophic and drastic improvements are needed in conservation and biomonitoring methods to track these changes [3]. Environmental DNA (eDNA) shows potential as a powerful monitoring tool. Referring to genetic material shed by organisms into their environment, eDNA can be isolated from either micro- or macroorganisms (Figure 1). For years, microbiologists have been extracting and reading the DNA of microorganisms such as bacteria and viruses from environmental samples, but this technology has been applied to macroscopic organisms (e.g., animals and plants) for just over a decade [4] (Figure 1).

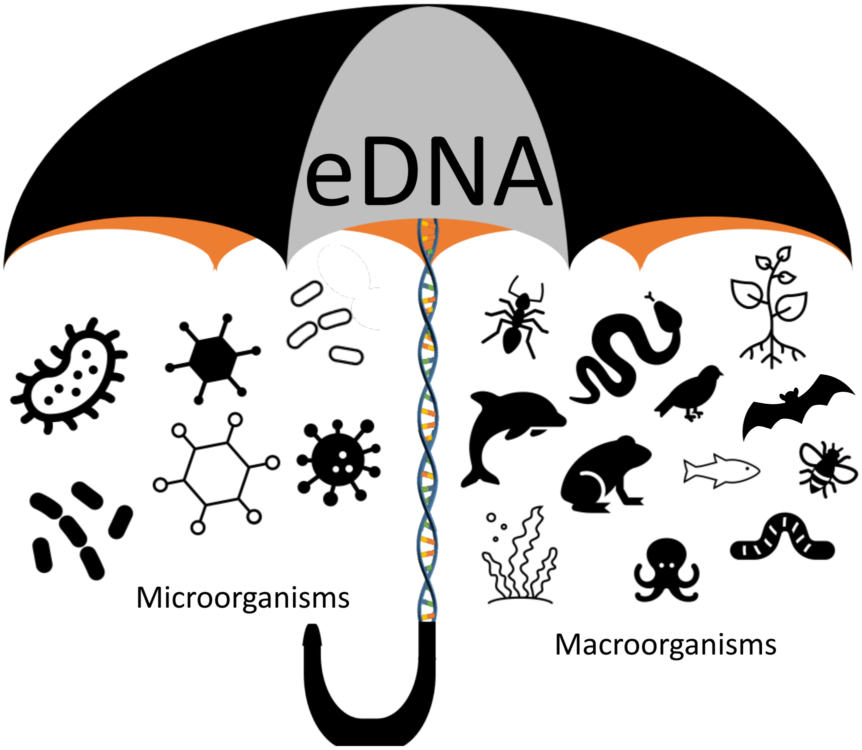


Figure 1. eDNA is an "umbrella" term that includes DNA isolated from microorganisms in environmental samples or DNA shed from macroorganisms that is released into the environment.

Through natural processes like shedding skin, excreting waste, or through reproductive fluids, organismal DNA is constantly being released into the environment. Extracting eDNA from easily obtained environmental samples has opened the door to asking myriad biological and ecological questions regarding species presence, richness, distribution, and abundance.

As advancements in environmental genomics continue to progress rapidly, eDNA tools are becoming increasingly powerful and applicable. In environments that are difficult to sample, or in finding elusive or cryptic species, the utility of eDNA is apparent. Additionally, eDNA sampling can often be more cost- and resource-effective in comparison with traditional methods, while offering greater species identification power [5,6]. Because of this, eDNA sampling is slowly being integrated into large-scale biomonitoring programs globally. As the sampling protocols, data sharing practices, and results become more standardized, eDNA data will also be useful for informing policy and management decisions.

1.2 eDNA Approaches

Currently, three broad approaches are applied to detect eDNA and infer species distribution: targeted analysis, and two community-based approaches—metabarcoding and metagenomics (Figure 2). A summary of the advantages and disadvantages of each method is presented in Table 1. Global applications of these methods are growing and addressing a broad range of biological questions. The present chapter provides an overview of the current state of the field, its opportunities and challenges, and projected outlook.

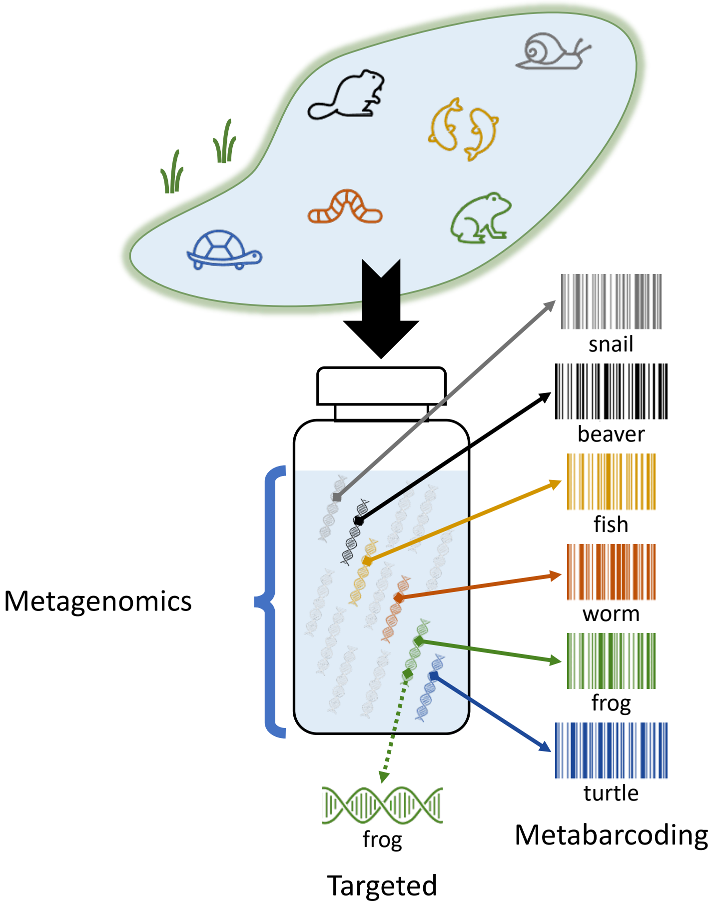


Figure 2. Depiction of three common approaches to the analysis of eDNA from environmental samples. Targeted approaches are derived from previous knowledge regarding specificity of a DNA fragment to identify the presence of the target species within a complex mixture. Metabarcoding uses "universal" primers to amplify specific regions of an organism's genome and then determine the genetic sequence of that region as a "barcode". Metagenomics involves sequencing and assembling the much larger portions of genomes or entire genomes found in an environmental sample.

1.2.1 Targeted eDNA Analysis

Targeted studies focus on questions revolving around certain target species. These types of studies use species-specific primers and probes that have been specially designed to detect gene sequences unique to the target species from environmental samples [7]. Targeted eDNA tests are powerful tools to find rare and elusive species and are relatively inexpensive to perform and interpret. For example, swabbing eDNA from artificial cover objects can be used to detect the elusive, endangered sharp-tailed snake [8]. The most common targeted eDNA analytical method uses quantitative real-time polymerase chain reaction (qPCR), which is an established, accessible, and robust technology when properly validated[7].

Due to their sensitivity, targeted eDNA tests have also begun to be used to quantify the amount of target species DNA found in the environmental sample that can subsequently estimate relative organismal abundance[9,10]. Statistical methods for quantifying copy number in low eDNA concentration samples using qPCR [11] and digital PCR (dPCR) methods [12,13] facilitate this application. Targeted approaches excel in sensitivity and quantitation, with the disadvantages that they require prior knowledge of DNA sequence information and will miss unexpected species presence.

1.2.2 Metabarcoding

In contrast, metabarcoding focuses on characterizing a community from an environmental sample by using “universal” primer sets that can amplify across species to examine a specific region of DNA that leads to the reading of its sequence or “barcode.” This method uses high-throughput sequencing techniques and bioinformatics analysis. The amplified barcode genes from bulk environmental samples are then sequenced and the resulting DNA sequences are then matched to the known genetic sequences of organisms present in DNA sequence databases. This method is highly useful[14-17] when the gene sequences of most organisms in the ecosystem of interest are present in DNA sequence databases. Metabarcoding can be used to simultaneously determine species presence and richness while enabling the detection of novel species.

Table 1. Summary of the advantages and disadvantages of the three main eDNA approaches.

Method	Advantages	Disadvantages
Targeted Analysis	• Reliably detects very low eDNA concentrations • Can estimate DNA particle abundance in the environment • Relatively inexpensive • Uses accessible technologies	• Relies on available sequence information for eDNA assay design • Detects a limited number of species at a time • High false-positive detection rates
Metabarcoding	• Detects many species at once • Assesses community biodiversity • Detects new species	• Relies on reference sequence availability for barcode identification • Is amplification biased and therefore cannot currently estimate abundance • High false-negative detection rates
Metagenomics	• Elucidates differences within species to understand population • Detects new species • Can assess biological state or other transcriptomic responses	• Relies on next-generation sequencing and extensive bioinformatics • Expensive and inaccessible to many

For example, a 2021 study used universal vertebrate primers to assess vertebrate diversity in the rivers of northern Colombia. They detected over 200 native taxa in multiple different phyla including endangered species like the Antillean manatee [16]. Despite its power, metabarcoding is prone to amplification bias causing high false-negative detection rates. This also broadly limits metabarcoding to species richness questions as abundance data may not be accurately and reliably inferred though there is work attempting to account for these limitations[18]. Furthermore, confident species identification relies on published genetic sequence information and the uniqueness of the barcode. Today, most organisms do not have published sequence information to allow for identification and most metabarcoding studies can only confidently characterize communities to the genus or family level. The ability to survey entire groups of species from a sample is extremely powerful but requires intensive verification of sequence databases and survey results to avoid misleading conclusions.

1.2.3 Metagenomics

The term “metagenomics” refers to the study of the collective genetic material from many organisms contained in each environment. Like metabarcoding, metagenomics methods use high-throughput sequencing and bioinformatics. However, this approach is more demanding as it aims to assemble large genome segments or whole genomes. Multiple recent studies have demonstrated the emerging utility of this approach in addressing complex population-level questions by identifying multiple mitochondrial haplotypes of the same species from environmental samples[19-25]. One example is a laboratory-based study that compared the allelic frequencies of the round goby detected from a water sample from a mesocosm to those detected from tissues of the animals within that mesocosm. Their results showed that intraspecific nuclear gene information could reliably reflect population differences [26]. As with metabarcoding, however, elevated scrutiny of the genetic resources is needed to mitigate erroneous results. With that said, improvements in long-read sequencing are expanding the possibilities for this type of work[27].

1.3 Challenges and Opportunities

Due to the numerous advantages offered by eDNA methods compared to conventional survey approaches, eDNA has been used in a wide range of applications including environmental assessment and monitoring, ecological recovery, species inventories in challenging environments, and conservation and resource management. While these applications and methodologies are broadening and progressing at a breakneck speed, specific challenges must be addressed to facilitate widespread acceptance and uptake to fully embrace their potential. Review of these challenges highlights the need for collection of comprehensive metadata alongside eDNA samples, the integration of ecosystem specific hydrological models into eDNA analysis and eDNA study design, careful selection of methodological approaches, data analyses methods that account for error, and other recommendations so that eDNA results can be assessed in a robust, comparative, meaningful way by experts and amateurs alike [28-31]. Below we present selected examples from around the world illustrating proof of concept for broad eDNA applications and expand on the challenges faced in the field that provide context for the four marine scenarios explored in this thesis.

1.3.1 Environmental Factors Influencing eDNA Detection

Overarchingly, there is a need to better understand how environmental factors, both biotic and abiotic, affect eDNA sources, dispersion, and degradation in differing environments. Abiotic factors such as salinity, acidity, temperature and UV exposure, and biotic factors such as microbial activity, or source animal metabolism or size can affect how much eDNA is shed from source organisms and how long eDNA can persist in the environment before degrading into undetectable components. Additionally, factors like wind, and rainfall can all affect how far and how fast eDNA spreads from its source. Though there have been many mesocosm studies and some *in situ* studies done to address these factors, overwhelmingly the extent to which each of these factors affects eDNA is ecosystem-dependent [29,32-44]. These factors influence the ability to link eDNA data to other pertinent factors like species abundance or range expansion. Marine ecosystems are particularly difficult to study due to the confluence of many abiotic factors, including those that are marine specific like tides, upwellings and currents. Nearshore environments are highly impacted by anthropogenic activities and are vital habitat for many marine species that we rely on. For example, the Olympia oyster is a native oyster species to British Columbia that is listed as a species at risk by the Canadian Species at Risk act. They are great candidates for eDNA monitoring because they are patchily distributed meaning, they can be difficult to locate, and their current stock assessments used by Fisheries and Oceans Canada are time and resource intensive[45]. However, as they are intertidal organisms, their eDNA signatures are likely impacted by the many abiotic factors that affect the intertidal zone. As the first marine scenario explored within this thesis, we used a targeted qPCR assay for the Olympia oyster to investigate the utility of eDNA monitoring for species detection in the context of multiple oceanographic effects.

1.3.2 Ecological Recovery

Early detection of organisms is vital for prediction and mitigation of change. eDNA has proven its worth as an early detection tool in many different contexts. For example, targeted eDNA analysis of water in Norway resulted in detection of *Batrachochytrium* *dendrobatidis* infecting native amphibian species, providing the first known occurrence of the pervasive pathogen in the country [46]. The utility of monitoring insect presence on forest plants using eDNA can be easily applied to a multitude of ecosystem recovery and protection scenarios, potentially providing an early warning against invasive species, and reducing the severity of pest outbreaks [47]. The potential for eDNA approaches for pandemic monitoring has been recognized and applied around the world [48,49]. For example, the SARS-CoV-2 virus can be detected in wastewater from cities providing advanced warning of surges in infection rate [50-52].

Similarly, these methods can be applied to determine the range expansions of invasive species or of the recovery of endangered and threatened species which has direct applications for informing conservation and remediation efforts [53-55]. In a targeted study of the endangered Mediterranean monk seal, eDNA detections showed the expansion of the known range of this species, informing scientists of the efficacy of their conservation efforts[56]. The effectiveness of a metagenomics approach for indicator species monitoring was demonstrated in a study that tracked the change in fungal communities over a ten year period of Australian woodland recovery after clearing and extensive use as grazing pasture [57]. As the woodland regained its native plant composition, the eDNA analysis of soil from the area reflected a shift toward native fungal diversity. In addition, metagenomic analysis of soil composition can elucidate the history of plant growth in fields [58], soil health [59], and impact on surrounding areas [60].

In British Columbia, the sea otter was hunted to extinction in the early 1900s. Since their reintroduction to the northern tip of the province in 1997, they have slowly been repopulating the coast and moving southwards[61,62]. As a patchily distributed marine mammal, using eDNA traces remaining in marine sediments to track their distribution is an exciting prospect. For this second marine scenario presented in this thesis, we developed a targeted eDNA assay and tested a series of sea water and sediment samples with varying levels of sea otter occupancy. We also performed a methodological extraction experiment to elucidate the best DNA extraction methods for highly inhibited marine sediments.

1.3.3 Environmental Assessment and Monitoring

In many countries when a proposed project or development is being considered, an environmental impact assessment that can quantify its ramifications on the surrounding environment and ecosystem must be completed. For example, extensive evaluation of the impacts on seabed ecosystems were required before an environmental license was granted to a sea floor sulphide deposit mining project in New Zealand [63]. These surveys are usually highly multidisciplinary, considering inter-related socio-economic, cultural, ecological, and human-health impacts, both beneficial and adverse. eDNA methods can be used in many ways to help achieve this: from detecting protected species triggering changes in development plans to identifying key habitats needing protection. For instance, the deep sea is under increasing anthropogenic stress as interest in deep sea mining grows. Being extremely inaccessible, characterizing what factors are important in maintaining biodiversity in the deep sea is very difficult. In one metabarcoding study, researchers collected eDNA using remotely operated vehicles (ROVs) in deep sea plains and sea mounts near proposed mining areas. They found that, though sea mounts were very important in terms of species endemism and richness as expected, the vast deep-sea plains in between them also held equally distinct communities dominated by nematodes, arthropods, molluscs, and cnidaria [64,65]. This study clearly demonstrates the utility of eDNA as a biomonitoring tool, an environmental impact assessment tool, and a tool to reach these highly inaccessible environments.

eDNA methods can also prove highly useful in increasing the efficiency of long-term site monitoring, which can be achieved using multiple approaches. Ecosystem health is commonly determined by monitoring the presence and abundance of indicator species whose life history is closely associated with healthy habitat functioning [66]. In Vancouver British Columbia, the Iona Island Wastewater Treatment plant treats sewage, stormwater and other metropolitan waste before it is released into the environment through the Iona Outfall. As part of the management plan for this infrastructure, there is a well-established monitoring program that has been in place for decades[67]. As an outcome of these years of research, certain benthic organisms – particularly polychaetes – can serve as indicator taxa, with specific taxa showing predictable responses to pollution. As the third marine scenario of this this thesis, we targeted these taxa with taxa specific eDNA assays to infer pollution impacts without having to physically find the organism itself. Using eDNA approaches for these broader purposes can increase the scope of monitoring efforts with minimal additional effort.

1.3.4 Species Inventories in Challenging Marine Environments

Conventional monitoring for aquatic stock assessment and management are frequently conducted using highly invasive catch methods [68,69] or hydroacoustic methods that lack resolution in species identification [70]. Application of eDNA tools presents an excellent opportunity as both a standalone and complementary detection methods [71-73].

For example, a metabarcoding study employing several universal fish primer sets assessing pelagic species diversity at multiple ocean water column depths, demonstrated that strong assays were able to detect fish up to at least 2,500 m below the ocean surface [72]. Another challenge in researching pelagic or transient fauna in the open ocean is their elusiveness. It is very difficult to define distinct populations and even more difficult to establish their connectivity. A 2016 metagenomic eDNA study of a whale shark aggregation in the Arabian Gulf enabled researchers to elucidate population-level information, providing a snapshot of population diversity not possible with traditional biopsy methods [30]. This information was invaluable to whale shark researchers as it indicated that populations were more interconnected than previously thought, and it was invaluable to eDNA researchers as it highlighted the benefits of designing studies to take advantage of natural aggregation events.

In British Columbia, rockfish of the genus *Sebastes* are an economically, ecologically and culturally important group. They have been used as food fish by First Nations on the coast for millennia, and they are a flagship species, meaning that their protection confers protection to associated habitats and species [74-76]. Routine monitoring of these important fish currently either occurs through long-line or SCUBA diving surveys. These methods, though informative, are invasive and cause high rates of mortality, or are depth limited and resource intensive requiring highly trained divers. eDNA monitoring provides a promising alternative as a minimally invasive, and scalable monitoring method that does not require species expertise and can test water samples taken from any depth. Within the fourth marine scenario of this thesis, we used a combination of targeted qPCR assays and metabarcoding to assess rockfish biodiversity in comparison to SCUBA diving surveys in a complex rocky reef ecosystem.

1.3.5 Conservation and Resource Management

An overarching aim of biomonitoring research is to provide compelling evidence to inform policy and management decisions for the conservation of species or of key habitats. eDNA has proven itself to be a very valuable tool that can fill in the gaps left by other biomonitoring techniques. For instance, a metabarcoding study aiming to quantify shark biodiversity in areas of differing anthropogenic impact neatly demonstrates that eDNA could detect more species than traditional underwater visual censuses or baited cameras. Additionally, they showed that decreased anthropogenic influence (either because of low human population in the area or because of mandated protection) corresponded with higher biodiversity [6,77]. This evidence is can be used to make decisions regarding monitoring protocols and the delineation of protected areas.

Another group based in Alaska took eDNA samples from close proximity to the harbour porpoise - an apex species whose healthy presence is a critical indicator for the health of the entire ecosystem. This study elucidated population-level data from the eDNA samples that demonstrated far more genetic differentiation in the population than was previously thought. This suggests that it may be better to manage the harbour porpoise population as two distinct populations rather than one. This type of indirect stock assessment has the potential to revolutionize population evaluation and subsequent management for inaccessible marine taxa [22].

In studies that seek to describe species range, the ability to detect threatened and cryptic species presence without visual observation or habitat destruction is extremely desirable for limiting survey invasiveness and reducing the requirement for sampling permits [22,78,79]. In British Columbia, Canada, targeted qPCR approaches have been successfully applied to threatened mountain stream-dwelling amphibians, whose conventional surveillance usually requires intensive time-constrained searches by expert biologists to properly identify the species [78,80]. In California, population recovery of white sharks was effectively monitored using targeted dPCR assays [81]. This approach provided a non-intrusive solution to the difficulty of monitoring a rare, protected marine species and demonstrated the effectiveness of eDNA in an open ocean environment.

Despite all this evidence, eDNA data has so far only been used in a handful of conservation decisions. This is largely due to the fact that eDNA methods are still relatively new, and that managers still lack trust in the data [82]. At this stage data pairing from different methodologies is the most promising way to overcome this barrier, and for eDNA to be reliably integrated into global monitoring programs. However, while ground-truthing with conventional methods is desired for independent use of eDNA methods, it is important to acknowledge the limitations of such comparisons particularly with respect to their relative strengths and weaknesses[83]. Undoubtedly, eDNA has its advantages over other methods in its ability to detect the genetic presence of organisms that would have been missed by methods which require physical presence of the organisms. However, eDNA has many limitations including difficulties in estimating organismal abundance. Many studies have shown varied evidence supporting the potential of quantitative data estimates from eDNA methods but variation around this positive correlation may be attributed to varying biotic and abiotic factors [84], poorly performing eDNA tests [85], or lack of standardized performance criteria [80]. Currently, the majority of eDNA studies focused on abundance compare eDNA results with known biomass data collected with conventional methods like beach seining, bottom trawls, electrofishing, or camera trapping [86]. These comparisons are necessary at this stage as the factors that impact strong correlations between eDNA surveys and biomass estimates determined by other survey methods are still being elucidated. Variation in technical methodologies – field and technical replication, sample volume, filtration method, extraction method, eDNA assay specificity and sensitivity – will also impact these correlations and the overall quality of eDNA research. This variation highlights the need for systematic experimentation to determine best practices [78,87-89]. Within this thesis we compare eDNA monitoring to multiple conventional methods including sediment sampling with subsequent microscopic species identification and enumeration for pollution indicator invertebrates, and SCUBA diving surveys for rockfish. We attempt to correlate eDNA data with traditionally estimated organismal abundance estimation with varied levels of success. We also systematically investigate field eDNA sampling protocols and DNA extraction methods and explore different analyses to increase trust and ease interpretation of eDNA data in the marine environment.

1.4 Thesis Overview and Objectives

As outlined above, there is vast potential in the use of eDNA methods, limited only by creativity and the necessity of systematic experimentation and experimental rigor. As new applications are explored, careful attention must be paid to each aspect of the methodological pipeline and collaboration encouraged between experts to maintain the highest quality of work and data interpretation. With these principles in mind this thesis had two overarching objectives:

1. *Develop powerful eDNA assays to reliably detect low quantities of DNA from species of interest in environmental samples*.

This objective was achievable due to the Helbing Lab's expertise for qPCR assay design and validation. We took a whole mitochondrial approach to expand the amount of genetic sequence information available from which we could design assays with good molecular characteristics[7]. Then, to identify areas within these entire mitochondrial genome that were unique to our target species - and thus good to target for assay design – we used a bioinformatic pipeline developed for this purpose [90]. The combination of these strategies, in addition to a robust validation pipeline resulted in highly sensitive and specific targeted eDNA assays that could be applied to environmental samples.

2. *Use these assays to assess ecosystem specific challenges using four marine scenarios*.

We applied the assays we developed to address challenges within four distinct marine scenarios outlined above. Due to the vast diversity of ecosystems in the oceans, we wanted to apply similar techniques to a variety of situations that demonstrated the vast and varied ways that eDNA methods can be used to assess marine environments. These four scenarios each form the data chapters that make up this thesis. Within each scenario we used different interpretation approaches and focused on different challenges that are highly relevant in the eDNA field.

Chapter 2: Using Environmental DNA (eDNA) to Assess Olympia Oyster Distribution in Barkley Sound, BC

Abstract

The Olympia oyster (*Ostrea lurida*) is native to British Columbia but faces threats from invasive species, environmental stressors, and habitat degradation. Although its population no longer faces commercial harvesting pressure, it remains listed as a species of 'Special Concern’, under the Canadian species at risk act. The oyster's patchy distribution makes it a good candidate for biomonitoring using environmental DNA (eDNA), which can detect species of interest through genetic material shed into the marine environment. This study aims to develop an eDNA assay to assess Olympia oyster distribution along the dynamic British Columbia coastline. Estimating organism density using eDNA is highly desirable, but eDNA signal strength is known to be impacted by many abiotic factors. Thus, we investigated tidal, topographic, reproductive phase and stratification effects on eDNA signal strengths. We found that none of these factors impacted eDNA signal strength in a predictable way, suggesting limitations for density estimation, and highlighting the need for comprehensive metadata and multi-method approaches for more accurate ecosystem monitoring. However, our developed eDNA assay can be used as a reliable early detection tool for the Olympia oyster in Barkley Sound, BC.

2.1 Introduction

The Olympia oyster (*Ostrea lurida)* is the only native oyster species that resides along the coast of British Columbia. It was commercially harvested until a near collapse of the population in the 1950s [45]. Since then, though there has been a lack of commercial harvesting pressure, this species is also highly sensitive to the increases in anthropogenic pressures apparent in the coastal, estuarine environments where they reside[91]. They are highly sensitive to cold temperature extremes and sedimentation. Additionally, recent introductions of exotic parasites and fouling ascidians have increased mortality via parasite load and smothering respectively[92]. They also face increased predation via newly invasive predatory snails and the voracious green crab, as well as competition from the commercially grown Pacific oysters[92]. It has been listed as 'Special Concern' under both the Species at Risk Act (SARA) and the Committee on the Status of Endangered Wildlife in Canada (COSEWIC)[93].

This species currently generally occurs at low densities in patchy distributions along the British Columbia coastline, it is very cryptic and is mainly found in the low intertidal zone. The fact that it isn't common, hard to find and in a habitat that's hard to survey make it an excellent candidate for detection via environmental DNA (eDNA) sampling. eDNA is DNA that can be isolated from environmental samples such as a scoop of water[4,94,95]. Organisms shed skin and other DNA into our environments. By detecting these biological traces through targeted eDNA testing, we can investigate biodiversity and species of interest through a molecular lens. Compared to many conventional methods, eDNA can greatly increase the scope of survey effort due to its relatively lower costs and ease of sampling[96]. Furthermore, eDNA has a low environmental impact and can allow researchers to "see the unseen". Using highly sensitive targeted qPCR assays for the detection of species presence is well-established and has potential to estimate relative organismal abundance information from an eDNA signal as well [9,10,12]. Abundance is a fundamental population parameter in ecology, conservation, and natural resource management, and thus would be invaluable from an eDNA methods perspective.

The aim of this work is to design and validate an eDNA assay that can reliably detect the Olympia oyster along the British Columbia coastline; a highly dynamic coastal environment that experiences high tidal exchange, large amounts of freshwater input, and is exposed to rough weather. Olympia oysters are filter feeding hermaphroditic broadcast spawners who spawn in water temperatures over 16 degrees C[92,97]. They are known to thrive at salinities higher than 25ppt, ranging from estuarine to marine environments. They generally inhabit the lower intertidal and upper subtidal regions but have been recorded to a depth of 50m[93]. Despite this, they can survive short bouts of freshwater and can survive above the low tide line [98]. The highly dynamic nature of the nearshore marine environment[99,100] they inhabit means that there are many factors affecting their eDNA signal detection strength[28,29]. We therefore also designed our field experiments to investigate parameters such as tidal phase, topographic features, depth, salinity, and reproductive status that may affect the accuracy or feasibility of relative abundance estimation using eDNA. Currently there have been few studies explicitly examining tides in the context of eDNA signals, and they have used metabarcoding approaches to assess ecological community differences at varied tidal phases [101-103]. Using a targeted qPCR approach allows us to investigate whether there is a necessary minimum proximity to target organisms, an optimal sampling time to maximize eDNA signal, or whether the signal remains relatively constant regardless of tidal phase, supporting the notion that eDNA signals do remain localized in space and time[95]. This approach is also relevant in terms of sampling depth and water composition. Multiple metabarcoding studies have identified differences in community, based on where in the water column samples were taken, with the most distinct communities present in surface water where there can be significant effects from stratification of the water column, or the presence of freshwater input[14,17,101,104]. Our study demonstrates that regardless of a highly sensitive qPCR assay, gleaning relative abundance information for a target species is extremely difficult in a highly dynamic coastal environment where so many factors can affect eDNA signal detection. Taking such factors into account will strengthen the utility of eDNA as a biomonitoring tool in the future.

2.2 Methods

2.2.1 Assay Validation

A targeted eDNA assay for *Ostrea lurida* was designed using *unikseq* [105] and validated using an established workflow described previously [7,105]. Reporting and performance characteristics were compliant with two Canadian national eDNA standards [106,107]. The assay was designed using a whole mitochondrial alignment including all available sequences for the target species, close relatives, and common geographically co-occurring bivalves accessed from the National Center for Biotechnology Information (NCBI) GenBank [108]. These sequences were supplemented by sequences from locally obtained target and relevant species that we assembled using *mtGrasp* version 0.1.0 [109].The full lists of all species mitogenomes and accession numbers used in the present study can be found in Appendix 1. The *unikseq* (v1.0.0) parameters used, as well as the relevant output sequence used for the assay is shown in Appendix 2.

To ensure that the candidate assay primer and probe sequences would not amplify DNA from other potentially interfering taxa, they were cross-referenced against the NCBI non-redundant (nr) sequence database and visualized against co-occurring species (Figure 3). The assay was then tested against tissue derived gDNA of target and non-target species (Appendix 3) and assay sensitivity was determined via standard curve construction with gBlocks® synthetic DNA obtained from Integrated DNA Technologies (Coralville, Iowa, United States). These synthetic DNA templates were specifically designed to match the target amplicons, and standard curve construction followed the methodology described previously [110]. Using the gBlocks® data, standard curves were created to establish a relationship between C_t values and the initial DNA copy number. The eLowQuant [11] method

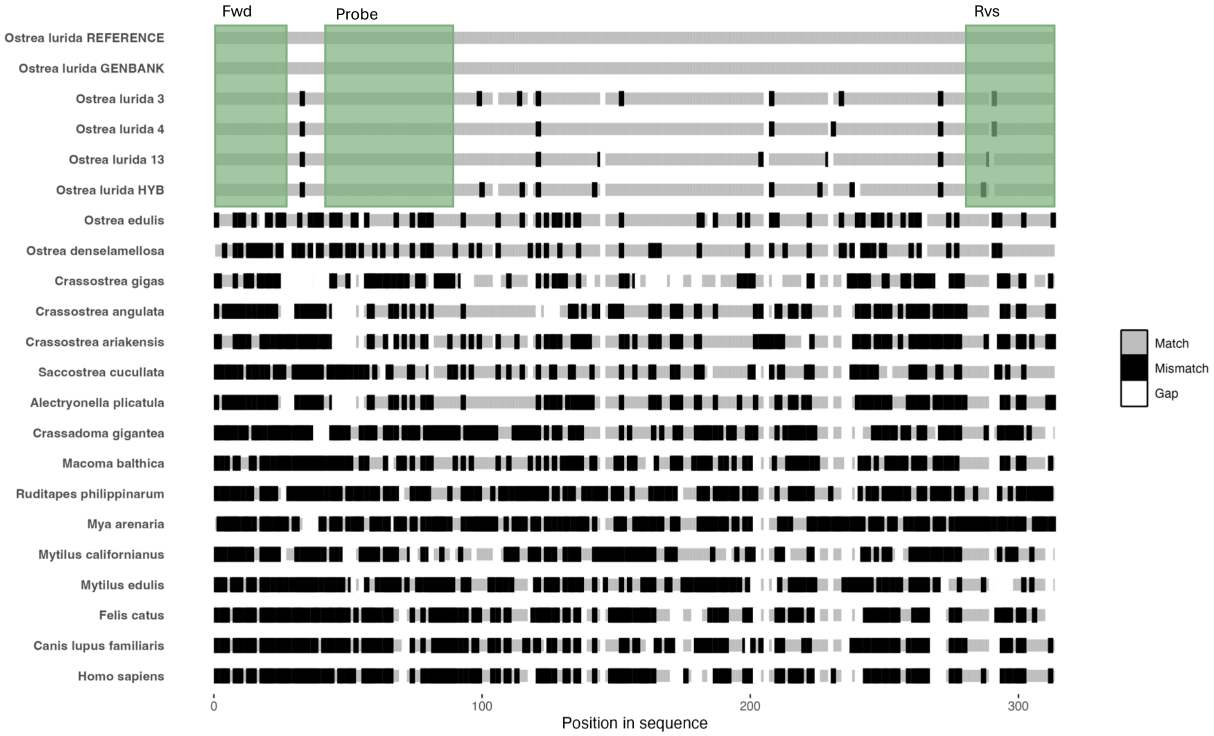


Figure 3. Gene sequence alignment of Olympia Oyster assay amplicon using sequences of target, and related and co-occurring taxa for which sequence information was available, as well as human, cat and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design.

was used to generate the limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) (Table 2).[105]. For the final validation step the assay was tested on known positive field environmental samples [107]. These samples were collected as part of the proof-of-concept pilot study we did for this work. Three 1L surface water sample replicates were collected from sites with high *O. lurida* density (Port Eliza), low *O. lurida* density (Hisnit Inlet) and no *O. lurida* present (Twin Islet). The samples were processed and tested as described below.

Table 2. eOSLU2 sequences, molecular characteristics, and calculated performance parameters

Target Species		**Ostrea lurida**
eDNA qPCR Assay name		eOSLU2
Target Gene		mt-cox-3
Optimal Ta (oC)		58.4
Amplicon Size (bp)		214
Assay Component Sequences (5' to 3')	**Fwd Primer**	CAGTCTTCTAATTTTACGCATA
	Rvs Primer	TTCATCCATACACAACCATA
	Probe	CACTCTTCTCATATACTTCCTCGTTCTCCT
	Amplicon	CAGTCTTCTAATTTTACGCATACTTCAGACGCGCACTCTTCTCATATACTTCCTCGTTCTCCTTACCATATCTTGGACC CTAGACCATGACCCGTTCTAATAGCAATTGGTTTATGGGGGCTAGCTATAACTTTTATTTGTTGAGCTAACGGAGTGC CTTGTAACAGGCTTCTTTTAGGGGCTGTGATTGCAGCTATGGTTGTGTATGGATGAA
Continuous Data where Ct >95%	**Slope**	-3.5893
	% Efficiency	90
	Y-Intercept	38.912
	R2 Value	0.9988
Binomial Data (Copy #/rxn)	**LOD (95% CI)**	0.3 (0.2-0.6)
	LOQ (95% CI)	1.3. (0.9-2.4)
	LOB	0

2.2.2 Field Methods

Over the course of this project, we ran a series of field surveys investigating multiple factors that could impact eDNA signal strength both spatially and temporally. In June of 2022, we visited nine sites distributed throughout Barkley Sound, British Columbia (Figure 4). This region is home to regularly well monitored populations of *O. lurida*. In fact, three of our study sites (Harris Island, Hillier Island, and Joes Bay) are index sites that have been monitored by Fisheries and Oceans Canada (DFO) since 2009[91]. At all nine sites, we collected three 1L water samples from the surface water as close to the main Oyster bed as possible on both the high and low end of tidal cycles (Appendix 4). *O. lurida* is known to spawn during the summer months, thus in June of 2022 we also randomly sampled individuals for dissection whereby spawning was confirmed.

In October of 2022 we revisited all these sites and took a series of three 1L water samples as an attempt to tease apart factors that could be affecting eDNA detection like sampling depth, topographic characteristics, and reproductive phase (Appendix 4). To investigate sampling depth, we took paired samples of surface and subsurface water at each site. Since these sites are quite shallow even at high tide, we used a sterilised bottle attached to a 3m sampling pole to collect subsurface (bottom) samples. Additionally, we wanted to investigate whether site characteristics like freshwater influence or the presence of a lagoon could impact eDNA signal. Pipestem Inlet and Lucky Creek both have sources of freshwater very close to their oyster beds. Thus, at each of those two sites we took three sets of surface samples 1. over the oyster bed, 2. right at the freshwater source, and 3. between the two. Harris and Hand Island each have a prominent lagoon adjacent to the main oyster bed. It is known that *O. lurida* accumulates in protected areas such as this, so we collected surface samples from the main bed and the lagoon[91]. During this field survey *O. lurida* individuals were again randomly selected for dissection to confirm lack of spawning during the winter. We also collected water samples from sites in the middle of a channel as negative field controls to ensure that *O. lurida* eDNA signal remained relatively local to study sites (Figure 4). Finally in August and October of 2023 we returned our focus to tidal effects and collected three 1L surface water samples at the high slack, low slack and high exchange times for six of the original sites (Appendix 4). A 1 L negative filtration control was also collected each field day by pouring instant ocean into a 1L Nalgene bottle aboard the field vessel. In addition to this, in October 2022, August 2023, and October 2023 all water sampling events were accompanied by a Castaway CTD ® (Xylem Inc., Washington, DC, Canada) to measure temperature and salinity profiles throughout the water column. All water samples collected were stored in a cooler with ice packs until filtration through a 0.45 µm mixed nitrocellulose filter (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) later that day. Filters were stored at -20ºC until DNA could be extracted in the Lab.

It should be noted that we surveyed six of our study sites using a traditional transect and quadrat methodology to generate *O. lurida* density estimates. This portion of the project is being led by collaborators and integrates drone imagery and spatial analysis to estimate the size of the oyster bed, and subsequently *O. lurida* population size. These data will allow us to model relationships between traditional and eDNA based organism relative abundance estimations.

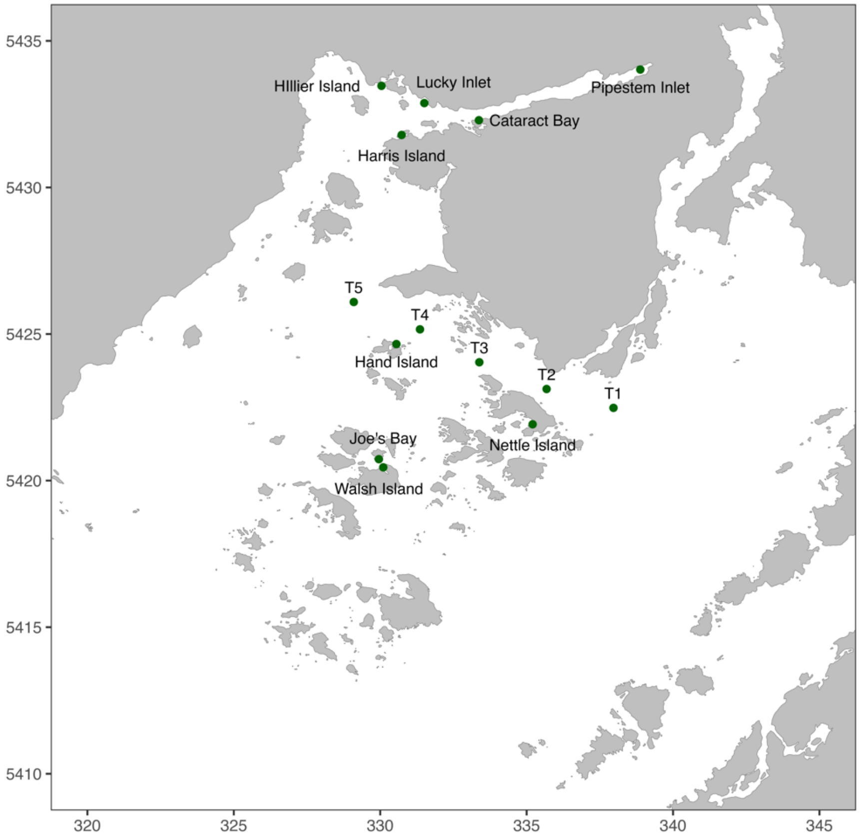


Figure 4. Study sites for O.lurida eDNA collection. Sites T1 to T5 indicate points chosen to act as negative field controls for eDNA collections.

2.2.3 Lab Methods

DNA was extracted from one quarter of each filter using the DNeasy Blood and Tissue kit (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506) as described previously [110,111]. All extractions were performed in a laminar flow hood wiped with 10% bleach (v/v) solution and 70% ethanol (v/v) solution prior to use. All eluates were stored at -20°C until analysis could be done. The integrity of DNA in each sample was assessed using the qPCR based IntegritE-DNA^{TM} test [110]. This test ensures that the eDNA samples are viable and no inhibitors were present that would affect PCR analysis. Any samples that failed this test (average qPCR C_t values >27) were cleaned with a OneStep PCR Inhibitor Removal Kit

(Zymo Research, Irvine, CA, USA, Cat # D60303) and retested. Only samples that passed this test were further tested (Appendix 4).

Each eDNA sample from the field surveys was tested with the eOSLU2 assay with eight technical replicates each using Immolase (FroggaBio scientific solutions (Cat # BIO-21048)). DNA amplification thermocycler conditions were an initial activation step of 2 min at 95°C followed by 50 cycles of 15 s denaturation at 95°C, 30 s annealing at 64°C, and 45 s extensions at 72°C. Each plate was run with eight NTCs and two positive controls comprised of synthetic DNA at a concentration of 20 copies/reaction. If any negative controls amplified, or if positive controls failed to amplify, the entire plate was re-run. These qPCR results were converted into an estimate of DNA copy number/L[11].

2.2.4 Statistical Analysis

All experimental data sets were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests respectively. If these requirements were not met, statistical analyses were performed using non-parametric methods. Samples from differing density sites from the field pilot study were compared using the Kruskal – Wallis rank sum test, and the post hoc analysis was done using the Dunn test with a Bonferroni correction. For samples collected in June 2022, August 2023 and October 2023 the experimental goal was to discern eDNA copy number differences between sampling at different tide times. For samples collected in October 2022 the experimental goals was to discern copy number differences between sampling depths, proximity to freshwater, or proximity to lagoon. For all above experiments a 2-way ANOVA was performed with site and the proposed effect (i.e. tide, depth, freshwater, lagoon) as factors, and post hoc analysis was done via the Tukey HSD test. Data was all rank transformed, with exception to the data for the freshwater and lagoon comparisons, where normality and homogeneity of variance requirements were met for an untransformed 2-Way ANOVA. Looking at sampling seasons and overall site variability, comparisons were completed using a Kruskal-Wallis rank sum test. To identify which sites drove these differences post-hoc testing was done using a paired Wilcoxon rank sum test with a continuity correction.

2.3 Results and Discussion

As demonstrated by targeted eDNA amplification at all expected sites the eOSLU2 assay is a highly sensitive and effected tool for Olympia oyster detection. During the pilot field study, the high-density site DNA copy number estimates were much higher than the lower-density site though this difference was not significant (Dunn test; p = 0.517). The high-density site DNA copy number estimates were significantly higher than the negative site (Dunn test; p = 0.019) (Figure 5). This pilot study indicated that eDNA copy number estimates may be able to provide an indication of oyster densities in the environment. However, the high-density pilot site has some of the densest Olympia Oyster beds known on the BC coast. Thus, during our subsequent experiments, this variation in eDNA signal strength was not so dramatic between sites.

Looking at tidal phase; for samples taken in June 2022 site explained a significant amount of variation in DNA copy number estimates (2-way rank transformed ANOVA; p = 0.0208) though in post hoc testing there were no significant difference between sites when aggregated by tidal phase. Joe’s Bay was the only site where there was a significant difference in DNA copy number estimation between tidal phase (Tukey HSD; p = 0.0323)

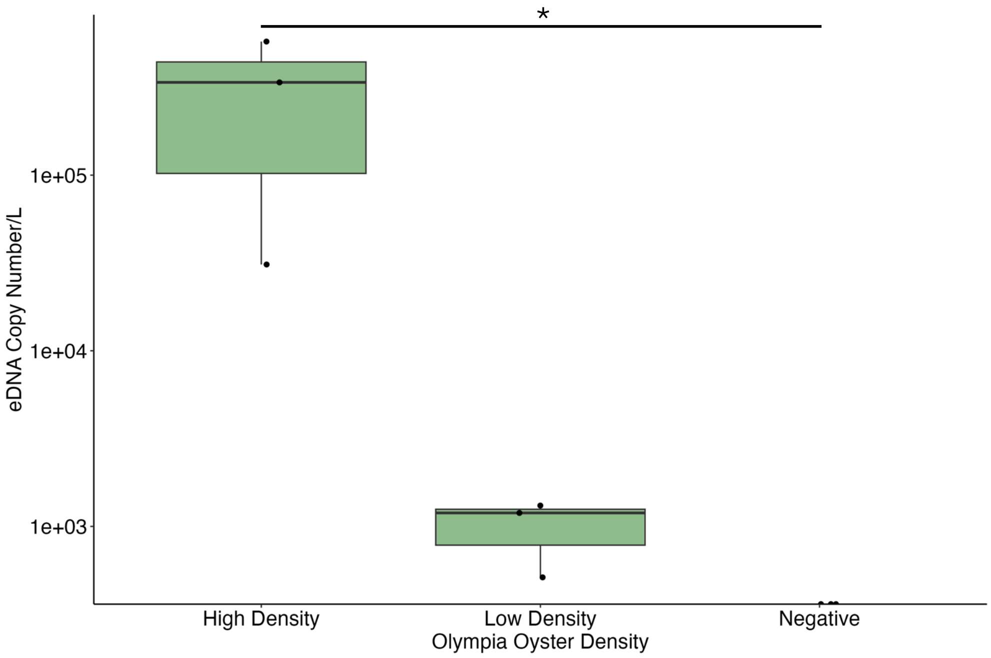


Figure 5. Olympia oyster eDNA copy number estimates from samples collected during the pilot study.

(Figure 6A). With that said, the samples taken during this time were not explicitly timed to be at the high and low slacks. Thus, when we returned in August and October of 2023, sampling was carefully timed to correspond to both slack tides, as well as at the time of highest exchange to reduce variability. Even with this alteration to methodology, for samples taken in August 2023 there were no significant difference found between any sample types (Figure 6B). For samples taken in October 2023 site and tide explained a significant amount of variation in DNA copy number estimates (2-way rank transformed ANOVA; $p = 0.02439$, $p = 0.01972$) though in post hoc testing there were no significant differences between sites when aggregated by tidal phase, or between tidal phase at each site (Figure 6C). The few studies that have attempted to investigate tidal phase on eDNA signal have found varied results. Taking a metabarcoding approach demonstrated no significant differences across tidal cycle in eukaryotic community composition in nearshore marine environments[101]. However, another recent metabarcoding study looking at arctic estuarine communities did observe differences in estuarine community composition over the tidal cycle [103]. Though these studies found opposing results, metabarcoding approaches take a broader approach themselves, and thus community composition changes may not correspond directly to changes in eDNA copy number[18]. The only other study currently in the literature that used a targeted qPCR approach investigated tidal impacts on eDNA signal attenuation away from salmon pens at fish farms. They did

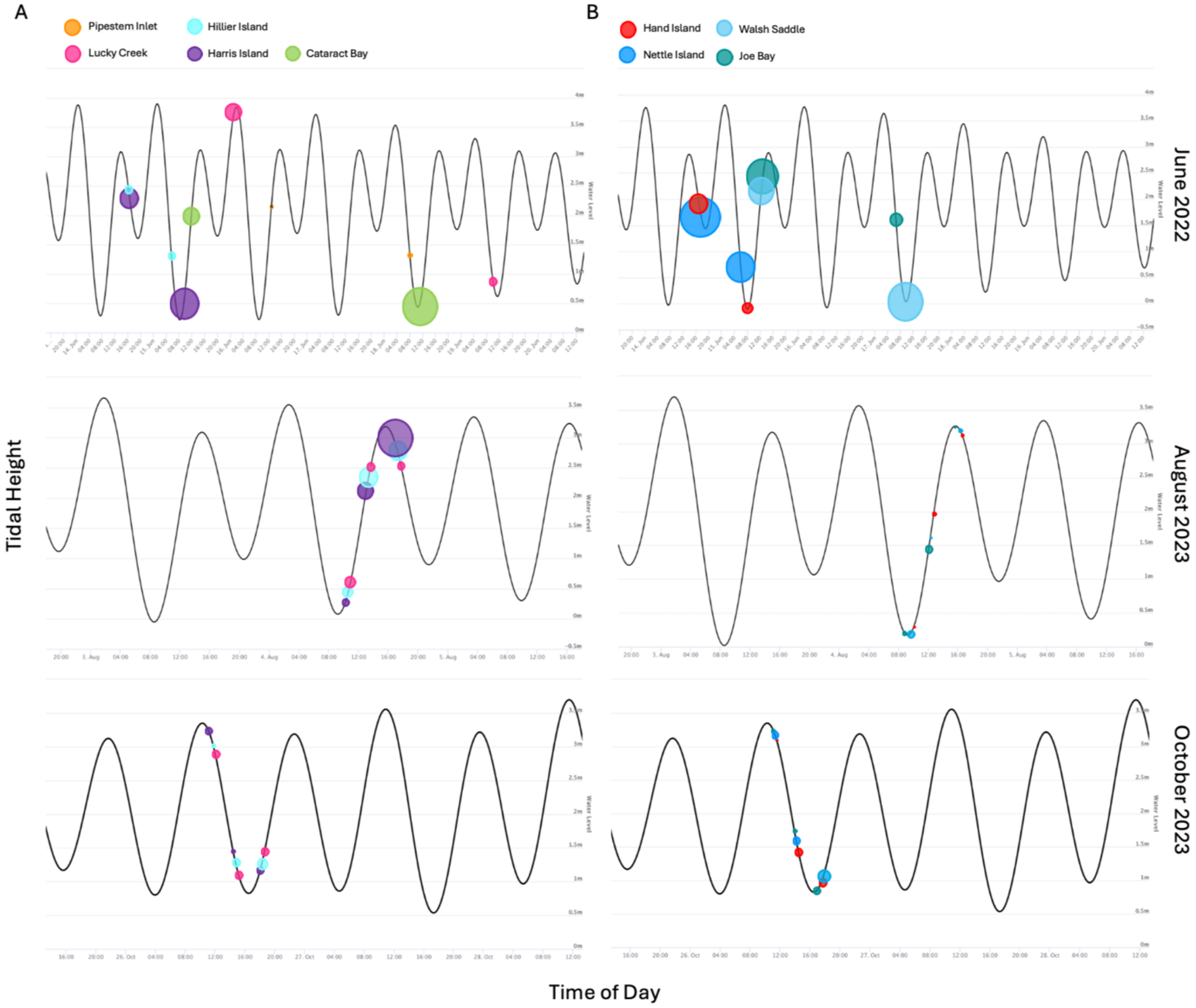


Figure 6. eDNA copy number estimates collected from different points in the tidal cycle. Circle size corresponds with from samples collected during the pilot study. Tidal data collected from A. Stopper Island Tide Station, B. Effingham Island Tide Station.

find significant differences in the distance away from salmon pens source eDNA was detectable based off tide, but did not find differences in eDNA copy number estimates at the eDNA source itself[112]. This study, though interesting is not comparable to our work since the salmon pens are not intertidal, and the amount of source DNA is much higher than what we were working with. Thus, our study is really the first that investigates tidal impacts on an intertidal species using targeted eDNA methodologies. Our results do concur with the previous studies showing that tidal phase does not impact eDNA signal strength.

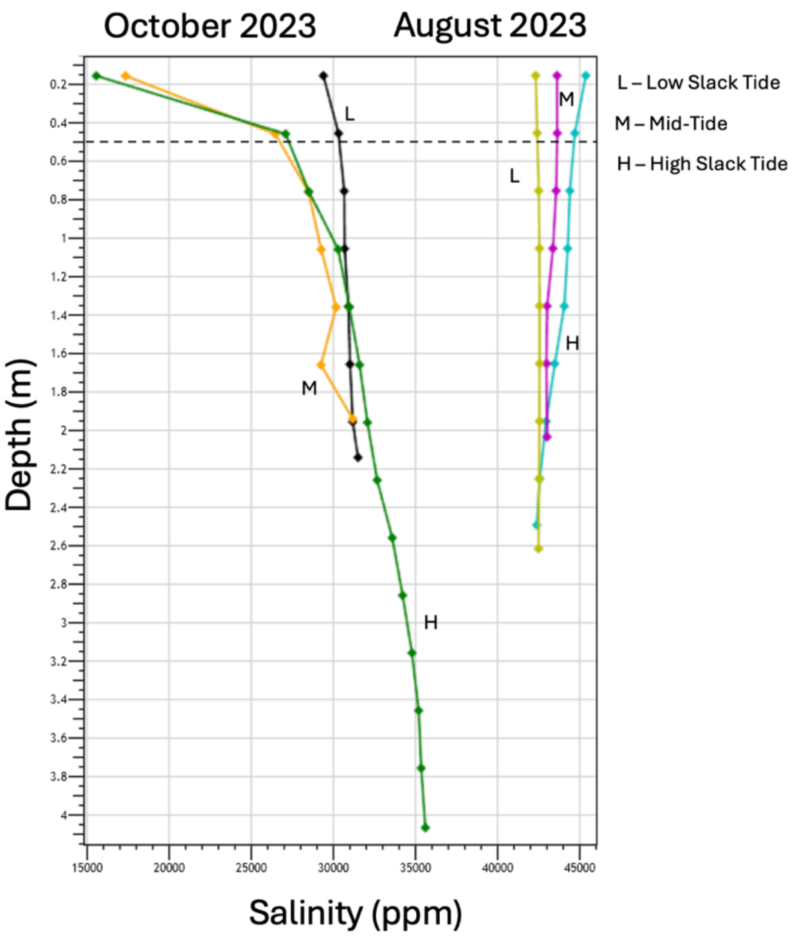


Figure 7. Salinity profiles of Lucky Creek (One of our study sites with a prominent freshwater source) from August and October 2023. Dashed line indicates depth of stratification at 0.5 m.

Another phenomena that is often associated with temperate marine environments is the strong stratification effects that are observed primarily in the winter when large amounts of precipitation lead to the formation of a distinct layer of freshwater laying on top of the denser seawater below. The Castaway CTD ® (Xylem Inc., Washington, DC, Canada) data we collected, confirmed that at some of our sites where there was a significant freshwater source this was indeed happening (Figure 7). Thus, comparing samples collected at the surface to those collected as close to the seafloor as possible allowed us to explore the impacts of stratification, and generally whether taking bottom water samples was beneficial at all in improving eDNA copy number estimates in relation to site specific density.

For the paired surface and bottom water samples we took in October 2022 site explained a significant amount of variation in DNA copy number estimates (2-way rank transformed ANOVA; p = 6.222 e-6). Most of this was explained by the significantly lower amount of target species DNA detected from Walsh Saddle in comparison to Lucky Creek (Tukey HSD: Surface: p = 0.0439, Bottom: p = 0.0039), Cataract Bay (Tukey HSD: Surface: p = 0.0004, Bottom: p = 0.0032), Harris Island (Tukey HSD: Bottom: p = 0.0074) and Pipestem Inlet (Tukey HSD: Bottom: p = 0.0219). Surface water samples from Hillier Island were also significantly lower than those of Cataract Bay (Tukey HSD; p = 0.0010). However, there were no significant differences between depths at any site (Figure 8). Other studies examining stratification have indeed seen significant difference in community composition between layers of the water column [14,104]. However, these studies occurred looking at vertical water profiles well over 20 m deep, thus the depth of stratification is not impacted by tide. Our deepest sample was taken at a depth of less than 5m, and the stratification depth measured at Lucky Creek is at 0.5m (Figure 7). At high slack and mid tide this stratification is detectable, but it is not detectable at low tide. Thus, it is likely that the stratification we saw is not consistent enough to prevent mixing in the intertidal zone.

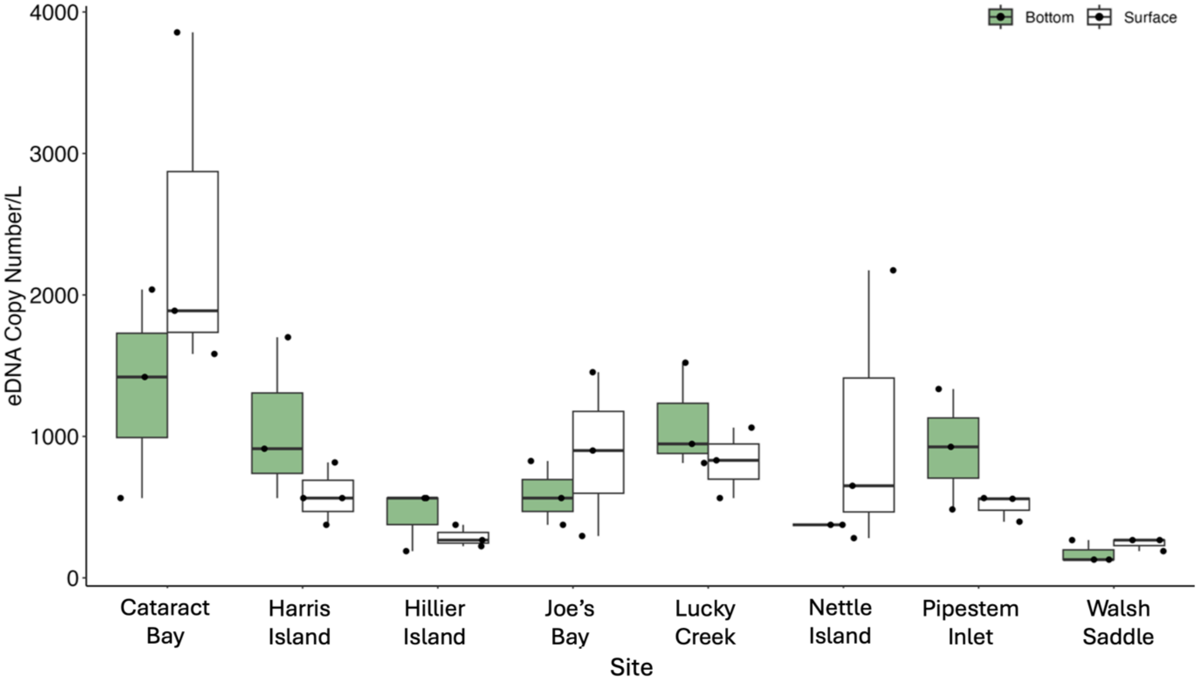


Figure 8. Comparison of eDNA copy number estimates between surface and subsurface (bottom) samples.

This is further supported by the fact that at two sites with prominent freshwater sources, proximity to those flows did not effect eDNA copy number estimates (Figure 9A). In fact, for both of the topographic site characteristics we investigated – proximity to freshwater and presence of a lagoon - the interaction between site and factor of interest explains a significant amount of variation in DNA copy number estimates (2-way ANOVA; p = 2.044 e-7, p = 0.0009386), though in post hoc testing there were no significant differences between any sites or sampling locations (Figure 9A,B).

We had hypothesized that lagoons could be functioning as eDNA reservoirs, since they are sheltered pools of water that are not fully exposed during tidal exchange. Additionally, it is well known that *O. lurida* tends to accumulate in lagoons[91]. However, at the two sites with a prominent lagoon, water collected in the lagoon had lower copy numbers of eDNA estimated in comparison to water collected from above the Oyster bed (Figure 9B), though

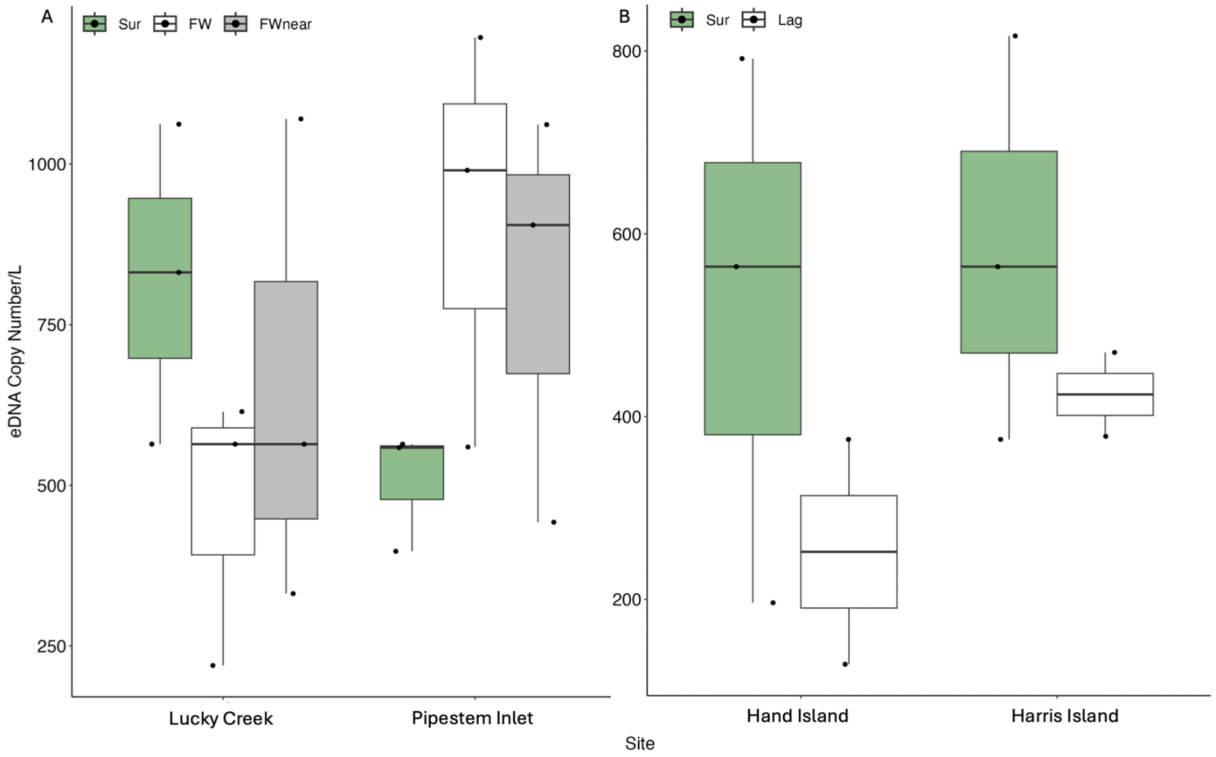


Figure 9. Comparison of eDNA copy number estimates based off **A.** proximity to a freshwater source. **B.** presence of a lagoon.

these differences were not significant. This suggests that in these lagoons the rates of eDNA degradation could be greater than eDNA accumulation, as lagoons can serve as hospitable environments for microorganisms that digest DNA. From a sampling standpoint, this is useful information for eDNA study design for intertidal organisms. Surface sampling requires much less effort and equipment than sampling from deeper depths. Knowing to avoid lagoons, and other topographic features that could have similar effects can help with site selection.

Finally, we compared eDNA copy number estimates across all sites including data from all sampling events. This showed that Cataract Bay did have a higher overall eDNA copy number estimate than sites Hillier Island, Lucky Creek, Pipestem Inlet, and Hand Island (Wilcoxon rank sum test; p = 0.0276, p = 0.0121, p = 0.0077, p = 0.0301) but not

Nettle Island, Joe’s Bay, Walsh Saddle, or Harris Island (Figure 10A). When samples from spawning times (June 2022, August 2023) were removed, these relationships shifted. Cataract Bay had significantly higher copy number estimates than all sites (Wilcoxon rank sum test; Nettle Island: p = 0.0462, Joe’s Bay: p = 0.00049, Walsh Saddle: p = 0.00462, Hillier Island: p = 0.00017, Harris Island: p = 0.00118, Lucky Creek: p = 0.00019, Pipestem Inlet: p = 0.00028, Hand Island: p = 0.00040). Cataract Bay is a small, sheltered bay that is relatively deeper than the lagoons tested at Harris and Hand Islands. Thus, it may in fact be acting as a reservoir for DNA since it does not completely empty at low tide. Additionally, Walsh Saddle had significantly lower copy numbers than Nettle Island, Harris Island, Lucky Creek and Pipestem Inlet (Wilcoxon rank sum test; p = 0.0238, p = 0.04250, p = 0.00862, p = 0.00153) and Pipestem had significantly higher copy numbers than Hand Island (Wilcoxon rank sum test; p = 0.03069) (Figure 10B). Once we have good estimates of *O. lurida* population size from out collaborators, it will be interesting to see if they correlate with the patterns estimated with eDNA based methods.

Regardless, of note is the change in variability in eDNA copy number estimates between samples collected in the summer (June 2022, and August 2023) during spawning versus samples collected in the winter (October 2022 and 2023) (Figure 10C). Though there is no significant difference in the mean eDNA copy number estimate (Kruskal-Wallis; p=0.3927), Levene’s test indicates a significant difference in variance between the two seasons (Pr(>5) = 0.001109). It is well known that eDNA sampling collection during spawning should be avoided unless spawning is specifically being studied [113]. Thus, another consideration that must be addressed is the feasibility of sample collection during certain seasons. It is obviously ideal to collect eDNA during the summer months when the weather is most favorable for reaching remote coastal field sites. However, spawning clearly skews eDNA copy number estimates. Thus, eDNA sample collection in the winter is better for this species, but much more difficult logistically. All the factors we investigated support that eDNA signals in the marine environment remain quite localised in space and time. Using eDNA to estimate relative abundance of an intertidal target organism in a dynamic coastal environment is immensely difficult.

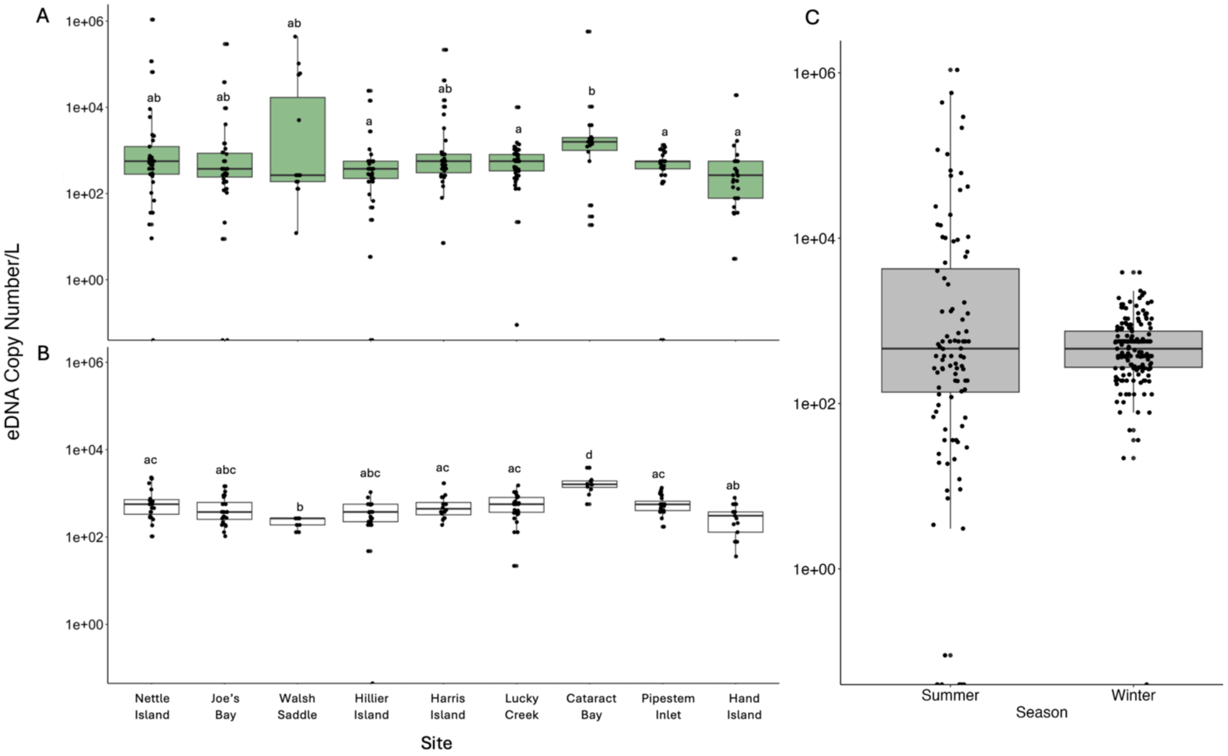


Figure 10. Comparison of eDNA copy number between sites**. A.** Includes all sampling events**, B.** Samples taken during spawning season removed. Significance between sites is indicated using letters**. C.** Compares eDNA copy number estimates between samples collected in winter months versus summer months.

2.4 Conclusions

For eDNA methods to be readily up taken into field programs and opportunistic sampling and decision-making processes there needs to be fundamental understanding of factors that can affect eDNA signal strength. There have been many studies that have investigated the effects of various abiotic parameters on the degradation and subsequent detection capabilities of eDNA in lab settings[29]. However, few studies have attempted to tease apart the many factors that can impact eDNA capture success and abundance estimation in highly dynamic coastal marine environments. Studying the Olympia oyster in Barkley Sound, BC provided an ideal study system to investigate some of these factors. Our targeted qPCR assay was consistently able to detect our target species, even when organismal abundance was relatively low. This observation aligns with the various publications that have demonstrated the efficacy of eDNA monitoring as a powerful early detection tool[110,114]. Since the Olympia Oyster is patchily distributed over the British Columbia Coastline, eDNA sampling with eOSLU2 will undoubtedly be useful in identifying areas of species occupancy.

It is surprising that over all the variables we examined there were no consistent and predictable impacts on eDNA copy number estimate. This supports the notion that there are many variables that all interact in complex ways that are immensely difficult to predict, reinforcing the importance of collecting associated metadata with samples to allow for the best interpretation of results. The best we can do at this point is to design field studies that can control for as many of these factors as possible and play to the strengths of the monitoring tools themselves. Currently, eDNA can be used as a reliable detection tool for the Olympia Oyster. As a highly cryptic species that inhabits an area that is difficult to survey (low intertidal), eDNA surveys will be particularly useful for exploratory purposes to detect unknown populations with minimal effort. However, combining multiple monitoring methods such as traditional stock assessments, eDNA and remote sensing can undoubtedly give us more nuanced ecosystem information.

Chapter 3: Comparison of DNA extraction methods for detecting the sea otter (Enhydra lutris) in marine sediment

Abstract

Sedimentary DNA (sedDNA), environmental DNA (eDNA) preserved in sediment, holds promise for improving our understanding of historical species occurrences and contemporary biomonitoring. However uneven DNA distribution, DNA fragmentation and Polymerase Chain Reaction (PCR) inhibition present challenges to species detection. In this study, we evaluate the efficacy of sedDNA methods to detect the presence of a patchily distributed marine mammal. To this end, we developed a targeted quantitative PCR (qPCR)-based assay for the sea otter (*Enhydra lutris*). This assay successfully amplified target DNA from aquaria occupied by sea otters, and from two of eight seawater samples from areas with sea otters. Additionally, we conducted an experiment to examine the utility of our assay in detecting sea otter DNA in sediment. We compared four sedDNA extraction techniques and two DNA cleaning protocols in surface sediment samples taken from areas with sea otters and using general fish and chloroplast DNA as endogenous controls. The quantity of DNA and the detection of sea otter, fish, and chloroplast DNA varied across the different extraction protocols. Sea otter DNA was detected at lower rates than expected, despite the confirmed presence of sea otters at the sampled sites. Cleaning protocols reduced sedDNA yield. Among the extraction methods tested, Qiagen

Powersoil Pro kit was most effective, yielding higher rates of target species detection with smaller input sediment amounts, and no need for cleaning to remove PCR inhibitors. The present study lays the groundwork for large-scale monitoring of marine mammals using sedDNA and advances the use of sedDNA detection as a valuable tool for reconstructing the temporal and spatial patterns of marine mammal presence. Importantly, this study identifies the need for a better understanding of the effects of marine sediment composition, mammal eDNA shedding rates, and DNA fragment size on detecting target sedDNA.

3.1 Introduction

Environmental DNA (eDNA) refers to the genetic materials shed by organisms into environmental matrices such as sediment, water, or air [37]. The use of eDNA for species detection contributes significantly to our understanding of terrestrial and aquatic species distribution, and has important implications for conservation [115]. In particular, the use of sedDNA, a form of eDNA isolated from sediment [116], provides a promising approach for assessing historical and contemporary species occurrences, and consequently, our understanding of biodiversity [117-120].

DNA can persist for thousands of years in sediment [121], suggesting that sedDNA offers a means to detect organisms over time and space [122,123]. Despite this promise, sedDNA poses some methodological challenges. Adsorption of sediment particles is necessary for the preservation of DNA in sediment; however, different properties of sediment, such as clay or borate content affect DNA persistence over time [124]. Sub-optimal sediment composition, in combination with factors known to degrade DNA, such as UV stress, high temperature, or high microbial activity that consumes DNA molecules, can result in substantial losses in the amount of DNA that is preserved in sediment [29,95,123]. The presence of significant amounts of organic matter such as humic acids, also present challenges in extracting sedDNA and affects downstream analysis through Polymerase Chain Reaction (PCR) inhibition. Moreover, compared to terrestrial or aquatic systems, a greater variety of forces determine how eDNA is aggregated and retained in marine ecosystems due to oceanographic forces that dictate particle movement[118]. To date, the majority of sedDNA studies carried out in the marine environment focus on unicellular or microbial organisms where the targeted DNA is intracellular, or where the target organisms are ubiquitous or have vast spatial coverage (i.e., eelgrass, alga, and many foundation species) [125,126]. Thus, the DNA of mobile organisms with patchy spatial distributions has a lower likelihood of detection [116]. Additionally, most multi-cellular, motile organisms differ from unicellular and microbial organisms in that their DNA is typically detected through shed DNA, rather than the whole organism being preserved in the sediment. Therefore, detecting macro-organismal target DNA in sediment requires a highly sensitive approach [122-124].

The few eDNA studies that have focused on marine mammal detections have extracted DNA from seawater rather than sediment [22,24,56,127-130]. The sea otter (*Enhydra lutris)* is a particularly good candidate for examining the potential of sedDNA detection for several reasons. Sea otters are listed as *endangered* by the International Union for Conservation of Nature (IUCN), and as a species of *special concern* under the Canadian Species at Risk Act [131,132]. Sea otters were hunted from roughly 300,000 to ~1500 individuals during the maritime fur trade that lasted from the mid-1700s until the early 1900s [133]. Sea otters have recovered to much of the Pacific Rim, but their distribution today forms a mosaic, with large swaths of habitat where sea otter populations have either become re-established or have been absent for more than a century. However, the pre-fur trade distribution of sea otters is not well understood. For example, prior to the fur trade sea otters may have resided in great numbers in sheltered and estuarine habitats, whereas today sea otter habitat is most-often associated with exposed rocky reef environments [27]. Thus, sedDNA could be an especially valuable tool for assessing historical sea otter distribution and ecological niche [134]. From a behavioural standpoint, sea otter individuals tend to aggregate in large groups, or rafts; some rafts in our study area comprise upwards of 100-200 individuals [61], and DNA shed through excrement, fur, or skin may be concentrated in the sediment below these floating sea otter aggregations. Finally, sea otter foraging often requires invertebrate prey to be excavated from the sediment (i.e., by digging for clams, worms; [62,135,136]) which may leave DNA from sea otter fur or skin.

Currently, no eDNA studies have specifically focused on sea otters. However, eDNA assays have been developed for the Himalayan River Otter (*Lutra lutra*) [137] and the Northern American river otter (*Lontra canadensis*) [138]. In some cases, studies have demonstrated that eDNA is more effective, practical, and accurate in detecting otters compared to traditional surveying methods [137].

Here, we designed and validated a targeted quantitative PCR (qPCR)-based assay (eENLU6) to determine the efficacy of using eDNA for the detection of sea otter in seawater and marine sediments. To address the paucity of data regarding marine sedDNA detection methods for macro-organismal target species, we compared the efficacy of four sedDNA extraction methods, and two DNA cleaning protocols for marine surface sediment. We assessed DNA extraction success through absolute DNA quantification, amplification of ubiquitous chloroplast DNA as an internal control, and amplification of general fish DNA to test our detection rates of a ubiquitous macro-organism, whose eDNA would likely be extracellular (i.e., shed) like our target species. Subsequently, the extracted sedDNA was used for targeted detection of sea otter using our validated eENLU6 assay. The present study aims to improve the methodological workflow for detection of macro-organisms in sedDNA, with the goal being to investigate the feasibility of using sedDNA to assess the historical and contemporary distribution of sea otter in British Columbia, Canada.

3.2 Methods

3.2.1 Assay Design and Validation

A targeted eDNA assay for the sea otter was designed using *unikseq* [105] and validated using an established workflow described previously [7,105] (Appendix 5). Reporting and performance characteristics were compliant with two Canadian national eDNA standards [106,107]. The assay was designed using a whole mitochondrial alignment including all available sequences for the target species, close relatives, and common geographically co-occurring marine mammals accessed from the National Center for Biotechnology Information (NCBI) GenBank [108]; all species mitogenomes and accession numbers are listed in (Appendix 6). The *unikseq* (v1.0.0) parameters, as well as the relevant output sequence used for the assay is shown in (Appendix 7).

To ensure that the candidate assay primer and probe sequences would not amplify DNA from other potentially interfering taxa, they were cross-referenced against the NCBI non-redundant (nr) sequence database using PrimerBlast and visualized against co-occurring species (Figure 11). The assay was then tested against tissue derived genomic DNA (gDNA) of target and non-target species and assay sensitivity was determined via standard curve construction with gBlocks synthetic DNA obtained from Integrated DNA Technologies (Coralville, Iowa, United States) using methodology described previously [110] (Appendix 8). The eLowQuant [11] method was used to generate the limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) (Appendix 8) [105].

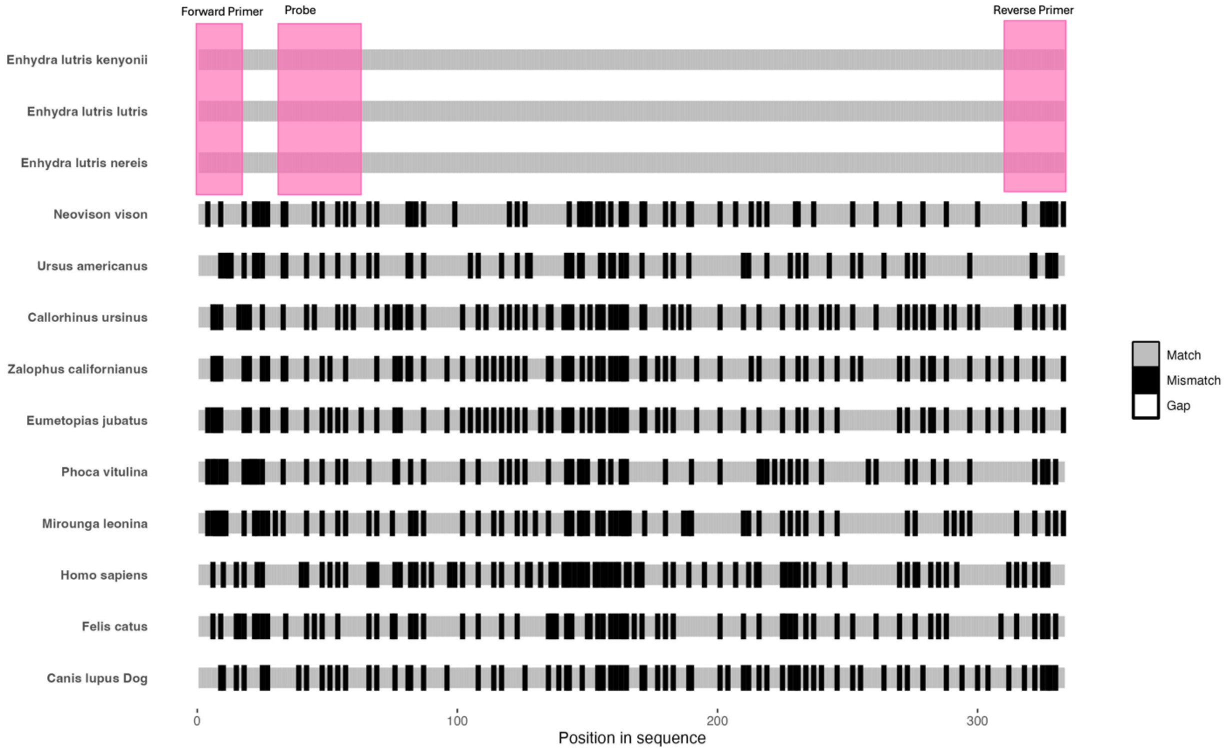


Figure 11. Gene sequence alignment of the eENLU6 amplicon using all three subspecies of the sea otter, and all other BC marine mammal species for which there is sequence data available, as well as human, cat and dog sequences. Grey indicates base pair conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. There is no intraspecific variability in any of the key assay regions.

As a field validation step, the assay was tested on water samples collected from tanks containing sea otter individuals from both the Vancouver Aquarium (British Columbia, Canada) and the Monterey Bay Aquarium (California, USA) to ensure that both the northern and southern sea otter subspecies (*E. l. kenyoni* and *E. l. nereis* respectively) were detectable with eENLU6. Three 1L water samples were taken at each aquarium. The water was immediately filtered through a 0.45 µm mixed nitrocellulose filter (Thermo Fisher Scientific Inc., Ottawa, ON, Canada). One quarter of each filter was then extracted using the DNeasy Blood and Tissue kit with a final elution volume of 150 µL (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506). Each extracted sample was tested with eENLU6 with eight technical replicates each using the QIAcuity Probe PCR kit (QIAGEN Inc. Mississauga, ON, Canada, Cat # 250102). DNA amplification thermocycler conditions were an initial activation step of 2 min at 95°C followed by 50 cycles of 15 s denaturation at 95°C, 30 s annealing at 64°C, and 45 s extensions at 72°C. Each plate was run with eight no template controls (NTCs) and two positive controls of synthetic DNA at a concentration of 20 copies/reaction. These qPCR results were converted into an estimate of DNA copy number/L.

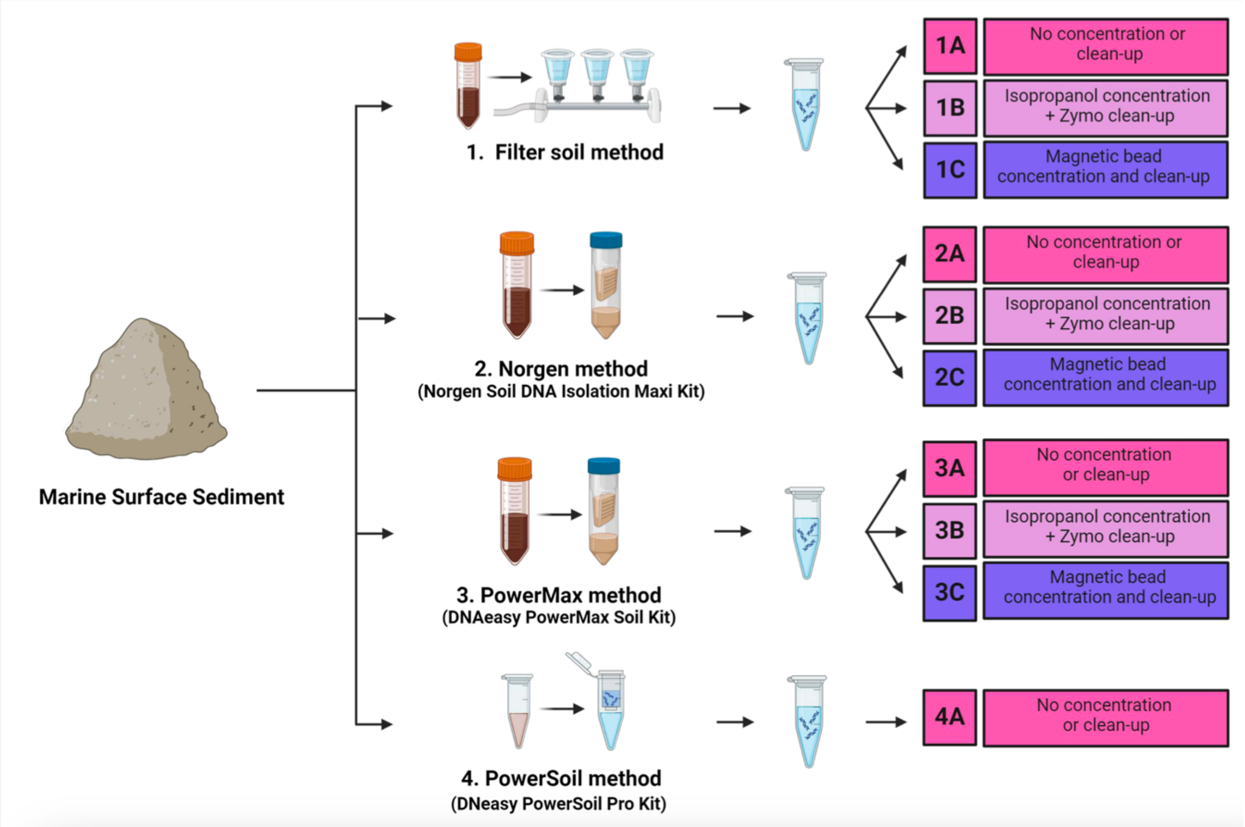
As an additional validation step, we collected 3 x 1L surface seawater samples from areas where we expected different intensities of sea otter use. Seawater samples were thus collected at three sites outside the current sea otter range [61], hereafter *Absent* sites; three inlet sites where otters had been reported in some years or seasons from 2009-2022 (J. Watson, July 10th 2023, pers. comm.), hereafter *Inlet* sites; and at six sites where sea otters have been established for >20 years [61,62], hereafter *Established* sites (Table 3). At the time of our sampling, sea otters were only visually confirmed at one site (Table 3). Field-collected seawater samples were processed as for the aquaria-collected samples, described above.

3.2.2 Sediment Sample Collection

As with seawater samples, surface sediment samples were collected at *Absent*, *Inlet*, and *Established* sea otter sites, inshore of seawater sample collections. At each site, six sample replicates were collected at low tide at random spots along the low intertidal zone of the beach. Samples were collected using a sterilized trowel to avoid contamination. Due to temporal and financial constraints, DNA extraction was only attempted for sediment samples collected at one *Absent* site (n=2 samples) and for eight sediment samples which were collected across three of the *Established* sites where we thought sea otter DNA detection was most likely (i.e., locations where sea otters are reliably sighted during surveys;[61]) (Table 4). Among the *Established* samples, two samples were taken directly from sea otter foraging pits, and one sample was taken from sediment with sea otter scat (Table 4). Together, this resulted in ten sediment samples from which DNA extraction was attempted (Table 4). To create a positive control to monitor extraction efficacy, one of the negative samples was spiked with a slurry of homogenized sea otter tissue consisting of 1 g sea otter tissue homogenized in 10 mL buffer ATL (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506). Marine sediment samples were assigned a randomized DNA processing number (DPN) and stored frozen at -20°C until DNA extraction.

3.2.3 Comparison of sedDNA Extraction Methods

Each field sample was split into four parts for extraction with each of four different methods (Figure 12). Each extraction method required different sample wet weights, extraction kits, and elution volumes. All extractions were performed in a laminar flow hood sterilized with 10% bleach (v/v) solution and 70% ethanol (v/v) solution prior to use. 1) *Filter* *soil method:* Sediment (50-100 mL) suspended in 1L of distilled water was allowed to settle at 4°C for 24 hours, followed by filtration through a 0.45 µm mixed nitrocellulose filter (Thermo Fisher Scientific Inc., Ottawa, ON, Canada). One quarter of each filter was then extracted using the DNeasy Blood and Tissue kit with a final elution volume of 150 µL, repeated three times per filter, for a total of 450 µL eluate (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506) as described previously [110,111]. 2*) Norgen method:* Sediment (10 g) was extracted using the Norgen Soil DNA Isolation Maxi Kit (Norgen Biotek Corp., Thorold, ON, Canada; Cat# 62000) with the final elution performed in 3 mL elution buffer B. 3) *PowerMax method:* Sediment (10 g) was processed using the DNeasy PowerMax Soil Kit (Qiagen Inc., Mississauga, ON, Canada; Cat# 12988-10) with the final elution in 3 mL solution C6. 4) *PowerSoil method:* DNA was extracted from 250 mg sediment using the DNeasy PowerSoil Pro Kit (Qiagen Inc., Mississauga, ON, Canada; Cat# 47014), with the final elution in 50 μL solution C6.



3.2.4 Comparison of sedDNA Cleaning Protocols

The sedDNA samples that were isolated with extraction methods 1-3 (Figure 12) were tested without cleaning (denoted with an “A” in Figure 12) and were also subjected to two cleaning protocols. For the first cleaning protocol (designated as “B” in Figure 12), the isolated DNA was concentrated and cleaned by a modified ethanol precipitation protocol [120] and further cleaned with Zymogen OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA, Cat # D60303). In the second cleaning protocol (designated as “C” in Figure 12), the isolated DNA was concentrated and cleaned using magnetic beads

Figure 12. Four extraction methods and three cleaning protocols compared in this study. All methods were tested on the same samples to allow for direct

following the manufacturer's instructions with a bead ratio of 1.8 (AMPure XP SPRI Reagent cat#A63881, Beckman Coulter). All samples were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The final resuspension volumes were 50 µL for the filter soil method, and 100 µL for the Norgen and PowerMax methods.

The total DNA within each eDNA extract was quantified using the Invitrogen Qubit 1x dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA, Cat #Q33230). All DNA samples were stored at -20°C until qPCR analysis could be performed.

Each of the extracted samples were then assessed using the qPCR-based IntegritE-DNA^{TM} assay [110,139] and eFISH1 assay [140] as endogenous controls, and our validated sea otter assay, eENLU6, with four to eight technical replicates each using the QIAcuity Probe PCR kit (QIAGEN Inc. Mississauga, ON, Canada, Cat # 250102). DNA amplification thermocycler conditions were an initial activation step of 2 min at 95°C followed by 50 cycles of 15 s denaturation at 95°C, 30 s annealing at 64°C, and 45 s extensions at 72°C. Each plate was run with eight NTCs and two positive controls of synthetic DNA at a concentration of 20 copies/reaction. These qPCR results were converted into an estimate of DNA copy number/L.

3.2.5 Statistical Analysis

Data were analyzed with R Studio© version 4.2.3. To compare the effects of the different extraction protocols (the unique grouping of both the extraction method and cleaning protocol used) on DNA yields, we grouped data from the ten sediment samples and standardized using the sediment input volume and DNA dilution factors. Since the data were non-normal and could not be transformed to meet the assumptions of parametric statistical tests, we used a Kruskal-Wallis test to determine whether different extraction protocols resulted in significant differences in each of the following DNA yields: DNA quantity estimates, IntegritE-DNA™ copy number estimates, or eFISH1 copy number estimates. To determine which extraction protocols resulted in significant differences in our DNA response variables, we used post-hoc Wilcoxon Signed Rank tests. Since sea otter could not be assumed to be ubiquitously present across all sample types eENLU6 copy number estimates were not included in this analysis.

3.3 Results

3.3.1 Validated eDNA Assay

The eENLU6 assay is a highly sensitive and specific assay with a reported LOD and LOQ of 0.3 (0.2 – 0.6 95% CI) and 1.3 (0.9 – 2.1 95% CI) copies/reaction respectively (Appendix 8). When tested on aquarium seawater samples from sea otter tanks there was ample amplification. For the Vancouver Aquarium enclosure containing five sea otter individuals we obtained mean copy number estimates of 753,136 copies/L. For the Monterey Bay Aquarium enclosure containing one sea otter individual we obtained a mean copy number estimate of 249, 899 copies/L. This demonstrates that the assay will amplify sea otter DNA if it is present in high enough quantities and is sufficiently intact. Of the twelve sites where seawater samples were collected, samples from three sites did not pass IntegritE testing, indicating that the samples had been compromised. Two of these samples were from *Absent* sites (where sea otters are absent) (Table 3). For the seven remaining seawater collections from *Inlet* and *Established* sea otter sites, we were able to detect sea otters at two sites with copy number estimates of 90 and 169 and copies/L (Table 3). This is notable because at one of these sites there were no sea otters present when we sampled. At the site with 169 copies/L a raft of 12-18 sea otters was observed roughly 200 m from our sampling location, although they dispersed before samples were collected. Even with a small sea otter raft present, DNA concentration was markedly lower than that observed in aquaria tanks. Sea otter DNA was not detected at our negative control seawater sites (i.e., at *Absent* sites), where sea otters have been absent for decades to more than a century (Table 3).

Table 3. Sea otter eDNA detection from surface seawater. *Y* indicates positive detection of sea otter eDNA; *N* indicates negative detection of sea otter eDNA.

Site	Sea otter occupancy	Sea otters present at sampling	eDNA detected
Smelt Bay	Absent	No	N
Marina Island	Absent	No	Samples Compromised
Open Bay	Absent	No	Samples Compromised
Head of Tahsish Inlet	Inlet	Yes	Y
Head of Kashutl Inlet	Inlet	No	N
Kauwinch River Beach	Inlet	No	N

Stryker Island	Established	No	N
Louise Channel	Established	No	Y
Spring Island	Established	No	N
Clinninick Cove	Established	No	N
Gay Passage	Established	No	N
McKay Lagoon	Established	No	Samples Compromised

Samples Compromised means that the samples did not pass IntegritE test

3.3.2 Comparison of sedDNA Extraction Methods

For DNA quantity estimates, IntegritE-DNA™, and eFISH1 copy number estimates, the Kruskal-Wallis test indicated there were significant differences between extraction protocols ($p < 0.001$) (Figure 13; Appendix 9) Samples extracted with the Filter Soil method, the Norgen method, and the PowerSoil method, all estimated comparable DNA quantities. Samples extracted with the PowerMax method and not cleaned, or cleaned with magnetic beads had significantly higher estimated quantities of total DNA than samples extracted with the other methods (Figure 13; Appendix 9).

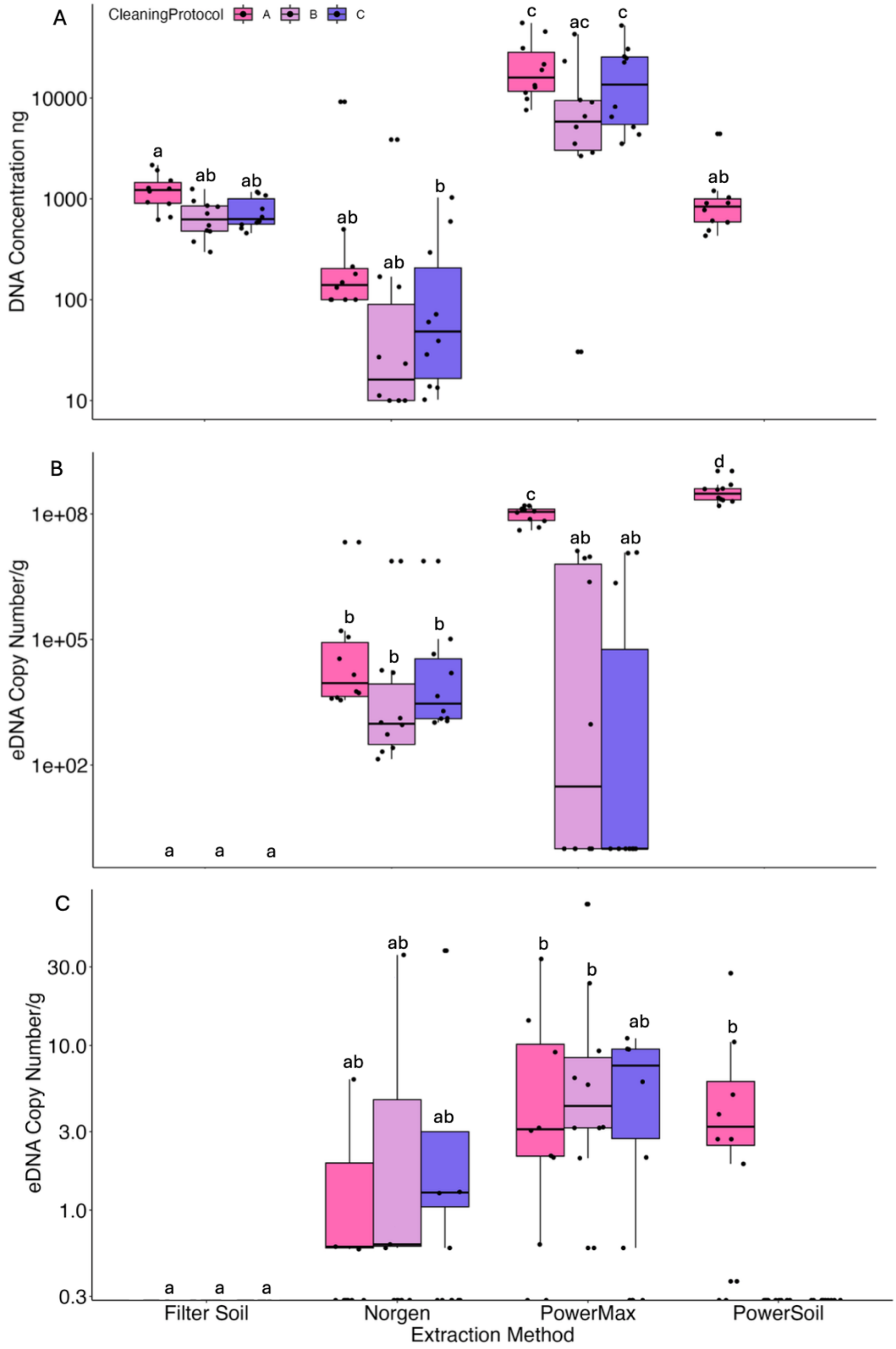


Figure 13. Comparison of extraction methods and cleaning protocols (described in Figure 12) represented by A) DNA quantity estimates, B) IntegritE-DNA^{TM} copy number estimates, C) eFISH1 copy number estimates. Significant pairwise comparisons are denoted by lowercase letters above each box (Paired Wilcoxon Test $p < 0.05$).

Samples extracted with the Filter Soil method did not have any DNA amplification with IntegritE-DNA™ regardless of cleaning protocol. Samples extracted with the Norgen method, and the cleaned samples from the PowerMax method had higher levels of chloroplast DNA amplification with IntegritE-DNA™ than the Filter Soil samples, though for the samples from the PowerMax method this was not significant. The uncleaned samples extracted from the PowerMax method had significantly higher amplification with IntegritE-DNA™ than all other sample types except for those extracted with the PowerSoil method, which were even higher (Figure 13; Appendix 9).

Samples extracted with the Filter Soil method also did not amplify fish DNA, with the eFISH1 assay regardless of cleaning protocol. Samples extracted with all other methods had higher levels of fish DNA amplification, though for samples extracted with the Norgen method, and the PowerMax method cleaned by magnetic beads, this difference was not significant. Based on our chosen metrics of amplification success, there is no significant difference between the two clean-up methods (Figure 13; Appendix 9).

Looking at targeted sedDNA detection*,* sea otter DNA was not detected in any of the samples extracted by the Filter Soil method, nor in any of the samples extracted with the PowerMax method cleaned by either protocol, even from samples that were spiked with tissue-derived DNA (Table 4). Samples extracted with the Norgen method detected *Sea otter* DNA in only the spiked samples. Sea otter DNA was detected in these samples as well as scat samples via uncleaned extracts from the PowerMax method, and via extracts from the PowerSoil method. These latter samples also detected *Sea otter* DNA from a site where the target species is known to be present (Table 4).

Table 4. Sea otter eDNA detection from surface sediment samples. Each sample was extracted using all four extraction methods and cleaning protocols outlined in Figure 12.

Site	**Substrate type**	**Field ID of Sample Replicate**	**Sea otter occupancy / sample information**	**Filter soil method**			**Norgen method**			**PowerMax method**			**PowerSoil method**
				1A	**1B**	**1C**	**2A**	**2B**	**2C**	**3A**	**3B**	**3C**	**4A**
Marina Island	Fine sand	B	Absent/negative control	N	N	N	N	N	N	N	N	N	N
	Fine sand	B	Absent/negative control spiked with sea otter tissue	N	N	N	**Y**	**Y**	**Y**	**Y**	N	N	**Y**
Spring Island	Mud	A	Established	N	N	N	N	N	N	N	N	N	N
	Mud	B	Established	N	N	N	N	N	N	N	N	N	N
Gay Passage	Gravel and mud	B	Established	N	N	N	N	N	N	N	N	N	N
	Gravel and sand	D	Established	N	N	N	N	N	N	N	N	N	N
	Gravel and sand	E	Established	N	N	N	N	N	N	N	N	N	**Y**
McKay Lagoon	Gravel	A	Established/ sample with otter scat	N	N	N	N	N	N	**Y**	N	N	**Y**
	Gravel	B	Established/ sampled fresh otter foraging pit	N	N	N	N	N	N	N	N	N	N
	Gravel	C	Established/ sampled fresh otter foraging pit	N	N	N	N	N	N	N	N	N	N

3.4 Discussion

Overall, there was much less target species detection than anticipated in both seawater and marine sediment samples, given the proximity of sea otters to sampling sites, and the evidence of recent sea otter digging where sediment was collected. From seawater, sea otter DNA was detected in one of three *Inlet* sites, where a raft of ~15 otters was present at the time of sampling, and one of six *Established* sites, even though otters were not present when we sampled. At the *Established* sites, sea otters have been routinely sighted in rafts or as individuals, during surveys, for the past >20 years (Nichol et al., 2020). Therefore, we expected to detect sea otter eDNA at the *Established* sites, even if sea otters were absent when we sampled. Our results differ from those of other studies that have found eDNA a reliable means to detect marine mammals, even when they are unable to be detected visually or are presumed absent at the time of sampling [56]. Sea otter DNA was very difficult to detect in sediment. At all the *Established,* and two of the three *Inlet* sites that we sampled, we observed ample evidence of recent sea otter foraging (i.e., sea otter foraging pits, clam shells showing evidence of otter predation;[62,135,136]). Despite this, we detected sea otter DNA in one *Established* sediment sample, in one *Established* sediment sample with sea otter scat, and failed to detect any sea otter DNA in the other six *Established* site sediment samples.

In some cases, DNA extraction kits failed to amplify *Sea otter* DNA even after the sample had been spiked with it, indicating that some kits were not well-suited to macro-organismal sedDNA detection. Once DNA has been shed into the environment, it is immediately susceptible to degradation through a plethora of factors [29]. Water temperature, pH, salinity, and UV radiation can affect DNA persistence in the environment. Microbial activity is also responsible for contributing to DNA degradation, especially in highly productive environments [24]. Successful detection of shed eDNA is affected by all these factors. Marine mammal DNA shedding rates are poorly understood, since manipulative experimental studies with marine mammals are difficult to conduct [24]. Factors like metabolic rate, body size, life history and behaviour can all affect DNA shedding [10]. Adding to this is the fact that marine mammals occupy large areas, so even once DNA has been shed, detection rates may be low due to spatial heterogeneity, and dilution of DNA molecules in the environment through particle movement driven by tides and currents. This may explain the high levels of sea otter eDNA detected in aquaria water samples, compared to the low levels of sea otter eDNA detection from field samples, even when sea otters were present within a few hundred metres of the sampling site.

Once DNA has been shed, its movement through the marine environment is difficult to predict. The nearshore coastal environments of British Columbia are well known as highly productive and dynamic places, with large tidal exchanges, upwelling, current flow, and freshwater influx all resulting in high water movement [141]. Thus, eDNA may become rapidly diluted in the water column, affecting accumulation in the sediment. Additionally, when eDNA reaches the sea floor, its retention is affected by sediment composition [118]. We were unable to control the type of sediment we sampled, given its variability across coastal BC [142]. However, across our ten sediment samples no sediment type demonstrated markedly different DNA conservation based on chloroplast and fish DNA detection. Future work could examine DNA concentrations from subtidal sediment, which would reduce the influence of changing tide heights and twice-daily exposure to weather on DNA preservation.

The results of our sedDNA extractions revealed that DNA quantity was comparable across all extraction methods, yet chloroplast and fish DNA was not detectable with the filter soil extracted samples suggesting high levels of PCR inhibitors in these samples that could not even be removed through cleaning protocols. This makes sense based off the filter soil sampling protocol where the sediment was fully resuspended in seawater and allowed to settle. This could allow for inhibitors to readsorb to DNA particles. For the other three extraction methods, chloroplast and fish DNA were consistently detected.

The eENLU6 assay has a target amplicon of 333 base pairs, which is on the long end of the recommended eDNA qPCR assay amplicon size (50 – 400 base pairs) [107,143]. Research regarding the recovery of DNA from ancient or deep core sediment samples have found that using smaller sized DNA targets, or hybridisation capture approaches with multiple target regions, can be more successful at species detection with partially degraded DNA than longer target amplicons. The extracellular DNA that is often relied on for detection of macro-organisms like fish or sea otters, is very susceptible to microbial DNA digestion, and physical degradation, which can lead to highly fragmented and shortened pieces of eDNA remaining in the sediment [118,125]. Within sediment samples, it is reasonable to expect that smaller DNA fragments are more abundant than larger DNA fragments, and in the case of hybridisation capture, if one fragment is degraded there is the potential that another region may have persisted [117,118]. Thus, investigating additional methods, better suited to targeting groups of fragments, or smaller fragments than that used in the present study may increase sea otter DNA detection from sediment. This hypothesis is supported by the higher amplification rate of fish DNA compared to sea otter DNA; the target amplicon of the eFISH1 assay was 153 bp.

The results from our sedDNA extraction experiment show that the greatest quantity of Sea otter DNA was detected from samples extracted using the PowerSoil method (Table 4). With a much smaller starting amount of sediment and no required cleaning, this kit was user friendly and produced samples with the highest copy numbers of chloroplast DNA among the methods we trialed, as well as providing high measurements of DNA quantity and fish DNA copy number estimates (Figure 13). The fact that sea otter DNA was not detected in spiked samples using the Filter Soil extraction method, or in samples cleaned after extraction with the PowerMax methods, demonstrates that the cleaning protocols we tested are not beneficial for the retention of sediment-bound DNA. Both tested cleaning steps reduced the sea otter DNA quantity and estimated IntegritE-DNA™ chloroplast, and fish DNA copy numbers across the PowerMax, Norgen and Filter Soil methods, likely because of DNA loss due to additional processing steps (Figure 13). This experiment demonstrated the importance of selecting an appropriate sedDNA extraction protocol to maximize DNA retention, and how DNA processing steps can dramatically influence detection of target species.

Within the present study we systematically tested four sediment extraction and DNA cleanup pipelines, however future work could examine a larger variety of pipelines. We did not explicitly account for sediment type, and we specifically used a targeted qPCR approach. Future studies investigating the potential of marine mammal detection in sedDNA could try multiple molecular approaches such as metabarcoding and hybridisation capture, or attempt to design a shorter target assay, and examine whether detection probabilities increase with such refinement. An experimental approach focused on spiking various sediment types with target-species DNA, would likely reveal further variables to consider or control when obtaining field samples.

Our study demonstrates that the eENLU6 assay is a highly sensitive qPCR eDNA assay that can be used to detect sea otter DNA in some contexts. The potential for assessing both historical and current distributions of marine organisms using sedDNA is highly exciting, especially where historical distributions or habitat use are uncertain. As molecular methods become more accessible, fundamental methodological experiments such as the current study will be necessary to build capacity for the use of these powerful tools.

Chapter 4: Monitoring marine pollution effects through targeted environmental DNA (eDNA) testing in the Pacific Northwest

Abstract

Globally, coastal waters experience degradation from pollution associated with multiple discharges, including industrial and agricultural runoff, and municipal wastewater. Certain benthic infaunal taxa are tolerant of high nutrient input and anoxic conditions, while others are sensitive to these conditions. Using these indicator taxa as proxies for assessing organic enrichment is well established to characterize subsequent pollution impacts. Conventional assessment of macroinfauna involves the detailed analysis of each individual specimen within a sample by taxonomic experts, a resource intensive process. Alternatively, we developed sensitive quantitative polymerase chain reaction (qPCR) assays to detect these indicator taxa in a scalable and reliable way. Using whole genome shotgun sequencing, we generated full mitogenome sequences of selected indicator taxa routinely used for monitoring programs in Pacific Northwest marine environments. These sequences were used to design new, rigorously validated environmental DNA (eDNA) assays capable of detecting low levels of eDNA - extra-organismal DNA that can be isolated from environmental samples. For nine sites at a Wastewater Treatment Plant Outfall in Vancouver, British Columbia, we tested three eDNA sample collection types: active filtration, a passive dip filter from water containing collected macroinfauna, and active filtration from water collected near the sea floor. Generalized linear models indicated that eDNA signal strength correlated with organism count particularly with passive dip sample collection type. eDNA occupancy modelling techniques estimated

detection probabilities corresponding with organism count. The present study emphasizes the value of integrating eDNA into marine outfall monitoring efforts to enhance the assessment of environmental effects.

4.1 Introduction

Globally, coastal waters experience degradation from pollution associated with multiple discharges including storm water, industrial and agricultural runoff, and municipal wastewater [144]. Organic enrichment from wastewater and industry can contribute to eutrophication of coastal environments resulting in algal blooms, increased sedimentation, depletion of bottom water oxygen, and dramatic shifts in the community structure, and/or death of benthic organisms [67,145]. The protection of marine habitats requires appropriate means to detect environmental effects accurately and consistently.

Despite challenges in drawing generalizations across diverse habitats, the responses of benthic invertebrate communities to organic inputs have some striking parallels worldwide. Certain opportunistic or tolerant taxa, usually polychaetes, proliferate in response to high nutrient input and corresponding anoxic conditions and thrive in polluted environments [146]; while sensitive taxa, such as some echinoderms and amphipods, may decline in their presence [147]. Meanwhile, unaffected taxa maintain relatively stable populations within a range of organic enrichment levels (barring extreme conditions). Macroinfaunal benthic invertebrate assemblages (i.e., invertebrates that live in soft sediment sea bottoms, and are >0.5mm) have long been the bioindicator of choice as their intimate association with sediments, relatively sedentary lifestyles, long lifespans, lack of marked seasonal variation in deeper habitats, and differing taxon-level tolerances to pollution make these organisms powerful and reliable sentinels of anthropogenic stress [67,146,148]. Accordingly, marine monitoring programs, such as those required for municipal outfalls, include an assessment of infaunal benthic macroinvertebrate communities to discern potential long-term effects of wastewater effluent. As a result, infaunal invertebrate taxa with strong responses to organic enrichment – both sensitive and tolerant – are relatively well known [145], particularly in regions where monitoring has been in place for a long time.

Conventional assessment of macroinfaunal benthos involves the microscopic identification of each individual specimen within a sample by taxonomic experts. This activity is labour intensive, requiring both the meticulous removal of organisms from the sample debris, and their identification and enumeration by highly trained personnel [149]. The resulting data yields powerful insights into coastal ecosystem health. However, both spatial and temporal resolution of monitoring surveys are limited due to the cost, time commitment, and skill required for taxonomic analysis, particularly for small local governments. In addition, data resolution is limited by specimen quality, maturity, and potential damage (and subsequent loss of identifying features) during collection. Coastal communities would therefore greatly benefit from the development of an accurate complementary tool that is faster, more efficient, and less expensive than conventional taxonomic analyses.

Environmental DNA (eDNA) is “genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material” [4,95]. In general, eDNA methods involve the collection and preservation of environmental samples, followed by DNA extraction and detection. As an efficient and non-invasive sampling approach, measurement of eDNA is rapidly becoming a popular method of taxon detection compared to conventional capture- and time-constrained surveying methods. Furthermore, eDNA methods do not require extensive training for sample collection, meaning that sampling is highly scalable and can provide valuable complementary data to conventional datasets [14,115,139,150,151].

Based on the ecological questions being posed, there are multiple eDNA approaches that can be used [152]. Within the present study, we focused on the use of eDNA for targeted taxa detection using quantitative polymerase chain reaction (qPCR). qPCR assays, when properly designed and validated, are extremely powerful molecular tools that are able to detect, and in some cases provide relative target taxon abundance estimations, from minute quantities of target taxon DNA in the environmental matrix [7,153]. Taking advantage of the long history of known indicator taxa responses and distributions at major municipal wastewater outfalls in British Columbia (BC), we selected a subset of original indicators for which we successfully obtained mitogenome sequences and subsequently designed and validated targeted eDNA assays according to the Canadian standards [107,143]. All chosen indictors were polychaetes (Annelida). *Glycera nana* (Glyceridae) and *Prionospio lighti* (Spionidae) were chosen as control (unaffected) taxa, since they were equally abundant regardless of proximity to discharge. *Heteromastus filiobranchus* (Capitellidae) and *Ophelina acuminata* (Opheliidae) were chosen as pollution-tolerant indicators, due to their proliferation in near-field stations, and *Decamastus* sp. nr. *gracilis* (Capitellidae; sp. nr indicating it conforms to this species with some variation and differences in distribution) was chosen as the pollution-sensitive indicator due to its apparent intolerance to pollution related conditions, such as low levels of oxygen.

These novel validated eDNA assays were used to test a series of environmental samples collected alongside samples processed with the conventional approach of taxonomic identification and enumeration. These experiments were conducted in conjunction with the ongoing receiving environment monitoring program for Metro Vancouver's largest primary treatment plant, which services a population of almost one million people. This wastewater treatment plant (WWTP) discharges about 500 million litres of treated wastewater per day through a deep-sea outfall adjacent to Sturgeon Bank, in the southern Strait of Georgia on the west coast of Canada, within the Salish Sea. This monitoring program has been conducted annually since 2001 and is comprised of a well characterized north-south arrangement of stations that experience decreasing pollution effects with increasing distance from the outfall modulated by the strong northward current [67]. This long history and straightforward survey design make the receiving environment of this outfall well suited to this experimental work. Nine stations were chosen, each previously being classified as one of "Near Field", "Mid Field" and "Far Field" zones based on their proximity to the outfall. We hypothesized that our eDNA assays would be able to detect and produce relative abundance estimates of our selected target taxa that correlated with the previous ecological knowledge of their distribution.

The use of molecular methods to estimate organismal abundance in nature is of high interest in the eDNA field, since abundance is a fundamental parameter in understanding taxa distribution [9]. Since the qPCR assays used in the present study are very sensitive, we wanted to take advantage of the paired study design by linking organismal counts of our indicator species to eDNA copy number estimates. To achieve this, we used a generalized linear modelling approach to approximate a relationship between the two parameters. Our objectives were to: 1) develop and validate novel eDNA tests for taxonomically verified voucher specimens of target indicator infaunal benthic macroinvertebrates; and 2) to create a robust, standardized eDNA field sampling approach for marine coastal community assessments using these indicator invertebrates. Over the four years of this project, we conducted multiple experiments pairing conventional benthic infaunal surveys with a variety of eDNA sampling types. We developed assays for our key indicator taxa and devised metrics for understanding the qPCR outputs. Our hope is that these assays can act as a toolkit for managers monitoring pollution impacts in the Pacific Northwest region of North America.

4.2 Methods

4.2.1 Indicator Species Selection

Various indicator species have been identified by the ongoing wastewater treatment plant outfall receiving environment monitoring program. This monitoring program has been conducted annually since 2001 and includes an annual assessment of the benthic infaunal macroinvertebrate community structure and monitoring of important indicator species.

Potential tolerant and sensitive indicator and control species were selected through a combinatorial process including a review of historical reports and compilation of data from 2001-2020. Preference for continuously distributed larger, abundant, soft-bodied organisms more likely to shed DNA. Indicator species were also selected based on their well-documented or presumed biological responses to organic loading from wastewater or nutrient enrichment [67,154].

Control (unaffected) taxa were selected based on high occurrence (in at least 95% of conventional samples), moderate abundances (mean of 5-100 individuals per station/year), and low to moderate variation (coefficient of variation <200%).

For all selected species, their distribution around other wastewater outfalls in the Salish Sea were also considered, to provide for broader-scale applicability of the selected indicator taxa [155,156].

4.2.2 eDNA Assay Design and Validation

Due to the obscurity of the selected indicator taxa and their lack of representation in public gene sequence databases, we generated whole mitogenome sequences through whole genome shotgun sequencing of voucher tissue specimens and subsequent mitogenome assembly with the *mtGrasp* (version 0.2.0) pipeline [109] (Appendix 10). These sequences were then compiled and supplemented by as many related non-target sequences already available on the National Center for Biotechnology Information (NCBI) GenBank [108] in addition to dog, cat, and human sequences as these are common sources of contamination from field and laboratory workspaces. All mitogenome sequences were aligned with MAFFT version 7.310 using default settings [157] and visualized using Geneious Prime (version 2202.1.1, Biomatters Ltd, Auckland, New Zealand). The full lists of all mitogenomes used in the present study and their NCBI accession numbers can be found in Appendix 11.

Unique mitogenome regions for subsequent assay design were identified using *unikseq v0.1.0* [90]. These regions were used to identify high scoring assay candidates comprised of forward/reverse primers and a probe selected using Beacon Designer 8.21 (Premier Biosoft

International, San Francisco, CA, USA). Candidate assay primer and probe sequences were queried against the NCBI non-redundant (nr) sequence database to ensure no other possible confounding taxa DNA would be amplified using the assays. From this point on assays were validated as described previously [7]. Reporting and performance characteristics met or exceeded the two Canadian national eDNA standards [106,107]. The assays were then tested against multiple voucher specimen-derived gDNA samples of target and non-target species to establish assay specificity (Appendix 12 -16). Standard curves were generated per assay using gBlocks® synthetic DNA obtained from Integrated DNA Technologies (Coralville, Iowa, United States) using methodology described previously [110]. The eLowQuant [11] method was used to generate the limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ) [105].

4.2.3 Sample Collection

We conducted four field experiments around the outfall in Vancouver, BC (Figure 14). The eDNA sampling protocol was designed so that it was easy to integrate into the conventional sampling protocol that is used for municipal wastewater treatment plant surveys on the BC Coast. This conventional sampling protocol was comprised of benthic sediment collection using a Van Veen grab (0.1m^2 sample area) deployed from a vessel at each sampling station. This sediment was then rinsed through screens (1.0 mm mesh size) and the organisms retained were preserved in 10% formalin(v/v) for subsequent identification and quantification. Upon arrival at the laboratory, samples were transferred into 70% ethanol (v/v) and stained with Rose Bengal. Trained technicians sorted all benthic invertebrate specimens into major taxonomic groups using dissecting microscopes at 10-40X magnification. Each sample was fully examined microscopically, and quality assurance procedures ensured >95% efficiency in organism removal. Organisms were then identified by qualified taxonomists to the lowest practicable taxonomic level using both dissecting (10–40X) and compound microscopes (100–1000X) with standard keys [158-161]. Mature specimens in good condition were generally identified to genus or species. Taxonomic characters used to identify species vary among families of polychaetes and include characteristics of body differentiation (numbers of anterior/posterior segments), gill morphology, chaetal morphology, and anterior features of the head (prostomium and associated segments). Taxonomic authorities include The Southern Association of Marine

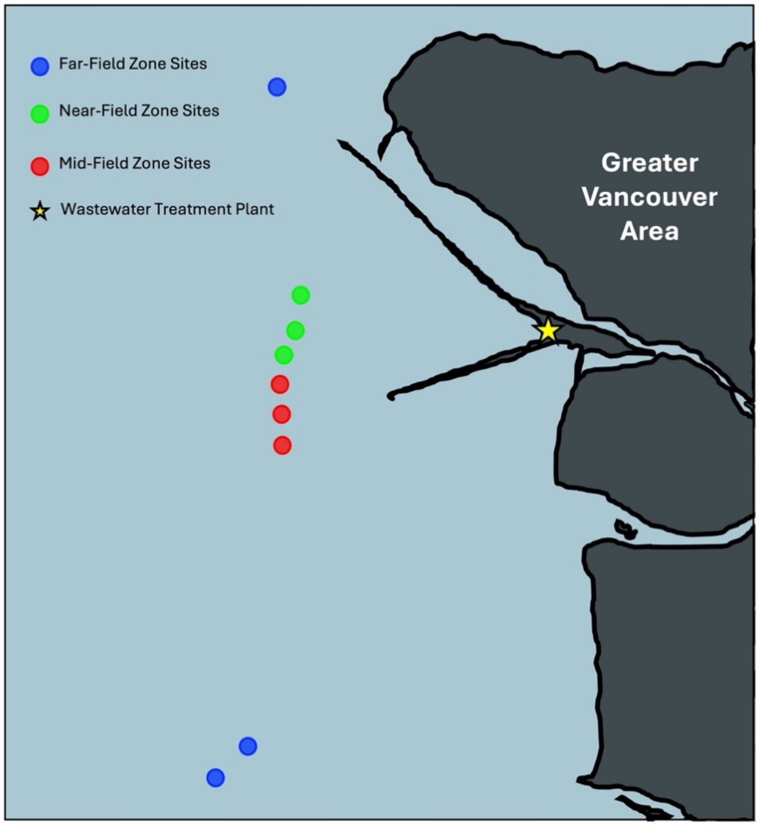


Figure 14. Map of sampling stations at the WWTP Outfall in Metro Vancouver, BC. Representative stations chosen for this study are indicated by color corresponding to zone.

Invertebrate Taxonomists (SCAMIT; scamit.org) and the World Register of Marine Species (marinespecies.org). Macroinvertebrates in British Columbia are poorly described and some species conform clearly to species descriptions while some do not, which are indicated by "sp. nr" or similar monikers.   A voucher collection was kept and maintained at Biologica Environmental Services, Ltd., with all specimens externally verified by an independent taxonomist.

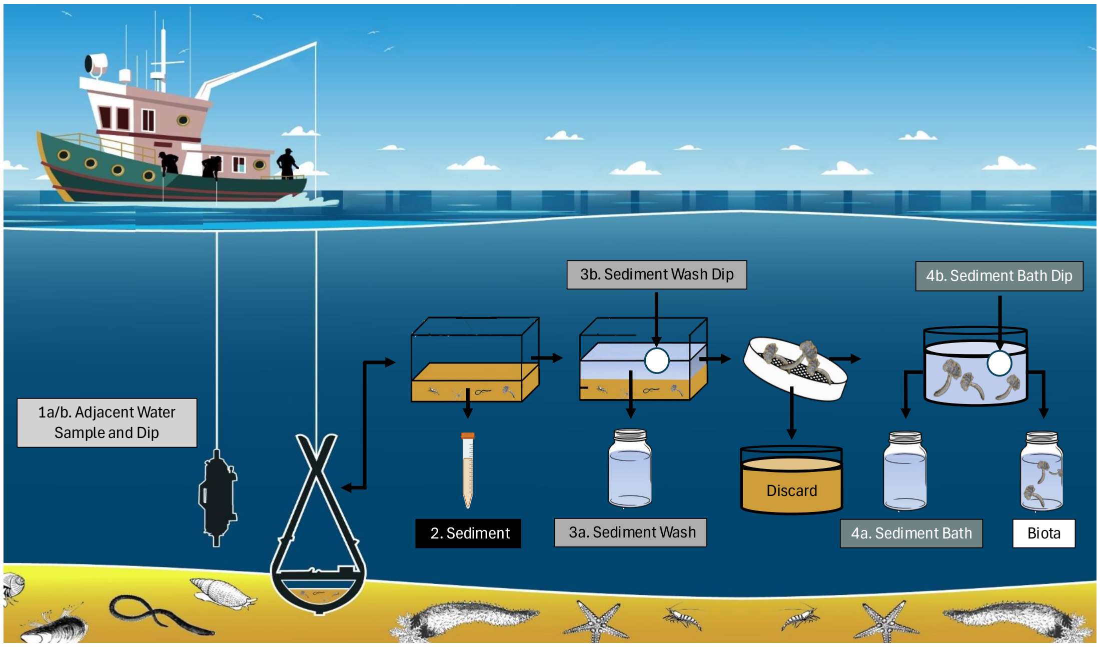


Figure 15. Infographic depiction of pilot field protocol. Conventional sampling consists solely of the Van Veen grab, and the removal of biota. The eDNA sampling pipeline was designed to fit within the scope of existing field protocols. Of all sample types tested, only Adjacent Water, Sediment Bath, and Sediment Bath Dip sample types were continued over the course of all four field experiments.

The first experiment tested different sampling protocols for maximizing eDNA output from these grabs (Figure 15). Once the grab was collected, 10 g sediment was immediately flash-frozen for sedimentary eDNA (sedDNA) (*Sediment)* extraction using the DNA extraction method described below. The remaining contents of the grab were then transferred using a small amount of Instant Ocean into a plastic 25 L tote containing 10 L of

Instant Ocean, a premixed solution of sea salt and deionised water (Spectrum Brand, Blacksburg, VA) and the sediment was manually stirred for 1 min. At this "wash stage" a dip filter was taken (*Sediment Wash Dip*). These dip filters were a 3 cm^2 square of positively charged nylon Zetaprobe membrane (BioRad, Mississauga, ON, Canada) that should theoretically attract negatively charged DNA particles. Each membrane square was placed into a resealable plastic bag into which 1 cup (254 mL) of sample water was placed. The bag was shaken for 3 min, after which the dip filter was removed and immediately placed into a prelabelled manila envelope and stored in another resealable baggie containing silica bead desiccant [162]. All samples were stored at -20°C until DNA extraction.

Following the collection of the *Sediment Wash Dip* sample, the sediment in the tote was allowed to settle for about five min and a 1 L water sample was taken from the supernatant (*Sediment Wash*). The remaining contents of the tote were then sieved through a 1 mm metal mesh frame so that all organisms could be isolated. These organisms were combined with 5 L of Instant Ocean and allowed to "bathe" for approximately 10 min. Another dip filter was taken at this point (*Sediment Bath Dip*) plus a 1 L water sample for filtration (*Sediment Bath)*. The sieve residue (debris and organisms) was then preserved in 10% formalin (v/v) for traditional morphological analysis.

In addition to the above samples, we were also interested to see whether a water sample taken very close to the sea floor, adjacent to the Van Veen grab site, could reflect the ecological community presented in the sediment. Thus, at each station where a sediment grab was taken, a 5L Niskin water bottle was also deployed to approximately one meter above the seafloor. A dip filter sample was taken from this water (*Adjacent Water*

Dip) and 1 L of water was collected from the Van Dorn bottle for filtration (*Adjacent Water*). This process was repeated at all nine stations (Figure 14).

4.2.4 DNA Extraction Methods

The *Sediment* samples were extracted from 10 g (wet weight) sediment using the Norgen Soil DNA Isolation Maxi Kit (Norgen Biotek Corp., Thorold, ON, Canada; Cat# 62000) with the final elution performed in 3 mL elution buffer.

The 1 L water samples (*Sediment Wash, Sediment Bath,* and *Adjacent Water*)*,* were vacuum filtered within 24 h through a 0.45 µm mixed nitrocellulose filter (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) and the filters stored in prelabelled manilla envelopes placed in resealable plastic bags containing silica desiccant [162]. The samples were stored at -20°C until DNA extraction.

One quarter of each filter from either the water samples, or the dip filter samples were then extracted using the DNeasy Blood and Tissue kit with a final elution volume of 150 µL (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506).

4.2.5 qPCR Analyses

Before testing the eDNA samples for presence of our indicator taxa, the DNA sample integrity was assessed using the IntegritE-DNA® test [110,139]. This test ensures that the eDNA samples are viable and no inhibitors or degradation were present that would affect qPCR analysis. Each sample was tested with the IntegritE-DNA® test with four technical replicates using the QIAcuity Probe PCR kit (QIAGEN Inc. Mississauga, ON, Canada, Cat # 250102). DNA amplification thermocycler conditions were an initial activation step of 2 min at 95°C followed by 50 cycles of 15 s denaturation at 95°C, 30 s annealing at 64°C, and 45 s extension at 72°C. Each plate was run with eight NTCs and two positive controls of the appropriate target synthetic DNA at a concentration of 20 copies/reaction. Any samples that failed this test (average qPCR C_t values > 30) were cleaned with a OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA, Cat #D60303) and retested. Samples that did not pass this threshold were removed from analysis due to poor quality (Appendix 17). Those that did pass the IntegritE-DNA® test were then tested with one tolerant, one sensitive, and one control indicator species (eDEGR2, eHEFI7, ePRLI4), with eight technical replicates using the amplification conditions described above. These qPCR results were converted into an estimate of DNA copy number/L using standard curves generated from continuous and binomial data using eLowQuant [11].

Based on the results of experiment 1 (Figure 16), we eliminated the *Sediment, Sediment Wash, Sediment Wash Dip* and *Adjacent Water Dip* samples due to the high degree of PCR inhibition and subsequent lack of target DNA amplification observed from those samples. Thus, for the second experiment, in the fall of 2022 we focused on the *Sediment Bath, Sediment Bath Dip,* and *Adjacent Water* samples, and increased sample replication. Three 1 L *Sediment Bath* samples and three 1 L *Adjacent Water* samples were taken at each of the nine stations. Unfortunately, we were unable to integrate dip samples during this experiment due to product shortages linked with the COVID-19 pandemic. However, in the third and fourth experiments in the spring of 2023 and 2024, respectively, we reintegrated bath dip samples. These samples were all processed as described above and tested for all five indicator taxa described herein. A 1 L negative Instant Ocean control was also taken on each field sampling day throughout all experiments. A summary of all samples taken appears in Appendix 18.

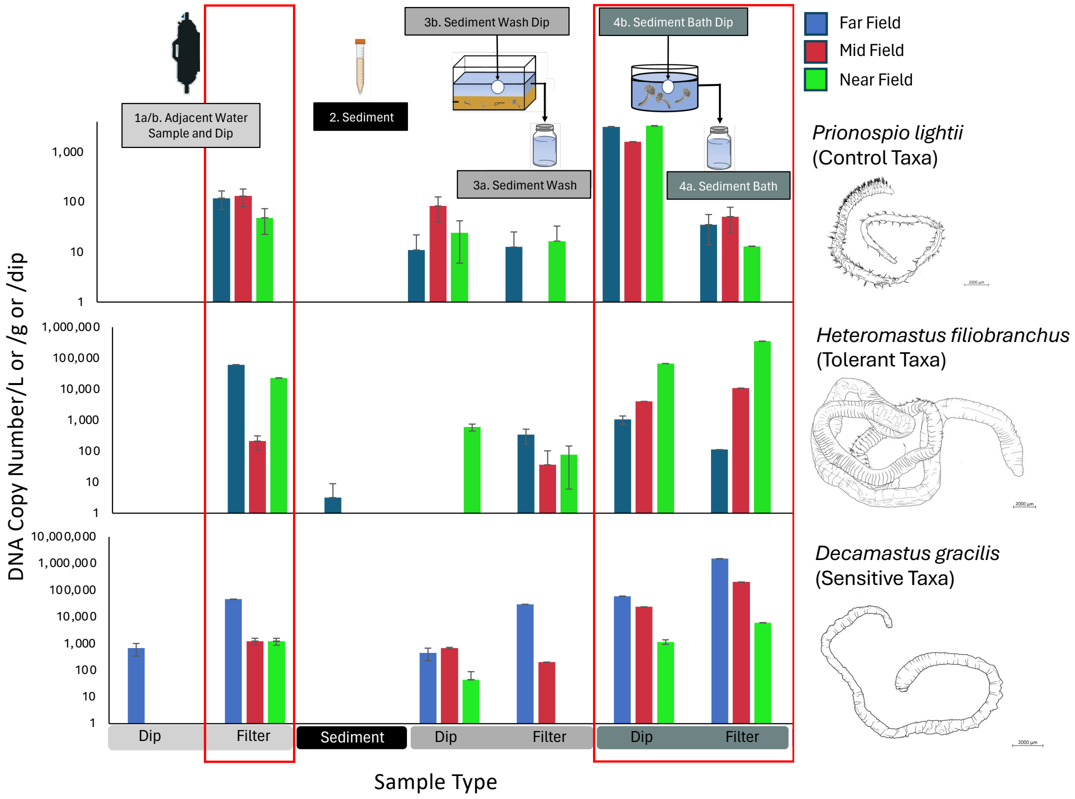


Figure 16. Pilot experiment results depicting eDNA copy number estimates for different eDNA sampling types for three different indicator taxa. Sample types chosen to continue with for subsequent experiments indicated with a red box.

4.2.6 Statistical Analyses

All data analyses were done with R, version 4.2.3, within the R Studio© environment. Data from all experiments were grouped since seasonality is not expected to influence adult taxon distributions more than inter-annual variation, especially for organisms >1.0mm . To determine whether the eDNA signal strength for the target species reflected the expected ecological pattern based off zone proximity to outfall, we further grouped data by zones and compared them to each other. After determining that requirements for normality and homogeneity were not met, non-parametric analyses were carried out. The Kruskal-Wallis test was used to determine whether there were significant differences between zones. Post-hoc analysis between zones was done using the Wilcoxon Signed

Rank test. These analyses were done for each sample type (*Sediment Bath, Sediment Bath Dip, and Adjacent Water*) and target taxa.

We investigated the relationship between eDNA signal strength (log-scale eDNA copy number) and species abundance using a generalized linear model (GLM) using the *MASS* package in R. Three separate GLMs were run independently for the three eDNA sampling methods, and an interaction term between eDNA copy number and taxon was used as the predictor to reflect the hypothesis that the relationship between eDNA signal strength and abundance is taxon specific. We tested both a Poisson and negative binomial distribution to describe the data-generating process for the species abundance and selected the most parsimonious model using AIC. We evaluated the significance of the predictor variables by calculating the 95% confidence interval with standard errors of the interaction terms.

We then used the software *eDNAjoint* to quantify the probability of a false-positive eDNA detection and compared the relative detection rates of both grab and eDNA sampling for the two least abundant species in the present study, *O. acuminata* and *G. nana* [163]. *eDNAjoint* jointly models binary eDNA detection/non-detection data and conventional, non-genetic observations in a Bayesian framework, quantifying uncertainty in both survey methods. We used paired eDNA detection and grab count observations of *O. acuminata* and *G. nana* at the nine sites to estimate the mean (expected) grab rate at each site and the false positive probability of eDNA detection, *p10*, using the joint model. We specified the prior distribution for *p10* in the model such that most of the probability density is less than 0.1 (beta distribution shape parameters: 1, 75; P(*p10* < 0.1) = 0.99), conservatively reflecting the lack of observed false positive detections in negative laboratory and field blank controls. To quantify the relative detection sensitivities of eDNA and grab survey methods, we used the model's posterior estimates to calculate the number of eDNA and grab samples necessary to detect presence with 90% probability.

We used the R package *eDNAOccupancy* [164] to generate multi-scale occupancy models to estimate posterior summaries of occurrence and detection probability of target taxa with our eDNA assays and sample types. We fit a series of multi-scale occupancy models. The first included no covariates, the second used organism count as a covariate of eDNA occurrence in samples (θ), while both the probability of eDNA occurrence at a site (φ), and probability of eDNA detection in a qPCR replicate (*p)* were assumed to be constant. For the third model we used organism count as a covariate informing both θ and *p.* Finally, since we have decades of data of indicator species occupancy at sites, we added the calculated probability of organism occurrence at each site to inform φ for the final model. For each taxon and sample type we compared the models using PPLC and WAIC as our model selection criteria.

4.3 Results

4.3.1 Indicator Taxa Selection

A list of potential indicator taxa was generated through examination of historical data, and was further narrowed due to sequencing and assay design success. The final set of indicator species used in the present study included: *Heteromastus filobranchus* (Polychaeta, Capitellidae; pollution-tolerant indicator); *Decamastus* sp. nr. *gracilis* (Polychaeta, Capitellidae; pollution-sensitive indicator); *Prionospio lighti* (Polychaeta, Spionidae; control indicator); *Ophelina acuminata* (Polychaeta, Opheliidae; tolerant indicator); and *Glycera nana* (Polychaeta, Glyceridae, control indicator) (Figure 17).

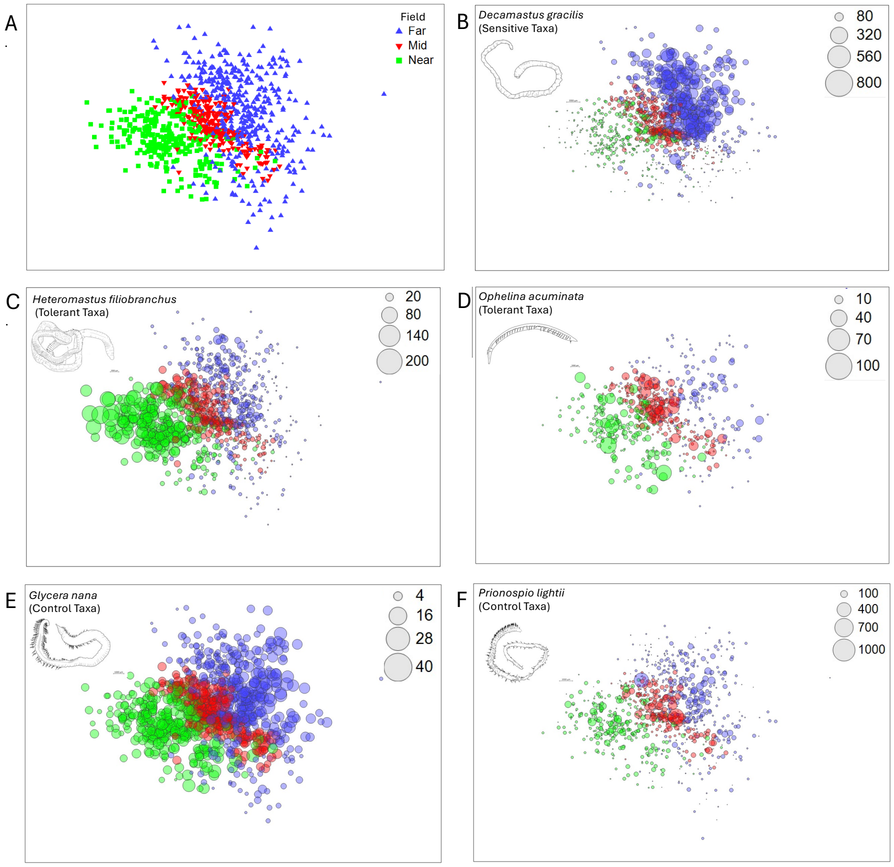


Figure 17. Non-metric multidimensional scaling (nMDS) plots representing benthic community structure across three zones within the WWTP receiving environment from 2001 to 2020. Analysis and plots were produced using PRIMER 7 software (PRIMER-e, Plymouth, UK). **A)** Non-metric multidimensional scaling (nMDS) plot showing all samples as points, color-coded by sample zone: blue for "Far," red for "Mid," and green for "Near." Data include samples from 16 stations along the 80 m depth contour in the WWTP receiving environment from 2001 to 2020. A fourth-root transformation was applied to species abundances, and the Bray-Curtis similarity was used as the distance metric. The 2D stress value is 0.24, indicating the fit of the ordination. **(B-F)** nMDS plots overlaid with species-specific abundances for the five benthic taxa of interest. Abundance is represented by point size, with sample zones colored as in panel **A**. **B)** Sensitive Taxa: *Decamastus* sp. nr. *Gracilis*, **C)** Tolerant Taxa: *Heteromastus filobranchus*, **D)** Tolerant Taxa: *Ophelina acuminata*, **E)** Control Species: *Glycera nana*, **F)** Control Species: *Prionospio lighti*.

4.3.2 Assay Validation

Due to the obscurity of the selected target taxa and their subsequent lack of representation in sequence databases, we sequenced their mitochondrial genomes using voucher specimens. However, because these organisms are so tiny, this process proved difficult. The successfully assembled mitogenomes can be found in Supplementary Table S1. The mitogenomes range from 13,689 base pairs to 20,410 base pairs long. We obtained at least one full mitogenome for each indicator taxa, and for many co-occurring relatives. The final validated assays were extremely sensitive with LODs ranging from 0.2-1.2 copies/reaction, LOQs ranging from 0.9-4.6 copies/reaction, and amplification efficiencies ranging from 82 - 102% (Table 5) [11]. Target assay specificity can be seen through amplicon alignments with co-occurring and potentially confounding species (Figure 18-22). For all taxa except for *O. acuminata* the assay region is conserved across all individuals of the target taxa. For *O. acuminata,* there is one base pair difference in the reverse primer binding region at ten base pairs away from the 3' end. To ensure that this did not affect assay performance we relied on empirical verification with gDNA from voucher specimens. This process was also especially important for the validation of the *D. gracilis,* and *H. filiobranchus* assays, since we only have one sequence for each species. For these taxa, we tested the assay on 4 and 6 individual voucher specimens respectively for which all amplified as expected (Appendix 12 and 14)

Table 5. Target assay sequences, molecular characteristics, and calculated performance parameters. Probes have the FAM fluorophore on the 5’ end and ZEN/IBQ quencher on the 3’ end.

Target Taxon		*Decamastus gracilis*	*Heteromastus filiobranchus*	*Ophelina acuminata*	*Glycera nana*	*Prionospio lighti*
Pollution Indicator Response		Sensitive	Tolerant	Tolerant	Control	Control
eDNA qPCR Assay name		eDEGR2	eHEFI7	eOPAC1	eGLNA3	ePRLI4
Optimal T_a (°C)		61.9	60.06	57.3	54.1	56.8
Amplicon Size (bp)		348	363	190	76	222
Assay Component Sequences (5’ → 3’)	**Forward Primer**	CGCCTATCATCCTATTCTG	CGGACTTCATGTAATTATCG	CAACCATATAGGATGAATACTA	ACTGCTATTCAACCAAAC	TATACTTCAACGGCAACA
	Reverse Primer	CTCAACAACGACACCATC	TATTCTTACTTCAGGCACATA	GAGGATAAGGATTGAGGA	GAAGCGTAATTGTTGTTATAG	ATCTGACCCTGTAATTCG
	Probe	CGAACCAGCAGACTACACAGATACAC	AACCTATTCCGAACTTATTGCTGCCAT	TCGTCATCCGCCATTGCCTA	TCCTGACGCCTACAATCGCC	TTGCTAAGGCTAATGCTGCTTCACA
	Amplicon	CGCCTATCATCCTATTCTGGAGATTGTTGGCGGGCGTAAGCTTGTTGACAGTACTCTGCATTGCGGTATATTTTTACGCAAGTCCTCGTTTTAATGACGAAAAAATAAACACTCCTGTACACGCGTCTGGCGATACGTCACCAGACTCTCTGGCGAAGAAGAAATAGGTCTAATAGACCTCGGATACCTTGGTGGAAGCGCTTGGTGCGGCTTGCTTGTAAGGGTGTTTCATTCTTAGGGCGGGGGTTTCTTTCTTTGGCGCGGGTCGTTGTCGCCCTAGACTTCGTTTGGGTTGACCTTTGGCTAATCGTCGCAGTGTGGTTGTGTATCTGTGTAGTCTGCTGGTTCGGGGAACCGGTGGGTCAGGATGGTGTCGTTGTTGAG	CGGACTTCATGTAATTATCGGCACTCTCTTCCTTTCCGCCAACCTATTCCGAACTTATTGCTGCCATTTTTCACCTGGCCACCATTTAGGGTTCGAATTCGGAGCATGATACTGACATTTTGTCGACGTCATCTGAATCTTCTTATACCTATTCCTTTATTGCTATAGCGCATGAGGCGCATAACCCCACTTCTCATGACACTGTCTCCCCTCACGAAAATACTACGCCACGTAACTCACCTTCCCGCCCCACGTAACCTATCAACACTCTGAAACTTTGGGTCCCTATTAGGCCTCTGCCTCGGAATCCAACTAGTAACTGGGCTATTTCTCGCAATACATTATGTGCCTGAAGTAAGAATA	CAACCATATAGGATGAATACTAGCCGCAAGAATCTCGTCATCCGCCATTGCCTACTCATACCTTTTTATTTATATTCTTATGGTTTTTCCGCTTATACTCTCCTTTTCAAAACTGGCCATGAAACCCCTATCAACCCACGAATCCCTTATATGAAACAATCCCTCACACCGATCCTCAATCCTTATCCTC	ACTGCTATTCAACCAAACCAGCAAATTAAAATCCTGACGCCTACAATCGCCGCGACTATAACAACAATTACGCTTC	ATACTTCAACGGCAACATGTGCTTATGTGCCTACTTACACTAGAAGCCGCCCTCTTATCCTTGGCATTTATTATAGGTGCCACTACCTCTTCCTACAACATAATTAACATATTCTTTTGTATTGTTATTTTAACATTAGGAGCATGTGAAGCAGCATTAGCCTTAGCAATCCTAGTGAATGTAACACGAATTACAGGGTCAGAT
Continuous Data where Ct >95%	**Slope**	-3.6925	-3.8319	-3.4653	-3.3553	-3.2798
	% Efficiency	87	82	94	99	102
	Y-Intercept	41.052	39.826	37.707	35.345	37.729
	R2 Value	0.9983	0.9898	0.9995	0.9998	0.9999
Binomial Data	**LOD (95% CI)**	1.2 (0.9-2.1)	0.7 (0.5-1.1)	0.5 (0.4-0.9)	0.2 (0.2-0.4)	0.3 (0.2-0.6)
	LOQ (95% CI)	4.6 (3.2-8.1)	2.8 (2.1-4.2)	2 (1.4-3.3)	0.9 (0.6-1.6)	1.2 (0.8-2.2)
	LOB	0	0	0	0	0

Figure 18. Gene sequence alignment of *Decamastus gracilis* assay amplicon using sequences of target, and related and co-occurring taxa for which sequence information was available, as well as human, cat and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. The length of alignment may be longer than amplicon sequence due to gaps in the alignment.

Figure 19. Gene sequence alignment of *Heteromastus filiobranchus* assay amplicon using sequences of target, and related and co-occurring taxa for which sequence information was available, as well as human, cat and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. The length of alignment may be longer than amplicon sequence due to gaps in the alignment.

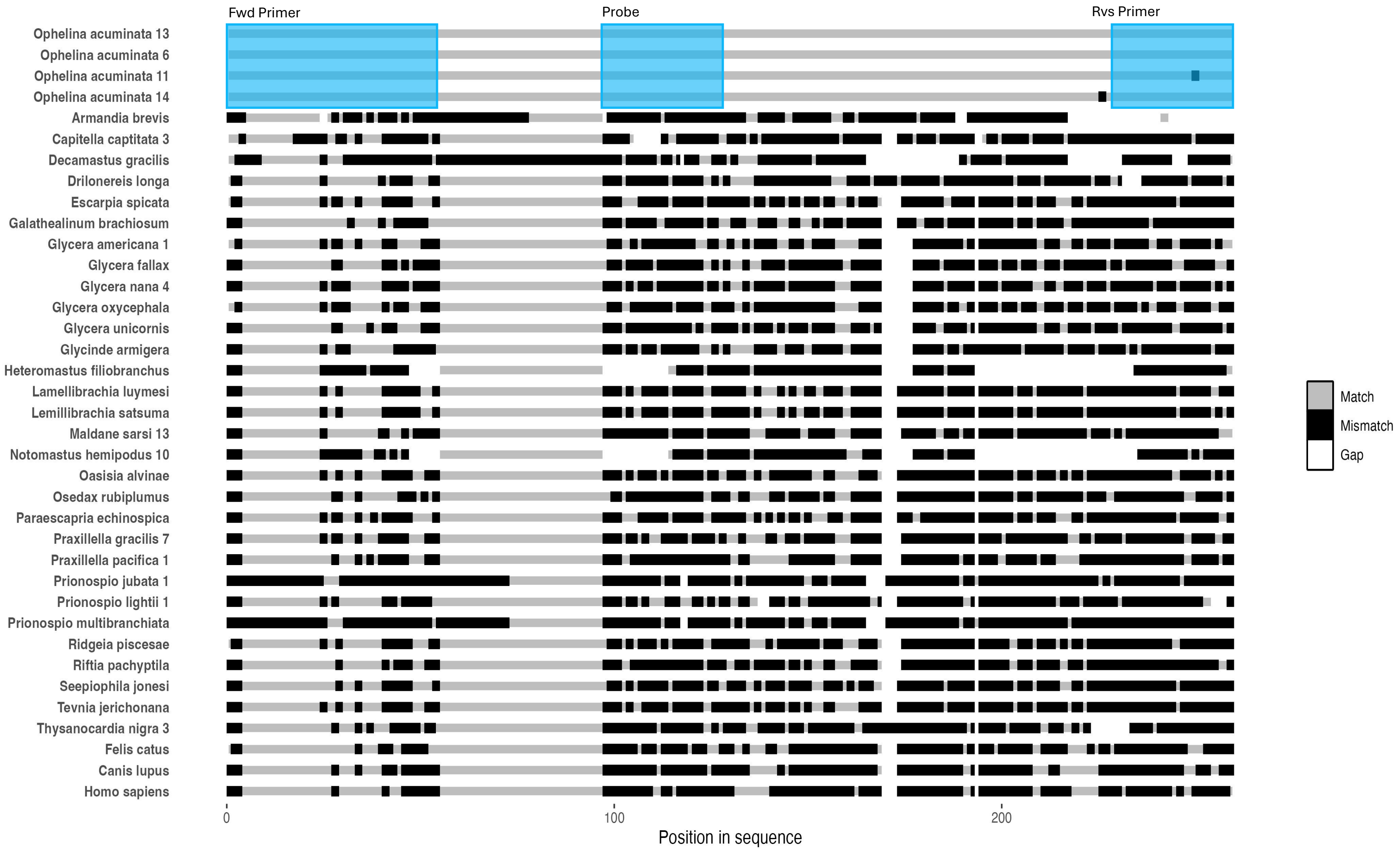


Figure 20. Gene sequence alignment of *Ophelina acuminata* assay amplicon using sequences of target, and related and co-occurring taxa for which sequence information was available, as well as human, cat and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. The length of alignment may be longer than amplicon sequence due to gaps in the alignment.

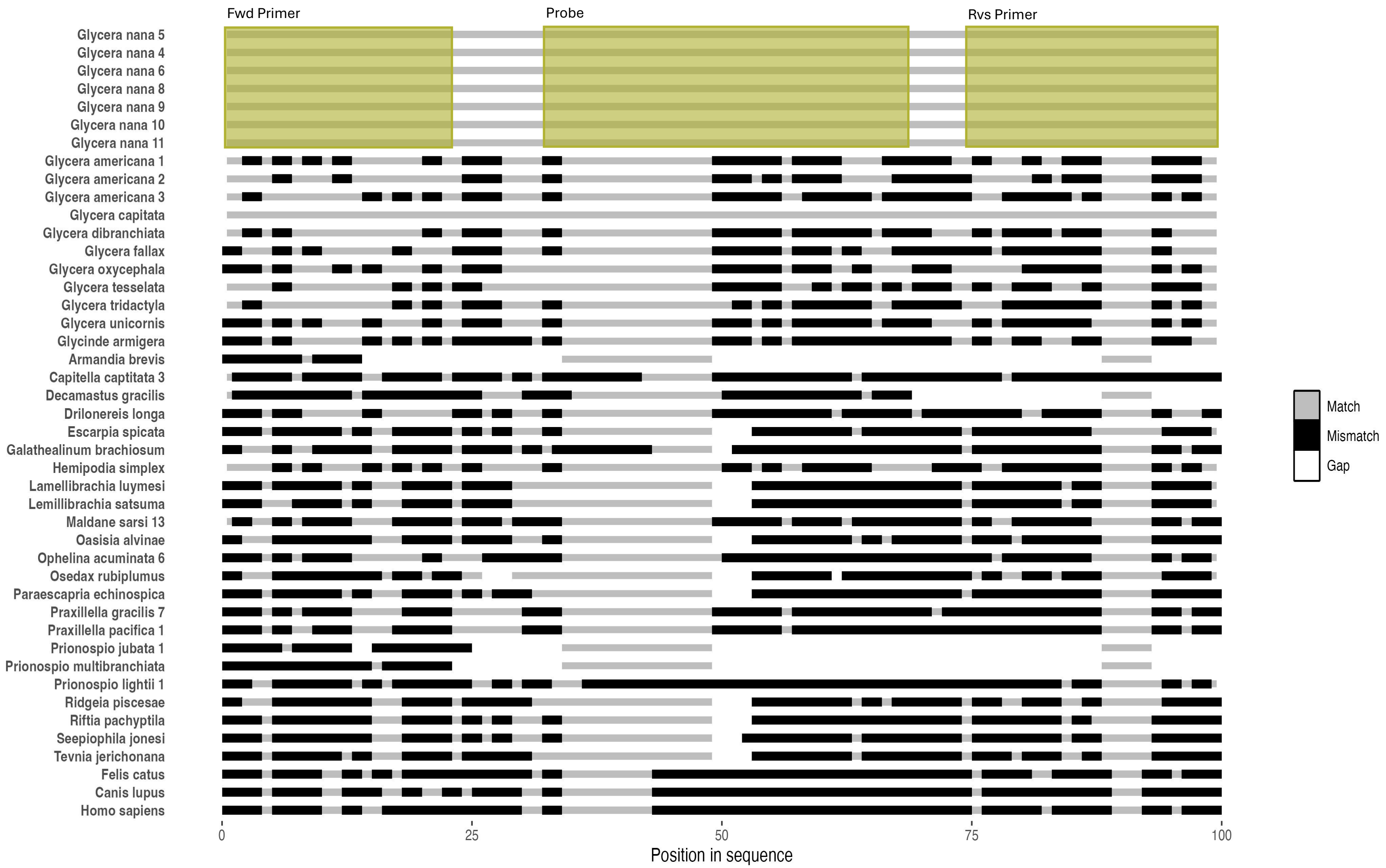


Figure 21. Gene sequence alignment of *Glycera nana* assay amplicon using sequences of target, and related and co-occurring taxa for which sequence information was available, as well as human, cat, and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. The length of alignment may be longer than amplicon sequence due to gaps in the alignment.

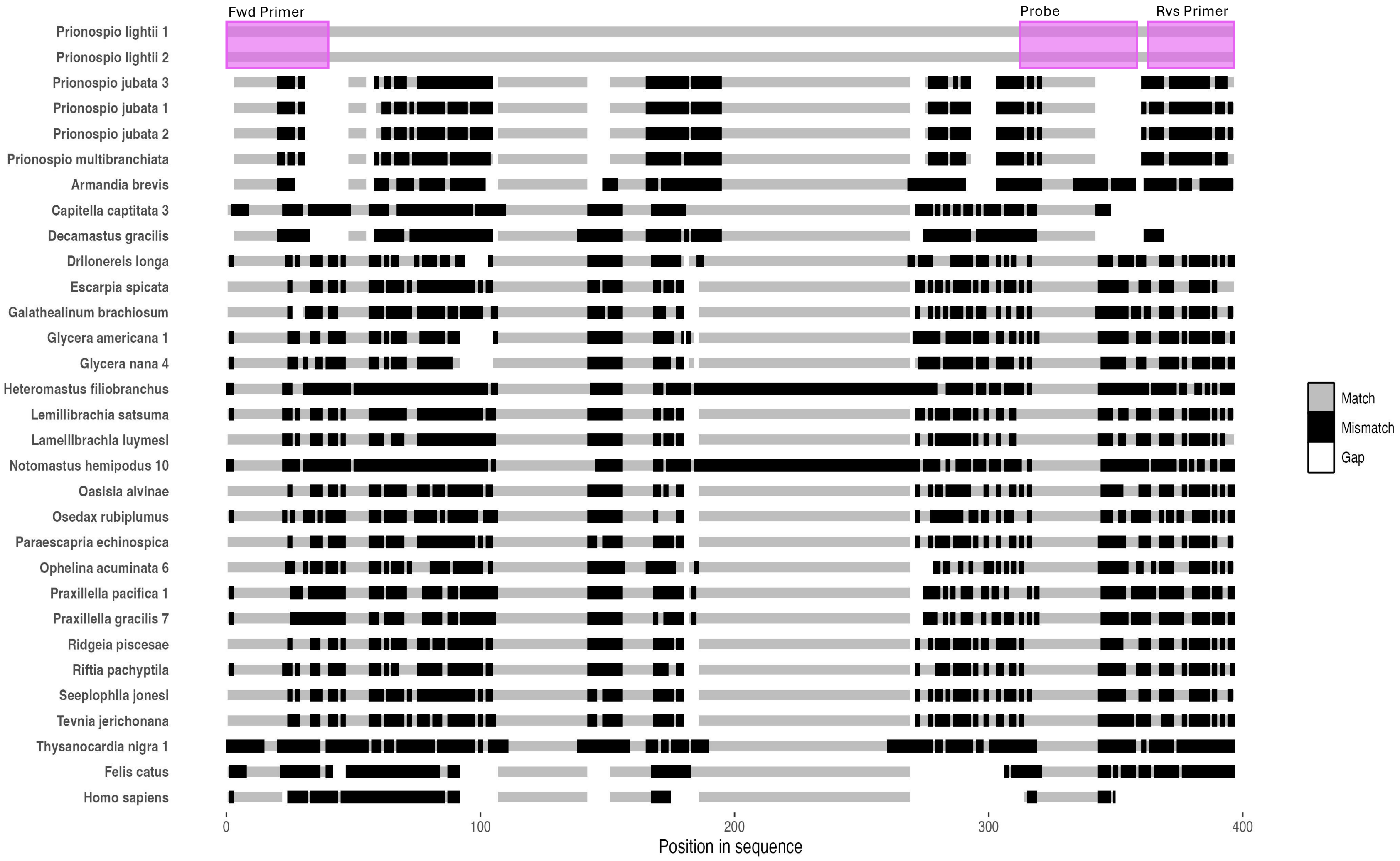


Figure 22. Gene sequence alignment of *Prionospio lighti* assay amplicon using sequences of target, and related and co-occurring taxa for which sequence information was available, as well as human, cat and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. The length of alignment may be longer than amplicon sequence due to gaps in the alignment.

4.3.3 Field Experiments

There were significant differences between zones for the *Sediment Bath* samples for *D. gracilis* (p = 0.002) and *O. acuminata* (p=0.02), for the *Sediment Bath Dip* samples for *D. gracilis* (p=0.003) and *H. filiobranchus* (p=0.001), and for the *Adjacent Water* samples for *H. filiobranchus* (p=0.006) and *O. acuminata* (p =0.008) (Figure 23).

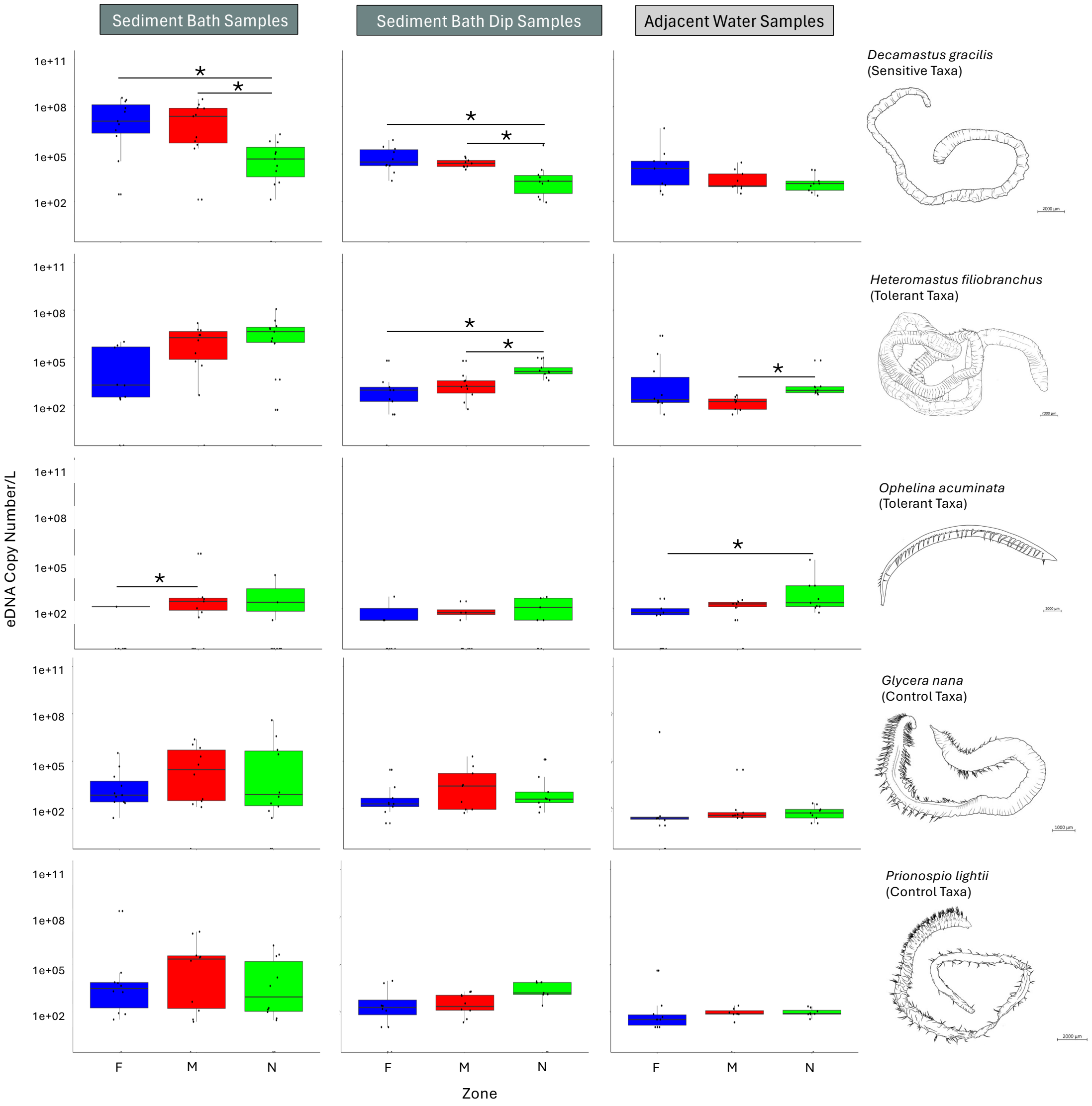


Figure 23. eDNA signal strength (Copy Number/L) for each indicator taxa in the far, mid and near field zones. Each column is a different eDNA sampling type, and each row is a different target taxa. Upper, mid, and lower line of box plots represent the 75th percentile, the median, and the 25th percentile eDNA copy number estimates from all sampling events. Significant differences (p < 0.05) between zones indicated by stars.

Post-hoc pairwise comparisons done using the Wilcoxon rank sum test indicated which zones contributed to these significant differences. For the *Sediment Bath* samples, *D. gracilis* demonstrated significant differences between Near and both Mid and Far Field zones (p = 0.0114, 0.0051). For the *Sediment Bath Dip* samples, there were also significant differences between Near and both Mid and Far-Field zones for both *D. gracilis* (p = 0.0063, 0.0117), and *H. filiobranchus* (p = 0.0063, 0.0065). For *Adjacent Water* samples, *H. filiobranchus* showed a significant difference between the Mid and Near-Field zones (p = 0.00025), and *O. acuminata* showed a significant difference between the Far and Near-Field zones (p = 0.0069) (Table 6 and Figure 23).

Table 6. Significance values of pairwise comparisons between zones of the Wastewater Treatment Plant Outfall using Wilcoxon rank sum test with continuity corrections. Values p <0.05 are bolded. F, Far-field; M, Mid-field; N, Near-field

Taxon	**Sample Type**								
	Sediment Bath			**Sediment Bath Dip**			**Adjacent Water**		
	F v M	**F v N**	**M v N**	**F v M**	**F v N**	**M v N**	**F v M**	**F v N**	**M v N**
Decamastus gracilis	1	**0.005**	**0.011**	1	**0.012**	**0.0063**	0.75	0.40	1
Heteromastus filiobranchus	0.26	0.14	1	0.42	**0.0065**	**0.0063**	0.80	0.40	**0.00025**
Ophelina acuminata	0.036	0.98	0.22	1	0.81	0.95	0.55	**0.0069**	0.37
Glycera nana	1	1	1	0.89	1	1	0.063	0.28	1
Prionospio lighti	1	1	1	1	0.65	0.81	1	0.61	1

We also calculated the percentage of species detections over all sampling events for all sample types and species (Conventional Samples [*D. gracilis:* 97%, *H. filiobranchus:* 92%, *O. acuminata:* 97%, *G. nana:* 100%, *P lighti:* 58%], *Sediment Bath* Samples [*D. gracilis: 95%*, *H. filiobranchus:* 91%, *O. acuminata:* 27%, *G. nana:* 86%, *P lighti:* 79%], *Sediment Bath Dip* Samples [*D. gracilis:* 97%, *H. filiobranchus:* 84%, *O. acuminata:* 22%, *G.*

nana: 81%, *P lighti:* 68%], *Adjacent Water* Samples [*D. gracilis:* 98%, *H. filiobranchus:* 83%, *O. acuminata:* 65%, *G. nana:* 62%, *P lightii:* 78%] (Table 7).

Table 7. Calculated percent detection success from all sampling events within this study.

Sampling Method	**Sample Type**	**Species**				
		Decamastus gracilis	***Heteromastus filiobranchus***	***Ophelina acuminata***	***Glycera nana***	***Prionospio lighti***
eDNA	**Sediment Bath**	95	91	27	86	79
	Sediment Bath Dip	97	84	22	81	68
	Adjacent Water	98	83	65	62	78
Conventional	**Biota**	97	92	97	100	58

Generalized linear models (GLMs) were used to evaluate the relationship between eDNA signal strength and species abundance (Figure 24A). The negative binomial GLM was much more parsimonious than the Poisson GLM for all eDNA sampling methods (ΔAIC = 6371, 3452, 5437 for sampling methods *Sediment Bath*, *Sediment Bath Dip*, and *Adjacent Water*, respectively). Variation in eDNA copy number generated through the *Sediment Bath Dip* sampling method explained a large proportion of variance in species abundance (R^2 = 0.65), while the *Sediment Bath* and *Adjacent Water* sampling methods explained less variance (R^2 = 0.47, 0.45, respectively).

Estimates of taxon-specific coefficients describing the relationship between log-scale eDNA copy number and taxon abundance were significantly positive for *D. gracilis*, *O. acuminata*, and *P. lighti* with all eDNA sampling methods, and was significantly positive for *H. filiobranchus* with only the *Sediment Bath Dip* sampling method (Figure 24B). Across all methods, the coefficient for *G. nana* was not significantly different than zero, suggesting no relationship between eDNA copy number and abundance for this taxa (Figure 24B).

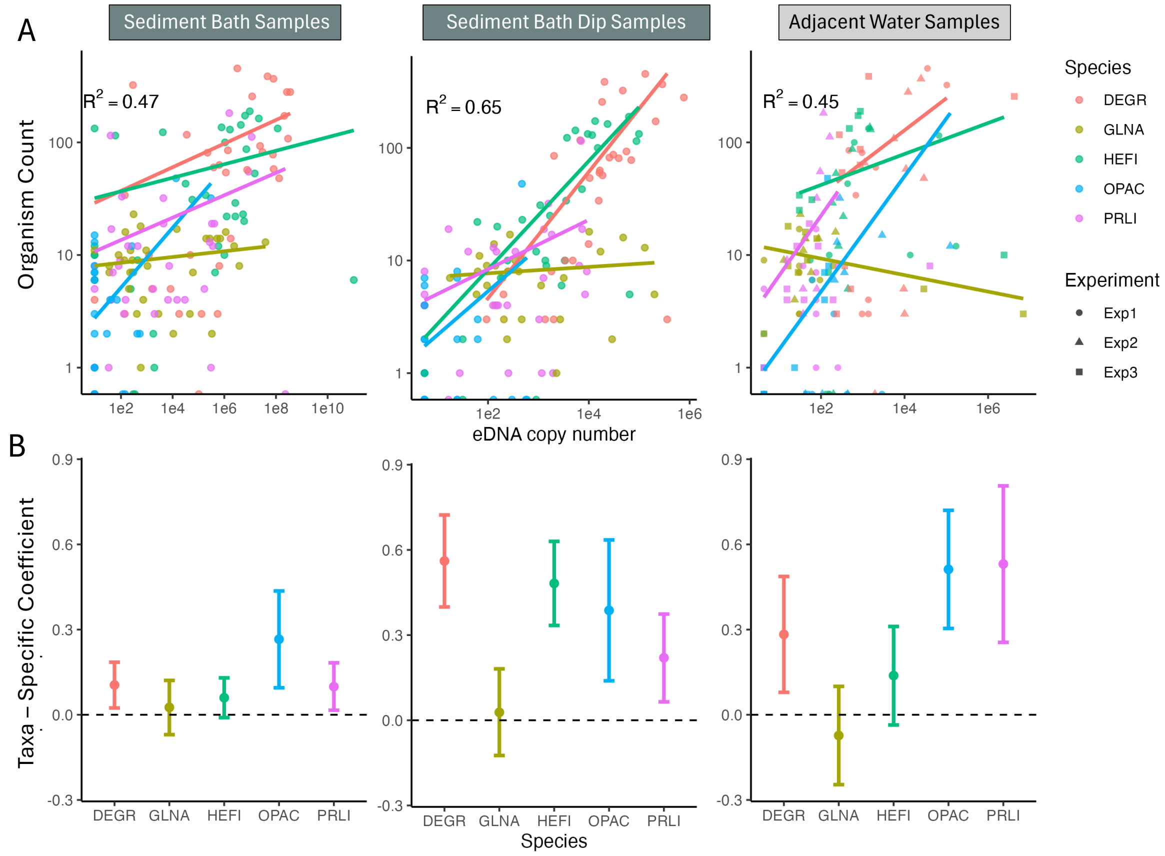


Figure 24. A) General Linear Model correlating eDNA signal strength and organismal count, with taxa as an interaction term. **B)** Species specific coefficents indicating whether the correlation between eDNA signal strength is significant. The null hypothesis of no significant correlation is indicated by the dashed line

We used *eDNAjoint* to quantify the probability of a false positive eDNA detection and compare the relative detection sensitivities of eDNA and conventional sampling for the two least abundant taxa, *O. acuminata* and *G. nana*. All models converged (Rhat < 1.01 for all model parameters), and posterior summaries are provided in Table 7. The estimated probability of a false positive eDNA detection was low for all eDNA sampling methods, with all mean posterior values less than 0.02 and 95% credibility intervals less than 0.07 (Table

8). We used the model parameter estimates to calculate the number of survey units (i.e., eDNA water samples or grab samples) needed to detect species presence and found that

Table 8. Posterior estimates and output values from eDNAJoint. p10 values indicate the probability of false positive eDNA detection,and beta is the model parameter that scales the sensitivity of eDNA sampling relative to traditional sampling. The mean and 95% credibility interval (in parenthesis) of posterior samples are provided. The number of survey units needed to detect species presence with 90% confidence when the expected grab rate (mu) is 0.1, 0.5, and 1 is calculated for all sampling methods. NA, not applicable.

Taxon	**Sample Type**	**p10**	**beta**	**Sampling effort to detect taxon presence (mu)**		
				0.1	**0.5**	**1**
Glycera nana	Sediment Bath	0.013 (0.002, 0.048)	1.49 (1.28, 1.74)	35	8	4
	Sediment Bath Dip	0.014 (0.002, 0.05)	2.11 (1.86, 2.36)	64	14	7
	Adjacent Water	0.014 (0.002, 0.051)	3.69 (3.40, 4.05)	301	61	31
	Conventional	NA		24	5	3
Ophelina acuminata	Sediment Bath	0.008 (0.002,0.025)	3.14. (2.87, 3.42)	177	36	19
	Sediment Bath Dip	0.019 (0.004, 0.046)	3.97 (3.55, 4.51)	403	81	41
	Adjacent Water	0.019 (0.002, 0.066)	2.07. (1.77, 2.42)	61	13	7
	Conventional	NA		24	5	3

the relative detection sensitivities between conventional and eDNA sampling varied by species and eDNA sampling method (Figure 25, Table 8). For *O. acuminata*, adjacent water eDNA surveys provided the same detection sensitivity as conventional surveys, while bath and bath dip eDNA surveys required more samples to detect *O. acuminata* presence than grab surveys. Conversely, adjacent water eDNA surveys were less sensitive than conventional surveys for *G. nana*, while bath and bath dip surveys required equal or fewer samples to detect presence than grab surveys (Figure 25).

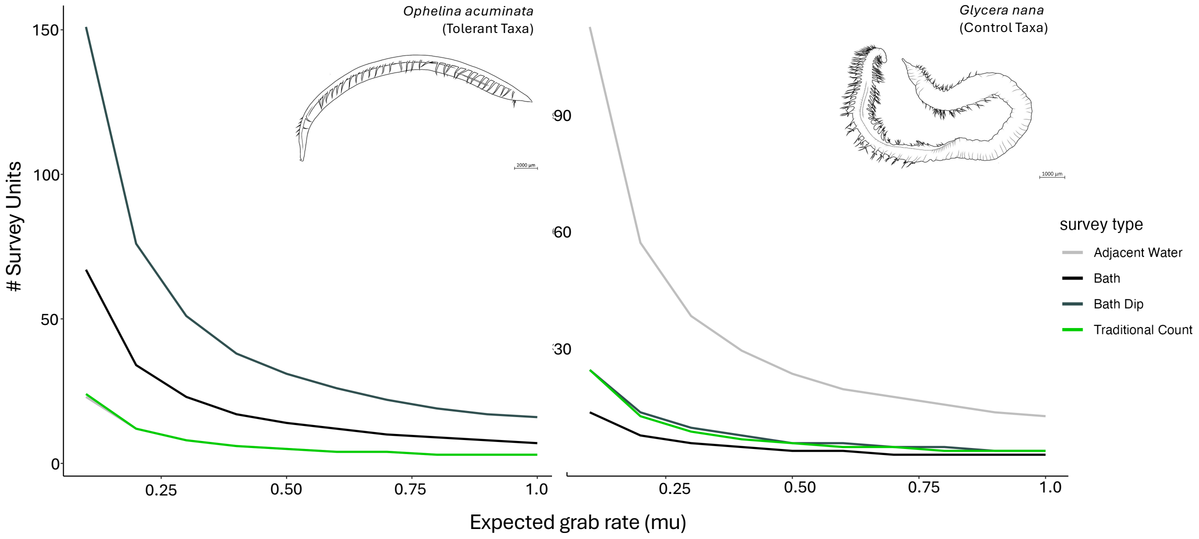


Figure 25. Comparison of the relative detection sensitivities of eDNA and traditional sampling for *O. acuminata* and *G. nana*. Lines indicate the number of survey units needed to detect taxa presence with 90% confidence, as a function of the mean (expected) grab rate, mu. Line colors indicate survey type.

We compared the four models run using *eDNAOccupancy* [164]. Generally, the third and fourth models we ran had the lowest values, suggesting a better fit to the data. In the cases where this wasn't true (i.e., *G. nana, Sediment Bath,* and *Sediment Bath Dip* samples), the differences in values were negligible, indicating that no specific model fit the data best. Thus, we visualised the probability of eDNA detection in a qPCR replicate (*p)* in relation to organismal count, using the parameter estimates generated by the fourth model for all taxa and sample types (Figure 26). The summary of these results including the mean parameter estimates of φ, θ, and *p,* and the PPLC and WAIC estimates can be found in Appendix 19.

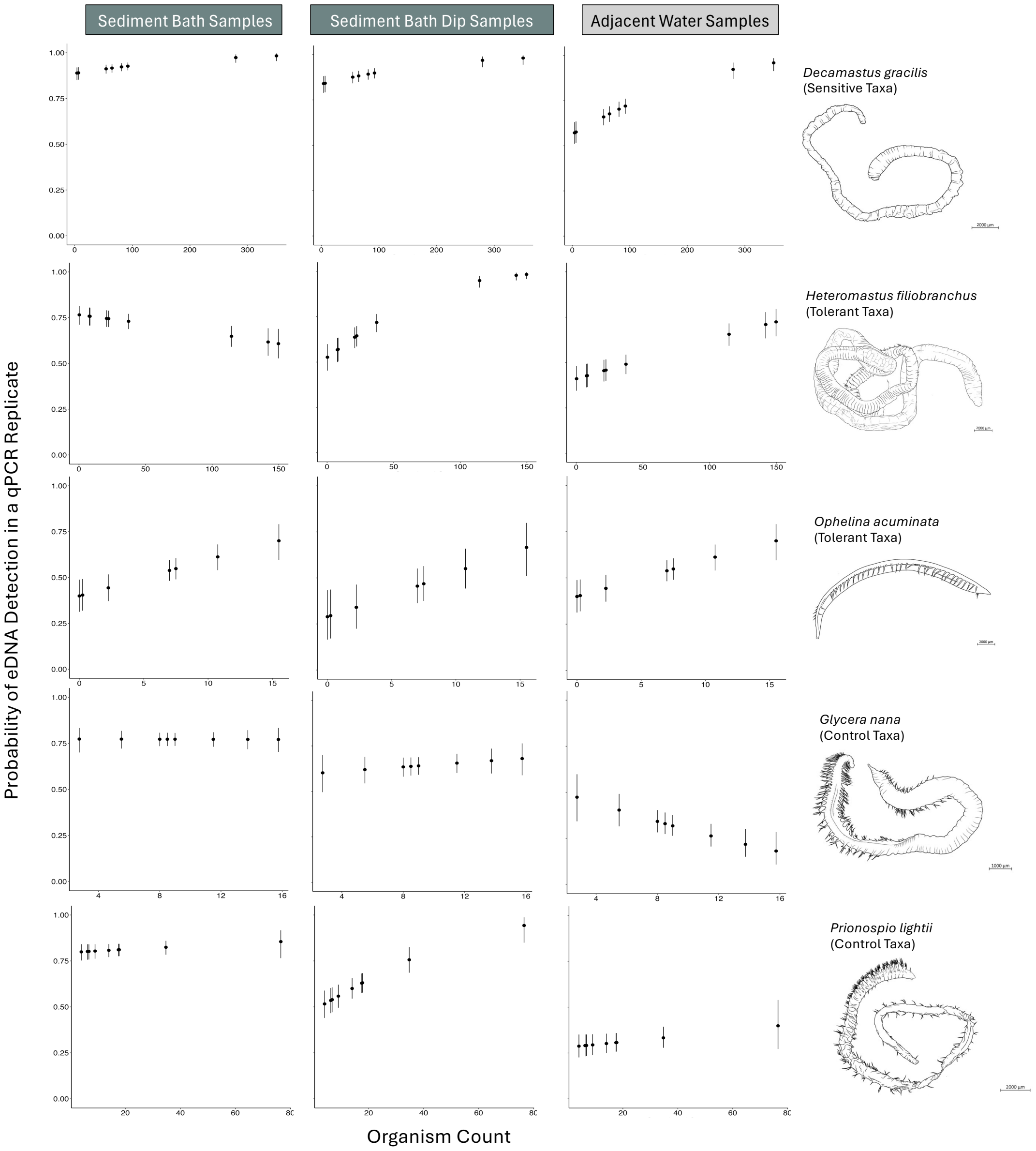


Figure 26. Probability of eDNA detection for each indicator taxa in relation to organismal count. Each column is a different eDNA sample type, and each row is a different target taxon. Error bars indicate the upper and lower estimates of p as calculated by eDNA occupancy.

4.4 Discussion

Composition changes of infaunal community assemblages in response to environmental stressors have been used to characterise marine pollution effects for decades [165,166]. Organismal responses to marine pollution are well characterized globally and can provide valuable information regarding the extent of pollution impacts [67,145]. The WWTP Outfall receiving environment is one of the major municipal wastewater outfalls serving Vancouver, BC, Canada. The macroinfaunal communities inhabiting the areas surrounding the outfall have been monitored for over 20 years using manual sediment processing and taxonomic identification [67]. This long-term dataset has provided invaluable information that we used to identify taxa that could serve as indicators of organic enrichment.

Using eDNA methods to detect broad community composition changes in response to pollution is not a new idea and has been employed successfully multiple times [167-170]. This is mainly because eDNA methods are highly sensitive and highly scalable in space and time [4,152,171]. Herein, we used our established expertise in targeted qPCR assay development [7,90,107,143] to design assays for our identified indicator taxa. This was particularly challenging due to the lack of sequence data for relatively obscure species. We obtained entire mitochondrial genome sequences of our target and potentially confounding species to design from. The resulting assays are highly sensitive and specific. However, it is imperative that as sequencing technology improves, and costs decline, sequences of as many taxa and individuals as possible are obtained [172,173]. This is important for creating a sequence archive for extant species, increases the annotation resolution for eDNA metabarcoding studies, and improves the strength of targeted eDNA qPCR assays.

The qPCR assays used herein are undoubtedly very powerful tools for target species detection and relative abundance estimation at the outfall. However, the utility of the assays may be limited to the geographic distribution of the target taxa to the coastal Pacific Northwest area of North America and can only be used elsewhere in the context of environmental effects monitoring if the taxa demonstrate the same marked response. Additionally, within this range, if there is any intrataxon variation, the sensitivity of the assay can fall dramatically [153]. For example, the base pair difference we found in the reverse primer binding region for the *O. acuminata* target assay shows that there is intraspecific variation within this region. And though this specific difference does not affect our assay efficacy, it is possible that there may be consequential intrataxa differences at other sites, so it is important to verify the effectiveness of the eDNA assays on samples from other sites to evaluate suitable performance.

Our target eDNA assays were extremely sensitive, with low false positive detection rates estimated by the *eDNAjoint* analysis and consistent levels of detection corresponding with predicted taxon distributions. The three sample types tested all generally reflected these patterns. However, false negative detection rates varied between target taxa and eDNA sampling types (Table 7), with the highest false negative detection rates unsurprisingly shown by the *Adjacent Water* sample type.

The *Sediment Bath* samples had higher DNA copy number estimates overall, but this did not correspond to more distinct differences between zones. In fact, the *Sediment Bath Dip* samples showed significant difference across zones in estimated DNA copy number for both *D. gracilis* and *H. filiobranchus* while also showing less variation amongst sample replicates for all species. The dip filters are positively charged membranes that attract negatively charged DNA particles in the environmental sample. These are passive samplers that are much more field amenable and require much less processing than filtration samples. That passive sampling can provide comparable results to filtration is a very exciting prospect within the field [174,175].

The justification for shifting to more field amenable sampling protocols is clear. Thus, the fact that the *Adjacent Water* samples reflected ecological distribution is very useful in terms of sampling scalability. Both the *Sediment Bath,* and the *Sediment Bath Dip* sample types require sediment collection and processing before they can be collected. *Adjacent Water* samples on the other hand, can be taken independently of sediment grabs. The importance of these results is reflected by the significant correlation found between eDNA copy number estimates and organism counts for all three sample types.

Using eDNA to estimate organismal abundance is very exciting, and highly challenging, especially in the context of a field-based study in a coastal environment [9,53,176]. The strongest correlation was found for the *Sediment Bath Dip* samples, again reflecting their lower variation ($R^2 = 0.65$). The R^2 values for the *Sediment Bath* and *Adjacent Water* sampling types are comparable. The taxon-specific coefficients are generally lowest for the *Sediment Bath* samples, reflecting the high variation in DNA copy number estimates observed. This could be due to patchy eDNA distribution captured on the filter, or due to different types of eDNA being captured (i.e., intracellular versus extracellular). This hypothesis is supported by the lower variation seen in the *Sediment Bath Dip* samples where only extracellular DNA particles would be free to bind to the membrane. The *Sediment Bath Dip* sampling method performs best of the three sample types tested herein, with low variability, high detection rates, and amenability to field sampling.

Across all sample types, *G. nana* showed no significant correlation between organism count and DNA copy number estimates. This is interesting because its targeted eDNA assay is extremely sensitive, and *G. nana* was chosen as a control species because it is ubiquitously and consistently distributed. However, it does occur at consistently low numbers. The most counted in any one sample was 23 individuals in comparison to the maximum 454 individuals of *D. gracilis* counted in a sample. *G. nana* may also be more likely to pass through the 1.0 mm mesh during screening due to its more active, crawling behaviour compared to more sedentary polychaetes. The same conclusion may be drawn for *O. acuminata*, which has a distinctive snake-like thrashing locomotion that makes it more likely to pass through the screen during field-sieving, which could impact its detection using conventional methods.

Additional differences in the ecology and behavior of the macroinfauna targeted in the present study could also influence these results. For example, during the indicator taxa selection process, very small crustaceans (e.g., amphipods) and shelled taxa (e.g., bivalves) were excluded. It was hypothesized that small or shelled organisms would shed less tissue and, consequently, DNA into the environment compared to some larger, soft-bodied annelid worms[177]. However, even within relatively large annelids, there are differences in life history such as whether they are tube-forming, or where they live in the sediment (i.e., surface dwelling or burrowing). This variability undoubtedly affects eDNA shedding. Spawning behaviour and status could also impact results. For instance, some Opheliidae (family to which *O. acuminata* belongs) tend to swarm when they free spawn, a behaviour that could increase the likelihood of detection from *Adjacent Water* samples, potentially providing the same detection sensitivity as conventional surveys, while for *G. nana* the *Sediment Bath* and *Sediment Bath Dip* samples better mirrored the grab surveys in taxon detection. Better understanding of the behavior and ecology of these animals will undoubtedly assist in the interpretation of these results.

Within the field of eDNA monitoring, there are many studies that demonstrate its sensitivity and reliability in comparison to conventional field biomonitoring methodologies [153,176,178]. However, eDNA data have not often been used by managers for conservation and resource management decisions due to a lack of trust in the new methodology; particularly due to concerns surrounding false negative and positive detections [82,179]. Though eDNA does have inherent limitations - as do all monitoring methods – there are still highly valuable data generated that should be used. Occupancy models are commonly used to account for imperfect detection in ecological studies by modelling the probability that a species occupying the site is detected [180,181]. Recently, several studies have used multi-scale modelling approaches to account for imperfect detection rates with eDNA data [182,183]. This approach works very well with eDNA, because of the natural three level nesting that occurs through multiple: 1) sampling stations; 2) field replicates per station; and 3) qPCR technical replicates per sample [164]. This study structure corresponds to the hierarchical relationships assumed between parameters used for conventional multi-scale occupancy modelling [21]. The occupancy modelling approach we used accounts for imperfect detection rates and provides detection probabilities based on our eDNA results in combination with two decades of conventional data. This method also supports the argument to use the *Sediment Bath Dip* sampling type with an increase in detection probability with organism count demonstrated across the taxa tested.

Using multiple monitoring methods together, in combination with statistical approaches that account for error, can assist in interpretation of eDNA data and increase the confidence needed by managers to uptake eDNA methods into long-term monitoring programs. In conclusion, the eDNA 'toolkit' we developed for marine managers in the

Pacific Northwest can enable faster and accurate indicator taxa detection and provide nuanced and reliable species distribution and relative abundance information. While indicator species may be modified depending on local habitat conditions and site-specific species proliferations, we demonstrate a tractable, creative, and informative way to use eDNA to measure marine pollution impacts. These methods are conducive to standardization and widescale adoption of eDNA sampling into marine environmental effects monitoring.

Chapter 5: Comparison of Environmental DNA and SCUBA Diving Methods to Survey Keystone Rockfish Species on the Central Coast of British Columbia, Canada

Abstract

The rocky reefs of British Columbia's (BC) coast are a productive ecosystem, home to 38 rockfish species (Genus: *Sebastes*) that are culturally and economically important. Quantitatively assessing rockfish populations is vital to support conservation and stock assessment needs. Self-contained underwater breathing apparatus (SCUBA) diving surveys are a commonly used monitoring method in BC. However, this resource-intensive approach is challenging, particularly for cryptic or deeper species. Herein, we compared environmental DNA (eDNA) detection methods with SCUBA diving surveys to capture overall rockfish biodiversity. We employed two eDNA methods: 1) a targeted quantitative real-time polymerase chain reaction (qPCR) approach to monitor species of particular importance to First Nations collaborators and decision makers, and 2) a metabarcoding approach for assessing community composition using the previously published MiSebastes assay. Both approaches are confounded by the little DNA sequence divergence among species and high sequence variation within species. Overcoming these challenges using a whole mitochondrial approach with the *mtGrasp* and *unikseq* pipelines, we generated highly useful eDNA tools. We found that eDNA methods were highly comparable to dive surveys, as both methods indicated a similar ecological reality, including species detections and distributions. Though there are certain species that cannot be distinguished by the MiSebastes assay, eDNA metabarcoding still detected more rockfish

species overall. Both eDNA methods show potential for use alongside conventional methods for scalable incorporation into community-based monitoring programs.

5.1 Introduction

Biodiversity is fundamental to the wellbeing of all life. Despite ongoing efforts, the world is losing biodiversity at an unprecedented rate due to climate change and anthropogenic development [1,2]. The global implications of these losses are catastrophic and drastic improvements are needed in conservation and biomonitoring methods to track these changes [3].

Rockfish populations of the genus *Sebastes* have been declining over the past decades, due to poorly regulated commercial and recreational fisheries, alongside rapid climate change [75,184]. They are a diverse, yet closely related group of fish that are mainly distributed throughout temperate regions of the Pacific Ocean. Globally there are over 120 species within the genus, with a variety of life histories and phenotypes [185]. Rockfish are long-lived, with certain species living over 200 years [186]. They grow slowly, require many years to reach sexual maturity, and often remain within restricted home territories [185]. These factors, in conjunction with high risk of barotrauma due to their deep sea habitats, make them very sensitive to population collapse, and loss of genetic diversity via overharvesting [186-188].

Rockfish have been a culturally important food fish for First Nations in the Pacific Northwest for millennia, and they are considered a “flagship” species meaning that their conservation confers protection to associated habitats and species [75]. In British Columbia, Canada there are 38 species that inhabit a range of habitats from the ocean surface to depths well over 1,000 m depending upon the species. To address declines in population size in the province Rockfish Conservation Areas (RCAs) have been established starting in 2002 [189].

Currently, First Nations of the Central Coast of British Columbia including the Kitasoo/Xai'xais Nation are using fishery-independent methods like SCUBA dive surveys or towed video surveys to monitor federally placed RCAs and areas of interest within their territories such as the newly established Gitdisdzu Lugyeks (Kitasu Bay) Indigenous Protected and Conserved Area (IPCA)) [75,190]. Dive surveys are a globally well-established method to determine marine biodiversity [14,191-193]. They not only provide species identification and abundance estimates, but also highly detailed associated observations on behaviour, water currents and visibility, and habitat characteristics like complexity and bottom composition. These are vital factors tied closely to biological productivity and therefore can provide a basis for good recommendations on the placement of protected areas. Despite their advantages, dive surveys are logistically challenging, prone to misidentification of species, and time and resource intensive. They require highly trained individuals who can reliably identify species, and who can handle adverse conditions that come with working in the field. In addition, SCUBA divers are depth-constrained, and due to the deep living range of rockfish, these methods can simply not access some key rockfish habitats [194].

A new approach that is increasingly utilized in biomonitoring is the detection and classification of environmental DNA (eDNA). This refers to DNA that can be isolated from environmental samples such as water [4,95]. Organisms shed DNA-containing skin, feces, gametes, etc. into their environments. By detecting these biological traces through eDNA testing, one can investigate biodiversity and monitor species of interest through a molecular lens. When used alongside conventional methods, eDNA can greatly increase the scope of monitoring efforts due to its relatively lower costs and ease of sampling[150,151] Furthermore, eDNA is minimally invasive to the organisms of interest, does not rely on time-constrained visual observation, and does not require extensive training for sample collection [14].

There are different eDNA approaches that can be used based on the ecological questions being posed [152]. Within the present study we employed two eDNA methods: 1) a targeted approach to monitor rockfish species of particular importance, and 2) a metabarcoding approach to assess community composition.

Targeted eDNA methods use species-specific assays that have been specially designed to detect gene sequences unique to the target species from environmental samples [7,152]. The most common targeted eDNA analytical method employs quantitative real-time polymerase chain reaction (qPCR) assays that, when properly validated [7,107], are powerful tools to identify species of interest. Using a recently developed whole mitochondrial genome (mitogenome) screening approach called *unikseq* [7,105] we validated targeted qPCR eDNA assays for three species: *Sebastes maliger* (Quillback rockfish; [105]) *Sebastes paucispinis* (Bocaccio rockfish) and *Sebastes ruberrimus* (Yelloweye rockfish). The species were chosen because they are particularly culturally, ecologically, and economically important [195]. Moreover, all three species are listed as threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) [196-198]. *S. ruberrimus* is also listed as a species of special concern under the Canadian Species at Risk Act (SARA) [199].

While targeted eDNA assays are particularly suited for reproducible, cost-effective, standardized evaluation of environmental samples, metabarcoding methods can provide valuable insight into community analysis. Metabarcoding assays rely on a hypervariable "barcode" region within the mitochondrial genome that is flanked by two shorter regions (20 – 30 bp) that are relatively conserved between species [200]. These barcode regions are amplified and sequenced with high throughput sequencing methods, then bioinformatically matched with known species sequences from a reference database [20,150,201]

The barcode currently most used for fish is the MiFish primer pair that amplifies a short fragment of the mitochondrially-encoded *12S* genic region (*mt-rnr1*) of the mitochondrial genome [200]. Despite the broad utility of the MiFish assay [200-202], it cannot differentiate between most rockfish species. This is because the *Sebastes* genus underwent a rapid divergence within the last million years, and *mt-rnr1* remains highly conserved in this genus [203,204]. Consequently, a different MiSebastes metabarcoding primer pair was designed using the mitochondrially-encoded *cytochrome b* genic region (*mt-cyb*), specifically for rockfish species along the coast of North America [205]. Since this assay performed well when tested on an aquarium population of California rockfishes [205], we used it in the present study.

If eDNA methods are to be integrated into largescale monitoring, there is a need for more systematic evaluation of both eDNA assay choice for target communities [205,206], and whether eDNA is the appropriate biomonitoring tool for each scenario [82]. The recent evolutionary history of *Sebastes* has resulted in a monophyletic group of many closely related species that overlap geographically. This means that there is high interspecific conservation within gene sequences [185,203]. At the same time, *Sebastes* species show considerable site fidelity as adults, and they are susceptible to regional isolation due to ocean currents or geographical barriers. This has resulted in distinct populations of rockfish species with high intraspecific variability [185,203]. This combination of high interspecific conservation and intraspecific variability makes *Sebastes* a challenging group to study with any genomics based methods including eDNA, especially since it is well known that the more overlap there is between these two factors, the less effective DNA barcoding becomes [207]. Thus, the overall goal of this work is to investigate the potential of targeted and metabarcoding based eDNA methods and compare them with conventional SCUBA diving surveys in their abilities to capture rockfish species of interest and overall rockfish biodiversity.

5.2 eDNA Assay Design and Validation

5.2.1 Targeted eDNA Assay Generation

Targeted eDNA assays were designed using *unikseq* [105] and validated using an established workflow described previously [7,105]. Reporting and performance characteristics were compliant with two Canadian national eDNA standards [106,107]. To design the *Sebastes maliger* (Quillback rockfish), *Sebastes paucispinis* (Bocaccio rockfish) and *Sebastes ruberrimus* (Yelloweye rockfish) targeted qPCR assays, all available full mitochondrial genome sequences for species within the *Sebastes* genus as well as the closely related *Sebastolobus* genus were accessed from the National Center for Biotechnology Information (NCBI) GenBank [108]. However, since there were not many available, additional *Sebastes* sequences were graciously supplied by Dr. Gregory Owens [186]. We also obtained voucher tissue DNA specimens of key *Sebastes* species that reflect haplotype variation in British Columbia and performed whole genome shotgun sequencing followed by mitogenome sequence assembly using *mtGrasp* version 0.1.0 [109]. The species that were sequenced included chosen target species, species that were most likely to co-occur, and relevant species for which there was no sequence data available. The full lists of all species mitogenomes and accession numbers used in the present study are in Appendix 20.

The *unikseq* (v1.0.0) parameters used for each assay are shown in Appendix 21. To ensure that the candidate assay primer and probe sequences would not amplify DNA from other potentially interfering taxa, they were cross-referenced against the NCBI non-redundant (nr) sequence database and visualized against co-occurring rockfish species (Appendix 23-25). They were then tested against tissue derived gDNA of target and non-target species (Appendix 22) [105].

Once promising assay candidates had been designed (Table 9) assay sensitivity was determined via standard curve construction with gBlocks® synthetic DNA obtained from Integrated DNA Technologies (Coralville, Iowa, United States) following the methodology described previously [110]. Using the gBlocks® data, standard curves were created to establish a relationship between C_t values and the initial DNA copy number. The eLowQuant [11] method was used to generate the limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) (Table 9).

For the final validation step each assay was tested on a verified positive environmental sample [107]. We used water samples from the Pacific Canada Pavilion tank (PacCan) and the Bella Bella tank at the Vancouver Aquarium for all our assay candidates. These recirculating tanks are home to several local *Sebastes* species and the exact content of each tank is known (Appendix 26).

Table 9. Target assay sequences, molecular characteristics, and calculated performance parameter.

Target Species		***Sebastes maliger***	***Sebastes paucispinis***	***Sebastes ruberrimus***
eDNA qPCR Assay name		eSEMA3	eSEPA9	eSERU5
Target Gene		*mt-d-loop*	*mt-nd5*	*mt-d-loop*
Optimal T_a (°C)		51.8	55.4	54.4
Amplicon Size (bp)		85	115	75
Assay Component Sequences (5' → 3')	**Forward Primer**	CGAAGGTATTACATAAAGCA	CCAGTAAACAATACAAAACTAC	CTACCAGTCCACTCATAA
	Reverse Primer	GAGTGTTTGTGTTAGGTCTTA	CCTAGAATTAAATTCAGCTTG	TGGGATTTCATATTCTACTTG
	Probe	CCAACAATCATTTATAAGGACTGAGCGAAT	TACCCACCATTTCTCCAACATGCTA	AACATAAGATATACGCCCATCAACCC
	Amplicon	CGAAGGTATTACATAAAGCAAAATAATAAGACCAACAATCATTTATAAGGACTGAGCGAATATTTAAGACCTAACACAAACACTC	CCAGTAAACAATACAAAACTACACCTAATCTAATTACCCACCATTTCTCCAACATGCTAGGCTTTTTCCCCTCAATCATCCACCGATTAACCCCCAAGCTGAATTTAATTCTAGG	CTACCAGTCCACTCATAAAAACATAAGATATACGCCCATCAACCCCCCGGTACCGCAAGTAGAATATGAAATCCCA
Continuous Data (C_t >95%)	**Slope**	-3.5083	-3.404	-4.0059
	% Efficiency	93	97	78
	Y-Intercept	40.028	36.206	41.248
	R^2 Value	0.9998	0.9997	0.9977
Binomial Data (Copy #/rxn)	**LOD (95% CI)**	1 (0.7-1.7)	0.2 (0.2-0.4)	0.2 (0.2-0.4)
	LOQ (95% CI)	3.9 (2.8-6.5)	0.9 (0.6-1.6)	0.9 (0.7-1.6)
	LOB	0	0	0

5.2.2 Metabarcoding Assay Validation and Assessment

Though the MiSebastes assay targets a mitochondrial region with much higher variability than the MiFish target region, there are still some redundancies between amplicons of very closely related rockfish species. We assessed these assays *in silico* for their ability to amplify and distinguish rockfish species in British Columbia using available sequence data as a proxy for environmental samples. First, we compiled the MiSebastes amplicon sequences from all rockfish species for which entire mitochondrial sequences were available. From each amplicon we aligned the primer sequences and identified all mismatches within the primer regions. We then aligned the entire amplicon DNA sequence regions to identify identical sequences across multiple *Sebastes* species.

5.3 Field Surveys

5.3.1 Field Sample Collection

Data collection for the present study did not require permits from federal agencies. The data collected were either from seawater samples or observational. The Kitasoo/Xai'xais First Nations hold Indigenous rights to their own territories, and all seawater samples were collected with the permission and logistical support of the Kitasoo/Xai’xais Stewardship Authority. In September of 2021 and 2022, 16 stations were cumulatively visited in the Kitasoo/Xai’ xais Territory along the central coast of British Columbia Canada (Figure 27). At each station visual data on rockfish and their habitats were collected as per the protocol developed by the Central Coast Indigenous Resource Alliance (CCIRA) [74]**.** This survey design uses three 30 m transects parallel to the shoreline shore, starting from the deepest at ~30 m, then at ~18 m, and finally at ~5 m below the surface. On each transect divers were responsible for identifying, counting, and size estimation of fish, as well as characterizing habitat complexity and invertebrates species richness (Figure 27).

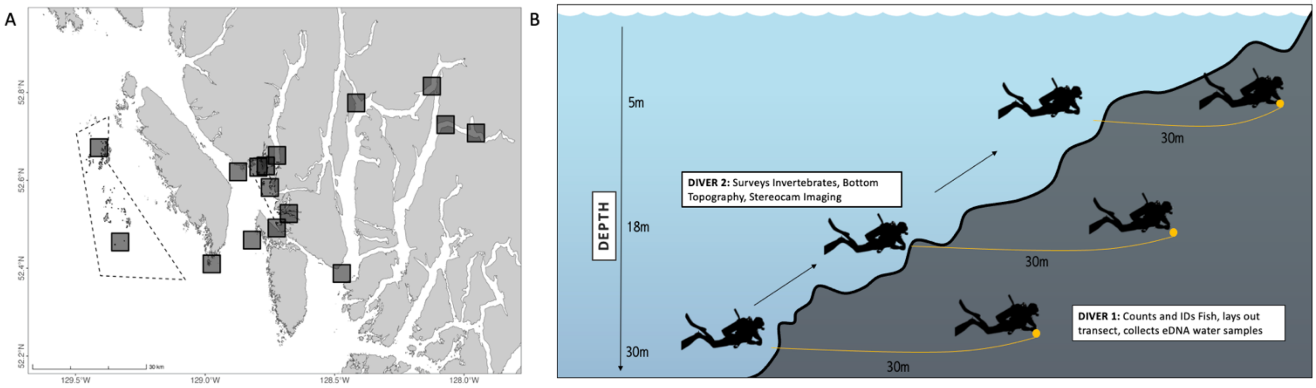


Figure 27. Field survey information. **A)** Map of survey stations, exact location masked on a scale of 16 km^2 **B**) Graphical depiction of field protocols. Each dive buddy pair descends to 30 m. The first diver rolls out a transect counting, identifying, and sizing rockfish. At the end of the transect the first diver then collects three water samples for eDNA analysis. The second diver records important substrate features, invertebrate life, and does opportunistic stereocam imaging of rockfish. This process is then repeated at 18 m and 5 m depths. All dives are done in accordance with CSA standards and using DCIEM Tables.

This protocol was modified with the addition of eDNA sample collection at each transect for a total of nine eDNA samples per station.

Water samples were collected using 1 L Kendall Kangaroo Joey™ bags (Medtronic, Brampton, ON, Canada). These bags are sterile and flexible, which makes them amenable to water collection while diving. At each transect the diver collecting eDNA would open the Kangaroo bag and swish the bag back and forth multiple times until it filled completely. Three 1L water samples per transect for a total of nine samples per dive were collected. On the last day of each trip, the divers' drysuits were soaked in a tote of freshwater and three 1L suit control samples were taken to account for any potential contamination from the divers' suits. All water samples collected were stored in a cooler with ice packs until filtration through a 0.45 µm mixed nitrocellulose filter (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) later that day. A 500 mL negative filtration control was also prepared by filtering bottled water in this manner every day filtering was done. Filters were stored at -20ºC until DNA could be extracted. One sample collected at South Aristazabal floated away and thus was not included in the analyses (Appendix 27).

5.3.2 Sample Preparation and Integrity testing

DNA was extracted from filters using the DNeasy Blood and Tissue kit (Qiagen Inc., Mississauga, ON, Canada; Cat# 69506) as described previously [110,111]. All extractions were performed in a laminar flow hood wiped with 10% bleach (v/v) solution and 70% ethanol (v/v) solution prior to use. For qPCR analysis one quarter of each filter was extracted. For metabarcoding analysis, two quarters of each filter were extracted, pooled, and concentrated to 50 µL. All eluates were stored at -20°C until analysis could be done.

Before testing the samples with rockfish specific approaches, the integrity of DNA in each sample was assessed using the qPCR based IntegritE-DNA™ test [110]. This test ensures that the eDNA samples are viable and no inhibitors were present that would affect PCR analysis. Any samples that failed this test (average qPCR C_t values >27) were cleaned with a OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA, Cat # D60303) and retested. Only samples that passed this test were further tested and sequenced (Appendix 28).

5.3.3 eDNA Assay Testing -qPCR

Each eDNA sample from the field surveys was tested with the eSEMA3, eSEPA9, and eSERU5 assays with eight technical replicates each using the QIAcuity Probe PCR kit from QIAGEN Inc on Bio-Rad CFX96 Real-Time PCR Detection Systems (Bio-Rad Laboratories

(Canada) Ltd, Mississauga, Ontario). DNA amplification thermocycler conditions were an initial activation step of 2 min at 95°C followed by 50 cycles of 15 s denaturation at 95°C, 30 s annealing at 64°C, and 45 s extensions at 72°C. Each plate was run with eight NTCs and two positive controls comprised of synthetic DNA at a concentration of 20 copies/reaction. If any negative controls amplified, or if positive controls failed to amplify, the entire plate was re-run. These qPCR results were converted into an estimate of DNA copy number/L.

Water sample replicates were grouped within each transect. and each transect was treated as a sample to enable comparison with the dive data. If the mean estimated DNA copy number/L across field replicates exceeded the calculated lower confidence interval of the assay's LOD [11], the transect was designated as a positive detection.

5.3.4 eDNA Assay Testing – Metabarcoding

Library preparation for the MiSebastes [205] metabarcoding assay was comprised of a commonly used two-step polymerase chain reaction [201,208]. The first PCR step is used to amplify target regions. Each sample was run in triplicate with a 25 µL reaction volume comprised of 5.5 µL sterile Nuclease-Free water, 0.6 µL of both the fwd and reverse MiSebastes primers (10 µM), 3.75 µl BSA (NEB), 12.5 µL 2X Taq (Froggabio), and 2 µL template. For the first PCR, DNA amplification conditions were an initial activation step of 5 min at 95°C, followed by 45 cycles of 30 s denaturation at 95°C, 30 s annealing at 52°C, and 45 s extensions at 72°C, with a final extension of 5 min at 72°C.

The triplicate PCR replicates were pooled and a SPRI bead (Beckman Coulter, Indianapolis, IN, USA) cleanup step (using 0.8X beads) was performed to remove excess primers and non-specific fragments. The SPRI beads cleanup followed the manufacturer's protocol, and samples were resuspended with 25 µL of sterile nuclease free water. For both assays, a negative DNase-free water control sample was included in all PCR reactions. The second PCR step was used to attach Illumina adapters (Illumina, San Diego, USA) with unique identifiers to the amplicons for sequencing. This PCR was carried out with 25 µL reaction volume, containing 5 µL of sterile Nuclease-Free water, 2.5 µL of each index (Nextera i7 and i5), 12.5 µL of 2X Taq (Froggabio), and 2.5 µL template (first PCR product). Different combinations of indices (i5 and i7) were used to assign a unique identification to each sample.

For the second PCR, DNA amplification thermocycler conditions were an initial denaturation for 3 min at 95°C, followed by eight to ten cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 30 s of 72°C, with a final extension for 5 min at 72°C. Another SPRI beads (Beckman Coulter, Indianapolis, IN, USA) cleanup was carried out (using 0.8X beads) and samples were resuspended with 25 µL of nuclease-free water. DNA was quantified with the Quant_IT dsDNA High Sensitivity Kit (Thermo Fisher Scientific Inc., Ottawa, ON, Canada), and then all samples were pooled so that there were 40 ng of DNA per sample. The quality of the library pool was accessed in the BioAnalyzer (Agilent) using the High Sensitivity DNA Analysis Kit and quantified using the NEBNext Library Quant Kit for Illumina. Prepared libraries were sequenced with the Illumina MiSeq at the Hakai Institute genomics facility [201]. The 2021 and 2022 samples were run separately with the Illumina V2 300 cycle kit and results were pooled.

5.3.5 Bioinformatics

The paired-end metabarcoding reads were processed using the dada2 library v1.22.0 in R v4.1.3 [209], and cutadapt v2.10 to trim primers [210]. After learning error rates, dereplication, sample inference, and read merging with dada2 using default parameters, we removed bimeras, amplicon sequence variants (ASVs) present in only a single sample with lower than 0.001 relative abundance, and two samples with fewer than 150 sequence reads present. Taxonomic assignment was carried out using a custom library of amplicons created from in-house sequence data and those from Dr. Greg Owens [186], as well as a BLASTn search of the NCBI nucleotide database. The Galaxy Tool LCA was used to determine lowest common ancestor (LCA) taxonomy strings for each ASV [211]. The top scoring blast hit was applied without using LCA if it had ≥ 98% similarity with the query, otherwise, LCA was employed to determine a consensus taxonomy.

Once the taxonomy classifications were verified, the Vegan package (R v3.6.3) was used to generate rarefaction curves [212]. These curves indicate whether sequencing depth was adequate to capture species representation and thus more reliable metabarcoding results. Then, the Decontam package (R v4.1.3) [213] was used with its suggested aggressive parameters to remove reads that were potentially contaminants based on the prevalence of certain ASVs detected from the diving suit control data and field blanks.

5.3.6 Rockfish Diversity Comparisons

Using the Vegan package (R v3.6.3), alpha diversity estimates, measured as species richness were compared between MiSebastes and dive survey data, and between depths using the non-parametric Wilcoxon signed-rank test [212]. Alpha diversity refers to the number of different species within a community, and is a key metric within biodiversity studies [214].

For each species detected by dive survey and by either eDNA method, a matrix was made indicating whether each transect was designated as a positive or negative detection. From these matrices the percentage of eDNA only detections, dive only detections, positive detection for both methods, and negative detection for both methods were calculated. At the station level, if there was at least one depth where the target species was detected, the station was designated as a positive detection. To quantitatively assess the difference between station designations via diving and eDNA methods, we performed the Fisher's exact test [215]. *Phyloseq* (R v3.6.3) [216] was used to generate taxonomic barplots for both the metabarcoding and dive data, aggregated by depth and location for visualization.

5.4 Results and Discussion

5.4.1 eDNA Assay Validation Results

eDNA Assay Performance – qPCR

Within the context of the present study, the full mitochondrial genomes of most rockfish species that are known to exist on the Pacific coast of North America were accessible [186]. To ensure that potential intraspecific variation was captured, five to six voucher specimens of each target species were sequenced (Figure 28), as well as other common species in British Columbia, from our area of study (Appendix 29). Although eSEPA9 and eSEMA3 show intraspecific variability in the assay region, probe and primer binding efficiency is not affected (Figures 29 and 30). This was confirmed through assay testing with tissue samples from the sequenced individuals. eSERU5 does not show any intraspecific variability in the assay region (Figure 31). However, the eSERU5 amplification efficiency at 78% (Table 9) is slightly lower than the 80% recommended by the new Canadian national standard on performance criteria for the analysis of eDNA by targeted qPCR [107]. However, given the considerable constraints for the design of this assay, it was the best possible we could do at this time.

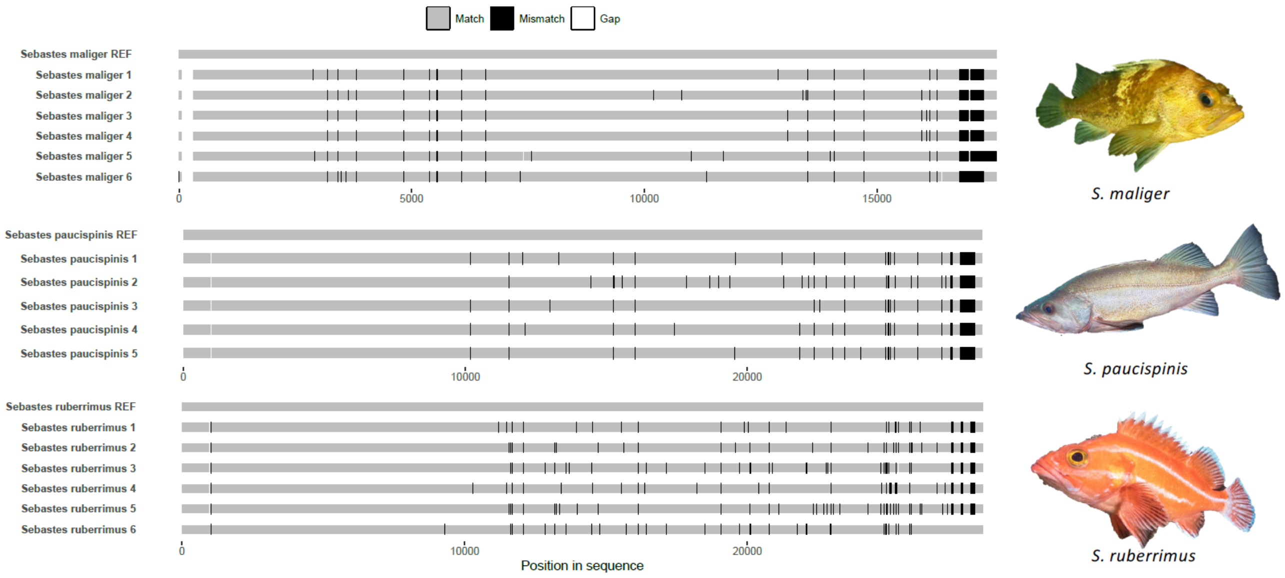


Figure 28. Whole mitochondrial genome alignments (~16,000 bp) of multiple individuals of each target species. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design. In all three target species, there are sequence differences between individuals across the entire mitochondrial genome. This indicates high intraspecific variability.

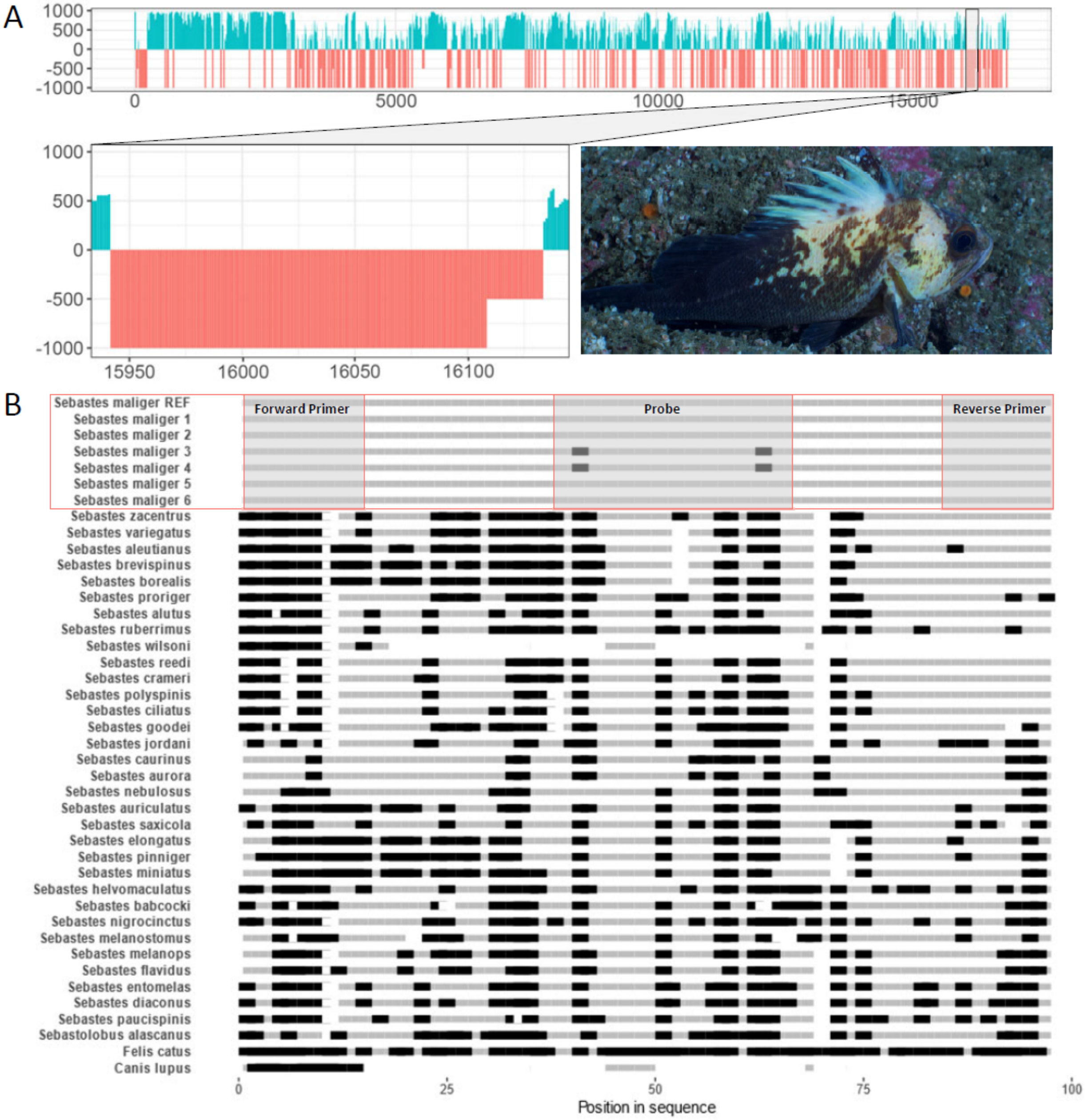


Figure 29. *Sebastes maliger* target assay design visualization. **A)** *Unikseq* output butterfly plot. The blue regions indicate high conservation between rockfish species, and the pink regions indicate regions of higher variability that are more amenable for assay design. The inset shows the *unikseq* output region that the assay was designed from. **B**) eSEMA3 amplicon alignment using multiple individuals of the target species, all other BC rockfish species for which there is sequence data available, and human, cat, and dog sequences. Grey indicates bp conservation between individuals and black lines indicate sequence differences. The first row is the reference sequence that was used for assay design.

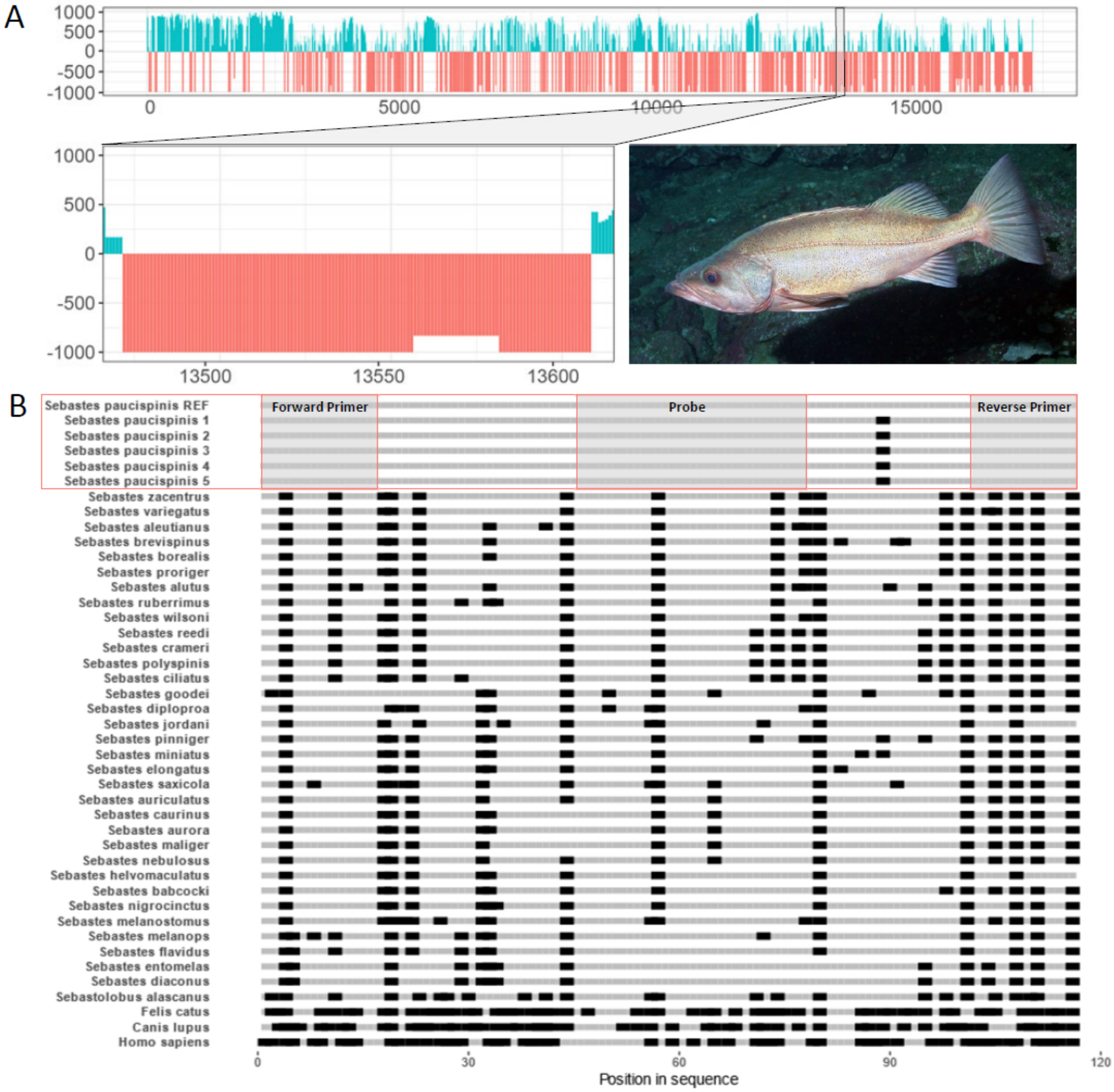


Figure 30. *Sebastes paucispinis* target assay design visualization. **A)** *Unikseq* output butterfly plot of the relevant regions for the design of the eSEPA9 targeted qPCR assay. **B)** eSEPA9 amplicon alignment. There is no intraspecific variability in any of the key assay regions. See the **Figure 29** legend for more details.

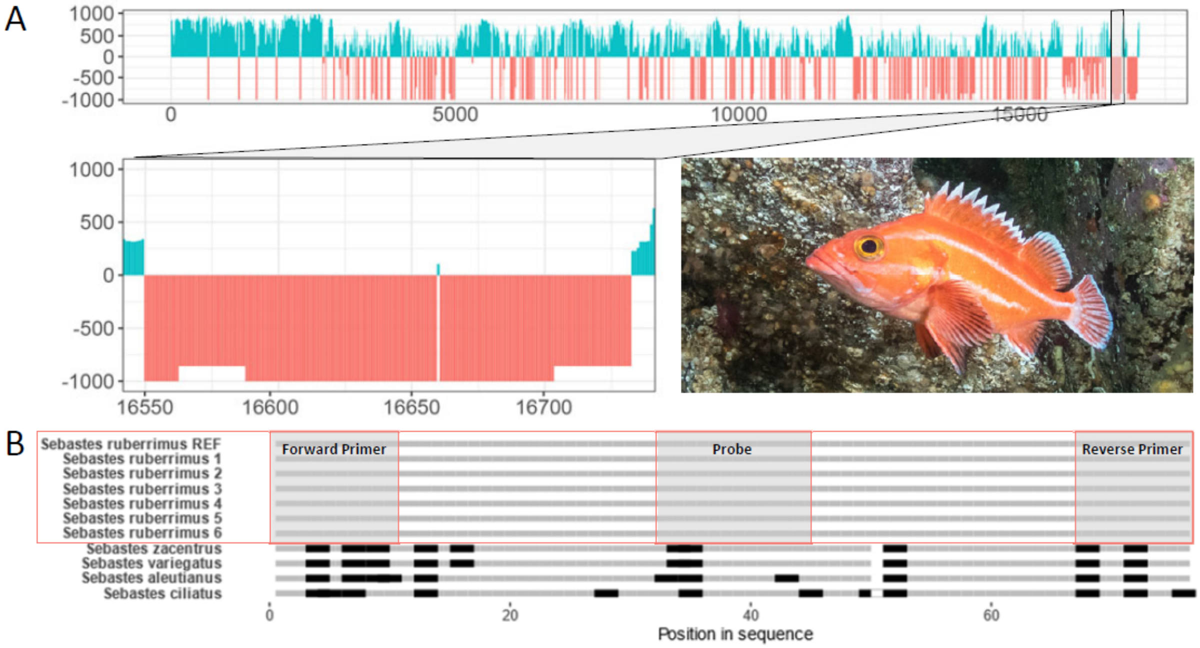


Figure 31. *Sebastes ruberrimus* target assay design visualization. **A)** *Unikseq* output butterfly plot of the relevant regions for the design of the eSERU5 targeted qPCR assay. **B)** eSERU5 amplicon alignment. Note that the eSERU5 region is out of range to align with most other species as well as human, cat, and dog sequences. *In situ* validation confirmed that species of concern were not amplified by this assay. There is no intraspecific variability in any of the key assay regions. See the **Figure 29** legend for more details.

The reference sequences originally used to design these assays were from animals in California. Including the voucher specimens from British Columbia in the assay design and validation procedure greatly strengthened our assay design capability to encompass common haplotypes occurring in our area of study. eSEMA3, eSEPA9, and eSERU5 all performed very well when tested on Vancouver Aquarium samples (Appendix 30), indicating that the assays would work well applied to field samples from British Columbia since the aquarium fish were collected locally. However, it is likely that all our assays will perform better in certain regions based on the dominant haplotypes present.

eDNA Assay Performance – Metabarcoding

For the MiSebastes assay, ten common primer mismatches in the forward primer region were found that were represented in the available rockfish sequences (Appendix 31). From the 80 globally distributed species for which an entire mitogenome was available, 63 species had a unique MiSebastes amplicon, and 17 species shared an identical MiSebastes amplicon with at least one other species (Appendix 32). This analysis demonstrated that there are certain rockfish species in BC that lack genetic variation at the MiSebastes region, and thus there are some species in BC that must be treated as complexes with this metabarcoding assay. Using the Tamura-Nei model on Geneious Prime, Neighbour-Joining phylogenetic trees for the British Columbia species were assembled from reference mitogenomes. This analysis confirmed that the indiscernible species complexes were also phylogenetic clades (Figure 32). It should be noted that there could be haplotypes within species for which sequence information is unknown that could allow species level identification in certain geographical regions.

To assess this phenomenon quantitatively, the custom library of amplicons for MiSebastes was queried with the BLAST tool [217]. Using the top hit for each species, the error percentage (16%), the average E-value (4.18E-100) and the average bit score (350) were generated. The error percentage refers to the proportion of top hits were assigned to the incorrect species. In this case, these hits were in fact unidentifiable species as all "incorrect hits" occurred for species within the previously identified species complexes. The barcode gap from the custom library was calculated using the MACER package (R v3.6.3) [218]. Forty-one percent of species did not have adequate sequence coverage for the assessment of barcode gap, 38% of species did have a barcode gap, and 20% did not. The species without a gap were within identified species complexes. These analyses confirm that except for the species complexes, most BC rockfishes (at least 24 of 38 based on available sequence data) can be identified to species level using the MiSebastes assay (Figure 32).

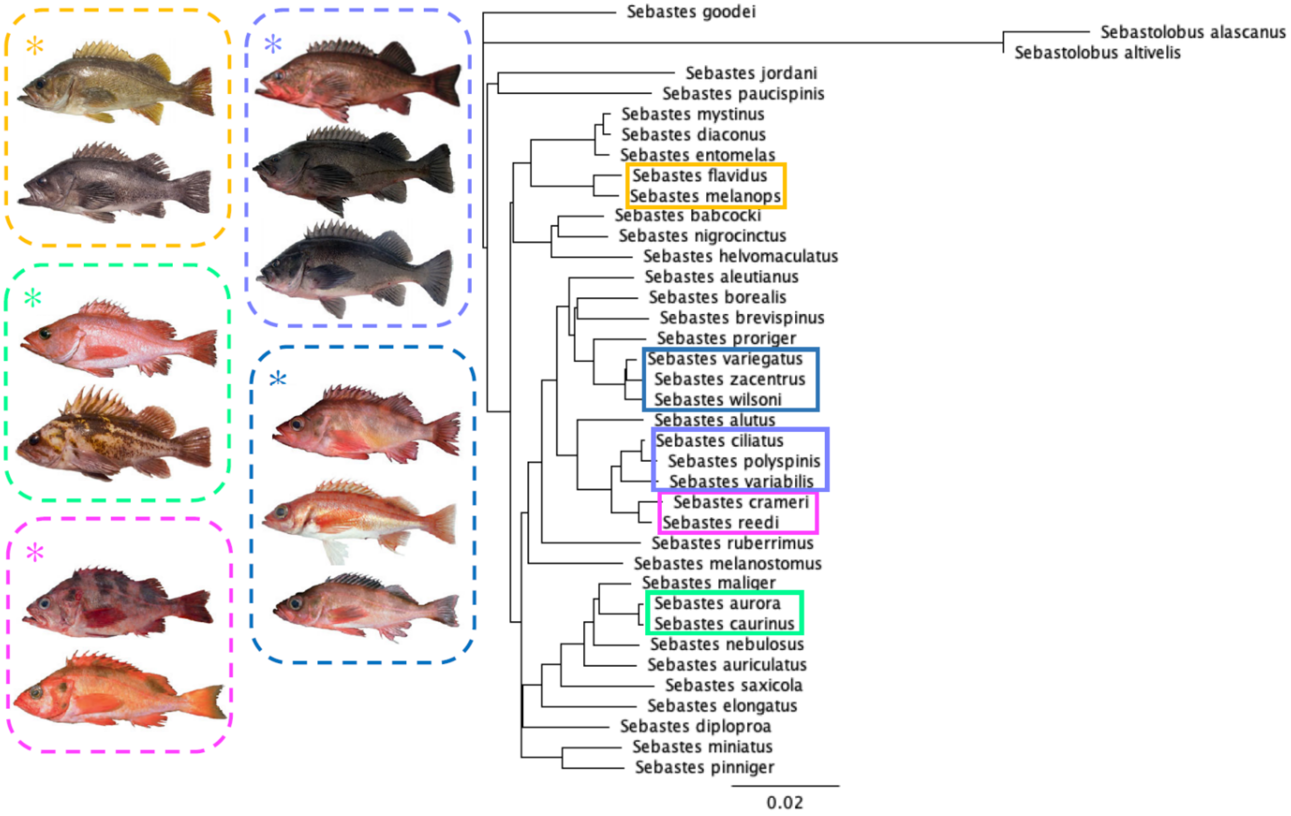


Figure 32. Neighbour-joining tree generated from entire reference mitogenomes of BC rockfish with indicated indiscernible groupings for the MiSebastes metabarcoding assay. Based off the available sequence data, the fish in each box have the identical sequences at the target amplicon and thus cannot be identified to species level. For the present study, they are thus treated as species complexes. The remainder of species can be discerned at the species level.

5.4.2 Field Survey Results

Metabarcoding Molecular Analyses

The MiSebastes amplicon sequencing produced 16,987,427 reads for downstream analyses, of which 6,304,907 reads were removed after quality filtering. Of the 10,682,520 reads (mean reads/sample = 67,185 from 1,798 ASVs) remaining, 1,416,204 unassigned reads and 64,056 non-*Sebastes* reads were removed, leaving 9,202,260 reads (mean reads/sample = 57,875 from 287 ASVs). The taxonomic classifications from these 287 ASVs were compared between the custom library and NCBI, and 224 ASVs (98.7%) had identical taxonomic assignments between the two reference databases. The annotation mismatches between the custom database and NCBI occurred for various reasons: The NCBI BLASTn search assigned 12 ASVs to species that were not within the geographical range of the present study and could assign one ASV only to the genus level. For these ASVs, taxonomic classification from the custom library was performed. Twenty-six ASVs were assigned with a higher percent identity from the NCBI BLASTN search than with the custom library. This is likely because the *cytb* region (mitochondrial gene targeted by the MiSebastes assay) has high coverage on NCBI and likely covers more haplotypes than are present in the custom library. Finally, 20 of the mismatched ASVs were within the known species complexes that could not be discerned by MiSebastes (Figure 32) resulting in equivalent blast scores to multiple different species. Those ASVs were therefore assigned to a species-complex rather than a single species. All rarefaction curves generated reached a plateau, confirming that there was adequate sequencing depth for species representation (Appendix 33). The MiSebastes assay did detect a few rockfish reads in the field blanks, however these should be accounted for using the decontamination pipeline which removed 2 ASVs from ecological analysis (MiSebastes ASV# 299, 958) (Appendix 34, 35)

SCUBA Diving and eDNA Results Comparison

Overall, the MiSebastes assay detected significantly higher alpha diversity than dive surveys in the observed number of species (Figure 33; Paired-Wilcoxon Test: p = 3.6e-11) It is also clear that there is more rockfish diversity on the deeper transects. This is consistent with what is known of rockfish ecology.

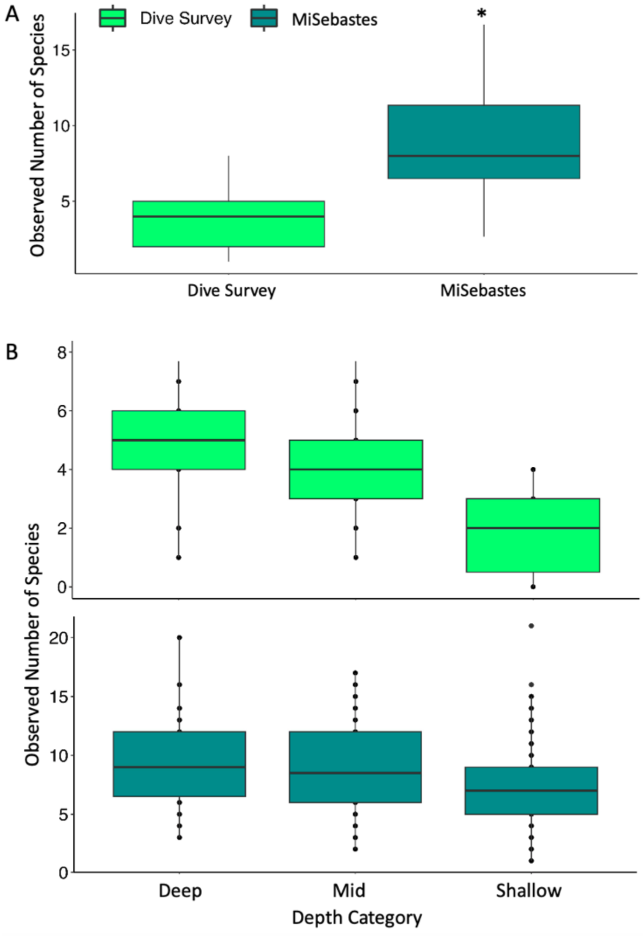


Figure 33. Alpha diversity estimates via observed number of species **A)** between detection methods (Paired-Wilcoxon Test: Observed p = 3.6e-11), and **B)** between depths. There is a consistent trend of higher rockfish diversity of at greater depths. No significant differences between depths for dive data and MiSebastes metabarcoding results using a Paired Wilcoxon Test.

Across all samples, both diver and metabarcoding-based detection methods identified eleven species (*S. melanops, S. flavidus, S. ruberrimus, S. maliger, S. caurinus, S. nigrocinctus, S. proriger, S. entomelas, S. paucispinis, S. ciliatus and S. pinniger*) (Figure 34).

Three of the identified species are complexes. MiSebastes cannot confidently distinguish between *S. melanops* and *S. flavidus; S. ciliatus,*

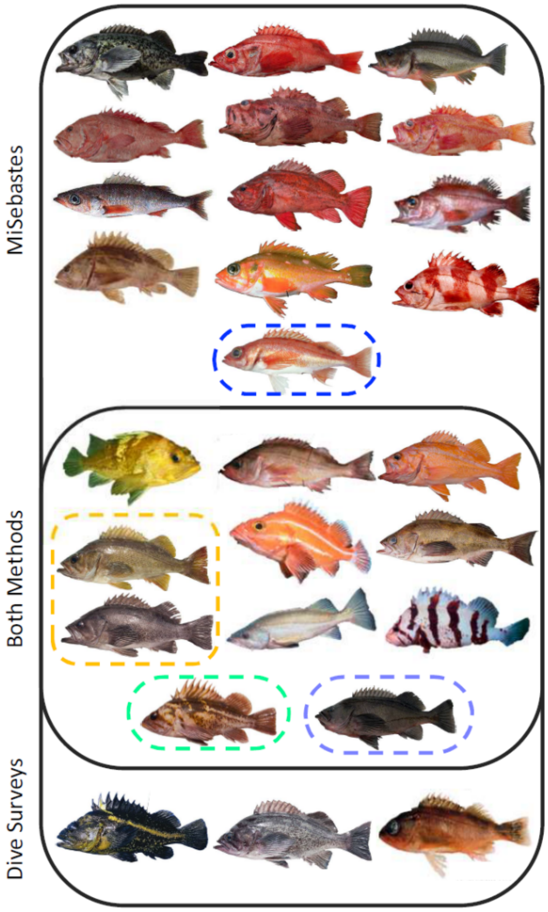


Figure 34. Overall detection of rockfish species. Colored boxes indicate species complexes corresponding to those identified **in Figure 32.**

S. polyspinis and *S. variabilis;* and *S. aurora* and *S. caurinus*. Since *S. aurora* generally occurs at depths greater than 80 m and is less common than *S. caurinus*, these classifications are likely the latter species. Though the MiSebastes assay cannot distinguish *S. ciliatus* from *S. polyspinis* and *S. variabilis,* and it is also hard to visually discriminate between these species. Thirteen additional species were detected solely by metabarcoding using the MiSebastes assay; *S. auriculatus, S. aleutianus, S. jordani, S. miniatus, S. alutus, S. helvomaculatus, S. babcocki, S. brevispinis, S. borealis, S. mystinus, S. melanostictus, S. diploproa,* and the *S. zacentrus, S. wilsoni,* and *S. variegatus* species complex.

There were also three species detected by diving alone: *S. emphaeus, S. nebulosus* and *S. diaconus*. *S. emphaeus* has very little genetic information available, so it may have been detected but not properly annotated. It is interesting that MiSebastes failed to detect *S. nebulosus* as there are no known primer mismatches for that species, and it was present at a number of sites surveyed. It is possible that the dominant haplotype in the region may not be known. It is likely that divers misidentified *S. mystinus* as *S. diaconus*. These two species are so difficult to visually distinguish that they were not formally classified as separate species until 2015 [204]. The MiSebastes amplicon sequence for these two species, however, is distinct. Therefore, the fact that divers did not detect *S. mystinus* and the MiSebastes assay did not detect *S. diaconus* suggests it was S. *mystinus* that the divers observed since it is more reliable to distinguish the two species genetically than visually.

Focusing on species detection at the station level considerable agreement was observed between diving and the MiSebastes metabarcoding assay (Figure 35). Using the Fisher's exact test we determined that for all species except *S.ruberrimus* (p=0.029)*,* and *S. proriger* (p<0.00001), there were no significant differences between station designations between diving and eDNA methods (*S.pinniger* (p=1), *S.caurinus* (p=0.48), *S. nigrocinctus* (p=1), *S.ciliatus* (p=1), *S. entomelas* (p=0.25), *S.maliger* (p=1), *S. paucispinis* (p=1)). Two noteworthy observations that aptly demonstrate the synthesis of a common ecological reality between methods include the clear lack of *S. nigrocinctus,* and *S. pinniger* in the fjords and the general ubiquity of *S. caurinus* and *S. maliger.* The fact that *S. proriger* eDNA seems ubiquitous but the species is rarely observed, is very interesting, and could be due to many different reasons such as a high amplification efficiency of this species by the MiSebastes assay or the species simply being missed or misidentified by divers.

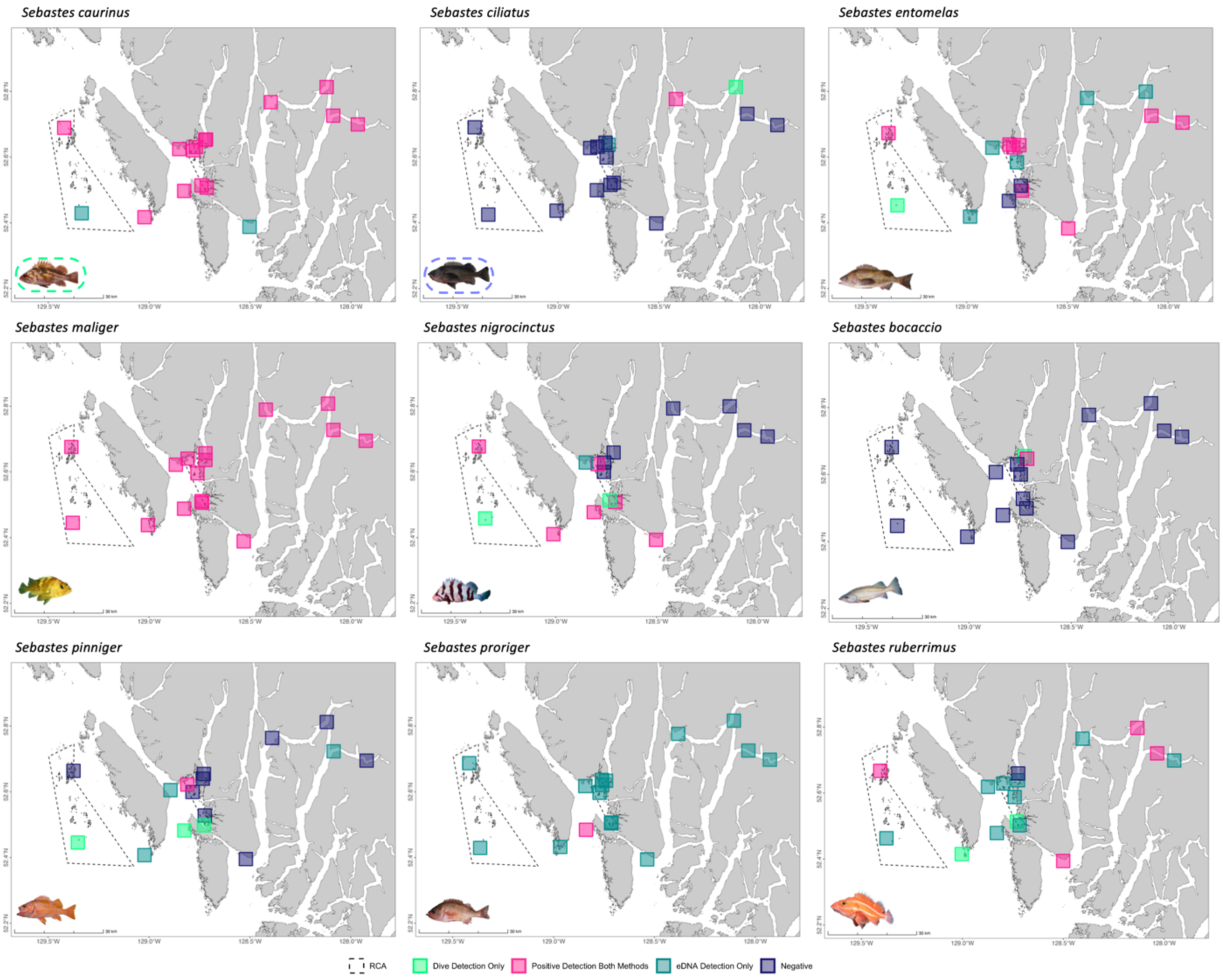


Figure 35. Each map depicts comparisons between detection by the MiSebastes assay and by dive survey. *S. caurinus,* and *S.ciliatus* are depicted as the more likely to occur species of their species complexes. The *S. melanops_flavidus* complex is not depicted because both species are equally likely to occur.

To compare detection agreement between methods at the transect level, the overall efficacy of each eDNA method in detecting the target species were calculated and reported as the percentage of transects where detections were eDNA only, dive only, or both negative or both positive (Figure 36A; Appendix 36). There was agreement between diving and eDNA methods for the majority of transects across target species. At the transect level for both *S. maliger* and *S. ruberrimus* there are more dive only detections of target species in comparison with the qPCR results, and there are generally more eDNA only detections in comparison with metabarcoding results. The high stringency of the qPCR assays results in the higher likelihood of false negative results, while lower stringency of metabarcoding results in higher likelihoods of false positive results. It is difficult to discern which method is closer to true species distribution.

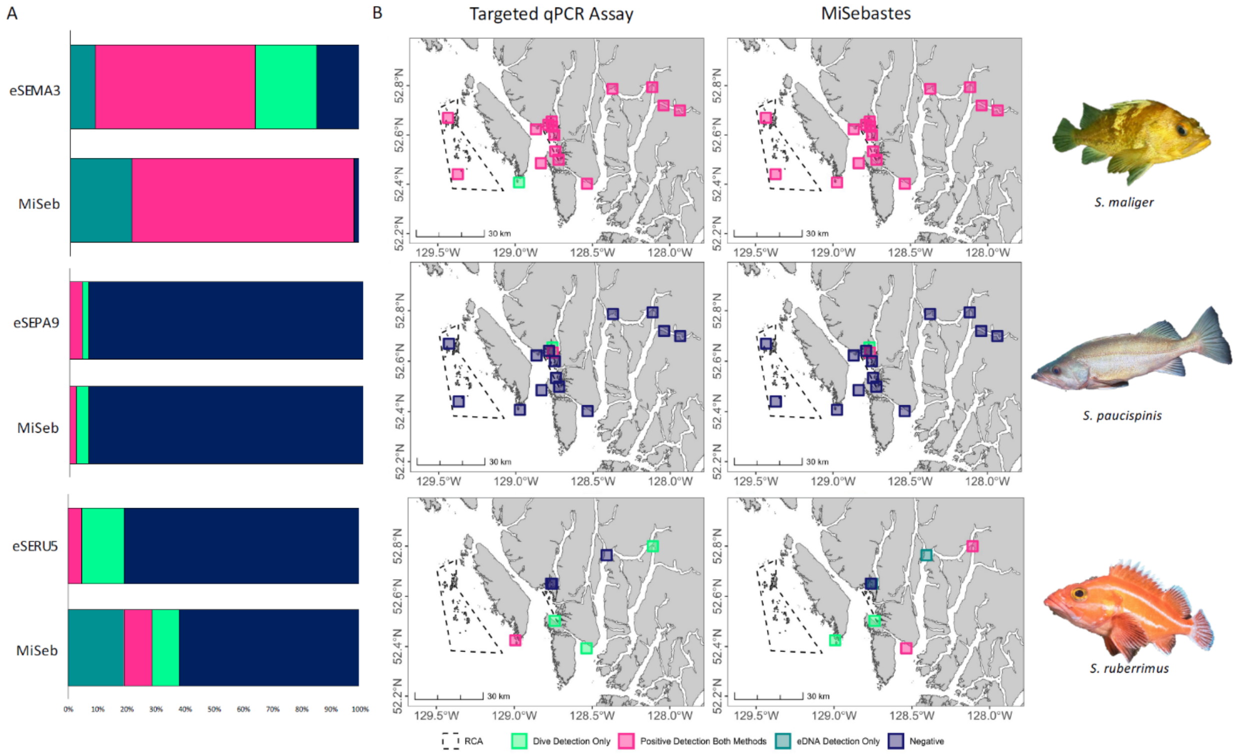


Figure 36. *Sebastes* target species detection comparison between dive surveys and eDNA methods. **A)** Depicts comparisons as percentage agreement between methods at the transect level, **B)** Depicts comparisons at the station level. For *S. ruberrimus*, only the 2021 data results are shown due to likely contamination of the 2022 samples (see text for details).

Closer examination of the target species indicates there were no significant differences between detection via diving or qPCR assay for *S.maliger* (eSEMA3; p=1), *S. paucispinis* (eSEPA9; p=1) or *S.ruberrimus* (eSERU5; p=0.48; Fisher's exact test) (Figure 36B). However, there was a surprising number of *S. ruberrimus* positive detections by both eDNA methods in the samples collected in 2022 even though this species generally does not occur at shallow depths (Fisher's exact test; eSERU5; p=0.015, MiSebastes; p=0.0023). There was no *S. ruberrimus* DNA detected in the field controls using eSERU5 assay. Though these signals may reflect true eDNA transported from deeper depths through natural upwelling, it is most likely due to fishing activities that were occurring during the time of sampling as rockfish were being pulled up through the water column. Unfortunately, the likely contamination of *S. ruberrimus* DNA confounds our results for this species. We therefore evaluated the 2021 data for *S. ruberrimus* separate from the 2022 out of caution and found excellent concordance between analytical methods (eSERU5; p=0.27, MiSebastes; p=1; Fisher's exact test). The above observations are a testament to the extreme sensitivity of targeted qPCR assays, and a subsequent field study focused on this species is warranted.

It should also be noted that *S. maliger* and *S. ruberrimus* were detected in low concentrations in the dive suit controls in both 2021 and 2022, but *S. paucispinis* was not. This indicates that despite efforts to minimize contamination, it is very challenging to control all sources of contamination in the field when using eDNA methods. Regardless, these three species are of conservation concern and these assays will undoubtedly be a useful tool for managers working with rockfish.

Although there was considerable overlap in the species detected by both methods, this does not extend to the estimated proportion of each species within the community (Figure 11, Appendix 37). The top five species detected by the MiSebastes metabarcoding assay by read proportion and species prevalence were the *S. flavidus_melanops* species complex (48% of total reads; 97% prevalence across samples), *S. ruberrimus* (22%; 55%), *S. maliger* (10%; 82%), the *S. caurinus_aurora* species complex (7%; 48%), and *S. auriculatus* (2%; 37%). Whereas the top five species detected by divers as a proportion of the total number of fish seen were *S. entomelas* (69% of all fish seen), *S. flavidus* (18%), *S. maliger* (4%), *S. paucispinis* (3%), and *S. caurinus* (1.5%) and, by species prevalence, *S. maliger* (71%), *S. flavidus* (60%), *S. caurinus* (50%), *S. nebulosus* (44%), and *S. entomelas* (35%). The other species detected can be found in Table 10.

Many factors can influence why relative species abundances are not always consistent between methods. However, primer bias is a major factor for consideration. This is a molecular bias whereby PCR assays can preferentially bind and amplify certain species based off genetic sequence characteristics, thereby not reflecting true community composition. This is a well-known concept in metabarcoding and is a reason why we cannot yet rely on eDNA metabarcoding data to infer relative abundance [18,152,219,220]. Species detection using eDNA metabarcoding, on the other hand, is quite reliable.

Thus, it is quite compelling that eDNA methods were able to detect many species that divers were not. A few common species found in the diveable depth range (*S. auriculatus, miniatus,* and *brevispinis*) were simply not observed by divers at the study stations as they were out of the visual range of the transect or concealed. Additional species detected by eDNA are known to be cryptic and have typical depth ranges starting near the deeper dive transects of the present study (*S. borealis* (24-1200 m), *S. helvomaculatus* (25-549 m), *S. zacentrus* (25-450 m) and *S. wilsoni* (30-2704 m)). Finally, there are several species that generally live much deeper in the water column (*S. diploproa* (90-80 m), *S. babcocki* (49-625 m), *S. alutus* (90-825 m), *S. jordani* (91-491 m), *S. aleutianus* (150-450 m), *S. melanostictus* (84-1000 m), and *S. variegatus* (70-560 m))[221,222].

These detections bring up the difficult questions regarding the ecology of eDNA [28,37]. The few studies that have attempted to address the complex fate of eDNA particles within the marine environment paint an intricate picture. Some studies found that eDNA detections will reflect the local community despite water movement caused by tides [101], and can discern communities separated by physical barriers like a kelp forest on a scale of only 60 m [223]. Other studies that have simulated eDNA particle movement through larger oceanographic processes estimate that eDNA could be detected 40 km away from the source on a timescale of four days [224]. This uncertainty has important implications on the confidence of the detection of improbable species in an environmental sample. While the current strength on each dive performed in the present study was recorded, this information is not useful without the context of *in situ* measurements such as upwelling, mixing, and stratification. Despite these limitations, eDNA surveying methods are still useful as divers are unable to survey the very deep rockfish habitats, and the methodology is far less invasive than commonly used long line survey methods with high rates of mortality. eDNA surveys from deeper water samples using these assays will be extremely informative and less invasive.

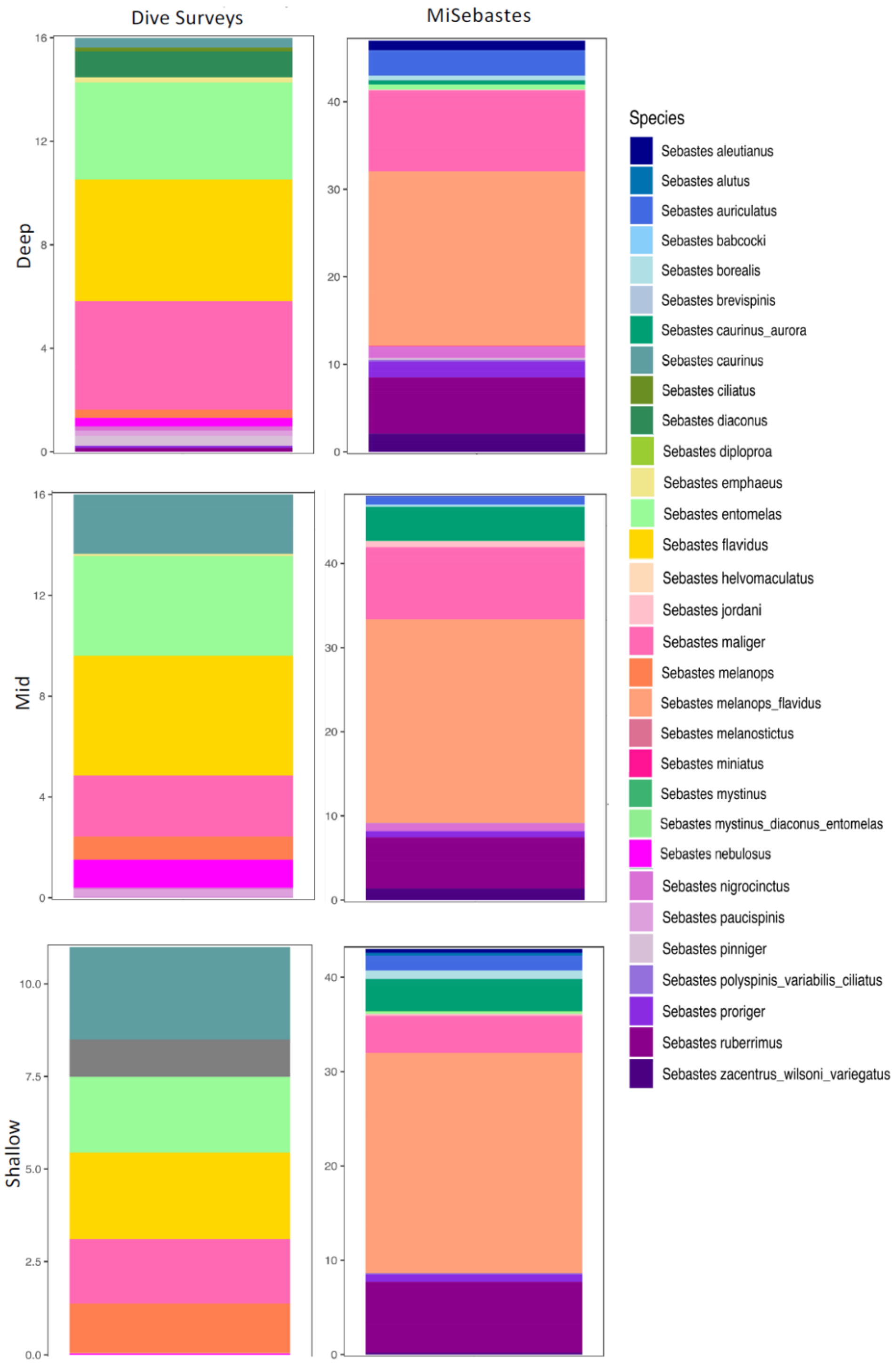


Figure 37. Visualization of the amplicon sequencing results per sample aggregated by depth. The colors show the relative proportion of rockfish species detected at each depth by MiSebastes metabarcoding and dive surveys. Note that MiSebastes cannot discern between certain species as indicated in the legend on the right.

Table 10. *Sebastes* species detected by diving and eDNA metabarcoding methods. The data are presented as relative abundance and as species prevalence.

Detection Method	**Species Detected**	**Proportion of total individuals seen or of total reads (%)**	**Proportion of positive detections across all eDNA samples or transects**
Dive Survey	*Sebastes entomelas*	68.75	35.42
	Sebastes flavidus	17.82	60.42
	Sebastes maliger	4.02	70.83
	Sebastes paucispinis	2.88	6.25
	Sebastes caurinus	1.42	50
	Sebastes melanops	1.32	16.67
	Sebastes diaconus	1.04	4.17
	Sebastes nebulosus	0.81	43.75
	Sebastes emphaeus	0.74	14.58
	Sebastes pinniger	0.43	8.33
	Sebastes ruberrimus	0.31	14.58
	Sebastes nigrocinctus	0.24	16.67
	Sebastes caurinus_maliger_auriculatus †	0.08	2.08
	Sebastes ciliatus	0.05	4.17
	Sebastes melanops_flavidus†	0.04	2.08
	Sebastes proriger	0.03	2.08
MiSebastes	*Sebastes melanops_flavidus*‡	48.25	97.4
	Sebastes ruberrimus	21.62	55.19
	Sebastes maliger	10.45	81.82
	Sebastes caurinus_aurora‡	6.94	48.05
	Sebastes auriculatus	3.11	37.01
	Sebastes zacentrus_wilsoni_variegatus‡	1.99	27.92
	Sebastes aleutianus	1.83	12.99
	Sebastes nigrocinctus	1.71	16.23
	Sebastes borealis	1.68	10.39
	Sebastes proriger	1.35	24.68
	Sebastes entomelas	0.3	19.48
	Sebastes pinniger	0.27	5.19
	Sebastes jordani	0.2	11.69
	Sebastes polyspinis_variabilis_ciliatus‡	0.1	3.25
	Sebastes miniatus	0.07	1.3
	Sebastes alutus	0.03	0.65

	Sebastes helvomaculatus	0.03	1.3
	Sebastes babcocki	0.03	0.65
	Sebastes brevispinis	0.03	3.25
	Sebastes mystinus	0.01	0.65
	Sebastes diploproa	0.01	0.65
	Sebastes paucispinis	0	0.65
	Sebastes melanostictus	0	1.95

†Cannot discern visually as juveniles

‡Cannot genetically differentiate

5.5 Conclusion

The genus *Sebastes* is of particular interest to many stakeholders and decision-making groups in the Pacific Northwest due to its cultural, ecological, and economic importance. However, the combination of high intraspecific variability and high interspecific conservation makes this genus particularly difficult to genetically distinguish between species, while also making it easy to miss haplotypes of individual species [185-188,203,204]. Fundamentally, eDNA methods rely on genetic sequence differences to discern species of interest from complex environmental samples. The integration of eDNA into larger scale projects necessitates a greater effort on the part of the eDNA scientist or practitioner to pre-emptively investigate the sequence availability, coverage, and quality that exists for the group of species being studied. To address intraspecific variability, more sequence data from different regions needs to be integrated into the assay or validation pipeline. We suggest that assays should also be tested on tissue-derived DNA from voucher specimens from the region where the assay is going to be applied. Indeed, this is encouraged by the Canadian national eDNA standard [11,106,107,111]. More sequence data will also help with resolving interspecific conservation. When the commonly used metabarcoding regions like *mt-co1* and *mt-rnr1* [14,200-202] are conserved within a group of interest – as they are within

Sebastes – using other regions like the *mt-cytb* region used in the MiSebastes metabarcoding assay described herein can be very helpful. However, as demonstrated in Figure 33, there are still important species like *S. flavidus* and *S. melanops* that cannot be discerned. Though outside the scope of this work, the *unikseq* [105] pipeline can also be used to find useful barcode regions as it systematically parses through entire mitochondrial genomes to find areas that are amenable to assay design. Thus, as sequencing costs continue to decline, and eDNA applications continue to increase, the argument for sequencing whole mitochondrial genomes – ideally multiple voucher specimens per species - is clear.

The purpose of the present work was to compare SCUBA diving methods and eDNA methods in their capabilities to assess rockfish biodiversity. Even though the MiSebastes assay cannot discern all the rockfish species in British Columbia, it can discern the majority, and the present work indicates which species must be treated as species complexes. Similarly, while diving there are some species that are very difficult to visually distinguish. This highlights the fact that all detection methods – even those that are well established – have limitations, but they still generate highly useful biodiversity data for decision makers [82,83].

It is important to remember that eDNA research is highly interdisciplinary. A field eDNA study carried out in the marine environment exists at the intersection of ecology, biochemistry, biology, oceanography, genomics, and bioinformatics. As eDNA studies expand in scale and scope, integrating these different forms of data will allow us to gain a clearer picture of biodiversity and species distribution. Overall, both SCUBA surveys and eDNA methods were comparable in their ability to assess rockfish diversity; however, it is also clear that each method had its own strengths and weaknesses. While dive surveys often miss species that tend to hide or have a depth range deeper than the survey depths, they can provide important ecological information like species abundance, size distribution, invertebrate biodiversity, and habitat complexity. These parameters are vital when making meaningful fisheries or protection-based management decisions. On the other hand, eDNA methods detected more species overall, particularly those that are unusual or live deeper. eDNA sampling is also much more scalable and, because of the ease of sampling, can easily be integrated into community-based monitoring programs. Using multiple approaches together will undoubtedly improve surveying capacity and understanding of areas important for rockfish biodiversity.

Chapter 6: Conclusions

Applying eDNA methods to diverse marine systems prone to various abiotic and biotic factors is immensely difficult. However, due to the dire need for scalable biomonitoring methods in the context of the global climate change and biodiversity loss crises, there is a huge push to overcome these challenges. Through fundamental eDNA studies such as those in this thesis we have improved our understanding of factors impacting eDNA recovery, detection and signal strength. Each application to a different marine system allowed us to address ecosystem specific challenges, apply different analyses methods, and provide recommendations to managers and end-users.

6.1 Importance of eDNA Methods Development

Marine eDNA monitoring is highly interdisciplinary, existing at the intersection of biochemistry, ecology, and physical and biological oceanography. Thus, collaboration between experts within each field leads to the most successful applications. With these principles in mind, we took effort to work with various collaborators to integrate their perspectives into study design and application. For example, though we only included the eDNA portion of the Olympia oyster study in the thesis, for publication, we plan to integrate remote sensing data to take advantage of the complementary strengths of both new-age, non-invasive monitoring methods. Or, for the rockfish work, we were able to optimize site selection in terms of rockfish diversity based off the local knowledge of our Kitasoo/X'ais x'ais collaborators.

6.1.1 Assay Design and Validation

On a more technical scale, eDNA assay design and validation is often overlooked in applied eDNA studies [7,225]. Scientists will often apply published eDNA assays without first doing the groundwork of ensuring that the selected assay has complete specificity to the target organism in the geographic region they are applying it (targeted eDNA studies), or if the target amplicon has enough variability to discern species of interest from each other (metabarcoding studies). For example, during assay design for the yelloweye rockfish, we found that, due to high levels of intraspecific variability, our assay has a limited geographic range where it is applicable. Additionally, for the metabarcoding component of that project we had to perform a meticulous barcode gap analysis for the miSebastes amplicon to best inform species annotation specifically in British Columbia.

Working in the Helbing lab has been extremely informative in this context due to our focus on rigorous assay design and our well-established pipeline for assay validation[7]. Though eDNA methods are diversifying rapidly, qPCR remains the most accessible technology for eDNA detection. It is most conducive to widespread global adoption and standardization due to its accessibility and cost-effectiveness [80,85,115]. With well-designed, robust assays, qPCR can be very sensitive and quantify very low eDNA copy numbers from environmental samples [85,226].

Using the whole mitochondrial approach described in each chapter allows us to identify good assay design regions in an unbiased way. The biochemistry expertise in our lab is then highly useful in ensuring that the assay has the molecular characteristics of an assay that works well. The amount of thought and effort that is put into this process before the assay is even applied to an environmental sample sets the bar high. Then, by pairing this process with *in silico* and *in situ* validation, we obtain powerful and sensitive eDNA assays.

6.1.2 Study Design and Interpretation

However, even with the best eDNA assay, the eDNA data output will be functionally useless without appropriate study design and interpretation techniques. Key challenges with eDNA data interpretation include improving the accuracy of species identification from eDNA, linking eDNA observations to species presence at various sites that are sampled since eDNA can be transported away from its source organisms, and developing modeling approaches to detect changes in eDNA accurately and precisely over time and space.

The work presented in this thesis supports the key observation made by many eDNA scientists, that eDNA persistence and movement is ecosystem specific. This is obviously, not ideal, but there are certain recommendations that can be applied to allow for optimal interpretation. This includes integration of hydrological models into understanding eDNA transport[28]. For freshwater and riverine systems this is largely possible and has allowed for amazing work that can strongly link eDNA signals to organismal abundance[31]. However, as described in the Olympia oyster and rockfish scenarios, this is more challenging in marine systems. Studies in areas with long term detailed oceanographic observation data like the Galapagos Islands or Monterey Bay have attempted to do this with interesting results[224,227]. However, this type of data is not readily available in most areas. Thus, another recommendation strongly echoed in the field is the meticulous collection and publication of environmental variables alongside eDNA samples. If this is done in a standardized and comparable way, environmentally driven variation can eventually be assessed and included in the eDNA interpretation pipeline[28]. One limitation of my thesis work was sub-optimal collection of these associated variables. For the rockfish project we did collect current and turbidity information, but since it was on a subjective scale and was not actually measured, this was not particularly useful for us during analysis. To remedy this oversight, I have been involved in a recently submitted publication that provides a comprehensive metadata checklist and data formatting guidelines that follow the FAIR (Findable, Accessible, Interoperable and Reusable) principles [228]. The output of this work is a well thought out checklist of standardised data templates for targeted and metabarcoding eDNA data and their associated metadata. These templates use Darwin Core Standard terminology that is an evolving community-developed biodiversity data standard that is already commonly used in large scale biodiversity studies.

In addition to careful study design and data collection, creative data analysis approaches can also assist with eDNA data interpretation. With these principles in mind, there have been multiple data analysis pipelines developed specifically to analyse targeted eDNA data[163,164]. For example, occupancy modelling approaches allow for species occurrence to be estimated accurately while accounting for false negative error rates. We used some of these techniques to help with interpretation for our pollution indicator species analysis. This was immediately met with positive feedback from our collaborators from a biological consulting company. As the diversity of eDNA studies and techniques continue to expand so too will the necessity for innovative data interpretation techniques that can account for error and increase trust.

6.1.3 Emerging Techniques and Technologies

Beyond qPCR and NGS approaches in common use, other methods are gaining traction for eDNA applications. Digital PCR (dPCR) can be more reliable and resistant to inhibitor presence for detecting and quantifying eDNA signals at low eDNA concentrations compared to conventional qPCR methods [12], but it is more expensive per sample, requires specialized consumables, and many institutions do not have access to dPCR machines. Third generation sequencing through Oxford Nanopore sequencers also show great promise for eDNA applications as they now have error rates that are comparable to Illumina platforms while generating longer reads in real time[229]. Other researchers are testing CRISPR-Cas9 with isothermal recombinase polymerase amplification (CRISPR/RPA) technology to amplify eDNA [230,231] instead of qPCR. This innovative approach is compatible for field use as a rapid bioassessment tool and has promise as an invaluable tool in species detection in remote environments without access to modern molecular equipment [230,231].

eDNA holds great promise as a revolutionary tool in molecular ecology because it enables sampling of difficult-to-access organisms and ecosystems. However, the majority of eDNA studies today still require the presence of humans to collect and process samples. Engineering advancements with eDNA in mind may change this. Robotic samplers, once tried and tested, have the potential to overcome the temporal and spatial limitations faced by humans [232]. For years now ROVs, unmanned aerial vehicles (UAVs) such as drones and autonomous underwater vehicles (AUVs) have been used to collect remote data. Some researchers have modified these tools to collect eDNA samples [25,64,65]. Others have even created samplers that are able to collect and preserve samples through carefully engineered instruments that capture and filter water followed by injection of preservation fluid into individual capsules to maintain eDNA sample integrity for up to three weeks [232]. Temporal and spatial constraints of ship- or shore-based sampling are alleviated through the higher resolution sampling enabled by ROVs, UAVs and AUVs. Through their use, time and spatial series data can be obtained at a frequency and resolution that is sufficient to reflect natural eDNA signal variation caused by the tide, spawning events, and vertical migrations of marine organisms. It is possible that one day this type of remote sampling collection tightly paired to oceanographic and geographic data will be widely used, and that even processes like extraction, amplification or even sequencing could be done remotely with creatively designed instrumentation in areas inaccessible to human sampling.

6.2 eDNA in Applied Conservation

Though eDNA methods continue to expand at a rapid rate, their uptake into conservation decision making is still very limited. This is due to many reasons including: 1) misrepresentation of uncertainties associated with monitoring results [82], 2) inflated claims of applications [233], and 3) inappropriate extrapolation of preliminary results [234,235]. To remedy the skepticism of managers and, and end-user communities, it is essential for scientists to clearly communicate both the limitations and benefits of emerging technologies and assessment methods in the context of management applications.

Creating a communication workflow plan that conveys information clearly, including uncertainty and appropriate data usage, can help prevent surprises when applying and interpreting eDNA-based methods. In fact, the first decision to be made by managers is whether eDNA is actually the right tool to be used at all, based off the many situation specific factors that must be considered. Figure 38 depicts a decision tree that can assist managers with this decision making process in a way that is easy to interpret, and does not require in-depth understanding of eDNA methodologies [82].

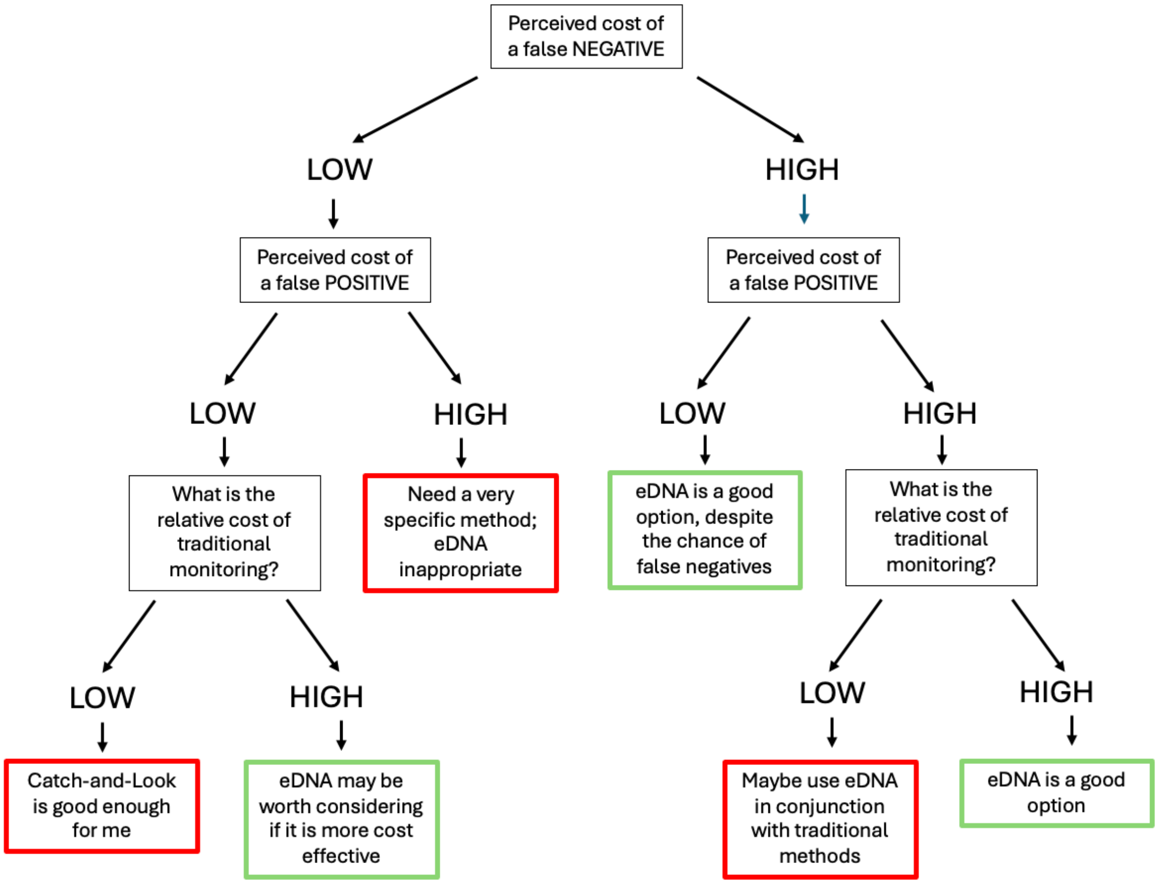


Figure 39. Decision pipeline for eDNA method used. eDNA methods have high expected sensitivity (low false negative rates), and thus are likely to be extremely valuable when the perceived cost of a false negative detection is very high. In these cases, the decision for which monitoring method to use will likely be based off cost. In certain cases, despite the risk of false negatives, eDNA might be the best or only available option. However, if the cost of a false negative is low, eDNA may not be the right choice. Figure adapted from [82].

An effective communication workflow plan must include aides that are understandable to a non-expert, like charts, decision trees, data visualizations, fact sheets, and criteria for outlining the next steps after both negative and positive detections.

These plans ensure transparent communication that meets the needs of end users, supporting timely, evidence-based decision-making. Such plans also define how, when, and what information will be shared with the public, and can collaboratively developed by scientists, decision-makers, and stakeholders before eDNA sampling begins (Figure 39).

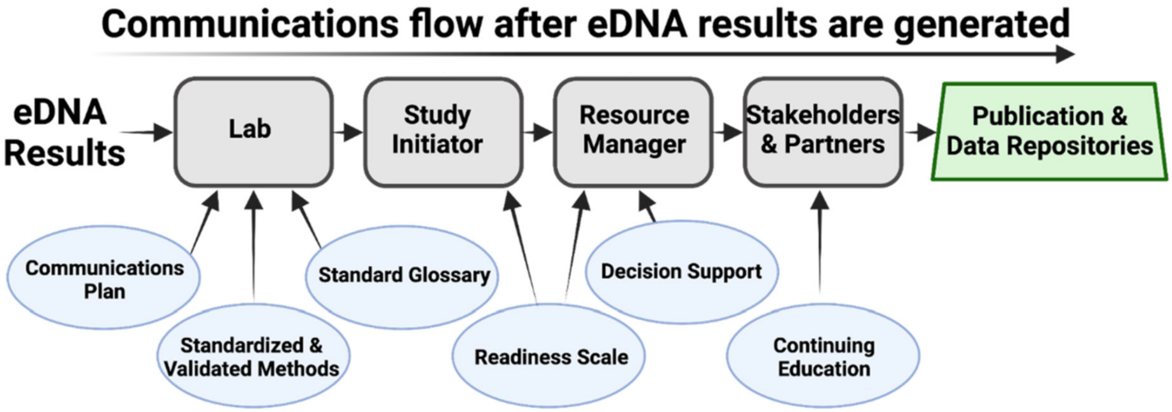


Figure 39. Communication workflow diagram showing key points of communication and associated communication strategies. Workflows are ideally developed jointly by scientists, managers, and other end-users and communication may be iterative between each step of the process. (Adapted from [236])

Undoubtedly, some eDNA methods are more ready for uptake into conservation decisions than others based on the intended application (e.g., species surveillance vs. abundance estimation) and the biological and environmental context. For example, targeted eDNA detection approaches may represent the best available technology for inferring the occurrence of rare species or estimating species distribution as shown in the Olympia oyster scenario where traditional methods are more resource intensive. However, it is important to constantly revisit, and revise expectations based on evolving science to ensure the messaging about the appropriate uses of eDNA reflects the most current science and readiness evaluations [171].

6.2.1 Data Standards, Methods and Harmonization

A big part of this process is the publication of best practise guidelines and adherence to standardized methods that reduce much of the influence of experimental unknowns, improve comparability between projects, and enable integration into policy. There is currently an international push for standardization in field and laboratory techniques for eDNA surveys. Scientific standards are an important aspect of ensuring quality and reliability and are often essential for developing trust in a scientific enterprise. They are also valuable because they help advance eDNA research tools and services and make them available, appropriate and trustworthy for institutional uptake for regulatory purposes[237]. Several groups have made notable steps in the publication of methods guidance documents, and the creation of eDNA resource repositories (Table 11). In Canada, we have two national eDNA standards, one for eDNA reporting requirements and terminology and the other for targeted qPCR assay performance criteria, that have recently been released [107,143] (Table 11).

As standards are established, eDNA application will be more globally accessible and used to inform and influence policy. A particular strength of eDNA is its amenability community-based science. As described above, the importance of community consultation and involvement in the development of environmental surveying and remediation projects is expanding. Community-based science initiatives increase ecological survey capacity without a large increase in project costs, and promotes better public understanding of the environment and its challenges [238]. Larger sampling capacity subsequently leads to more data that are helpful for academic and management initiatives. Effective citizen science projects have been completed by groups such as

EnviroDNA [239], NatureMetrics [240], Living Lakes Canada [241] , Invasive Species Centre [242], and the Hakai Institute [243]. However, global standardization of best practices are needed to make these community-based initiatives more reliable and comparable [244]. As the Anthropocene continues, harmonized research is vital to identify global patterns of change in biodiversity and species distribution .

Table 11. Examples of eDNA standards, guidance documents, and repositories

Category	Type	Location
National Standard	Environmental DNA (eDNA) reporting requirement and terminology[143]	Canada
	Performance criteria for the analyses of environmental DNA by targeted quantitative polymerase chain reaction[107]	Canada
	National Aquatic Environmental DNA Strategy[245]	USA
Guidance Documents	Guidance on the use of targeted environmental DNA (eDNA) analysis for the management of aquatic invasive species and species at risk [246]	Canada
	Environmental DNA protocol for freshwater aquatic ecosystems [247]	Canada
	A validation scale to determine the readiness of environmental DNA assays for routine species monitoring[225]	International
	Implementation option for DNA-based identification into ecological status assessment under the European Water Framework Directive [248]	Europe
	Environmental DNA sampling and experiment manual [249]	Japan
	A protocol for collecting environmental DNA samples from streams [250]	USA
	Quality assurance project plan eDNA monitoring of bighead and silver carps [251]	USA
Repositories	Atlas of Living Australia [252]	Australia
	Barcode of life data systems [253]	Canada
	Mitochondrial genomes of Danish vertebrate species generated for the national DNA reference database, DNAmark [254]	Denmark
	The aquatic eDNAtlas project [255]	USA
	eDNA Explorer[256]	USA
	Global Biodiversity Information Facility (GBIF) [257]	International

Globalized research also generates immense amounts of data. In this age of information, having comprehensive, accessible, and well-organized data repositories is necessary to facilitate meaningful worldwide collaboration. For example, a single metabarcoding study may generate millions of DNA barcodes and identify many different taxa. This information is often published in the scientific literature in a way that is not comprehensive or comparable to other similar work. There are many examples of open-access repositories aiming to address this problem to provide the data depth that is needed for eDNA studies (Table 2). The barcode of life database (BOLD) is working to create reference genetic libraries of barcode regions for as many species as possible, and the Atlas of Living Australia contains occurrence data from thousands of different contributors [258]. There are also repositories of different primer sets that have been designed and used, and repositories of longer gene sequences like the mitogenome repository of Danish Vertebrates (Table 2). For targeted eDNA studies these long gene sequence repositories are immensely valuable because they provide ample sequence information for powerful assay design. Additionally, eDNA collections offer the benefit that samples are easily preserved, and can be tested with multiple primer sets, enabling the characterization of multiple taxonomic groups from the same environmental sample[22]. Once sequence data has been generated, the archival sample can always be re-classified, using the ever-growing global sequence databases as more and more species are added to them.

Though these are all important steps, the value of eDNA to industry, government and decision makers would be greatly improved through standardized reporting, and incorporation of eDNA data with pertinent metadata into widely used, accessible and curated repositories. The Global Biodiversity Information Facility (GBIF) is attempting to fill this gap. They are a massive user-friendly, open access database for global biodiversity data, that now allow molecular eDNA data to be visualized alongside traditional biodiversity observations. In fact the metabarcoding dataset from the rockfish project was one of the first eDNA datasets to be published through their platform[259].

Data repositories facilitate global sequence availability. Increasing the accessibility of genetic sequences will aid creation of targeted assays and improve the ability of metabarcoding and metagenomics approaches to recognize species diversity. As the relative costs of some molecular biology technologies - including sequencing - decreases, the global demand and accessibility will increase. However, financial and technical barriers continue to exist, particularly impacting widespread access to new promising sequencing and molecular technologies.

6.2.2 Equity and Accessibility

As emphasized many times within this thesis, global biodiversity monitoring is vital to understand the rate and implications of biodiversity loss. However, it is also clear that the largest gaps in biodiversity data exist in areas that are hard to access, or do not have the resources to conduct large scale biodiversity studies such as the Global South, and in areas beyond national jurisdiction (ABNJs) like the high seas. If we are to meaningfully attempt large scale biodiversity monitoring it must be done in an equitable and fair way that has often been overlooked by exploration into new frontiers in the past. In fact, the equitable benefits sharing of marine genetics resources like eDNA biodiversity data is one of the goals outlined in the Kunming-Montreal Global Biodiversity Framework[3].

The standardisation efforts described in the previous section, though immensely valuable, are largely led by researchers in the Global North with the best labs spaces, equipment, technologies, and funding. This means that proposed “International Standards” are currently not truly global, ironically leading to areas with the most biodiversity not being represented in the process. I participated in a recent study where eDNA scientists from around the world were surveyed with a series of open-ended questions to highlight themes that need to be seriously considered for International eDNA standards to be truly equitable[237]. From this work, four overarching themes emerged:

1. **Create Adaptable Standards:** Standards should be flexible, with different protocols for varying contexts (e.g., in-field vs. in-lab), materials, and goals. Pragmatic guidelines are needed to ensure comparability without stifling innovation. Flexibility fosters shared understanding across communities and regulatory bodies, enabling broader use while adapting to evolving technologies and diverse applications.
2. **Develop Regional Capacity:** Building regional capacity in the Global South is crucial to avoid "parachute" or colonial science and ensure equitable access to eDNA technologies. Initiatives like mentorship programs between the Global North and South, regional workshops, and local lab development can help address knowledge and infrastructure gaps. For example, the African BioGenome Project has trained over 400 African scientists and aims to create a biodiversity genome database for endemic species[260]. These efforts increase

access to sequencing technology, support capacity building, and foster locally relevant genetic research and data sharing.

3. **Address Data Sovereignty:** eDNA data publishing standards should be flexible, recognizing that not all data can be geolocated and allowing communities to opt out of sharing sensitive data due to sovereignty or security concerns. Ethical engagement with indigenous communities is essential, incorporating frameworks like UNDRIP (UN Declaration on the Rights of Indigenous Peoples) and the CARE (Collective Benefit, Authority to Control, Responsibility, Ethics), and OCAP (Ownership, Control, Acess and Posession) Principles for Indigenous Data Governance to balance open data with protecting traditional knowledge. Researchers should also value local expertise equally to genetic data, ensuring equitable participation and addressing historical harms of scientific colonialism and exploitation in research practices. In accordance with the CARE principles, the rockfish biodiversity data shared on GBIF was only done with permission from our Kitasoo/X'ais x'ais collaborators, and specific locations are masked to scale deemed appropriate by the nation.
4. **Standardisation should uphold global capacity building:** International eDNA standards should be accessible and adaptable to practitioners worldwide, considering local biological and social contexts. Standardisation should build capacity, promoting an inclusive, global network of researchers. Open, affordable standards can democratize research, addressing the biodiversity crisis through global collaboration, training, and mentorship, with broad, inclusive dialogues.

These considerations are immensely important, especially in the context of current global conservation goals to protect 30% of the worlds land and oceans by 2030. As a new scientific field that is not yet deeply mired in bureaucracy and tradition, I have hope that global eDNA monitoring will be truly accessible and useful on a global scale.

6.3 Conclusions

Virtually no area of environmental surveying lacks the potential to benefit from the many forms of eDNA analysis, provided the methods are appropriately applied. The rapid pace of globalization and climate change emphasizes the immediate need for rapid monitoring methods. Reliable and accessible eDNA methods have that ability to assess current biodiversity enabling effective estimation of future trajectories and negative impact mitigation. Through attentive implementation of these techniques, we add an entirely new set of tools to supplement conventional methods, providing a high calibre of resolution and confidence in all fields of environmental assessment.

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Appendices

Appendix 1. Identification and Accession numbers for sequences used for eOSLU2 assay design from the National Center for Biotechnology Information (NCBI), and from in house sequencing

Abbreviation	Species	Accession Number	Source
ma-CALUfa	Dog (*Canis lupus familiaris*)	NC_002008.4	NCBI
ma-FECA	Cat (*Felis catus*)	NC_001700.1	NCBI
ma-HOSA	Human (*Homo sapiens*)	NC_012920.1	NCBI
mo-OSLU	Olympia Oyster (*Ostrea lurida*)	KC768038.1	NCBI
mo-OSLU	Olympia Oyster (*Ostrea lurida*)	NC_022688.1	NCBI
mo-OSLU	Olympia Oyster (*Ostrea lurida*)	F112697-mo-OSLU-3	Helbing
mo-OSLU	Olympia Oyster (*Ostrea lurida*)	F112698-mo-OSLU-4	Helbing
mo-OSLU	Olympia Oyster (*Ostrea lurida*)	F112699-mo-OSLU-13	Helbing
mo-OSCR	Olympia Oyster (*Ostrea lurida* (potential hybrid))	F112700-mo-OSCR-1	Helbing
mo-OSCR	Olympia Oyster (*Ostrea lurida* (potential hybrid))	F112702-mo-OSCR-3	Helbing
mo-OSCR	Olympia Oyster (*Ostrea lurida* (potential hybrid))	F112703-mo-OSCR-4	Helbing
mo-OSCR	Olympia Oyster (*Ostrea lurida* (potential hybrid))	F112704-mo-OSCR-5	Helbing
mo-OSCR	Olympia Oyster (*Ostrea lurida* (potential hybrid))	F112701-mo-OSCR-2	Helbing
mo-OSCR	Olympia Oyster (*Ostrea lurida* (potential hybrid))	F112705-mo-OSCR-6	Helbing
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	EU672831.1	NCBI
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	KJ855241.1	NCBI
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	KJ855242.1	NCBI
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	KJ855243.1	NCBI
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	KJ855244.1	NCBI
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	KJ855245.1	NCBI
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	F112706-mo-CRGI-1	Helbing
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	F112707-mo-CRGI-3	Helbing
mo-CRGI	Pacific Oyster (*Crassostrea gigas*)	F112708-mo-CRGI-9	Helbing
mo-OSDE	Lamellated Oyster (*Ostrea denselamellosa*)	HM015199.1	NCBI
mo-OSDE	Lamellated Oyster (*Ostrea denselamellosa*)	NC_015231.1	NCBI
mo-OSED	European Flat Oyster (*Ostrea edulis*)	JF274008.1	NCBI
mo-CRAN	Portuguese Oyster (*Crassostrea angulata*)	EU672832.1	NCBI
mo-CRAN	Portuguese Oyster (*Crassostrea angulata*)	FJ841965.1_	NCBI
mo-CRAN	Portuguese Oyster (*Crassostrea angulata*)	KJ855249.1	NCBI
mo-CRAN	Portuguese Oyster (*Crassostrea angulata*)	NC_012648.1	NCBI
mo-CRAR	Suminoe Oyster (*Crassostrea ariakensis*)	EU672835.1	NCBI
mo-CRAR	Suminoe Oyster (*Crassostrea ariakensis*)	FJ841964.1	NCBI
mo-CRAR	Suminoe Oyster (*Crassostrea ariakensis*)	KJ855254.1	NCBI
mo-CRAR	Suminoe Oyster (*Crassostrea ariakensis*)	NC_012650.1	NCBI

mo-SACU	Hooded Oyster (*Saccostrea cucullata*)	KT992044.1	NCBI
mo-SACU	Hooded Oyster (*Saccostrea cucullata*)	KT992045.1	NCBI
mo-SACU	Hooded Oyster (*Saccostrea cucullata*)	MF198445.1	NCBI
mo-SACU	Hooded Oyster (*Saccostrea cucullata*)	MF198445.1	NCBI
mo-ALPL	Fingerprint Oyster (*Alectryonella plicatula*)	MW143047.1	NCBI
mo-CRGA	Giant Rock Scallop (*Crassadoma gigantea*)	MH016739.1	NCBI
mo-CRGA	Giant Rock Scallop (*Crassadoma gigantea*)	MN316631.1	NCBI
mo-MABA	Baltic Macoma (*Macoma balthica*)	KM373203.1	NCBI
mo-MABA	Baltic Macoma (*Macoma balthica*)	KM373204.1	NCBI
mo-MABA	Baltic Macoma (*Macoma balthica*)	KM373205.1	NCBI
mo-MYAR	Soft-Shell Clam (*Mya arenaria*)	KJ755996.1	NCBI
mo-MYAR	Soft-Shell Clam (*Mya arenaria*)	NC_024738.1	NCBI
mo-MYCA	California Mussel (*Mytilus californianus*)	JX486123.1	NCBI
mo-MYCA	California Mussel (*Mytilus californianus*)	JX486124.1	NCBI
mo-MYCA	California Mussel (*Mytilus californianus*)	NC_015993.1	NCBI
mo-MYED	Blue Mussel (*Mytilus edulis*)	AY484747.1	NCBI
mo-MYED	Blue Mussel (*Mytilus edulis*)	HM489873.2	NCBI
mo-MYED	Blue Mussel (*Mytilus edulis*)	MF407676.1	NCBI
mo-MYED	Blue Mussel (*Mytilus edulis*)	NC_006161.1	NCBI
mo-RUPH	Manila Clam (*Ruditapes philippinarum*)	NC_031332.1	NCBI

Appendix 2**.** Running parameters, and output used for *uniksesq* v0.1.0 program for eOSLU2 design

Length of K-mers	25
Threshold Length	100
% of Average ID in Ingroup Sequences	25
Tolerated # of consecutive K-mers in Outgroup	1
% Unique	90
Maximum Proportion of Outgroup Sequences Tolerated	0

Output Region	**Sequence**
29-300	GTTTTATATAGTATTTGGATTTTGGGCCGTTTTAGCCGGAACTAGGCTTAGGTCCTTAATTCGGTGAAGGCTATTTAATCCTGGGTCTAAATTTCTAGACCCTGTATGTTATAATGCTGTAGTAACTATGCATGCTTTAGTGATAATTTTCTTTTTTGTCATGCCGGTAATAATTGGCGGGTTTGGAAATTGACTAGTGCCTTTAATACTTCAAGTGCCTGATATACAGTTCCCTCGAATGAATGCCTTTAGATTTTGAGTTTTGCCGGTTT
302-515	TCTTTATTTTATAGTAGTTTCTGCTTTTGTTGAAAATGGTGTTGGGACGGGTTGAACTGTTTACCCACCGTTATCAACTTTCTCGTACCATGGAATGTGTATAGACTTAGCAATTTTAAGGCTTCATTTAGCTGGTATTAGGTCAATTTTTAGATCAATTAATTTTATGGTTACAATTACTAATATGCGGTCAGTAGACGGGCATTTATTAGCG
518-723	ATTTCCGTGATCAATTAAAGTAACATCATTTTTACTACTAACTACTTTACCTGTGCTAGCTGGAGGTTTGACTATGCTATTTACAGATCGTCATTTTAATACTTCTTTCTTTGATCCTGTTGGGGGCGGAGACCCGGTATTATTCCAGCACTTATTCTGGTTCTTTGGCCACCCAGAAGTTTATGTACTAATTCTTCCTGGATTTG
725-1181	TATGATTTCACATGTCTTATGTTTCTGATCTAGAAAAAAAACTGCTTATGGTAACATAGGAATGTTTTATGCTATGTTAAATATTGGATTTCTAGGCTTCATTGTGTGAGGTCATCATATGTTTGTTGCTGGAATGGACATTGACACGCGTGCTTATTTTAGCGCTGCAACCGTTATTATTGCCGTGCCTACTGGTATTAAAGTGTTTGCCTGATTAGCTACAATAATGGGTTCTAAAGTGTCAACACAAGCACCTATGTTGTGAAGAATAGGATTTATTACTCTTTTCACTATTGGTGGTTTAACCGGATTAATTTTGTCAAGTGCTTCAGTTGACGTGACTCTGCATGATACCTATTTTGTAACGGGTCATTTTCATTACGTGCTGTCTATGGGGGCTGTGTTTACTATTTTAGCGGGTTTTACCCACTGGTCTCCTTTGTTTTCACGGGTTATAATACACCGGC
1193-1422	AAAGATGAAGAGACATTTTATTGCAATATTCTTAGGTGTTAATGTAGCATTTTTGCCTCATCACTTTTTAGGATTAGCTGGAATGCCTCGGCGAGTTGTTGACTATCCTGATCAATTTTGATTTTGAAACAAAATCTCAACGTTTGGGTCTCATTTAAGAACCGCATCTCTTTTGTTGTTTGTATTTCTAATTTGAGAGGCTTTTATGTCTCATCGACCTGTAGTAAGTG
1427-1537	AAATAGTTCAAGGTCTCCTGAATGGTCTGTAATTGCGAATCTTCCAAAACACGCAGCTTTGGAGAGAGCAAAAATATCAGACGGTAAGATTTAAGTTTAAGTGTTTTTAGT

1572-1951	**CTGTAAAAAGAAAAAACATATAAATCATATATTCATAGCTGACATAGAGCTATTCAGGCAGTAAATAAAAAATACTAGTGTTATAAATCTAGAGTGCTGTATAAAATAAATAATACACTGCTTAATAATGAAATTGTATAGACTTACATCCAAAAAATTATTCTCTGGTTTTAAGAATCAGTCTTCTAATTTTACGCATAGAAGACTGCGCCACTCTTCTCATATACTTCCTCGTTCTCCTTACCATATCTTGGACCCTAGACCATGACCCGTTCTAATAGCAATTGGTTTATGGGGGCTAGCTATAACTTTTATTTGTTGAGCTAACGGAGTGCCTTGTAACAGGCTTCTTTTAGGGGCTGTGATTGCAGCTATGGTTG**
1962-2302	AACTCGTGATCTTGTAAATGAAGGAACTTTCCAGGGTTTTCATACTAAAAAAGTTCAAAGCGGTCTTACTATAGGGTTTATTTTATTCTTAGTATCTGAGTTGATATTATTCTTCTCTTTCTTCTGAGGGTTTTTCCATTCGGCTTTGTCTCCTTCATTGGAAATTGGTTGTTGTTGACCCCCGGCTGGGATTGACTGCTTAGATTGAAGTAAGGCTCCGCTTCATAATACAGCTCTTTTAGTTGCTTCTTCTTGTACTGTTACATCTAGTCACAAGTACTTAAAAACCGGAAACTTTTCTAGAGCAGTTGGAATGCTACTTCTTACTGTGCTACTCTCTG
2307-2515	ATTTGTGAAAAATCAATATGGGGAATACGCATGATCCTCTTTTACTATTGCCGATGGTGTTTACGGCAGGTGTTTTTTTATGCTGACCGGGTTACATGGAATGCATGTAATAGGCGGAACTTCTGGTTTATTATACTGCCTTGTTCGTATGTTAGCTCGACAGTTCTCTACAACACACCATCTTGCTTACACTTTTGCTATTTGGTATT
2603-2732	TGTTGATGTCAAAAACTACAGGGAGTTATCCCTCAATACCTTAAAATCAGTTTTAGTATAATTTAATTACAATGGTCTTGTAAACCTTAAATCTAAACTTTTAGAGGCTGATTTAATAAGTCTAGACTAG
2745-3761	CATAAAATTTTCGTTTTTATAGAGTGGGTTATCCTCGCCTAGATTGCTTTTAAATTTTAAAGTGAGTTCACAACTAAAAAACTATTACTATAGCGCTCTAAAGTACCCGTGTCCTCCAAATTTAAATATCTGATGAAATATGGGATCTTTACTATTTCTAATTCTAAGGGTACAAATTGTAAGGGGGTTACTTCTTAGAGTTAGCTATACGAGCGATGAGTTGTTAGCTGTAAAAAGGATGAACTATATTCTTCGTGATGCCAATTATGGATGGGCGATACGCGTAATTCATATAGGAGGGGCTTCTTTATTTTTCGCCCTAATTTACATGCATATTTGTCGTGGTGTATATTATGGTAGCTATATTAAAAACCCCCACCTTTGATACAGTGGTGTAACTTTATATTTTATCTCTATGGGAATTGCTTTTCTTGGGTACGTCCTTCCATGAGGAGTGATATCTTACTGAGGTTTAACAGTGATTACTAATATGTTAACTGTGGTTCCAGGAGGAACTCGTATACTGCAGTTGTTTCAGGGCAGATTTATTATTGGGTCTGTTACTCTAAAACGTGTTTTCATTTTACATTTTTTACTACCTTTTGTAGTTTTAGGGTTGAGGGTGGTCCATGTCTTATTATTACATGAAAAAGGATCTAGAAATCCTTTGGGGTTAGATCTTAGAAATTTCAGGATTCCGTTCCATCCATATTACACAAGGAAGGATTTAGTAGGGTTTGCATTTGGGCTAGCTATTTTAGTAGGACTAGCATTTACATTTCCAAAAGTAACAGCAGATCCTTTACAATGACAAATTGTAAATAAAGCACAAACTCCTAAGGTAATTAAGCCGGAGTGATACTTTTTATTTGCTTATGCAATTCTTCGATCTATTCCGTACAAAGCAGCTGGTGTTAGTATGATGTTTCTTTCTATTGTTAGGATTGCGGTACTACCCCTTCTGGGGAATCGATCTAAAACTCAAAGGATAGTAATTTGTCCAATTGCAGAGCTCGT
3768-3928	TATTTGAGTGGCAAATTTTATATTCCTTACGATTGTAGGTGGGCAAGAACCGGCTGGTGTTTGAGTTATAGGAGGCCAAATTGGGACTTTTGTTCACTTTTTTTGTCTGTTAATTATACCACTTAGAAGACTAGCTTGAGAGTCCTTGCTTTTTGATACAC
4042-4283	TGTTTTTGTAGCTCTTAGAATGGCTACTTCTTGTTTTTCTCCTTATAGATTCCTTTTTATACAAACTAACCATACGCTTGAACGAATTTGGGGTTTAGTTCCTTTGGTTATTTTAACCTTTTTGTCATGGCCTTCTATAGGTTTGTTATACGCAATAGCAGAACTGGAGACTCCGTTAATCACATTAAAGTGTGTAGGTCATCAATGATACTGAGAGTATGAATACTCGGATTTTGGGCTGG

4285-4657	TTCTTATAACTCTTACATAGTACCAGAAGAAGACTTAAAGCTAGGGGAGCCCCGAATGTTAACAGTAGATAAATCAGTAGTTTTACCGTTTTCAGTTTGATGTCGAGTATTAACTACATCTTTTGATGTGATTCACTCTTGAGCAGTTCCGGCTTTAGGTGTAAAATCAGACGCTGTTCCTGGTCGACTTAGAGAAAGGAGAATTAAAGTTGAAACTCCTGGTGTTTTTTGAGGCCAATGTTCAGAAATTTGCGGGGCATTACACAGATTTATACCTATTCGAGTAGAGGTTGTAAGATGTGAAATTTTTGGTAAGTGAATTAATTTTATGGAAGAAATGAATTAAAATATTTAGTCAGATAGATTAAGCTAT
4755-4944	AAGGGCAGAGCTTTTGACTTTCTTGTGATTCTGGGTTTAACTATAGCAACATTTTTATTAATTTCAATACTGTGGGCATGGGGGGTATATAAAGACCCTTCTAACATTATGTCTGATATTGAATCGGATTTAAGCTCTTTATAAAAGTCAAGTTTTGTATACAATTATGAAAGAAAGGTAGTTTATTCCA
4953-5059	GGTTTCATAATCCGAAAGTAGCAACGGCTTCTTTCTGAATATTGCGTGATAGCACATGTTAACAAAACCTAAGAAAATTTGACGTCAATAAATCTAAGGGGGATAAG
5216-5386	TGTTTTGGGGACACTAGTTCCTTTCATCGGGGTCTTAGAGCTAAGGATGCTATAATATTAGGCAGTTTATATAAAAAACTTTAAATGAACAAAGTATGGGAACATCAGAAATGTTAGTTAGTATTTATAGGTCTGTAGAGATTTTCATAAAAATTAAAAAGCTTTAGTTAA
5390-5504	AGTACCTTTTGTATAATGGCCTTTTAAGAGAGTTTGCTTTATAAGCTTATCCCGAATTAAGCGGATTTTTTAAGGAACGTGAAAGGTTTTCGATGTGAGAGATCTTTAGATTTCT
5507-5680	AAGGATGTGATATGCAATATCGAAGCTTAAGATAGCTGGTTAAGTGATAAATGCCTTTAAGTGCAGCTTTGAAGTAATATTTTGTAATGGTTAAGATTGCAGATTAACTTTAAAAGTGAAAAGCCTGGCTAAAGGTCGGGTTTATTAATTTTCTTAAAATTTGTTCTTCGTAGA
5710-6420	ATAAGAGTATTTAACAATTTATCTCTGTATAAAAAAGAATAATTTATGGTATTTAAGAGGTTAAAAAATCGCTTAATCTCCAAAAATGGTCTTATGGTAAGTTTTCATGAACAGGATTAGTAGTAACTGTGCAAAATCTTGTGTTATAGCAAGGATTTTGAGTGAGTTCTTTTGGTGGTATTATATTTGTGGTTTTAAGGTTAATATTTAGGGTATTTATAATCTTTTCATTAAGACTTTTTTGAGTTTGGGTCATATTAGATTTAAGCACTATTTTTCTAATTCCTTACTTAGTGTGCCAAGGGTCCTCTAGAGGGCGTTGGTATAAAGGAGTGGCTATATATTTTGTAGTCCAGTTTATAGGAAGTGCTCTGATTTTATATTCTGTTGTAACACTTTCAATTACCAGGATACAACTATATGGGGAAGTGTTAGTTCTTGTCGGGTTTATGCTAAAACTTGGGCTATTTCCGATACATTTCTGGGTACCGCGTGTATTCGCGAACCTTCACTATGGAGGTATTTTTATTGTAGGAGCGTTACAAAAGGTATTTATGATTTGTGCTGTGCCTTGTACATTTCAGAATATTGGATGCAATTCTCTTGTGTATTTTATAGCTTTAACAAGTGCAATCTATGGCCCCATTGCCATGTTTAACAGAAACGACGTAAAAACTTTTTTAGCATACTCATCTGTAAATAATACAGGTC

6428-6905	TATTTGTAATTTAGTTAGAACTACGCTAATAGGAATTTATGCATTTATCTACGTAGTTAGAATAGTGTTCCTAGTGATAATTTTTTCAAGGTATAATGGAGATAGAATCTCAGGAATCGGTTTTGGCATGCCGGATTTTTATAGTGCTGGGTTTATATCTATTGCTACTAGATTTTCAGGCTTCCCGCCATTTACTGACTTCTGCGTTAAGTTTATAGTGTTAATATCCTGTGCTGAAAAAAACTTATGAGTGTTAGTATACGCGGTCTTGATTAGCGGTTTTATTAATTTATTAGTGTGAATTTGATTATTAGTTATAAATATACCAACTTTAGGCGATATAGGTTTTACATTGCAAGCCTCTGTTGGTATGTATTCTGAGGTTTTAAATAAAACATGTGTTTTATGGAATATTTGTGGTGGAATAATCGTAATGAGATTATTATAAAACTTTAATTAGTTCGTGGGGTAGTTTAAT
6979-7214	GAAAGACTGTAAATCTTTAAGAGCTGAAAAGCTGTGATGTGTGAGTACACAGTTTCTAGGTTTAATTTTATTTTTTGCAATTTTTGCATACTTCTATTCTTCGTACTTTTCTAATTTTCCGTGTGTCACTGCTTGTAATCAAAGCATGCATGATTTGTATAAAAAAGCATATACGGGCACTATGCATAGAAAGTCCCAACGATTCGGTGCTTTAGGTATAGGTGTTGGAGAGGGTT
7216-7631	AAGATGGGTATTTGACTTTGCAATCACAGCGGTTTTAATTGGAGTACTACCCTGAGGATTCCCAGTTTTAGCTTCTTGGGAAGTGGTGGCAGGTTTAATACCGAGACTTTTTTATAGTGTAAATATCATACAAACAGATACAAGTATTTTTAGTAAGTCACTTAAATTTAAACAATCCGTTTTAAAATCGTTTTTGTACTTCCCTCTTATATTCCTTAGAGTTGTAATTCGGCCGGTAACTCTAACAGTTCGAATGTTGGCCAATGCTTTAGTTGGTGCTATTATCTGTCATTCTGTAGGAAAGAAGGTAGTATACGGATTTGTGTATGGGTGAGGTATTGATCCGCGCCCGTTTTTCTATTTATCAATTGTAATGATTTGGGAGTTAGTCGTAATGTTAGTTCAGAGGTCTCTAT
7726-7870	TTGTTAATCAAAAGTTACAAAAATCTACTTTGAGCCTCTCTGAATTGGTAAGAAGTGAATAATTTACGATCGGTTTCGGCCCGAATCTTGGCTAATTTAGGCCCTTGCTATAAAAATGTGGGGAATAGTATAATAAATTACTTAG
8076-9351	TAAATTTATTTGCAATTTGGACTGCTTATAGCCTTACTATGTGAGGTGGGCCTGGTGTATCTTGATCTTCTTTAAGAAATATGATTTGCGTAGATGTATTTAGAATAAGTCTAGTTAGCCTGACGTTTTGAGTATGCGGGATTAGGGTTATAGCTAGAGGCGCTATATGCACACTTCGTGGTAATTACTTAAGATTTTGTTGGCTTACTGTTTTGTTGTGCTTACTTTTGGTCGGGTGTTTTATGGTAAATAGTTTTATCGCATTTTATATCTTATTTGAAAGAACCTTGGTCCCTTTGGTTGTTATTATTGCCCTTTGAGGCCAGCAACCAGAGCGTATTTCGGCTATTCGGTATATTAGGGCATACACAGTAGGAGGCTCTTTTCCTCTTTTAGTTGTTTTGGTTACTATGGAATCTAAAATTGGTAGTAGGTTTTTTTGGTATGTAAGAGCTAAAACACAATTTGAGTGATGATTTTGAGCTATGTGCTTTTTGGGTTTTTTGGTAAAACTCCCAGCTTATCCTGTACACACATGACTTCCTAAAGCTCATGTTCAAGCCCCTGTTGGTGGTTCAGTAGTTCTAGCTGGAATTTTACTGAAATTAGGAGGTTATGGAATTTTTCGTTTGATAATAACATTTTCCTATGGGTTAGAATCATTCGGTTTATTTGCTGTGTCTTTGTCTGTTTTTGGTAGTCTTTACGCAGCTGTAATATGCGCCTGTCAAAGAGATGTTAAAAAACTCGTAGCTTACTCATCTGTTTCTCATATAGCTTTTCCAATTATTGGGTTATTTAGGTGTAGGGAAGTTGGAATTTCTTCGGCATTTATTATATTAGTAAGGCATGGATTCATTTCCTCAGGGCTATTTGCTTTGTGTGGGATTAGCTCTGAGCTTACGGCTACTCGTAATCTAAGACTAATAAGAGGAGCCTGTCGAGCTGTGCCAATTCTCGGTTTTATGTGGCTAGTATTTATTATATCGAATTTAGGGGTTCCTCCATGCCCTAGGTTTATTAGTGAGGTATTAACTGTAGTTGCAGCTAGATCTATAGGTCCTTGAGTTTTTGTGATTCTAGCAGTTTATTTAGTAGTAAGGGGTGTATACAGTTTTAGGTTATACTGCCAGCTTACTCATGGAAGACAAGTAAGAGACAGATGAATAGGATTTGATATAGTAAGACCACGGGATATATTATCTTTATTATTGTTGTTTTATCCCTTGGTTGAGGTATTATTTAAGTGAGATTTATGAAGAATAATTTAAAATGA

9368-9502	CTGGTATAATGTCAGCCTAGAGTTGCCTAAAATACACAATGTAGAACACTTTCGTAATGTGCTAAATAGATAAAAGAGATAAGCTAGTGTGACTCCTAAAAGGAGTAGTAAGGCAGCATAAGCATAAGTCTAAGA
10257-10394	GTAGGGGAACCTGCTCTTATGATACTTACTTATATTAAACTTTTTGTTACTTGAGTTAAATAAAAAAGTTAAAAAGAATTAAATTATAAAGGTATAAGTTAGGCCATTTTAATAGAAATGTTTGAATAAAATTAACCA
10999-13276	ACGAAAGGACCGTTAAAAGAGGAAGTTTCCTTTTAAAAGAAATAGATTTAAATTGGAGTAAGTATAGTCTGGAGCTGTAATAATAGCAAACTAATTAATTAGTGTTAAATTTTAGGCAGACTTATATGGTTGTATTAAATTCTTATATTTTAGTTTTTTTTAGATGTCTATTTGGTTTACTAGCTGGGATTTTAGCTAACTGAGGGTTGCTGTTTTGGTTCTCTTGGGAGTTAGGACATGTTTCACTGTTATCAGTAAGCTTGGAGCTACGGGTTGACTGAATAGCAGCATTGTTTGTAGCTACCATCTTCCTTATTTCTGGTTGTGTTGGGTTTTTCATAAATGTATATATAAGAGGAGAAGATAGAGTAAATCGTTTTAATGTTATGTTATATCTTTTTATTCTTTCTATAATTGTTTTAGTAGTTGCTAGTTCCATGCCTGTGGTTATATTAGGATGGGATTGGTTGGGGGTAACATCTTTTCTTCTTGTCATGTATTACGAAGGTAAAAAATCTTTCGATGCGGCTATAATCACCGGTCTAACTAACCGTATTGGTGATGCTTTGTTGATTTGCAGGGTGGCTGGTATGTGTTTTTCTTTTGACTTAAGGTTGGATATAAAGCCCTATTGCTGGGCTTTAATTTTTGTAGTAGGATGTGCTACTAAGAGGGCTCAAATTCCTTTTAGAAGATGACTTCCAGCTGCTATAATAGCCCCAACTCCTGTGTCTTCATTAGTTCATTCTTCTACTTTAGTTACAGCTGGGATTTATCTTTTAATTCGGTCAAATTTAATTTGAATGAATTCTTCAACTACTTGTTCTTTGCTTGTGGGAGCTGGTTTATCAACTGCTATTATAGCAGGGGTGATAGCTGTTACAGAAAGTGACATAAAAAAAGTAATTGCTCTTTCTACCCTAAGCCAGCTAGGGCTAATGGCATTTAGTCTAGGATTGGGTGAAATTAAATTAGCTTTCTTACATTTACTATGTCACGCCTTTTTAAGGCAGGTATATTCTTAAGTGTAGGTTCTATAATCAGTTTTAATTCCGGAAATCAGTCTTTTAGCACATTTAGAGTTGCTACAATAAATCTTTGCCCAGCAGCTGTATTAGGTTTATTTATGGGGAGGGTTTCTTTAGCAGGTATTCCCGGAACAGCTGGATATATTTCAAAAGAGTCTATCATTGCATCAGGCTATAGCTGTACACCTTGGTTGTCTAGGATCTTGTTGTGGCTAAGTGTTGCATTAACTACCCTTTACTCTTTACGTGTAATTTTAGGATTAACTATAGTAGTAAAGTCCGGTTTGCCGAACTTTACGGCGTCTCGAGAAGTGTTTAGTTTGTCTGTGCCTGGTGTAGTGTTAGTATTTATAGGGTTAGTAGGAGGGGAATTAGTTTTGTTAAGAAGGTCTGGTGGATTTTTTTTCGAAGCAGCTCCTTCAAGTGAGGCTTGATTTATTCCTTATACTATGATTCTGATTGGTACGGCAGTAGCAGTTAGGTTAAAGTTCTTCTACTCAACATTTTATGAATTAGGGTTTAAGTGAATTTATAGAATATTATTTTTAGACTCTGTTTCCCGTTCTGTCTTAAGTGACAGGTCTTACTCAGGTTTTATTCACTCATCTAAAGACGGTGAAAATATGTCTGAGGTTGCTATCCCTCAAACAGTTTTGACTACTGGTTTACAGTATTTAACTTCTATTCATAACTTTGTTCAACGGGCATCTGTACCTAGAGGAGTTGCGGCAGTAGGTAGATTATTTGTAGCAAGTATTTTGGTTTAAATGAAATTAATCGTGTGTATGATTCTAAGCTTTTTAGTTTTATGCTCCATTTATCCAGTACCCTTGTTCTTTTTAAGAGTTTTGTTGAGGCTTTGCATGCTTGCGGAGATTGGCGCTAATTTCAGAGATTTTCTAGCAATATTGGCTTTCATGGTGTATATTAGAGGAATTATAGGGGTTATTGGCTACTTTATTACCTTTTTTCCAAAGAAGTTTGAAAGAGCAGGACAGGAGTGGTGCTTTTACAGCTTAGCTTATATAGGAGGTTTAATTTTTTTTACGCTTTGGGCATTTTCTAGGTATTACCCAGGGTCTCAGCTTTATAGCGTTTGGTATTCAGAAGCTCCTGCAACTTTCAGAGGAGAAGTAGTTCTGTTTCTTGCCCCAATCTTACTTATTGTGATAGTGGCTGTTGTAGTGATATCTAAGCCTGAGCTAAAAACAGTCCGACGTTGGGTTTGAAATAATTCATAAACTAGATT

13295-13756	CACGAAAGTTTTTGGTACTTTAGGGGCTTACTTTAAAAGCTAACACTAAGTGAGTTTAGATGTAATTTTGTTTGTAGTATTCCTATCTTTACTACCGATTTTCTTTTCTACTTGATTTTCAGGTTTACTTCAAGAGTTTTATGATTGAGAAAAATCTTCCCCATATGAGTGTGGGTTTATTATGACTAGAAATCCTGGTGACTTTTCTTCTCGTTTTTTTCATTTAGTAATTCTCTTTTTAGTCTGGGATGTTGAAATTGTATTATTGGTGCCATGTTTTCATGATCTATTGGTATGGTCAGTAGGGGCTTTAGCTGCAAGCAGGTTTATTTTAGTGCTTATTTATGGTCTGTACTATGAAATAATTGAAGGCACTATTAAATGGACTCTGCATACAAGCTAGAGGTTGTAGCTTAATTTAGAGCTTTGAGCTTTTAACTCAACGGTGGGATTTTCCTCGAC
13928-14360	AGATTTGCATTTTCGTAATGAGTAATACTCTCGAGCTAATATCATTGACATATGTCAGACTACCTTACACTATTAATAATATAAAAAAAAATTTAACCTTTTTACATAGGTTAAAAAGTCAAAATTAAAATGGGTTTATACTTACTAATTACAGCAGTACTTGTTTACTTAGGGGTTTTGTTAAGGGTGGCTTATTTTACTTTAATAGAACGAAAATGCCTTAGATCTTTAGGAATTCGGCTAGGCCCTAATAAGGTTAGAGTAGGGGGGATTTTACAACCTGTCACTGACGGAATAAAGCTATTTGTTAAAGAATTTGCTATTCCAGCTAACGCTGCTAAAATTTTTCTGGTTGTTCCTTCAATTTCTCTTCTTTTATGTTTTGTTGGTTGGCAGCTTTTCCCTTCTAGTGGTTTCTCAGCTAGGGGTTCTA
14362-15346	TGATATTTTATTTTTCTTAGTAGTAACCAGGCTTAATGCTCATGTTGCTATTATAAGAGGGTGGGCTTCTAATTCTAAATACGCTCGAGTTGGAAGGGTTCGAGGTTTCGCTCAGGTTATTTCATATGAAATTTGTTTAAGAATTACTCTTGTAATTGTGATCTTTCTTAGAGGAGGTGTGAGGTTTTTCTCATCATCAGAGAACGGGTGATCTTTATGATGAGGTTTAGCTTGGCTTCCTATTATTCTATCTTGAATAACTATTTGCTTAGCTGAGACAAATCGAGCCCCGTTTGACATGGTAGAAGCTGAGTCAGAGCTGGTTTCTGGGTTTAATGTTGAATACTCCGCCGGTGGTTTCGCTGTACTTTTTATTGCCGAATACGGGATGATTATGCTAATATGTACTGTCACTACTTGGAGATTTCTTCAAAACCCTAAAGTGTGGGCTGTTTCTGGAGTGGTTTGGATGTTTTTAAAAATTGGAGCACTTATATTCTTTTTTATTTGAGTACGGGCGTCTTTACCGCGGCTTCGATATGATTTATTAATGCTAATATGCTGAGTAAGTCTTCTTCCAGCTGTTTTAGGTGTTTATGGGTTTATTACATATGCAATAAAAATGTAAAATGAGTTGATCTTTTAGTATATTTAGACTAGTATTGTGGACAATACTGGGAAGTATAATAAAGCACTTTTCAAATTTTTTAAGTTACTTGATTTTGGTAGAAGGAGCTTGTGTAAGACTTGGTGCTATAATAATTCAGGCCAATAGTACACTGCCAGTGCTAGGTTTATTTGTGTTCTTAGTTTTGGTGGCTTCTGAGACTTCGTTAGGTTTAGGATTAATAGTTGGACTGATGCGTAATGCCGAGTCTGGTATTTATTCCTTATATAGCCAAGGGGAGTAAGGCTAGTATATAATATTTAGAGTGTTTACGTAACTGTATATATACATACATAAAGATCGGGTCATAAGTTAAAT

Appendix 3. Technical Bulletin for the eOSLU2 Assay

Helbing Lab
eDNA Inventory

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity [1-3].

General eDNA Assay Information

Target Species: Olympia Oyster (*Ostrea lurida*)
Species Code: mo-OSLU
eDNA qPCR Tool: eOSLU2
eDNA qPCR Format: TaqMan
Gene Target: MT-CO3
Published in:

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 0.3 95% CI 0.2-0.6 Copies/Rxn LOQ 1.3 95% CI 0.9-2.4 Copies/Rxn LOB 0 hits/8

$\text{LOQ}_{\text{continuous}}$ 4 Copies/Rxn

Binomial-Poisson model for 8 technical replicates determined using eLowQuant R code [4]. When the LOQ < LOD, use the LOD for the LOQ. Enzyme: Immolase

eDNA Assay Specificity Test Information

Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

Species	Common Name (*Species*)	Detection	# Voucher Specimens	Sample Sources/Locations
mo-OSLU	Olympia Oyster (*Ostrea lurida*)	Yes	9	British Columbia
mo-CRGI	Pacific Oyster (C*rassostrea gigas*)	No	5	British Columbia
mo-MAIN	Pointed Macoma (*Macoma inquinita*)	No	2	British Columbia
mo-MYAR	Softshell clam (*Mya aremaria*)	No	2	British Columbia
mo-MYSP	Mussel (*Mytilus spp.*)	No	4	British Columbia
mo-NUOB	Mahogany clam (*Nuttalia obscurata*)	No	1	British Columbia
mo-VEPH	Manila clam/Japanese littleneck (*Venerupis philippinarum*)	No	2	British Columbia
ma-CALUfa	Domestic dog (*Canis lupus familiaris*)	No	1	British Columbia
ma-FECA	Domestic cat (*Felis catus*)	No	1	British Columbia
ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands

References

1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus*, in British Columbia, Canada, using environmental DNA methods. Environmental DNA, 2: 350-361. https://doi.org/10.1002/edn3.82

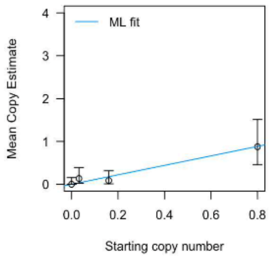
2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei*, in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849

3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164

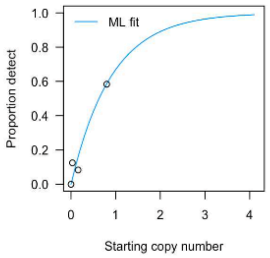
4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA

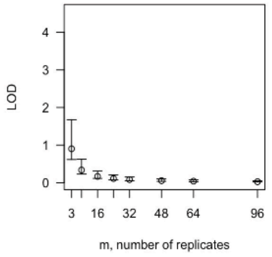
eOSLU2



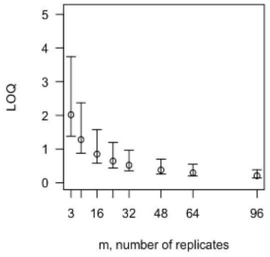
eOSLU2



eOSLU2



eOSLU2



Binomial-Poisson model: No intercept

Determined using eLowQuant R code[4].

Based on a 2 µL DNA input in a total 15 µL reaction

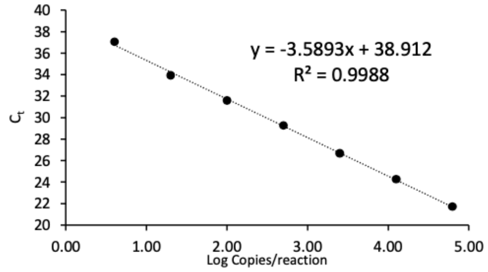
To calculate tables for different numbers of replicates, raw csv data files can be accessed here:
https://onlineacademiccommunity.uvic.ca/helbinglab/edna/

From 8 Technical Replicates

# Detects	# Copies	SE
0	0	0
1	0.12	0.12
2	0.26	0.19
3	0.43	0.27
4	0.63	0.35
5	0.89	0.46
6	1.25	0.63
7	1.88	0.95

Determined using eLowQuant R code[4].

Applied to reactions with 100% positive hits



Efficiency	90%

Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location
Water	Y	2	Y	Sugarloaf Mountain, British Columbia
Water	Y	4	Y	University of British Columbia Campus

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-CO3	Mitochondrial cytochrome c oxidase subunit III gene
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 4. Olympia oyster field sample metadata and tracking information.

Site Name	Site Code	Latitude	Longitude	Sample Replicate	Collection Date	Collection Time	Tidal Height (ft)	Tide	Tide Station	Sample Depth	Purpose	Sample Cleaned	IntegritE passed
Hillier Island	HI	49 01.878	125 19.493	A	2022-06-14	16:15	2.40	H	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.873	125 19.495	B	2022-06-14	16:15	2.40	H	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.876	125 19.494	C	2022-06-14	16:15	2.40	H	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.946	125 18.903	A	2022-06-14	16:30	2.30	H	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.946	125 18.903	B	2022-06-14	16:30	2.30	H	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.948	125 18.904	C	2022-06-14	16:30	2.30	H	Stopper Island	S	Tide Comparison	N	Y
Hand Island	HN	48 57.096	125 18.871	A	2022-06-14	17:05	1.60	H	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.095	125 18.866	B	2022-06-14	17:05	1.60	H	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.094	125 18.863	C	2022-06-14	17:05	1.60	H	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.687	125 14.979	A	2022-06-14	17:30	1.50	H	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.687	125 14.979	B	2022-06-14	17:30	1.50	H	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.687	125 14.979	C	2022-06-14	17:30	1.50	H	Effingham	S	Tide Comparison	N	Y
Field Blank	FB				2022-06-14					FB	Tide Comparison	N	Y
Nettle Island	NI	48 55.687	125 14.979	A	2022-06-15	5:55	0.68	L	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.687	125 14.979	B	2022-06-15	5:55	0.68	L	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.687	125 14.979	C	2022-06-15	5:55	0.68	L	Effingham	S	Tide Comparison	N	Y
Harris Island	HA	48 57.121	125 18.863	A	2022-06-15	8:08	-0.11	L	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	48 57.121	125 18.863	B	2022-06-15	8:08	-0.11	L	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	48 57.121	125 18.863	C	2022-06-15	8:08	-0.11	L	Stopper Island	S	Tide Comparison	N	Y
Walsh Saddle	WS	48 54.982	125 19.279	A	2022-06-15	12:22	2.19	H	Effingham	S	Tide Comparison	N	Y
Walsh Saddle	WS	48 54.982	125 19.279	B	2022-06-15	12:22	2.19	H	Effingham	S	Tide Comparison	N	Y
Walsh Saddle	WS	48 54.982	125 19.279	C	2022-06-15	12:22	2.19	H	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.982	125 189.095	A	2022-06-15	12:30	2.30	H	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.982	125 189.095	B	2022-06-15	12:30	2.30	H	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.982	125 189.095	C	2022-06-15	12:30	2.30	H	Effingham	S	Tide Comparison	N	Y
Field Blank	FB				2022-06-15					FB	Tide Comparison	N	Y
Hillier Island	HI	49 01.885	125 19.471	A	2022-06-16	6:18	1.31	L	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.885	125 19.471	B	2022-06-16	6:18	1.31	L	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.885	125 19.471	C	2022-06-16	6:18	1.31	L	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.945	125 18.904	A	2022-06-16	10:09	0.52	L	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.945	125 18.904	B	2022-06-16	10:09	0.52	L	Stopper Island	S	Tide Comparison	N	Y

Harris Island	HA	49 00.945	125 18.904	C	2022-06-16	10:09	0.52	L	Stopper Island	S	Tide Comparison	N	Y
Cataract Bay	CB	49 01.2546	125 16.783	A	2022-06-16	12:23	1.93	H	Stopper Island	S	Tide Comparison	N	Y
Cataract Bay	CB	49 01.2546	125 16.783	B	2022-06-16	12:23	1.93	H	Stopper Island	S	Tide Comparison	N	Y
Cataract Bay	CB	49 01.2546	125 16.783	C	2022-06-16	12:23	1.93	H	Stopper Island	S	Tide Comparison	N	Y
Pipestem Inlet	PI	49 02.2990	125 12.1802	A	2022-06-16	12:44	2.18	H	Stopper Island	S	Tide Comparison	N	Y
Pipestem Inlet	PI	49 02.2990	125 12.1802	B	2022-06-16	12:44	2.18	H	Stopper Island	S	Tide Comparison	N	Y
Pipestem Inlet	PI	49 02.2990	125 12.1802	C	2022-06-16	12:44	2.18	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.5576	125 18.3301	A	2022-06-16	13:09	2.42	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.5576	125 18.3301	B	2022-06-16	13:09	2.42	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.5576	125 18.3301	C	2022-06-16	13:09	2.42	H	Stopper Island	S	Tide Comparison	N	Y
Field Blank	FB				2022-06-16	13:10				FB	Tide Comparison	N	Y
Joes Bay	JB	48 54.8312	125 19.1266	A	2022-06-17	6:36	1.61	L	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.8312	125 19.1266	B	2022-06-17	6:36	1.61	L	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.8312	125 19.1266	C	2022-06-17	6:36	1.61	L	Effingham	S	Tide Comparison	N	Y
Walsh Saddle	WS	48 54.9722	125 19.2624	A	2022-06-17	9:38	0.04	L	Effingham	S	Tide Comparison	N	Y
Walsh Saddle	WS	48 54.9722	125 19.2624	B	2022-06-17	9:38	0.04	L	Effingham	S	Tide Comparison	N	Y
Walsh Saddle	WS	48 54.9722	125 19.2624	C	2022-06-17	9:38	0.04	L	Effingham	S	Tide Comparison	N	Y
Field Blank	FB				2022-06-17	9:38				FB	Tide Comparison	N	Y
Pipestem Inlet	PI	49.03830	125.20339	A	2022-06-19	8:35	1.56	L	Stopper Island	S	Tide Comparison	N	Y
Pipestem Inlet	PI	49.03830	125.20339	B	2022-06-19	8:35	1.56	L	Stopper Island	S	Tide Comparison	N	Y
Pipestem Inlet	PI	49.03830	125.20339	C	2022-06-19	8:35	1.56	L	Stopper Island	S	Tide Comparison	N	Y
Cataract Bay	CB	49.02084	125.27908	A	2022-06-19	8:58	1.28	L	Stopper Island	S	Tide Comparison	N	Y
Cataract Bay	CB	49.02084	125.27908	B	2022-06-19	8:58	1.28	L	Stopper Island	S	Tide Comparison	N	Y
Cataract Bay	CB	49.02084	125.27908	C	2022-06-19	8:58	1.28	L	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49.02557	125.30592	A	2022-06-19	9:19	1.15	L	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49.02557	125.30592	B	2022-06-19	9:19	1.15	L	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49.02557	125.30592	C	2022-06-19	9:19	1.15	L	Stopper Island	S	Tide Comparison	N	Y
Hand Island	HN	48 57.096	125 18.871	A	2022-10-11	9:32	2.073	M	Effingham	S	Lagoon	N	Y
Hand Island	HN	48 57.095	125 18.866	B	2022-10-11	9:33	2.073	M	Effingham	S	Lagoon	Y	Y
Hand Island	HN	48 57.094	125 18.863	C	2022-10-11	9:34	2.074	M	Effingham	S	Lagoon	Y	Y
Hand Island	HN	48 95.087	125 31.728	A	2022-10-11	9:47	2.23	M	Effingham	S-Lag	Lagoon	Y	Y
Hand Island	HN	48 95.087	125 31.728	B	2022-10-11	9:48	2.232	M	Effingham	S-Lag	Lagoon	Y	Y
Hand Island	HN	48 95.087	125 31.728	C	2022-10-11	9:49	2.3	M	Effingham	S-Lag	Lagoon	Y	N
Field Blank	FB				2022-10-11	10:44				FB	Blank	N	Y
Harris Island	HA	49 00.946	125 18.903	A	2022-10-12	8:30	1.578	L	Stopper Island	S	Abundance	N	Y
Harris Island	HA	49 00.946	125 18.903	B	2022-10-12	8:31	1.58	L	Stopper Island	S	Abundance	Y	Y

Harris Island	HA	49 00.948	125 18.904	C	2022-10-12	8:32	1.58	L	Stopper Island	S	Abundance	N	Y
Harris Island	HA			A	2022-10-12	8:42	1.6	L	Stopper Island	S-Lag	Abundance	Y	N
Harris Island	HA			B	2022-10-12	8:44	1.65	L	Stopper Island	S-Lag	Abundance	Y	Y
Harris Island	HA			C	2022-10-12	8:45	1.653	L	Stopper Island	S-Lag	Abundance	Y	Y
Pipestem Inlet	PI	49 02.262	125 12.190	A	2022-10-13	8:07	1.69	L	Stopper Island	S-FW	FW	N	Y
Pipestem Inlet	PI	49 02.261	125 12.188	B	2022-10-13	8:08	1.69	L	Stopper Island	S-FW	FW	N	Y
Pipestem Inlet	PI	49 02.261	125 12.188	C	2022-10-13	8:09	1.691	L	Stopper Island	S-FW	FW	N	Y
Pipestem Inlet	PI	49 02.290	125 12.200	A	2022-10-13	8:12	1.692	L	Stopper Island	S-FWNear	FW	N	Y
Pipestem Inlet	PI	49 02.295	125 12.194	B	2022-10-13	8:13	1.692	L	Stopper Island	S-FWNear	FW	N	Y
Pipestem Inlet	PI	49 02.296	125 12.202	C	2022-10-13	8:14	1.692	L	Stopper Island	S-FWNear	FW	N	Y
Lucky Creek	LC	49 01.607	125 18.350	A	2022-10-13	8:32	1.71	L	Stopper Island	S-FW	FW	N	Y
Lucky Creek	LC	49 01.607	125 18.353	B	2022-10-13	8:33	1.71	L	Stopper Island	S-FW	FW	N	Y
Lucky Creek	LC	49 01.612	125 18.353	C	2022-10-13	8:34	1.711	L	Stopper Island	S-FW	FW	N	Y
Lucky Creek	LC	49 01.582	125 18.296	A	2022-10-13	8:39	1.72	L	Stopper Island	S-FWNear	FW	N	Y
Lucky Creek	LC	49 01.581	125 18.296	B	2022-10-13	8:41	1.73	L	Stopper Island	S-FWNear	FW	N	Y
Lucky Creek	LC	49 01.581	125 18.296	C	2022-10-13	8:42	1.745	L	Stopper Island	S-FWNear	FW	N	Y
Imperial Eagle Channel	IEC	48 56.350	125 10.043	A	2022-10-13	12:16	3.19	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC	48 56.356	125 10.045	B	2022-10-13	12:18	3.195	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC	48 56.360	125 10.046	C	2022-10-13	12:19	3.197	H	Stopper Island	S	Field Negative	N	Y
Field Blank	FB				2022-10-13	12:20	3.199	H	Stopper Island	FB	Blank	Y	N
Imperial Eagle Channel	IEC-1	48 56.0416	125 12.7488	1	2023-02-15	9:19	3.297	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-1	48 56.0335	125 12.7324	2	2023-02-15	9:22	3.297	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-1	48 56.0264	125 12.7332	3	2023-02-15	9:24	3.297	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-2	48 56.3524	125 14.6336	1	2023-02-15	9:32	3.237	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-2	48 56.3525	125 14.6624	2	2023-02-15	9:33	3.237	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-2	48 56.3553	125 14.6988	3	2023-02-15	9:34	3.237	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-3	48 56.8080	125 16.5374	1	2023-02-15	10:00	3.076	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-3	48 56.8012	125 16.5617	2	2023-02-15	10:01	3.076	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-3	48 56.8049	125 16.5756	3	2023-02-15	10:02	3.073	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-4	48 57.3804	125 18.2241	1	2023-02-15	10:13	2.976	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-4	48 57.3746	125 18.2240	2	2023-02-15	10:15	2.976	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-4	48 57.3721	125 18.2240	3	2023-02-15	10:15	2.972	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-5	48 57.8459	125 20.0992	1	2023-02-15	10:27	2.865	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-5	48 57.8851	125 20.1311	2	2023-02-15	10:28	2.865	H	Stopper Island	S	Field Negative	N	Y
Imperial Eagle Channel	IEC-5	48 57.8570	125 20.1572	3	2023-02-15	10:29	2.865	H	Stopper Island	S	Field Negative	N	Y
Joes Bay	JB	48 54.8208	125 19.1078	A	2023-08-04	8:43	1.05	L	Effingham	S	Tide Comparison	Y	Y

Joes Bay	JB	48 54.824	125 19.1123	B	2023-08-04	8:45	1.03	L	Effingham	S	Tide Comparison	Y	Y
Joes Bay	JB	48 54824	125 19.1117	C	2023-08-04	8:46	1.02	L	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.687	12 14.9	A	2023-08-04	9:30	0.29	L	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.689	125 14.973	B	2023-08-04	9:30	0.29	L	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.689	125 14.974	C	2023-08-04	9:31	0.29	L	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.1117	125 18.8540	A	2023-08-04	9:56	0.37	L	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.1088	125 18.8513	B	2023-08-04	9:57	0.38	L	Effingham	S	Tide Comparison	Y	N
Hand Island	HN	48 57.1053	125 18.8480	C	2023-08-04	9:58	0.38	L	Effingham	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9508	125 18.910	A	2023-08-04	10:32	0.6	L	Stopper Island	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9518	125 18.9105	B	2023-08-04	10:33	0.61	L	Stopper Island	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9569	125 18.9027	C	2023-08-04	10:34	0.61	L	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.8955	125 19.4680	A	2023-08-04	10:48	1.94	L	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01. 8993	125 19.4667	B	2023-08-04	10:49	1.96	L	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.8994	125 19.4661	C	2023-08-04	10:51	2	L	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6131	125 18.2557	A	2023-08-04	11:07	2.52	L	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6179	125 18.2586	B	2023-08-04	11:09	2.6	L	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6216	125 18.2649	C	2023-08-04	11:10	2.6	L	Stopper Island	S	Tide Comparison	Y	Y
Joes Bay	JB	48 54.8174	125 19.1048	A	2023-08-04	12:02	1.56	M	Effingham	S	Tide Comparison	Y	Y
Joes Bay	JB	48 54.8182	125 19.1058	B	2023-08-04	12:03	1.57	M	Effingham	S	Tide Comparison	Y	Y
Joes Bay	JB	48 54.8170	125 19.1073	C	2023-08-04	12:04	1.58	M	Effingham	S	Tide Comparison	Y	N
Nettle Island	NI	48 55.6891	125 15.9755	A	2023-08-04	12:26	5.54	M	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.6922	125 15.9744	B	2023-08-04	12:27	5.57	M	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.6908	125 15.9730	C	2023-08-04	12:28	5.6	M	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.1006	125 18.8897	A	2023-08-04	12:43	1.9	M	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.1014	125 18.8862	B	2023-08-04	12:44	1.91	M	Effingham	S	Tide Comparison	Y	N
Hand Island	HN	48 57.1002	125 18.8856	C	2023-08-04	12:45	1.92	M	Effingham	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9528	125 18.9002	A	2023-08-04	13:03	2.14	M	Stopper Island	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9516	125 18.8987	B	2023-08-04	13:04	2.15	M	Stopper Island	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9597	125 18.8965	C	2023-08-04	13:06	2.16	M	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.9070	125 19.4613	A	2023-08-04	13:15	2.27	M	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.9091	125 19.4588	B	2023-08-04	13:16	2.28	M	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.9091	125 19.4588	C	2023-08-04	13:16	2.29	M	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6121	125 18.2599	A	2023-08-04	13:30	2.43	M	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6121	125 18.2578	B	2023-08-04	13:31	2.44	M	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.6139	125 18.2534	C	2023-08-04	13:32	2.45	M	Stopper Island	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.8181	125 19.1056	A	2023-08-04	15:34	3.12	H	Effingham	S	Tide Comparison	Y	Y

Joes Bay	JB	48 54.8150	125 19.1029	B	2023-08-04	15:36	3.12	H	Effingham	S	Tide Comparison	Y	Y
Joes Bay	JB	48 54.8081	125 19.1036	C	2023-08-04	15:40	3.12	H	Effingham	S	Tide Comparison	Y	N
Nettle Island	NI	48 55.6917	125 15.9731	A	2023-08-04	16:05	3.08	H	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.6890	125 15.9685	B	2023-08-04	16:07	3.08	H	Effingham	S	Tide Comparison	Y	Y
Nettle Island	NI	48 55.6910	125 15.9681	C	2023-08-04	16:09	3.07	H	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.1023	125 18.8660	A	2023-08-04	16:26	3.01	H	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.0903	125 18.8762	B	2023-08-04	16:29	3	H	Effingham	S	Tide Comparison	Y	Y
Hand Island	HN	48 57.0900	125 18.8746	C	2023-08-04	16:30	299	H	Effingham	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9521	125 19.3020	A	2023-08-04	16:52	2.87	H	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.9529	125 19.8988	B	2023-08-04	16:54	2.88	H	Stopper Island	S	Tide Comparison	Y	Y
Harris Island	HA	49 00.9510	125 19.8952	C	2023-08-04	16:55	2.85	H	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.8958	125 19.4701	A	2023-08-04	17:08	2.76	H	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.8971	125 19.4628	B	2023-08-04	17:09	2.76	H	Stopper Island	S	Tide Comparison	Y	Y
Hillier Island	HI	49 01.9046	125 19.4596	C	2023-08-04	17:10	2.75	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.6168	125 18.2675	A	2023-08-04	17:21	2.67	H	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6204	125 18.2591	B	2023-08-04	17:22	2.65	H	Stopper Island	S	Tide Comparison	Y	Y
Lucky Creek	LC	49 01.6266	125 18.2622	C	2023-08-04	17:24	2.64	H	Stopper Island	S	Tide Comparison	Y	Y
Joes Bay	JB	48 54.824	125 19.098	A	2023-10-26	11:05	3.59	H	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.820	125 19.101	B	2023-10-26	11:07	3.6	H	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.822	125 19.101	C	2023-10-26	11:08	3.6	H	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.684	125 14.972	A	2023-10-26	11:23	3.61	H	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.683	125 14.972	B	2023-10-26	11:24	3.61	H	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.684	125 14.972	C	2023-10-26	11:25	3.61	H	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.102	125 18.888	A	2023-10-26	11:41	3.6	H	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.100	125 18.897	B	2023-10-26	11:42	3.6	H	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.099	125 18.888	C	2023-10-26	11:44	3.6	H	Effingham	S	Tide Comparison	N	Y
Harris Island	HA	49 00.951	125 18.892	A	2023-10-26	11:57	3.57	H	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.951	125 18.894	B	2023-10-26	11:59	3.56	H	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.95	125 18.895	C	2023-10-26	12:00	3.56	H	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.902	125 19.471	A	2023-10-26	12:06	3.54	H	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.903	125 19.467	B	2023-10-26	12:07	3.54	H	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.909	125 19.462	C	2023-10-26	12:09	3.53	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.606	125 18.260	A	2023-10-26	12:17	3.5	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.603	125 18.261	B	2023-10-26	12:18	3.49	H	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.600	125 18.261	C	2023-10-26	12:19	3.49	H	Stopper Island	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.818	125 19.099	A	2023-10-26	13:51	2.78	M	Effingham	S	Tide Comparison	N	Y

Joes Bay	JB	48 54.819	125 19.102	B	2023-10-26	13:52	2.78	M	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.824	125 19.104	C	2023-10-26	13:53	2.76	M	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.683	125 14.973	A	2023-10-26	14:05	2.64	M	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.684	125 14.971	B	2023-10-26	14:06	2.63	M	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.685	125 14.973	C	2023-10-26	14:08	2.61	M	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.100	125 18.883	A	2023-10-26	14:20	2.48	M	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.104	125 18.886	B	2023-10-26	14:21	2.46	M	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.107	125 18.878	C	2023-10-26	14:23	2.45	M	Effingham	S	Tide Comparison	N	Y
Harris Island	HA	49 00.951	125 18.893	A	2023-10-26	14:36	2.31	M	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.951	125 18.893	B	2023-10-26	14:37	2.29	M	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.949	125 18.894	C	2023-10-26	14:39	2.28	M	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.900	125 19.476	A	2023-10-26	14:45	2.28	M	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.899	125 19.476	B	2023-10-26	14:47	2.2	M	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.900	125 19.475	C	2023-10-26	14:48	2.19	M	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.603	125 18.256	A	2023-10-26	14:54	2.13	M	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.606	125 18.258	B	2023-10-26	14:55	2.12	M	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.596	125 18.256	C	2023-10-26	14:56	2.1	M	Stopper Island	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.824	125 19.106	A	2023-10-26	16:59	1.16	L	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.825	125 19.110	B	2023-10-26	17:02	1.15	L	Effingham	S	Tide Comparison	N	Y
Joes Bay	JB	48 54.824	125 19.115	C	2023-10-26	17:03	1.14	L	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.684	125 14.974	A	2023-10-26	17:16	1.11	L	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.683	125 14.977	B	2023-10-26	17:17	1.1	L	Effingham	S	Tide Comparison	N	Y
Nettle Island	NI	48 55.685	125 14.976	C	2023-10-26	17:18	1.1	L	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.112	125 18. 865	A	2023-10-26	17:36	1.08	L	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.113	125 18.865	B	2023-10-26	17:37	1.07	L	Effingham	S	Tide Comparison	N	Y
Hand Island	HN	48 57.114	125 18.864	C	2023-10-26	17:38	1.07	L	Effingham	S	Tide Comparison	N	Y
Harris Island	HA	49 00.950	125 18.901	A	2023-10-26	18:06	1.09	L	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.949	125 18.902	B	2023-10-26	18:08	1.1	L	Stopper Island	S	Tide Comparison	N	Y
Harris Island	HA	49 00.948	125 18.905	C	2023-10-26	18:09	1.1	L	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.898	125 19.470	A	2023-10-26	18:16	1.11	L	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.893	125 19.471	B	2023-10-26	18:18	1.12	L	Stopper Island	S	Tide Comparison	N	Y
Hillier Island	HI	49 01.891	125 19.471	C	2023-10-26	18:19	1.12	L	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.589	125 18.262	A	2023-10-26	18:27	1.15	L	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.588	125 18.260	B	2023-10-26	18:28	1.16	L	Stopper Island	S	Tide Comparison	N	Y
Lucky Creek	LC	49 01.588	125 18.259	C	2023-10-26	18:29	1.16	L	Stopper Island	S	Tide Comparison	N	Y

Appendix 5. eENLU6 sequenced, molecular characteristics, and calculated performance parameters.

Target Species		**Enhydra lutris**
eDNA qPCR Assay name		eENLU6
Target Gene		mt-*cytb*
Amplicon Size (bp)		142
Assay Component Sequences (5' to 3')	**Forward Primer**	TTCATCATTTCAGCACTG
	Reverse Primer	GGATAGGATTAAGGCTAGTA
	Probe	ATAATCCACCTCCTATTCCTCCACGA
	Amplicon	TTCATCATTTCAGCACTGGCAATAATCCACCTCCTATTCCTCCACGAGACAGGGTCTAACAACCCATCCGGAATCCCATCCAACTCTGACAAAATCCCATTTCACCCCTACTATACCATCAAGGACATCCTAGGCGCCCTATCCCTAGTCCTAGCGCTGACAACGCTAGTGCTATTCTCCCCCGACCTGCTAGGAGACCCCGACAACTATATCCCCGCCAACCCACTCAGTACACCACCCCATATCAAACCCGAATGATATTTCCTATTTGCATATGCAATCCTACGGTCCATCCCTAACAAACTAGGAGGAGTACTAGCCTTAATCCTATCC

Appendix 6. Mitochondrial Genome Sequences used for assay design alignment. All sequences accessed from National Center for Biotechnnology Information (NCBI)

Abbreviation	Species	Accession Number
ma-CALUfa	Dog (*Canis lupus familiaris*)	NC_002008.4
ma-FECA	Cat (*Felis catus*)	NC_001700.1
ma-HOSA	Human (*Homo sapiens*)	NC_012920.1
ma-ENLU	Northern Sea Otter (*Enhydra lutris kenyonii*)	NC_009692.1
ma-ENLU	Japanese Sea Otter (*Enhydra lutris lutris*)	AB291077.1
ma-ENLU	Southern Sea Otter (*Enhydra lutris nereis*)	SAMN09238016
ma-NEVI	American mink (*Neogale vison*)	KU146454.1
ma-NEVI	American mink (*Neovision vision*)	KM488625.1
ma-NEVI	American mink (Neogale vison)	MT410953.1
ma-URAR	Grizzly bear (*Ursus arctos*)	CM035080.1
ma-URAM	American black bear (*Ursus americanus*)	AF303109.1
ma-URAM	American black bear (*Ursus americanus*)	KM257059.1
ma-CAUR	Northern fur seal (*Callorhinus ursinus*)	MG916809.1
ma-CAUR	Northern fur seal (*Callorhinus ursinus*)	NC_008415.3
ma-ZACA	California sea lion (*Zalophus californianus*)	CM019821.1
ma-ZACA	California sea lion (*Zalophus californianus*)	NC_008416.1
ma-EUJU	Stellar sea lion (*Eumetopias jubatus*)	GU475464.1
ma-EUJU	Stellar sea lion (*Eumetopias jubatus*)	NC_004030.2

ma-MILE	Southern Elephant Seal (*Mirounga leonina)*	NC_008422.1
ma-PHVI	Harbour seal (*Phoca vitulina*)	NC_008422.1
ma-PHVI	Harbour seal (*Phoca vitulina*)	MT410883.1

Appendix 7. Running parameters, and output used for unikseq v0.1.0 program for eENLU6 design

Length of K-mers	25
Threshold Length	100
% of Average ID in Ingroup Sequences	25
Tolerated # of consecutive K-mers in Outgroup	1
% Unique	90
Maximum Proportion of Outgroup Sequences Tolerated	0

Output Region	**Sequence**
318-484	CACCGCGGTCATACGATTAACCCAGATTAATAGGCCCACGGCGTAAAACGTGTCAAGGATTACAAAATACTAAAGTTAAAATTTAACTAGGCCGTAAAAAGCTACTGTTAATATAAAATATACCACGAAAGTGACCTTACTATTTCCGACTACACGACAGCTGAGGC
1091-1197	CACTTTGAACTAAAGCTAGCCCAACCAACCACCAACTTAACTATCACAACAACCACAATTAAAACATTTAATTATACCATTACAGTATAGGAGATAGAAATTCTATT
2722-2873	AGGTTCAAATCCTCTTCCTAACAATATGCTCATAATCAACATCATTTCACTAATTGTACCAATCTTGCTCGCCGTAGCTTTCCTGACATTAGTGGAACGAAAAATCCTAGGATACATACAACTCCGCAAGGGCCCAAATATTGTAGGACCCT
2875-3038	CGGCCTCCTACAACCAATTGCAGACGCCGTGAAACTTTTTACTAAAGAACCCCTACGACCCTTAACATCATCTATTACTATGTTCGTAATAGCCCCAATCCTAGCCCTAGCACTAGCCCTAACCATGTGAGTCCCATTACCTATACCTTACCCCCTCGTTAACA
3145-3279	ACGAGCTGTAGCCCAAACAATCTCCTATGAGGTCACACTAGCTATCATTCTTCTTTCAGTACTGCTAATAAATGGCTCATTCACCCTATCCACATTAATCACCACACAAGAACACCTATGACTAATCCTCCCCGC
3482-3718	TTCCACACCCCCTACCTACCAGAACTATACACCATCAACTTCACCGTGAAAACACTCCTACTAACAGCCTCTTTCCTATGAATTCGAGCATCTTACCCACGATTCCGCTATGACCAACTTATACACCTACTATGAAAAAACTTCCTCCCTCTAACACTAGCTCTATGCATATGACACATGGCCCTACCTATTATAACAGCAAGCATTCCTCCCCAAACATAAGAAATATGTCTGATA
3987-4423	CTGACTGATAATCTGGATCGGATTTGAAATGAATATACTAGCCATTATCCCAGTCCTAATAAAAAGTTTCAACCCACGAACCATAGAAGCATCAACAAAATATTTCCTCGCCCAAGCCACCGCATCCATACTCCTCATACTAGGAGTCATTATCAACCTACTACTGACAGGCCAATGAACAGTACTAAACACCCCACACCCAATCACATCAAACATAATAACAGTGGCCATAGCAATAAAACTAGGACTATCACCCTTTCACTTCTGAATACCCGAAGTAGCCCAAGGAATCTCACTATCATCAGGAATAATCCTACTTACCTGACAAAAAATTGCCCCTCTATCCGTTCTATACCAAATCTCACCCTCTGTAAACCCAAACCTACTAATGACTATAGCAGCCATATCCGTGCTGGTAGGTGGATGAGGGGGTCTTA
4425-4578	CCAAACACAACTACGAAAAATCCTAGCTTATTCATCAATCGCTCATATGGGATGAATAGTCGCCGTAACAACATACAACCCCACCCTAATATTACTAAACCTTACAATCTACATTATAATAACACTAGGAACATTCATACTATTTATCCTCAGC

4583-4842	CCACAACTACACTATCATTATCCCACATATGAAACAAAACCCCATTAATCACTTCACTAGTCCTAGCAATTATACTCTCCTTAGGAGGCCTGCCTCCTCTGTCAGGATTTATCCCCAAATGAATAATCACTTACGAACTTACAAAAAATAACATGGTCACTGCAGCAATATTCATGGCAATAACAGCCCTACTAAACCTATATTTCTACATACGACTAACATATGCAACAGCACTAACCATGTTCCCTTCAACAAACGCC
4847-4967	AAATAAAATGACAATTCGAAAACATAAAAAACACCACCCTAATGTCTCCTCTAATTGTATCCTCAACTATACTACTCCCACTAACCCCAATAATACTAGCAATATTCTAGAAGTTTAGGTT
5432-5874	CACCGCTCTCAGCCTATTAATCCGTGCTGAATTAGGTCAACCTGGCGCTCTATTAGGGGATGACCAGATTTATAATGTTATCGTCACCGCCCACGCATTCGTGATAATTTTCTTTATAGTAATACCAATCATGATCGGTGGGTTTGGAAACTGACTAGTACCCCTAATAATTGGTGCGCCTGACATAGCATTTCCACGAATAAACAATATAAGCTTTTGACTCCTACCCCCCTCCTTTCTACTTCTTCTAGCCTCATCTATGGTAGAAGCGGGTGCAGGAACAGGATGAACCGTATACCCCCCTTTAGCAGGTAATCTAGCACATGCAGGAGCATCGGTAGACCTGACAATTTTTCTCTACACCTGGCAGGTGTATCGTCCATCCTAGGAGCTATCAATTTTATCACCACTATCATTAACATAAAACCCCCCGCAATATCAC
6050-6510	ATTCTTCGGACACCCAGAAGTATACATTCTAATTCTGCCTGGATTCGGAATTATCTCACACGTCGTTACATACTACTCAGGAAAGAAAGAACCGTTTGGTTATATAGGAATGGTATGGGCAATAATATCAATTGGCTTCCTAGGCTTTATTGTGTGAGCCCACCACATATTTACTGTAGGTATGGATGTCGATACACGAGCATACTTTACATCAGCCACTATAATCATTGCTATCCCTACAGGGGTAAAAGTATTTAGCTGACTGGCTACTCTACACGGAGGAAATATTAAATGATCCCCAGCTATGCTATGAGCTTTAGGGTTTATTTTTCTATTTACAGTGGGTGGTCTAACGGGTATCGTATTATCAAACTCATCACTGGATATTGTTCTTCACGACACATACTATGTAGTAGCACACTTTCATTACGTTCTCTCAATGGGGGCAGTATTTGCAATTA
6518-6681	ATTTGTCCACTGATTCCCACTATTCACGGGTTATATACTGAATGATACTTGAGCGAAAATCCACTTCACAATTATATTTGTCGGAGTAAACATAACATTCTTTCCTCAACACTTCCTAGGCCTATCAGGTATGCCTCGACGTTACTCCGACTATCCAGATGCTT
6698-6882	AGTATCTTCCATAGGCTCATTCATCTCACTAACAGCAGTAATATTAATGATCTTCATAATCTGAGAAGCCTTCGCATCCAAACGAGAAGTACTAACAGTACAACTCACTTCAACAAACATTGAATGGTTACACGGGTGTCCTCCACCATATCACACCTTTGAGGAGCCAACCTATGTACTATCGA
7111-7262	AATAATCGTATTCTTAATTAGCTCTCTCGTTCTCTACATTATTTCACTGATATTGACCACTAAACTCACGCATACCAGCACCATGGACGCCCAGGCAGTCGAAACAATCTGAACCATCTTACCCGCCATCATTCTAATCCTAATCGCCCTTC
7435-7604	AGAAGTAGACAACCGAGTAGTTCTCCCAATAGAAATAACAATCCGGATGCTAATTTCATCTGAAGACGTATTACACTCATGAGCCGTGCCATCCCTAGGACTAAAAACTGATGCCATCCCTGGACGCCTGAACCAAACTACCCTAATGGCCATGCGACCGGGATTATACT
7621-7729	AATCTGCGGCTCTAATCACAGCTTCATACCAATTGTTCTCGAGCTAGTGCCTCTGTCATACTTTGAAAAATGATCTGCCTCAATGCTATAAATTCGTTGAGAAGCTAAA
7782-7928	TATGCCACAACTAGACACTTCAACCTGGCTTATCACGATTATATCGATAATTATCACCCTATTCATTATATTCCAATTAAAAGTATCCAAGCATTGTTTTCCAGATAACCCTGAGCCAAAATTAACGACCACATCAAAACCCACCAC

7937-8846	AAGAAAAATGAACGAAAATCTATTTTCCTCTTTCATTACCCCTGTAGTAATAGGACTTCCTATTGTTATCGCCATTGTAATATTCCCAAGTATTATATTCCCATCACCTAGTCGACTAATCAACAACCGACTCATCTCTATTCAACAATGACTAGTACAACTAACATCAAAACAAATGTTGTCCATTCACAACCAAAAAGGACAAACCTGGGCATTAATATTAATATCCCTCATCTTATTTATTGGCTCCACCAACCTATTGGGCCTACTACCCCACTCATTTACCCCTACTACTCAATTATCCATAAACTTAGGAATGGCTATCCCCTTATGAGCAGGCACAGTAATTACTGGCTTCCGATACAAAACAAAAGCCTCCCTAGCCCATTTCTTACCACAAGGAACACCCATCCCCCTAATCCCCATACTTATTATCATCGAAACTATTAGTCTGTTCATCCAACCAATGGCTCTAGCTGTACGGCTAACAGCTAACATCACTGCAGGCCACTTATTAATCCACCTAATCGGGGGAGCTACCCTAGCCCTAATAAACATTAGCACTATCACAGCAGCAGTAACCTTCATCATCCTTATCCTCTTGACCATCCTGGAATTTGCAGTAGCCCTTATTCAAGCCTATGTTTTCACTCTGCTAGTAAGCTTATACTTACATGATAATACTTAATGACCCACCAAACCCATTCGTACCATATAGTTAACCCAAGCCCTTGACCCCTAACAGGGGCTCTCTCAGCACTCCTCACAACATCAGGACTAGTGATATGATTTCATTTTAACTCGATATTCCTTCTAACCCTAGGTATAGTAACCAACGTACTAACTATGTATCAATGGTGACGAGATATTGTCCGAGAAGGGACATTCCAAGGCCACCACACTCCTACCG
8851-8951	AAAAGGCCTACGATACGGAATAATCCTATTTATTACATCAGAAGTCTTCTTCTTTGCGGGCTTCTTTTGAGCTTTTTACCATTCAAGCCTAGCACCAACCC
8955-9380	AACTAGGAGGATGCTGACCCCCTACGGGCATCACACCCTTGAACCCACTAGAAGTACCACTACTTAATACTTCAGTCCTCCTAGCCTCCGGAGTCTCCATTACCTGAGCCCATCACAGCCTAATGGAAGGAGACCGCAAACACATACTCCAGGCCCTATTCATTACAATCTCCTTAGGCCTATACTTTACCCTGTTGCAAGCCTCAGAATACTACGAAGCACCATTTGCAATCTCTGACGGGATTTACGGCTCCACATTTTTCATGGCCACAGGATTCCACGGTCTCCATGTCATCATCGGATCTACCTTCCTTATTGTATGCTTCCTGCGACAATTAAACTATCACTTCACATCTAGCCATCATTTTGGATTCGAAGCAGCTGCCTGATATTGACATTTCGTAGATGTTGTGTGACTATTCCTGT
9428-9843	TACAGTTGACTTCCAATCAACTAGTTCTGGTGATAGTCCAGAGAGAAATAATAAACATAATACTGACCCTACTCACTAACATATCGCTAGCATCTCTACTCATCCTAATCGCATTCTGACTACCCCAACTAAATATCTACATAGAAAAAGCAAGTCCATATGAATGCGGTTTTGACCCACTAGGATCAGCACGTCTGCCTTTCTCTATAAAATTCTTCCTAGTAGCCATCACATTCTTACTGTTTGACTTAGAAATCGCATTACTTCTCCCATTACCATGAGCCTCACAATCAAACAACCTAGAAACGACACTCATTACAGCACTAACATTAATTTCCATCCTAGCCGTAAGCTTAGCCTACGAGTGGACCGAAGAAGGCTTAGAATGGAATGAATAATGATAATTAGTTTAACAA
9881-10544	TTACAATTATCAAATGTCCATAGTGTATATTAATATCTTCCTAGCCTTTATCTTATCCTTCATAGGACTACTCATATACCGATCTCACCTCATATCCTCCCTACTCTGTCTAGAAGGTATAATACTATCCCTATTCGTCATAATAACAATCACAATCCTGGTAAACCACTTCACACTAGCCAGTATAACCCCTATCATCTTGCTTGTATTCGCGGCCTGTGAAGCAGCCCTAGGACTATCACTGCTAGTCATAGTCTCCAACACATATGGAACGGATTACGTACAAAACTTAAATCTACTACAATGTTAAAAATTATTCTCCCAACCATAATACTGATTCCCCTGGCGTGACTATCAAAATATAACATAATCTGAATCAATACAACAGCTTATAGCATACTAATCAGCCTAATTAGCCTAACATATTTTAATCAGTCCCCAGACAATAATCTAAGCTTCTCATTACTCTTTTTCGCAGACTCCTTATCAGCACCCCTACTAACACTCACAACATGACTTCTCCCTCTAATGCTTATAGCAAGCCAACACCACCTATCGAAAGAAACCCTCATTCGTAAAAAACTGTATATCACAATATTAATCACACTTCAACTACTCCTAATTATAACATTCACCTCCACAGAACTAATTATGTTCTACATCC

10547-11054	TTTGAAGCCACACTCATACCAACACTAATTATTATCACCCGATGAGGCAACCAAACAGAACGCCTAAACGCTGGCCTATACTTCCTATTCTACACCCTAGTAGGCTCTTTACCCCTTCTGATCGCTCTATTATGAACCCAGAACAATCTAGGCACCCTACACCTACTGGTAATCCAATTCTGAGTTCAACCCCTGCCAAACACCTGATCCAACACACTACTATGATTAGCATGCATAATGGCATTCATAGTAAAAATACCGCTATACGGCCTTCACCTATGACTCCCGAAAGCCCACGTAGAAGCCCCTATTGCGGGATCCATAGTCCTTGCCGCCGTACTCCTAAAACTGGGAGGATATGGAATAATACGAATTACCATACTACTAAACCCACTAACAAACCACATAGCCTATCCCTTCATGATACTATCCCTATGAGGGATAATCATGACAAGCTCTATCTGCTTGCGCCAAACAGACTTAAAATCCCTAATTGCCTACTCCTCAG
11120-11315	ACAGCCCTAATAATCGCCCACGGTCTAACATCATCCCTATTATTCTGCTTAGCCAATTCCAACTATGAACGTATCCACAGCCGCACTATAATCCTCGCACGAGGCTTACAAATACTCTTACCATTAATAGCTGCATGATGATTACTCGCTAGCCTAACCAACCTAGCCCTACCACCCACTATCAACTTAGTAGGGG
11323-11558	CGTAGTAATAGCCTCATTCTCATGGTCCAACATCACCATCATCCTACTAGGAATTAACATCACCATCACTGCCCTATACTCCCTGTTCATACTAATCACCACGCAACGCGGGAAACTCACACATCATATCAAAAATGTTAACCCATCATTCACACGGGAAAATTCTCTAATGGCCCTCCACCTATTACCCCTACTACTCCTCTCACTCAACCCTAAAATTATCTTAGGCCCCATCT
11576-11680	ACAAAAACATTAGATTGTGAATCTGATAATGAAAGCTCGAGCCTTTCTATTTACCGAAAAAGTACCACGCAAGAACTGCTAACTCATGCTCCCATGCATAAAAGC
11744-12048	GCAACTCCAAATAAAAGTAATCAACCTATTCACTTCCTCCATCCTCGTAACACTATTCATACTAACCCTCCCCATCATAATAACCAACACTACCCTTTATACCAATAAACTATATCCCCAATACGTAAAAACCACTATCTCACATGCTTTTATAATAAGCCTGATCCCCACAATAATATTTCTCTACTTAGGGCAAGACACAATAATTTCAAACTGACACTGAATTACAATTCAAACTGTAAAACTCTCCCTCAACTTTAAACTTGATTACTTCTCAATAATCTTCATACCGGTAGCACTATTCG
12071-12243	CTCAATATGATACATACACTCAGACCCCTACATCAACCGATTCTTCAAATATTTACTTCTTTTCCTAATCACTATAATAATCCTAGTCACCGCCAACAATATATTTCAACTATTTATCGGATGGGAAGGAGTAGGTATTATATCATTTCTACTTATCGGATGATGATACGGGC
12248-12417	AGACGCCAACACAGCCGCACTACAGGCCATCTTATACAACCGTATTGGAGATGTAGGGTTCATCTTAGCCATGGCCTGATTCCTAGCCAACCTAAACACCTGAGATCTACAACAAATCCTCATAACCGACCACAAAAATCTGAACCTCCCTCTCACAGGCCTATTATTGG
12425-12595	CGGAAAATCCGCACAATTTGGCCTACACCCATGATTGCCCTCGGCCATAGAAGGTCCAACCCCTGTATCAGCCCTACTGCATTCAAGCACTATGGTTGTAGCAGGAGTATTTTTGCTAATCCGATTCCATCCCCTAATAGAACATAACAAAACAATTCAAACCATCACACT
12698-12816	AATAATCGTAACCATCGGCATCAATCAGCCCTACCTAGCGTTTCTCCATATCTGTACACATGCATTCTTCAAAGCCATATTATTCATGTGCTCTGGATCAATCATTCACAACCTAAACG
12971-13162	TACCAACGCCTGAGCCCTTCTAATTACCCTCATTGCCACCTCCATAACAGCTGCCTATAGCACTCGAATCCTATTTTTCGCACTCCTAGGACAACCCCGCTTCAACCCTGTCGTCGCAATCAACGAGAATAATCCCCTCCTAATCAACTCCATTAAACGTCTCCTACTCGGGAGTATCTTCGCAGGATTCCT
13196-13652	ACAGATAACTATGCCTCATTATCTCAAAATAATAGCCCTCACCATAACCATCTTAGGTTTCATTTTGGCACTAGAACTTAACCTCACAATACAAAGCCTAAAATTTAAATATCCCTCGAACCTATTCAAATTCTCAAACATACTAGGCTACTTCCCAACTACTATTCACCGCCTAATACCCAAAATAAATCTACTCATAAGCCAAAAATCAGCATCAACACTGTTAGATATAATTTGACTAGAAAAGATCTTACCAAAATCCATCTCCCACTTCCAAATAAAATCTTCAATCACCGTCACTAACCAAAAAGGCCTAATCAAACTATACTTCTTATCATTTATACTAACCCTGACTCTAAGCCTACTCGTACTTAACTTCCACGAGTAACCTCCATAATTACCAACACCCCAATAAGAAGTGACCAGCCAGTGACAACAACCAACCAAGTCCCATAAC

13654-14015	ATATAAAGCTGCAATTCCCATAGCTTCCTCACTAAAAAATCCTGAATCACCTGTATCATAAATAACCCAATCGCCCGCTCCATTAAACTTCAATACAACTTCAATGTCAATATCATCACCCTTTAAAACATAACAAGCAGTTAATAACTCAGACAACAAGCCAACGATAAAAGCTCCTAAAACAGCCTTATTAGAGGCCCAAACTTCAGGATACTGCTCAGTAGCCATGGCTGTAGTGTACCCAAAAACAACTAGCATACCCCCTAAATAAATCAAAAATACCATTAAACCCAAAAACGATCCTCCAAAACTCAACACAATCCCACAACCAACAGCCCCACTAATAATTAAAACCAGCCCGC
14383-14543	CCGAGACGTCAACTACGGCTGAATTATCCGGTACATACATGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCCTACATGTAGGACGAGGATTATACTATGGATCTTACATATTTTCCGAAACATGAAACATTGGTATTGTCTTACTATTCACAGTTA
14545-14693	AGCAACAGCATTCATAGGTTACGTCTTACCATGAGGACAAATATCCTTTTGAGGCGCAACCGTAATTACTAACCTACTATCAGCCATCCCATACATCGGAACTAACCTTGTAGAATGAATCTGGGGTGGGTTCTCAGTAGACAAAGCTA
14704-14978	**ATTCTTCGCCTTCCACTTTATCCTGCCCTTCATCATTTCAGCACTGGCAATAATCCACCTCCTATTCCTCCACGAGACAGGGTCTAACAACCCATCCGGAATCCCATCCAACTCTGACAAAATCCCATTTCACCCCTACTATACCATCAAGGACATCCTAGGCGCCCTATCCCTAGTCCTAGCGCTGACAACGCTAGTGCTATTCTCCCCCGACCTGCTAGGAGACCCCGACAACTATATCCCCGCCAACCCACTCAGTACACCACCCCATATCA**
14980-15104	**ACCCGAATGATATTTCCTATTTGCATATGCAATCCTACGGTCCATCCCTAACAAACTAGGAGGAGTACTAGCCTTAATCCTATCCATCCTAATCCTAGCTATCATTCCCCTACTACACACCTCTA**
15109-15299	ACGAAGCATGATATTCCGCCCACTCAGCCAATGCCTGTTCTGACTATTAGTAGCTGACCTCCTTACCCTAACCTGAATTGGAGGCCAACCAGTAGAGCACCCATTCGTCGTCATCGGCCAATTGGCCTCAATTCTTTACTTTATAATTCTCCTAGTACTAATACCAATCACCAGCATTATCGAAAACAACC
15372-15527	CCCTAAGACTCAAGGAAGAGGCAAGAACCCCACCATCAGTACCCAAAACTGACATTCTAACTAAACTATTCCCTGATTTCCTCACCCTATTGTCCTAATTCATATATTTAATAATACTTACTGTGCTTGCCCAGTAAGTATTACCCGCGCACCTCC
15846-15989	GTGTCATGCATTTGGTATTTTTAACTTTTGGGGGGGGAGAACTTGGTGTCACTCAGCTGTGGCCGTGGCGGCCTCGTAGCAGTCAGATAACTTGTAGCTGGACTTGATCTTCATCATTTATCCGCATTACATAGCCATAAGGTG
15995-16211	CAGTCAATGGTTACAGGACATATACCTACATATACCCACGTACACCCACGTACGTACACGTACACCCACGTACGTACACGTACACCCACGTACGTACACGTACACCCACGTACGTACACGTACACCCACGTACGTACACGTACACCCACGTACGTACACGTACACCCACGTACGTACACGTACACCCACACACGTATTCAACAGATAGGGATTGATT

Appendix 8. Technical Bulletin for the eENLU6 Assay

Helbing Lab
eDNA Inventory

Supplementary Figure 1: Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity[1-3].

General eDNA Assay Information

Target Species: Northern sea otter (*Enhydra lutris*)
eDNA qPCR Tool: eENLU6
Gene Target: MT-CYB
Species Code: ma-ENLU
eDNA qPCR Format: TaqMan
Published in:

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 0.3 95% CI 0.2-0.6 Copies/Rxn
LOQ 1.3 95% CI 0.9-2.1 Copies/Rxn
LOB 0 hits/8
Binomial-Poisson model for 8 technical replicates
Determined using eLowQuant R code[4].
When the LOQ < LOD, use the LOD for the LOQ.
Enzyme: QIAcuity

eDNA Assay Specificity Test Information

Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

Species	**Common Name (*Species*)**	**Detection**	**# Voucher Specimens**	**Sample Sources/Locations**
ENLU	Northern sea otter (*Enhydra lutris*)	Yes	5	North Pacific Ocean
NEVI	American mink (*Neovision vision*)	No	5	Vancouver Island
BAAC	Minke whale (*Balaenoptera acutorostrata*)	No	2	Southwestern British Columbia
CAUR	Northern fur seal (*Callorhinus ursinus*)	No	1	North Pacific Ocean
ESRO	Gray whale (*Eschruchtius robustus*)	No	2	Vancouver Island
EUJU	Stellar sea lion (*Eumetopias jubatus*)	No	2	Vancouver Island
LAOB	Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)	No	2	Vancouver Island
LOCA	River otter (*Lontra canadensis*)	No	2	Vancouver Island
MENO	Humpback whale (*Megaptera movaeangliae*)	No	2	Langara Island
PHDA	Dall's porpoise (*Phocoenoides dalli*)	No	2	Vancouver Island
PHLE	Southern elephant seal (*Phoca leonina*)	No	1	Antarctica
PHPH	Harbour porpoise (*Phocoena phocoena*)	No	2	Vancouver Island
PHVI	Harbour seal (*Phoca vitulina*)	No	2	Vancouver Island
ZACA	California sea lion (*Zalophus californianus*)	No	2	Nanaimo
URAM	American black bear (*Ursus americanus*)	No	2	British Columbia
URAR	Grizzly bear (*Ursus arctos*)	No	2	British Columbia
HOSA	Human (*Homo sapiens*)	No	1	Netherlands
CAFA	Canine (*Canis lupus familiaris*)	No	1	Southwestern British Columbia
FECA	Cat (*Felis catus*)	No	1	Southwestern British Columbia

References

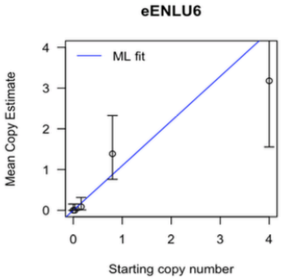
1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus* , in British Columbia, Canada, using environmental DNA methods. Environmental DNA, 2: 350-361. https://doi.org/10.1002/edn3.82

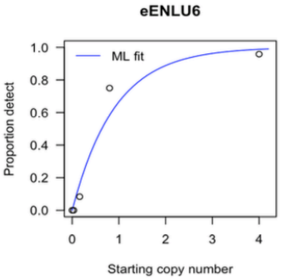
2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei* , in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849

3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164

4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA

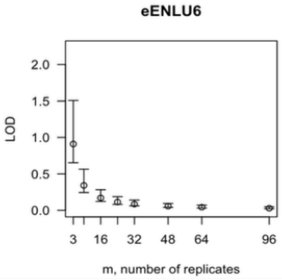


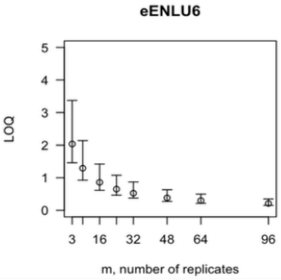


From 8 Technical Replicates

# Detects	# Copies	SE
0	0	0
1	0.12	0.12
2	0.26	0.19
3	0.43	0.26
4	0.63	0.35
5	0.9	0.45
6	1.26	0.61
7	1.9	0.94

Determined using eLowQuant R code[4].



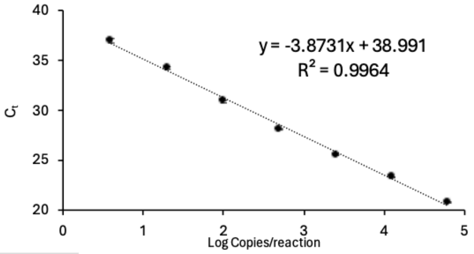


Binomial-Poisson model: No intercept

Determined using eLowQuant R code[4].

Based on a 2 µL DNA input in a total 15 µL reaction

Applied to reactions with 100% positive hits



Efficiency 93%

Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location	Notes;
Water	Y	3	Y	Vancouver Aquarium Sea Otter Tank	Represented Northern subspecies
Water	Y	4	Y	Monterey Bay Aquarium Sea Otter Rehabilitation Tank	Represented Southern subspecies

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-CYB	Mitochondrial cytochrome-b gene
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 9. Reporting of statistical test output values. Significant results for the pairwise comparisons are bolded.

eDNA Amplification Success Metrics	Shapiro-Wilk Normality Test		Levene's Test For Homogeneity of Variance			Kruskal-Wallis Rank Sum Test			Pairwise Comparisons using Wilcoxon rank sum exact test									
	W	p-Value	Df	F Value	Pr (>F)	chi-squared	Df	p-Value		Filter Soil_A	Filter Soil_B	Filter Soil_C	Norgen_A	Norgen_B	Norgen_C	PowerMax_A	PowerMax_B	PowerMax_C
DNA Concentration	0.507	<2.2 e-16	11	7.547	1.659 e -9	100.96	11	<2.2 e-16	Filter Soil_B	0.4447	-	-	-	-	-	-	-	-
									Filter Soil_C	0.6589	1	-	-	-	-	-	-	-
									Norgen_A	0.1771	0.4593	0.2266	-	-	-	-	-	-
									Norgen_B	0.1706	0.1706	0.1706	1	-	-	-	-	-
									Norgen_C	**0.0372**	0.4678	0.2602	1	1	-	-	-	-
									PowerMax_A	**0.0007**	**0.0007**	**0.0007**	**0.0152**	**0.0106**	**0.0116**	-	-	-
									PowerMax_B	0.0978	0.0978	0.0978	0.7166	0.0683	0.0733	0.7473	-	-
									PowerMax_C	**0.0007**	**0.0007**	**0.0007**	**0.0477**	**0.0143**	**0.0116**	1	1	-
									PowerSoil_A	1	1	1	0.2873	0.1319	0.2034	**0.0118**	0.3755	**0.0213**
IntegritE Amplification	0.36	<2.2 e-16	11	6.657	2.075 e -8	99.714	11	<2.2 e-16		Filter Soil_A	Filter Soil_B	Filter Soil_C	Norgen_A	Norgen_B	Norgen_C	PowerMax_A	PowerMax_B	PowerMax_C
									Filter Soil_B	-	-	-	-	-	-	-	-	-
									Filter Soil_C	-	-	-	-	-	-	-	-	-
									Norgen_A	**0.00358**	**0.00358**	**0.00358**	-	-	-	-	-	-
									Norgen_B	**0.00358**	**0.00358**	**0.00358**	1	-	-	-	-	-
									Norgen_C	**0.00358**	**0.00358**	**0.00358**	1	1	-	-	-	-
									PowerMax_A	**0.00358**	**0.00358**	**0.00358**	**0.00061**	**0.00061**	**0.00061**	-	-	-
									PowerMax_B	0.83616	0.83616	0.83616	1	1	1	**0.00913**	-	-
									PowerMax_C	1	1	1	1	1	1	**0.00738**	1	-
									PowerSoil_A	**0.00358**	**0.00358**	**0.00358**	**0.00061**	**0.00061**	**0.00061**	**0.00242**	**0.00913**	**0.00738**
eFISH1 Amplification	0.38	<2.2 e-16	11	1.5	0.142	66.409	11	5.849 e -10		Filter Soil_A	Filter Soil_B	Filter Soil_C	Norgen_A	Norgen_B	Norgen_C	PowerMax_A	PowerMax_B	PowerMax_C
									Filter Soil_B	-	-	-	-	-	-	-	-	-
									Filter Soil_C	-	-	-	-	-	-	-	-	-
									Norgen_A	1	1	1	-	-	-	-	-	-
									Norgen_B	1	1	1	1	-	-	-	-	-
									Norgen_C	1	1	1	1	1	-	-	-	-
									PowerMax_A	**0.0421**	**0.0421**	**0.0421**	0.7025	1	1	-	-	-
									PowerMax_B	**0.0036**	**0.0036**	**0.0036**	0.0573	0.1441	0.2002	1	-	-
									PowerMax_C	0.3344	0.3344	0.3344	1	1	1	1	1	-
									PowerSoil_A	**0.0418**	**0.0418**	**0.0418**	1	1	1	1	1	1

Appendix 10. Whole mitochondrial genome sequences of indicator and co-occurring taxa successfully generated through Illumina sequencing from tissue voucher specimens. Sequences were assembled with *mtGrasp V0.2.0.*[261]. Sequences are now published by the National Center for Biotechnology Information (NCBI).

Sample ID	**Taxon Name**	**Library ID **Will be switched to Accession Number**	**Scaffold Length (bp)**
an-ARBR-11	*Armandia brevis*	F121992	13,689
an-CACA-3	*Capitella capitata*	F23063	16,945
an-CACA-6	*Capitella capitata*	F112628	18,785
an-CACA-17	*Capitella capitata*	F112633	16,128
an-CACAW-6	*Capitella capitata*	F121995	14,071
an-DEGR-3	*Decamastus gracilis*	F129301	17,421
an-DRLO-8	*Drilonereis longa*	F126743	15,772
an-GLAM-1	*Glycera americana*	F112684	15,331
an-GLNA-4	*Glycera nana*	F112637	14,772
an-GLNA-5	*Glycera nana*	F112638	15,211
an-GLNA-6	*Glycera nana*	F112639	15,205
an-GLNA-8	*Glycera nana*	F112641	15,203
an-GLNA-9	*Glycera nana*	F112642	15,761
an-GLNA-10	*Glycera nana*	F112643	15,215
an-GLNA-11	*Glycera nana*	F112644	15,212
an-HEFI-1	*Heteromastus filiobranchus*	F258579	17,452
an-MASA-13	*Maldane sarsi*	F126741	15,224
an-MASA-14	*Maldane sarsi*	F126739	15,520
an-NOHE-10	*Notomastus hemipodus*	F126827	16,276
an-NOHE-12	*Notomastus hemipodus*	F126744	15,674
an-OPAC-11	*Ophelina acuminata*	F122001	15,464
an-OPAC-13	*Ophelina acuminata*	F122002	15,467
an-OPAC-14	*Ophelina acuminata*	F122003	15,460
an-OPAC-6	*Ophelina acuminata*	F112674	15,464
an-PRGR-7	*Praxillella gracilis*	F121986	16,644
an-PRGR-10	*Praxillella gracilis*	F121985	17,044
an-PRGR-11	*Praxillella gracilis*	F126738	16,176
an-PRPA-1	*Praxillella pacifica*	F112685	17,943
an-PRPA-3	*Praxillella pacifica*	F112687	20,410

an-PRPA-5	*Praxillella pacifica*	F112681	18,817
an-PRPA-9	*Praxillella pacifica*	F112693	19,921
an-PRJU-1	*Prionospio jubata*	F898	15,274
an-PRJU-2	*Prionospio jubata*	F148743	15,434
an-PRLI-1	*Prionospio lighti*	F87418	14,879
an-PRLI-2	*Prionospio lighti*	F87418	14,875
an-PRLI-3	*Prionospio lighti*	F88198	14,941
an-PRMU-1	*Prionospio multibranchiata*	F93922	16,283
si-THNI-1	*Thysanocardia nigra*	F112661	15,364
si-THNI-3	*Thysanocardia nigra*	F112663	16,347
si-THNI-4	*Thysanocardia nigra*	F112664	16,126
si-THNI-5	*Thysanocardia nigra*	F112665	16,023
si-THNI-7	*Thysanocardia nigra*	F112667	15,960

Appendix 11. National Center for Biotechnology Information (NCBI) accession numbers for species sequences used in the present study.

Species ID	**Species Name**	**Accession Number**
an-ESSP	*Escarpia spicata*	NC_026856.1
an-GABR	*Galathealinum brachiosum*	NC_026857.1
an-GLAM	*Glycera americana*	KT989321.1
an-GLAM	*Glycera americana*	KT989330.1
an-GLAR	*Glycinde armigera*	KT989325.1
an-GLCA	*Glycera capitata*	KT989319.1
an-GLCA	*Glycera capitata*	KT989320.1
an-GLDI	*Glycera dibranchiata*	KT989318.1
an-GLFA	*Glycera fallax*	KT989323.1
an-GLOX	*Glycera oxycephala*	KT989329.1
an-GLTE	*Glycera tesselata*	KT989326.1
an-GLTR	*Glycera tridactyla*	KT989327.1
an-GLTR	*Glycera tridactyla*	KT989328.1
an-GLTR	*Glycera tridactyla*	KT989331.1
an-GLUN	*Glycera unicornis*	KT989324.1
an-HESI	*Hemipoda simplex*	KT989322.1
an-LALU	*Lamellibrachia luymesi*	NC_026858.1
an-LESA	*Lamellibrachia satsuma*	NC_027854.1

an-OAAL	*Oasisia alvinae*	NC_026859.1
an-OSRU	*Osedax rubiplumus*	MT108937.1
an-PAEC	*Paraescapria echinospica*	NC_037085.1
an-RIPA	*Riftia pachyptila*	KJ789166.1
an-RIPA	*Riftia pachyptila*	NC_026860.1
an-RIPI	*Ridgeia piscesae*	KJ872501.1
an-RIPI	*Ridgeia piscesae*	NC_024653.1
an-SEJO	*Seepiophila jonesi*	NC_026861.1
an-TEJE	*Tevnia jerichonana*	KJ789172.1
an-TEJE	*Tevnia jerichonana*	NC_026862.1
ma-CALUfa	Dog (*Canis lupus familiaris*)	NC_002008.4
ma-FECA	Cat (*Felis catus*)	NC_001700.1
ma-HOSA	Human (*Homo sapiens*)	NC_012920.1

Appendix 12. Technical Bulletin for the eDEGR2 Assay

Helbing Lab

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity[1-3].

General eDNA Assay Information

Target Species:	*Decamastus Gracilis*	eDNA qPCR Tool:	eDEGR2	Gene Target:	MT-ND4
Species Code:	an-DEGR	eDNA qPCR Format:	TaqMan	Published in:	

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 1.2 95% CI 0.9-2.1 Copies/Rxn LOQ 4.6 95% CI 3.2-8.1 Copies/Rxn LOB 0 hits/8

Binomial-Poisson model for 8 technical replicates determined using eLowQuant R code[4]. When the LOQ < LOD, use the LOD for the LOQ. Enzyme: QIAcuity

eDNA Assay Specificity Test Information

Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

Species	Common Name (*Species*)	Detection	# Voucher Specimens	Sample Sources/Locations
an-DEGR	Decamastus (*Decamastus gracilis*)	Yes	6	CRD/METVAN BC
an-HEFI	Heteromastus (*Heteromastus filobranchus*)	No	6	CRD/METVAN BC
an-GLNA	Glycera (*Glycera nana*)	No	*2*	CRD/METVAN BC
an-CACAW	Capitella (*Capitella capitata*)	No	4	CRD/METVAN BC
an-PRMU	Prionospio (*Prionospio multibranchiata*)	No	1	CRD/METVAN BC
an-PRLI	Prionospio (*Prionospio lighti*)	No	6	CRD/METVAN BC
an-PRJU	Prionospio (*Prionospio (Prionospio) jubata*)	No	6	CRD/METVAN BC
an-NOHE	Notomastus (*Notomastus hemipodus*)	No	2	CRD/METVAN BC
an-RIPI	Hydrothermal vent worm (*Ridgeia picescae*)	No	4	Endeavor Hydrothermal Vent
ma-HOSA	Human (*Homo sapiens*)	No	1	
ma-CAFA	Domestic dog (Canis lupus familiaris)	No	1	
ma-FECA	Cat (*Felis catus*)	No	1	

References

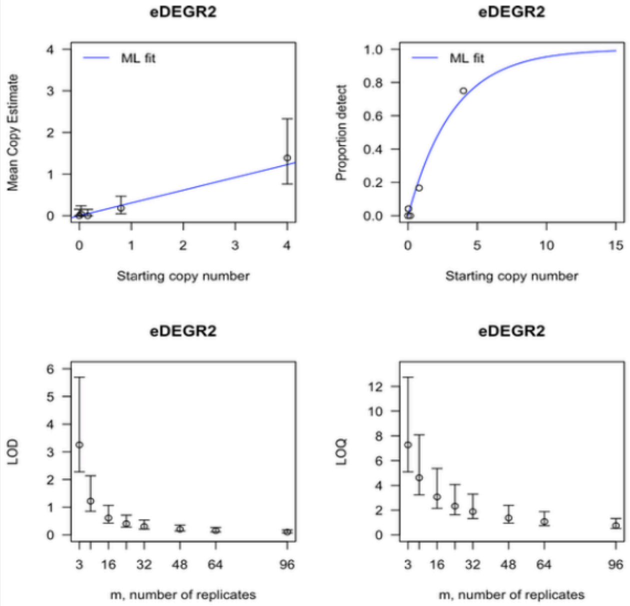
1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus*, in British Columbia, Canada, using environmental DNA methods. Environmental DNA, 2: 350-361. https://doi.org/10.1002/edn3.82

2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei*, in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849

3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164

4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA



Determined using eLowQuant R code[4].

Based on a 2 µL DNA input in a total 15 µL reaction

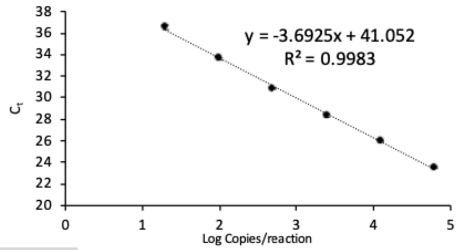
To calculate tables for different numbers of replicates, use raw csv data files.

From 8 Technical Replicates

# Detects	# Copies	SE
0	0	0
1	0.44	0.45
2	0.94	0.7
3	1.53	0.95
4	2.26	1.25
5	3.2	1.64
6	4.52	2.23
7	6.78	3.39

Determined using eLowQuant R code[4].

Applied to reactions with ≥95% positive hits



Efficiency	87%

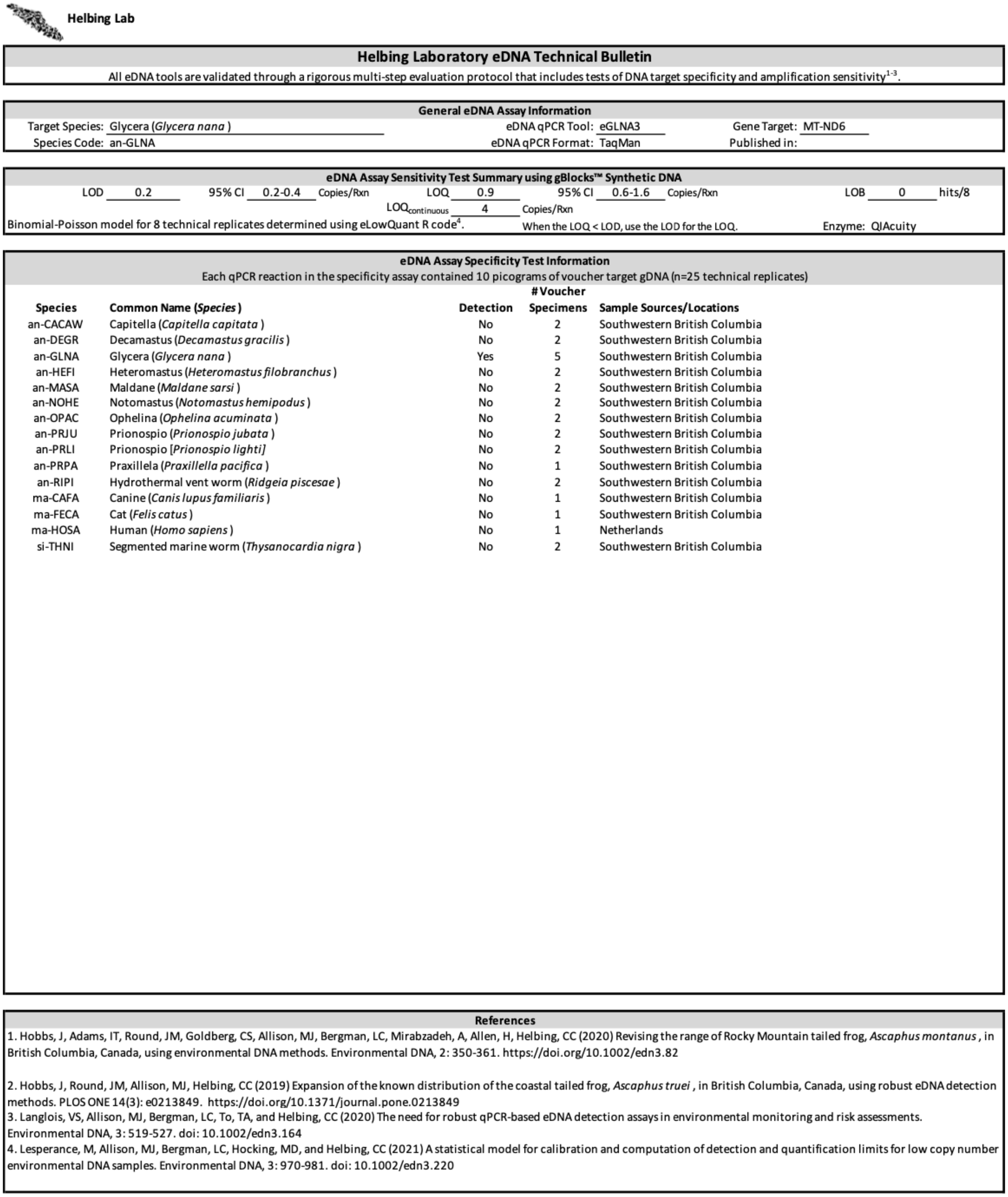
Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location
Water	Y	280	Y	Vancouver, BC, Canada

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-ND4	Mitochondrial NADH Dehydrogenase subunit 4
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 13. Technical Bulletin for the eGLNA3 Assay



Helbing Lab

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity[1-3].

General eDNA Assay Information

Target Species: Glycera (*Glycera nana*)
Species Code: an-GLNA
eDNA qPCR Tool: eGLNA3
eDNA qPCR Format: TaqMan
Gene Target: MT-ND6
Published in:

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 0.2 95% CI 0.2-0.4 Copies/Rxn
LOQ 0.9 95% CI 0.6-1.6 Copies/Rxn
LOB 0 hits/8
$LOQ_{continuous}$ 4 Copies/Rxn
Binomial-Poisson model for 8 technical replicates determined using eLowQuant R code[4].
When the LOQ < LOD, use the LOD for the LOQ.
Enzyme: QIAcuity

eDNA Assay Specificity Test Information

Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

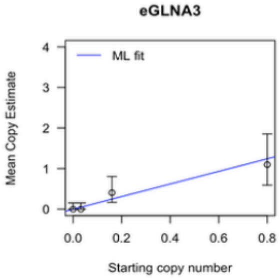
Species	Common Name (*Species*)	Detection	# Voucher Specimens	Sample Sources/Locations
an-CACAW	Capitella (*Capitella capitata*)	No	2	Southwestern British Columbia
an-DEGR	Decamastus (*Decamastus gracilis*)	No	2	Southwestern British Columbia
an-GLNA	Glycera (*Glycera nana*)	Yes	5	Southwestern British Columbia
an-HEFI	Heteromastus (*Heteromastus filobranchus*)	No	2	Southwestern British Columbia
an-MASA	Maldane (*Maldane sarsi*)	No	2	Southwestern British Columbia
an-NOHE	Notomastus (*Notomastus hemipodus*)	No	2	Southwestern British Columbia
an-OPAC	Ophelina (*Ophelina acuminata*)	No	2	Southwestern British Columbia
an-PRJU	Prionospio (*Prionospio jubata*)	No	2	Southwestern British Columbia
an-PRLI	Prionospio [*Prionospio lighti]*	No	2	Southwestern British Columbia
an-PRPA	Praxillela (*Praxillella pacifica*)	No	1	Southwestern British Columbia
an-RIPI	Hydrothermal vent worm (*Ridgeia piscesae*)	No	2	Southwestern British Columbia
ma-CAFA	Canine (*Canis lupus familiaris*)	No	1	Southwestern British Columbia
ma-FECA	Cat (*Felis catus*)	No	1	Southwestern British Columbia
ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands
si-THNI	Segmented marine worm (*Thysanocardia nigra*)	No	2	Southwestern British Columbia

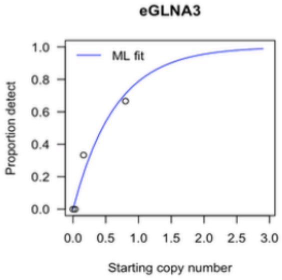
References

1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus* , in British Columbia, Canada, using environmental DNA methods. Environmental DNA, 2: 350-361. https://doi.org/10.1002/edn3.82

2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei* , in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849
3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164
4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA



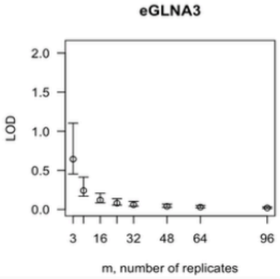


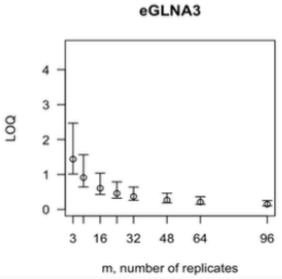
To calculate tables for different numbers of replicates, raw csv data files can be accessed here:
https://onlineacademiccommunity.uvic.ca/helbinglab/edna/

From 8 Technical Replicates

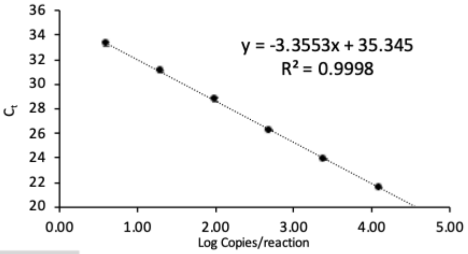
# Detects	# Copies	SE
0	0	0
1	0.086	0.088
2	0.19	0.14
3	0.3	0.19
4	0.45	0.25
5	0.63	0.32
6	0.89	0.44
7	1.34	0.67

Determined using eLowQuant R code[4].





Applied to reactions with 100% positive hits



Binomial-Poisson model: No intercept

Determined using eLowQuant R code[4].

Based on a 2 µL DNA input in a total 15 µL reaction

Efficiency 99%

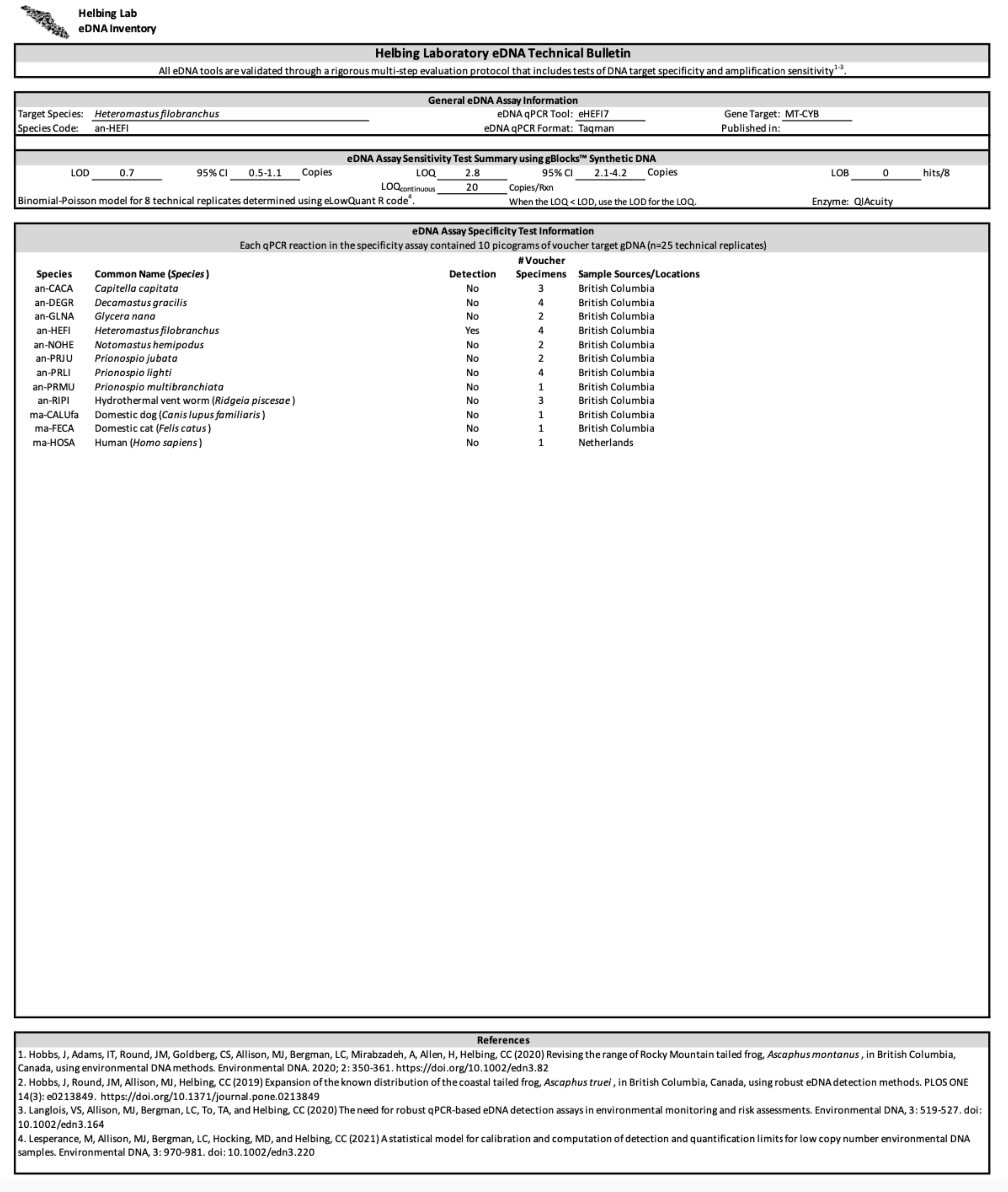
Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location
Water	Y	280	Y	Vancouver, BC, Canada

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-ND6	Mitochondrial NADH dehydrogenase subunit 6 gene
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 14. Technical Bulletin for the eHEFI7 Assay



Helbing Lab
eDNA Inventory

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity[1-3].

General eDNA Assay Information

Target Species: *Heteromastus filobranchus*
Species Code: an-HEFI
eDNA qPCR Tool: eHEFI7
eDNA qPCR Format: Taqman
Gene Target: MT-CYB
Published in:

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 0.7 95% CI 0.5-1.1 Copies
LOQ 2.8 95% CI 2.1-4.2 Copies
LOB 0 hits/8
$\text{LOQ}_{\text{continuous}}$ 20 Copies/Rxn
Binomial-Poisson model for 8 technical replicates determined using eLowQuant R code[4].
When the LOQ < LOD, use the LOD for the LOQ.
Enzyme: QIAcuity

eDNA Assay Specificity Test Information

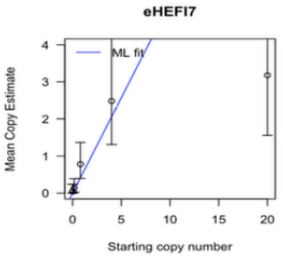
Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

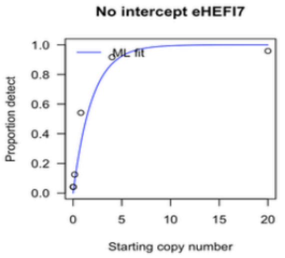
Species	Common Name (*Species*)	Detection	# Voucher Specimens	Sample Sources/Locations
an-CACA	*Capitella capitata*	No	3	British Columbia
an-DEGR	*Decamastus gracilis*	No	4	British Columbia
an-GLNA	*Glycera nana*	No	2	British Columbia
an-HEFI	*Heteromastus filobranchus*	Yes	4	British Columbia
an-NOHE	*Notomastus hemipodus*	No	2	British Columbia
an-PRJU	*Prionospio jubata*	No	2	British Columbia
an-PRLI	*Prionospio lighti*	No	4	British Columbia
an-PRMU	*Prionospio multibranchiata*	No	1	British Columbia
an-RIPI	Hydrothermal vent worm (*Ridgeia piscesae*)	No	3	British Columbia
ma-CALUfa	Domestic dog (*Canis lupus familiaris*)	No	1	British Columbia
ma-FECA	Domestic cat (*Felis catus*)	No	1	British Columbia
ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands

References

1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus*, in British Columbia, Canada, using environmental DNA methods. Environmental DNA. 2020; 2: 350-361. https://doi.org/10.1002/edn3.82
2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei*, in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849
3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164
4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA

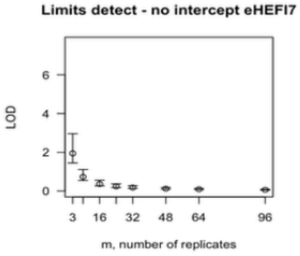

eHEFI7

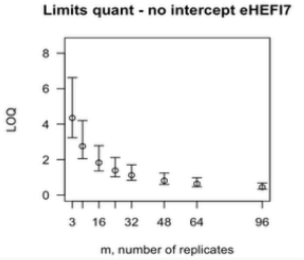

No intercept eHEFI7

To calculate tables for different numbers of replicates, raw csv data files can be accessed here:
https://onlineacademiccommunity.uvic.ca/helbinglab/edna/
From 8 Technical Replicates

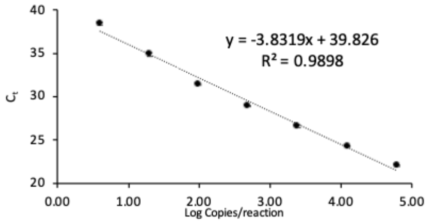
# Detects	# Copies	SE
0	0	0
1	0.26	0.26
2	0.56	0.41
3	0.91	0.56
4	1.35	0.73
5	1.91	0.95
6	2.7	1.28
7	4.05	1.95

Determined using eLowQuant R code[4].


Limits detect - no intercept eHEFI7


Limits quant - no intercept eHEFI7

Applied to reactions with 100% positive hits



Efficiency	82%

Binomial-Poisson model: No intercept
Determined using eLowQuant R code[4].
Based on a 2 µL DNA input in a total 15 µL reaction

Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location
Water	Y	280	Y	Vancouver, BC, Canada

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-CYB	Mitochondrial cytochrome b gene
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 15. Technical Bulletin for the eOPAC1 Assay

Helbing Lab
eDNA Inventory

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity[1-3].

General eDNA Assay Information

Target Species:	Ophelina (*Ophelina acuminata*)	eDNA qPCR Tool:	eOPAC1	Gene Target:	MT-ND2
Species Code:	an-OPAC	eDNA qPCR Format:	TaqMan	Published in:	

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 0.5 95% CI 0.4-0.9 Copies/Rxn LOQ 2 95% CI 1.4-3.3 Copies/Rxn LOB 0 hits/8

$LOQ_{continuous}$ 4 Copies/Rxn

Binomial-Poisson model for 8 technical replicates determined using eLowQuant R code[4]. When the LOQ < LOD, use the LOD for the LOQ. Enzyme: QIAcuity

eDNA Assay Specificity Test Information

Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

Species	Common Name (*Species*)	Detection	# Voucher Specimens	Sample Sources/Locations
an-CACAW	Capitella (*Capitella capitata*)	No	2	Netherlands
an-DEGR	Decamastus (*Decamastus gracilis*)	No	2	British Columbia
an-GLNA	Glycera (*Glycera nana*)	No	2	British Columbia
an-HEFI	Heteromastus (*Heteromastus filobranchus*)	No	2	British Columbia
an-MASA	Maldane (*Maldane sarsi*)	No	2	British Columbia
an-NOHE	Notomastus (*Notomastus hemipodus*)	No	2	British Columbia
an-OPAC	Ophelina (*Ophelina acuminata*)	Yes	5	British Columbia
an-PRJU	Prionospio (*Prionospio jubata*)	No	2	British Columbia
an-PRLI	Prionospio [*Prionospio lighti]*	No	2	British Columbia
an-PRPA	Praxillela (*Praxillella pacifica*)	No	1	British Columbia
an-RIPI	Hydrothermal vent worm (*Ridgeia piscesae*)	No	2	British Columbia
ma-CALUfa	Domestic dog (*Canis lupus familiaris*)	No	1	British Columbia
ma-FECA	Domestic cat (*Felis catus*)	No	1	British Columbia
ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands
si-THNI	Segmented marine worm (*Thysanocardia nigra*)	No	2	Alberta

References

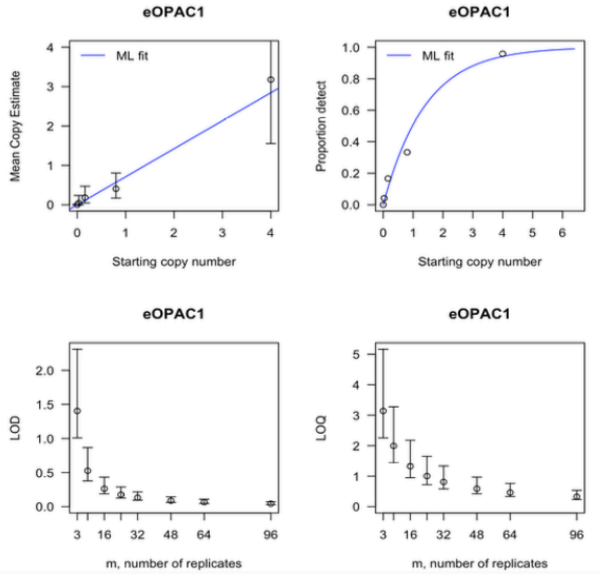
1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus* , in British Columbia, Canada, using environmental DNA methods. Environmental DNA, 2: 350-361. https://doi.org/10.1002/edn3.82

2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei* , in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849

3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164

4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA



Binomial-Poisson model: No intercept

Determined using eLowQuant R code[4].

Based on a 2 µL DNA input in a total 15 µL reaction

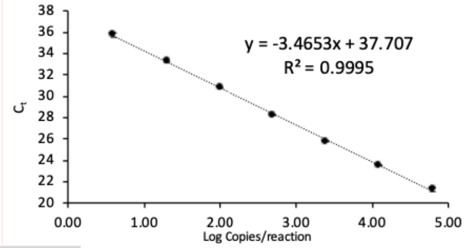
To calculate tables for different numbers of replicates, raw csv data files can be accessed here:
https://onlineacademiccommunity.uvic.ca/helbinglab/edna/

From 8 Technical Replicates

# Detects	# Copies	SE
0	0	0
1	0.19	0.19
2	0.41	0.3
3	0.66	0.41
4	0.98	0.53
5	1.38	0.7
6	1.95	0.95
7	2.92	1.44

Determined using eLowQuant R code[4].

Applied to reactions with 100% positive hits



Efficiency 94%

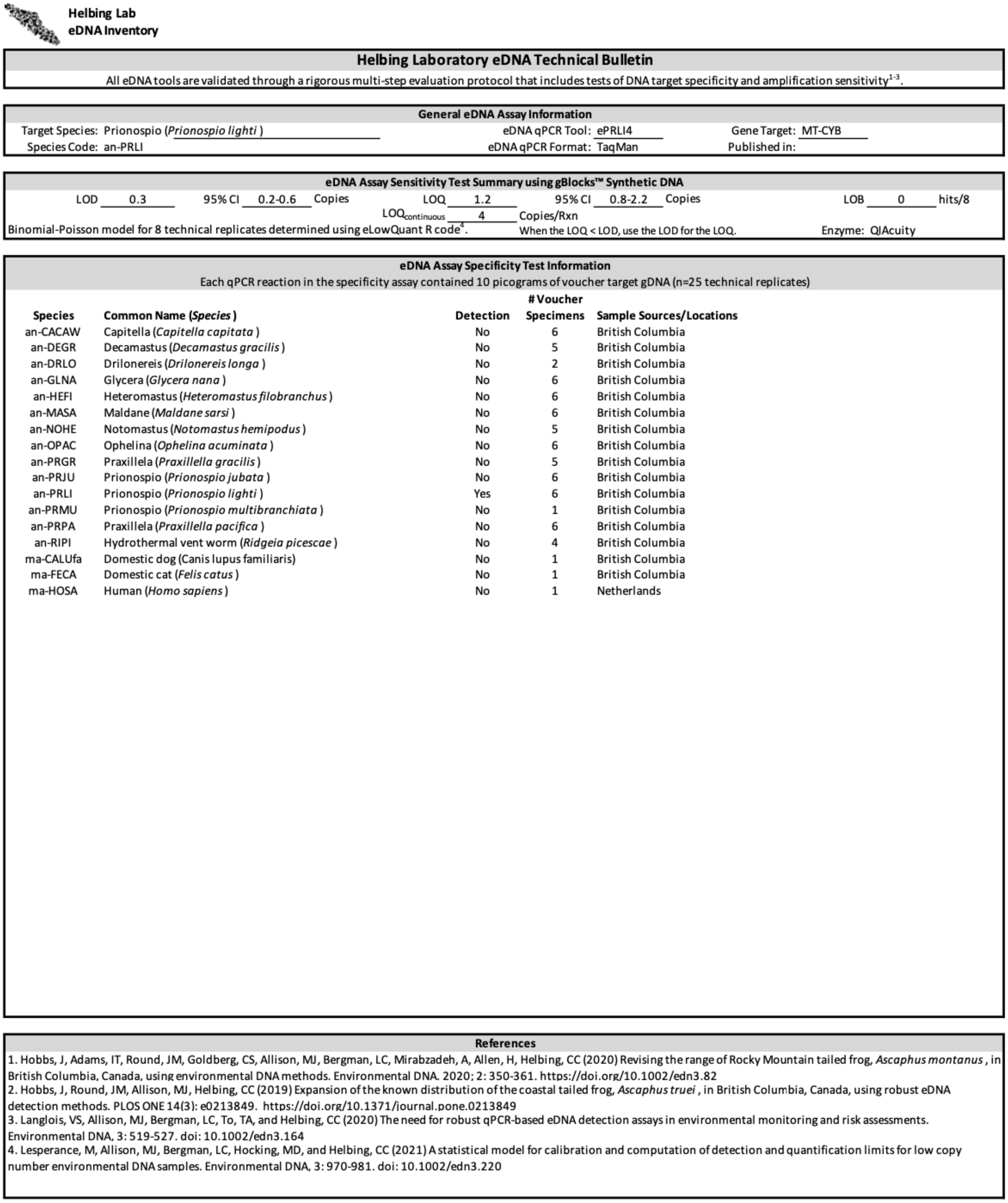
Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location
Water	Y	280	Y	Vancouver, BC, Canada

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-ND2	Mitochondrial NADH dehydrogenase subunit 2 gene
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 16. Technical Bulletin for the ePRLI4 Assay



Helbing Lab
eDNA Inventory

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity[1-3].

General eDNA Assay Information

Target Species: Prionospio (*Prionospio lighti*)
Species Code: an-PRLI
eDNA qPCR Tool: ePRLI4
eDNA qPCR Format: TaqMan
Gene Target: MT-CYB
Published in:

eDNA Assay Sensitivity Test Summary using gBlocks™ Synthetic DNA

LOD 0.3 95% CI 0.2-0.6 Copies
LOQ 1.2 95% CI 0.8-2.2 Copies
LOB 0 hits/8
$\mathrm{LOQ}_{\mathrm{continuous}}$ 4 Copies/Rxn
Binomial-Poisson model for 8 technical replicates determined using eLowQuant R code[4].
When the LOQ < LOD, use the LOD for the LOQ.
Enzyme: QIAcuity

eDNA Assay Specificity Test Information

Each qPCR reaction in the specificity assay contained 10 picograms of voucher target gDNA (n=25 technical replicates)

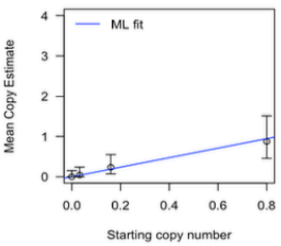
Species	Common Name (*Species*)	Detection	# Voucher Specimens	Sample Sources/Locations
an-CACAW	Capitella (*Capitella capitata*)	No	6	British Columbia
an-DEGR	Decamastus (*Decamastus gracilis*)	No	5	British Columbia
an-DRLO	Drilonereis (*Drilonereis longa*)	No	2	British Columbia
an-GLNA	Glycera (*Glycera nana*)	No	6	British Columbia
an-HEFI	Heteromastus (*Heteromastus filobranchus*)	No	6	British Columbia
an-MASA	Maldane (*Maldane sarsi*)	No	6	British Columbia
an-NOHE	Notomastus (*Notomastus hemipodus*)	No	5	British Columbia
an-OPAC	Ophelina (*Ophelina acuminata*)	No	6	British Columbia
an-PRGR	Praxillela (*Praxillella gracilis*)	No	5	British Columbia
an-PRJU	Prionospio (*Prionospio jubata*)	No	6	British Columbia
an-PRLI	Prionospio (*Prionospio lighti*)	Yes	6	British Columbia
an-PRMU	Prionospio (*Prionospio multibranchiata*)	No	1	British Columbia
an-PRPA	Praxillela (*Praxillella pacifica*)	No	6	British Columbia
an-RIPI	Hydrothermal vent worm (*Ridgeia picescae*)	No	4	British Columbia
ma-CALUfa	Domestic dog (Canis lupus familiaris)	No	1	British Columbia
ma-FECA	Domestic cat (*Felis catus*)	No	1	British Columbia
ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands

References

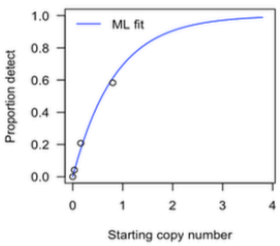
1. Hobbs, J, Adams, IT, Round, JM, Goldberg, CS, Allison, MJ, Bergman, LC, Mirabzadeh, A, Allen, H, Helbing, CC (2020) Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus* , in British Columbia, Canada, using environmental DNA methods. Environmental DNA. 2020; 2: 350-361. https://doi.org/10.1002/edn3.82
2. Hobbs, J, Round, JM, Allison, MJ, Helbing, CC (2019) Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei* , in British Columbia, Canada, using robust eDNA detection methods. PLOS ONE 14(3): e0213849. https://doi.org/10.1371/journal.pone.0213849
3. Langlois, VS, Allison, MJ, Bergman, LC, To, TA, and Helbing, CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and risk assessments. Environmental DNA, 3: 519-527. doi: 10.1002/edn3.164
4. Lesperance, M, Allison, MJ, Bergman, LC, Hocking, MD, and Helbing, CC (2021) A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Environmental DNA, 3: 970-981. doi: 10.1002/edn3.220

eDNA Assay Sensitivity Test Details using gBlocks™ synthetic DNA

ePRLI4



ePRLI4



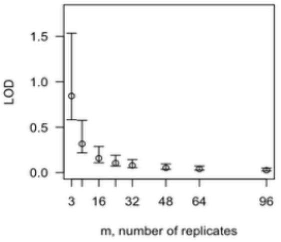
To calculate tables for different numbers of replicates, raw csv data files can be accessed here:
https://onlineacademiccommunity.uvic.ca/helbinglab/edna/

From 8 Technical Replicates

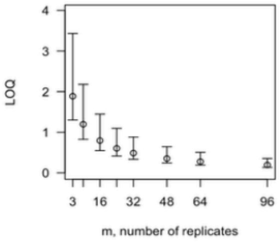
# Detects	# Copies	SE
0	0	0
1	0.11	0.11
2	0.24	0.18
3	0.4	0.25
4	0.59	0.33
5	0.83	0.43
6	1.17	0.58
7	1.76	0.89

Determined using eLowQuant R code[4].

ePRLI4

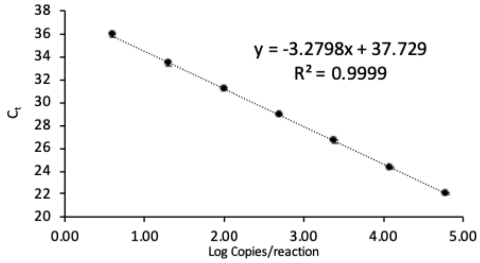


ePRLI4



Binomial-Poisson model: No intercept
Determined using eLowQuant R code[4].
Based on a 2 µL DNA input in a total 15 µL reaction

Applied to reactions with 100% positive hits



Efficiency	102%

Field Sample Validation

Sample Type	Known Presence	# Samples	Detected	Location
Water	Y	280	Y	Vancouver, BC, Canada

Abbreviations

95% CI	95% Confidence interval	LOQ	Limit of quantification
eDNA	Environmental DNA	MT-CYB	Mitochondrial cytochrome-B gene
gDNA	Total genomic DNA extracted from voucher specimen	NTC	qPCR no template control
LOB	Limit of blank	qPCR	Quantitative real-time polymerase chain reaction
LOD	Limit of detection	SE	Standard error

Appendix 17. Samples removed from analysis because of poor sample integrity.

Experiment	Site	Sample Type	Sample replicate	Cleaned	Passed IntegritE
1	8	Sediment Wash	A	Yes	No
1	9	Sediment Wash	A	Yes	No
1	9	Field Control	A	Yes	No
2	1	Sediment Bath	A	Yes	No
2	1	Sediment Bath	B	Yes	No
2	1	Sediment Bath	C	Yes	No
2	8	Sediment Bath	B	Yes	No
2	15	Sediment Bath	B	Yes	No
2	15	Sediment Bath	C	Yes	No
3	1	Sediment Bath	B	Yes	No
3	7	Sediment Bath	B	Yes	No
3	9	Sediment Bath	C	Yes	No
3	11	Sediment Bath	A	Yes	No
3	11	Sediment Bath	B	Yes	No
3	11	Sediment Bath	C	Yes	No
4	NA	Field Control	3	Yes	No

*A filtration blank that does not pass the IntegritE-DNA® test indicates no contamination

Appendix 18. Field experiment sampling summary '-', no sample taken.

Date	Site	Latitude	Longitude	Experiment #	# of Sample Type Replicates								
					Sediment	Sediment Wash		Sediment Bath		Adjacent Water		Instant Ocean Field Blank	
						Dip	Filter	Dip	Filter	Dip	Filter	Dip	Filter
27-03-2022	6	478 400	5 452 248	1	1	1	1	1	1	1	1	1	1
28-03-2022	15	476 840	5 441 000	1	1	1	1	1	1	1	1	1	1
29-03-2022	16	477 283	5 442 000	1	1	1	1	1	1	1	1	1	1
22-04-2022	1	478 105	5 457000	1	1	1	1	1	1	1	1	1	1
22-04-2022	7	478 235	5 451 229	1	1	1	1	1	1	1	1	1	1
22-04-2022	8	478 152	5 450 673	1	1	1	1	1	1	1	1	1	1
27-03-2022	6	478 400	5 452 248	1	1	1	1	1	1	1	1	1	1
28-03-2022	15	476 840	5 441 000	1	1	1	1	1	1	1	1	1	1
29-03-2022	16	477 283	5 442 000	1	1	1	1	1	1	1	1	1	1
05-10-2022	1	478 105	5 457000	2	-	-	-	-	3	-	3	-	-
05-10-2022	6	478 400	5 452 248	2	-	-	-	-	3	-	3	-	3
05-10-2022	7	478 235	5 451 229	2	-	-	-	-	3	-	3	-	-
05-10-2022	8	478 152	5 450 673	2	-	-	-	-	3	-	3	-	-
05-10-2022	9	478 104	5 450 157	2	-	-	-	-	3	-	3	-	-
06-10-2022	10	478 052	5 449 136	2	-	-	-	-	3	-	3	-	-
06-10-2022	11	478 076	5 449 657	2	-	-	-	-	3	-	3	-	3
06-10-2022	15	476 840	5 441 000	2	-	-	-	-	3	-	3	-	-
06-10-2022	16	477 283	5 442 000	2	-	-	-	-	3	-	3	-	-
11-04-2023	1	478 105	5 457000	3	-	-	-	3	3	-	3	-	-
11-04-2023	9	478 104	5 450 157	3	-	-	-	3	3	-	3	-	1
11-04-2023	16	477 283	5 442 000	3	-	-	-	3	3	-	3	-	-

12-04-2023	6	478 400	5 452 248	3	-	-	-	3	3	-	3	-	1
12-04-2023	10	478 052	5 449 136	3	-	-	-	3	3	-	3	-	-
12-04-2023	11	478 076	5 449 657	3	-	-	-	3	3	-	3	-	-
13-04-2023	7	478 235	5 451 229	3	-	-	-	3	3	-	3	-	1
13-04-2023	8	478 152	5 450 673	3	-	-	-	3	3	-	3	-	-
13-04-2023	15	476 840	5 441 000	3	-	-	-	3	3	-	3	-	-
16-02-2024	9	478 104	5 450 157	4	-	-	-	3	3	-	-	-	-
16-02-2024	10	478 052	5 449 136	4	-	-	-	3	3	-	-	-	-
16-02-2024	11	478 076	5 449 657	4	-	-	-	3	3	-	-	-	-
16-02-2024	15	476 840	5 441 000	4	-	-	-	3	3	-	-	-	-
16-02-2024	16	477 283	5 442 000	4	-	-	-	3	3	-	-	-	-
17-02-2024	1	478 105	5 457000	4	-	-	-	3	3	-	-	-	-
17-02-2024	6	478 400	5 452 248	4	-	-	-	3	3	-	-	-	1
17-02-2024	7	478 235	5 451 229	4	-	-	-	3	3	-	-	-	-
17-02-2024	8	478 152	5 450 673	4	-	-	-	3	3	-	-	-	-

Appendix 19. Parameter estimates (posterior means) and model-selection criteria (Posterior predictive loss criterion (PPLC) and Watanabe - Akaike information criteria (WAIC)) for each model. Each model was fit by running the MCMC algorithm for 6000 iterations and retaining the last 5000 for estimating posterior summaries. Bold font indicates lowest values of PPLC and WAIC.

Taxon	**Model**	**Sediment Bath Samples**					**Sediment Bath Dip Samples**					**Adjacent Water Samples**				
		Occupancy at Site (ψ)	**Occupancy within Sample (θ)**	**Detection in a Replicate (ρ)**	**PPLC**	**WAIC**	**Occupancy at Site (ψ)**	**Occupancy within Sample (θ)**	**Detection in a Replicate (ρ)**	**PPLC**	**WAIC**	**Occupancy at Site (ψ)**	**Occupancy within Sample (θ)**	**Detection in a Replicate (ρ)**	**PPLC**	**WAIC**
Decamastus gracilis	ψ (·), θ (·), ρ (·)	0.93	0.94	0.92	230.23	123.22	0.93	0.96	0.89	269.57	125.53	0.93	0.97	0.71	502.49	186.21
	ψ (·), θ (*count*), ρ (·)	0.93	0.95	0.92	230.20	123.16	0.93	0.96	0.89	269.67	125.45	0.93	0.98	0.71	501.94	186.20
	ψ (·), θ (count), ρ (count)	0.93	0.94	0.92	222.30	122.07	0.93	0.96	0.89	258.60	119.60	0.93	0.98	0.71	423.62	161.12
	ψ (prob), θ (count), ρ (count)	1.00	0.94	**0.92**	**222.88**	**121.82**	1.00	0.96	0.89	**259.16**	**119.50**	1.00	0.98	0.71	**421.63**	**95.77**
Glycera nana	ψ (·), θ (·), ρ (·)	0.85	0.83	0.82	478.78	189.30	0.93	0.80	0.64	**477.27**	**168.58**	0.93	0.65	0.31	291.11	121.92
	ψ (·), θ (*count*), ρ (·)	0.86	0.83	0.82	**478.32**	**189.34**	0.94	0.80	0.64	477.30	168.65	0.93	0.65	0.31	292.05	123.47
	ψ (·), θ (count), ρ (count)	0.86	0.83	0.82	479.42	192.19	0.93	0.80	0.64	479.18	171.12	0.93	0.68	0.32	285.30	122.26

	ψ (prob), θ (count), ρ (count)	0.88	0.84	**0.83**	480.98	191.98	1.00	0.81	0.64	479.36	171.31	1.00	0.68	0.32	**284.06**	**121.95**
Heteromastus filiobranchus	ψ (·), θ (·), ρ (·)	0.93	0.97	0.71	502.49	186.21	0.93	0.84	0.76	390.55	151.49	0.93	0.82	0.54	389.87	143.94
	ψ (·), θ (*count*), ρ (·)	0.93	1.00	0.71	502.41	186.16	0.93	0.84	0.76	390.30	151.42	0.93	0.82	0.55	388.32	142.71
	ψ (·), θ (count), ρ (count)	0.93	0.99	0.71	487.13	184.10	0.93	0.83	0.73	260.57	111.77	0.93	0.83	0.53	333.11	135.19
	ψ (prob), θ (count), ρ (count)	1.00	0.93	0.71	**486.07**	**183.89**	1.00	0.84	**0.73**	**261.36**	**111.33**	1.00	0.83	0.53	**334.11**	**134.98**
Ophelina acuminata	ψ (·), θ (·), ρ (·)	0.66	0.39	0.65	213.64	75.18	0.89	0.24	0.47	169.59	58.90	0.93	0.65	0.53	405.77	140.89
	ψ (·), θ (*count*), ρ (·)	0.69	0.36	0.65	213.22	75.24	0.89	0.25	0.47	170.60	59.26	0.93	0.65	0.53	407.58	143.02
	ψ (·), θ (count), ρ (count)	0.69	0.36	0.69	206.16	75.64	0.89	0.25	0.41	153.02	59.29	0.93	0.65	0.50	**380.71**	**139.41**
	ψ (prob), θ (count), ρ (count)	0.69	0.37	**0.69**	**205.14**	**75.53**	0.89	0.25	0.41	**152.40**	**59.06**	1.00	0.65	0.50	380.72	139.57
Prionospio lightii	ψ (·), θ (·), ρ (·)	0.93	0.79	0.81	446.90	179.05	0.93	0.68	0.64	388.75	139.01	0.93	0.81	0.32	304.39	133.59
	ψ (·), θ (*count*), ρ (·)	0.93	0.79	0.81	446.56	179.03	0.93	0.68	0.64	389.90	139.23	0.93	0.82	0.31	306.90	135.33
	ψ (·), θ (count), ρ (count)	0.93	0.79	0.81	447.62	182.48	0.93	0.68	0.63	**354.43**	**130.02**	0.93	0.82	0.31	**304.90**	**136.85**
	ψ (prob), θ (count), ρ (count)	1.00	0.79	**0.81**	**446.18**	**181.95**	1.00	0.68	0.63	354.69	130.29	1.00	0.82	0.31	305.09	136.28

Appendix 20. Mitochondrial genome sequences used in the present study

Abbreviation	Common Name	Species Name	Unique ID	GenBank Accession Number	Sequence Origin
ma-CALUfa	Dog	*Canis lupus familiaris*	NA	NC_002008.4	NCBI
ma-FECA	Cat	*Felis catus*	NA	NC_001700.1	NCBI
ma-HOSA	Human	*Homo sapiens*	NA	NC_012920.1	NCBI
te-ANFI	Sablefish	*Anoplopoma fimbria*	NA	NC_018119.1	NCBI
te-CLPA	Pacific herring	*Clupea pallasii*	NA	NC_009578.1	NCBI
te-CYAG	shiner perch	*Cymatogaster aggregata*	NA	NC_009059.1	NCBI
te-GACH	Alaska pollock	*Gadus chalcogrammus*	NA	NC_004449.1	NCBI
te-GAMA	Pacific cod	*Gadus macrocephalus*	NA	NC_036931.1	NCBI
te-HIST	Pacific halibut	*Hippoglossus stenolepis*	NA	NC_009710.1	NCBI
te-MOSA	Striped bass	*Morone saxatilis*	NA	NC_014353.1	NCBI
te-ONGO	Pink salmon	*Oncorhynchus gorbuscha*	NA	NC_010959.1	NCBI
te-ONKE	Chum salmon	*Oncorhynchus keta*	NA	NC_017838.1	NCBI
te-ONKI	Coho salmon	*Oncorhynchus kisutch*	NA	NC_009263.1	NCBI

te-ONMY	Rainbow trout	*Oncorhynchus mykiss*	NA	NC_001717.1	NCBI
te-ONNE	Sockeye salmon	*Oncorhynchus nerka*	NA	NC_008615.1	NCBI
te-ONTS	Chinook salmon	*Oncorhynchus tschawytscha*	NA	NC_002980.1	NCBI
te-OPEL	Ling cod	*Ophiodon elongatus*	NA	NC_026887.1	NCBI
te-OSMO	Rainbow smelt	*Osmerus mordax*	NA	NC_015246.1	NCBI
te-SAAL	Arctic char	*Salvelinus alpinus*	NA	NC_000861.1	NCBI
te-SASA	Atlantic slamon	*Salmo salar*	NA	NC_001960.1	NCBI
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	SEB-31	GCA_916701645.2	Kolora et. Al
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	SEB-32	GCA_916701685.1	Kolora et. Al
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	F112779-te-SEALA-218	OR641559	Yang et. Al
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	F112776-te-SEALA-219	OR641556	Yang et. Al
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	F112775-te-SEALA-1	OR641555	Yang et. Al
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	F112777-te-SEALA-2	OR641557	Yang et. Al
te-SEAA	Shortspine thornyhead	*Sebastolobus alascanus*	F112778-te-SEALA-3	OR641558	Yang et. Al
te-SEAB	Yellowbarred red rockfish	*Sebastes albofasciatus*	133423	GCA_916701655.1	Kolora et. Al
te-SEAI	Brown rockfish	*Sebastes auriculatus*	S-1A	GCA_916700975.1	Kolora et. Al
te-SEAL	Rougheye rockfish	*Sebastes aleutianus*	SEB-111	GCA_916700875.1	Kolora et. Al
te-SEAL	Rougheye rockfish	*Sebastes aleutianus*	NA	NC_039779.1	NCBI
te-SEAL	Rougheye rockfish	***Sebastes aleutianus***	F112709-te-SEALE-1	OR641492	Yang et. Al
te-SEAL	Rougheye rockfish	***Sebastes aleutianus***	F112710-te-SEALE-2	OR641493	Yang et. Al
te-SEAL	Rougheye rockfish	***Sebastes aleutianus***	F112711-te-SEALE-3	OR641494	Yang et. Al
te-SEAL	Rougheye rockfish	***Sebastes aleutianus***	F112713-te-SEALE-5	OR641495	Yang et. Al
te-SEAO	Aurora rockfish	*Sebastes aurora*	NA	KUI448	NCBI
te-SEAR	Kelp rockfish	*Sebastes atrovirens*	xSEB-24	GCA_916700955.1	Kolora et. Al
te-SEAT	Longspine thornyhead	*Sebastolobus altivelis*	xSEB-32	GCA_916701675.1	Kolora et. Al
te-SEAU	Pacific ocean perch	*Sebastes alutus*	NA	KUI2069	NCBI
te-SEAU	Pacific ocean perch	*Sebastes alutus*	F112760-te-SEAU-2	OR641502	Yang et. Al
te-SEAU	Pacific ocean perch	*Sebastes alutus*	F112759-te-SEAU-1	OR641501	Yang et. Al
te-SEBA	Redbanded rockfish	*Sebastes babcocki*	HO-4	GCA_916700865.1	Kolora et. Al

te-SEBA	Redbanded rockfish	*Sebastes babcocki*	F112762-te-SEBA-2	OR641504	Yang et. Al
te-SEBA	Redbanded rockfish	*Sebastes babcocki*	F112761-te-SEBA-1	OR641503	Yang et. Al
te-SEBO	Shortraker rockfish	*Sebastes borealis*	NA	KUI2095	NCBI
te-SEBR	No common name	*Sebastes baramenuke*	131606	GCA_916700835.1	Kolora et. Al
te-SEBR	Silvergrey Rockfish	*Sebastes brevispinis*	F112730-te-SEBR-4	OR641505	Yang et. Al
te-SECA	Copper rockfish	*Sebastes caurinus*	SEB-191	GCA_916700845.1	Kolora et. Al
te-SECA	Copper rockfish	*Sebastes caurinus*	F112754-te-SECA-94	OR641510	Yang et. Al
te-SECA	Copper rockfish	*Sebastes caurinus*	F112753-te-SECA-93	OR641509	Yang et. Al
te-SECA	Copper rockfish	*Sebastes caurinus*	F112752-te-SECA-3	OR641508	Yang et. Al
te-SECA	Copper rockfish	*Sebastes caurinus*	F112751-te-SECA-2	OR641507	Yang et. Al
te-SECA	Copper rockfish	*Sebastes caurinus*	F112750-te-SECA-1	OR641506	Yang et. Al
te-SECH	Japanese white seaperch	*Sebastes cheni*	135386	GCA_916700745.1	Kolora et. Al
te-SECI	Dusky rockfish	*Sebastes ciliatus*	NA	KUI2032	NCBI
te-SECL	Greenspotted rockfish	*Sebastes chlorostictus*	xSEB-10	GCA_916700915.1	Kolora et. Al
te-SECN	Gopher rockfish	*Sebastes carnatus*	xSEB-21	GCA_916700855.1	Kolora et. Al
te-SECO	Starry rockfish	*Sebastes constellatus*	SEB-71	GCA_916700895.1	Kolora et. Al
te-SECR	Darkblotched rockfish	*Sebastes crameri*	SEB-4	GCA_916700755.1	Kolora et. Al
te-SECR	Darkblotched rockfish	*Sebastes crameri*	SEB-6	GCA_916700715.1	Kolora et. Al
te-SECR	Darkblotched rockfish	*Sebastes crameri*	F112724-te-SECR-2	OR641512	Yang et. Al
te-SECR	Darkblotched rockfish	*Sebastes crameri*	F112723-te-SECR-1	OR641511	Yang et. Al
te-SEDA	Calico rockfish	*Sebastes dalli*	xSEB-31	GCA_916700725.1	Kolora et. Al
te-SEDI	Deacon rockfish	*Sebastes diaconus*	SEB-21	GCA_916700945.1	Kolora et. Al
te-SEDP	Splitnose rockfish	*Sebastes diploproa*	NA	KUI2154	NCBI
te-SEDP	Splitnose rockfish	*Sebastes diploproa*	F112774-te-SEDI-2	OR641514	Yang et. Al
te-SEDP	Splitnose rockfish	*Sebastes diploproa*	F112773-te-SEDI-1	OR641513	Yang et. Al
te-SEEL	Greenstriped rockfish	*Sebastes elongatus*	SEB-146	GCA_916700925.1	Kolora et. Al
te-SEEL	Greenstriped rockfish	*Sebastes elongatus*	F112756-te-SEEL-2	OR641516	Yang et. Al
te-SEEL	Greenstriped rockfish	*Sebastes elongatus*	F112755-te-SEEL-1	OR641515	Yang et. Al
te-SEEN	Widow rockfish	*Sebastes entomelas*	SEB-16	GCA_916701365.1	Kolora et. Al

te-SEEN	Widow rockfish	*Sebastes entomelas*	SEB-8	GCA_916700775.2	Kolora et. Al
te-SEEN	Widow rockfish	*Sebastes entomelas*	F112735-te-SEEN-213	OR641520	Yang et. Al
te-SEEN	Widow rockfish	*Sebastes entomelas*	F112733-te-SEEN-214	OR641518	Yang et. Al
te-SEEN	Widow rockfish	*Sebastes entomelas*	F112734-te-SEEN-3	OR641519	Yang et. Al
te-SEEN	Widow rockfish	*Sebastes entomelas*	F112732-te-SEEN-2	OR641517	Yang et. Al
te-SEES	Swordspine rockfish	*Sebastes ensifer*	SEB-163	GCA_916700965.1	Kolora et. Al
te-SEEX	Buccaneer rockfish	*Sebastes exsul*	SEB-BAJA-1	GCA_916700705.1	Kolora et. Al
te-SEFA	Acadian redfish	*Sebastes fasciatus*	NA	KUI2796	NCBI
te-SEFL	Yellowtail rockfish	*Sebastes flavidus*	SEB-19	GCA_916700785.1	Kolora et. Al
te-SEFL	Yellowtail rockfish	*Sebastes flavidus*	F112742-te-SEFL-5	OR641525	Yang et. Al
te-SEFL	Yellowtail rockfish	*Sebastes flavidus*	F112741-te-SEFL-4	OR641524	Yang et. Al
te-SEFL	Yellowtail rockfish	*Sebastes flavidus*	F112740-te-SEFL-3	OR641523	Yang et. Al
te-SEFL	Yellowtail rockfish	*Sebastes flavidus*	F112739-te-SEFL-2	OR641522	Yang et. Al
te-SEFL	Yellowtail rockfish	*Sebastes flavidus*	F112738-te-SEFL-1	OR641521	Yang et. Al
te-SEGL	Gray rockfish	*Sebastes glaucus*	134638	GCA_916700795.1	Kolora et. Al
te-SEGO	Chilipepper rockfish	*Sebastes goodei*	SEB-148	GCA_916700735.1	Kolora et. Al
te-SEGO	Chilipepper rockfish	*Sebastes goodei*	F112749-te-SEGO-2	OR641527	Yang et. Al
te-SEGO	Chilipepper rockfish	*Sebastes goodei*	F112748-te-SEGO-1	OR641526	Yang et. Al
te-SEHE	Rosethorn rockfish	*Sebastes helvomaculatus*	NA	KUI479	NCBI
te-SEHE	Rosethorn rockfish	*Sebastes helvomaculatus*	F112765-te-SEHE-1	OR641528	Yang et. Al
te-SEHE	Rosethorn rockfish	*Sebastes helvomaculatus*	F112766-te-SEHE-2	OR641529	Yang et. Al
te-SEHO	Squarespot rockfish	*Sebastes hopkinsi*	xSEB-7	GCA_916701215.1	Kolora et. Al
te-SEHU	No common name	*Sebastes hubbsi*	137692	GCA_916701285.1	Kolora et. Al
te-SEIN	Japanese red seaperch	*Sebastes inermis*	136187	GCA_916701085.1	Kolora et. Al
te-SEIR	No common name	*Sebastes iracundus*	136139	GCA_916701015.1	Kolora et. Al
te-SEIT	No common name	*Sebastes itinus*	130931	GCA_916701335.1	Kolora et. Al
te-SEJO	Shorbelly rockfish	*Sebastes jordani*	NA	KUI517	NCBI
te-SEJO	Shorbelly rockfish	*Sebastes jordani*	F112770-te-SEJO-2	OR641531	Yang et. Al
te-SEJO	Shorbelly rockfish	*Sebastes jordani*	F112769-te-SEJO-1	OR641530	Yang et. Al

te-SEJY	Togot seaperch	*Sebastes joyneri*	137149	GCA_916701105.1	Kolora et. Al
te-SEKL	No common name	*Sebastes kiyomatsui*	145871	GCA_916701135.1	Kolora et. Al
te-SEKO	No common name	*Sebastes koreanus*	134992	GCA_916701025.1	Kolora et. Al
te-SELE	Cow rockfish	*Sebastes levis*		KUI567	NCBI
te-SEMA	No common name	*Sebastes matsubarae*	146680	GCA_916701035.1	Kolora et. Al
te-SEMA	Quillback rockfish	*Sebastes maliger*	NA	KUI3233	NCBI
te-SEMA	Quillback rockfish	*Sebastes maliger*	SEB-721	GCA_916701265.1	Kolora et. Al
te-SEMA	Quillback rockfish	*Sebastes maliger*	F112725-te-SEMA-1	OR641532	Yang et. Al
te-SEMA	Quillback rockfish	*Sebastes maliger*	F112727-te-SEMA-3	OR641534	Yang et. Al
te-SEMA	Quillback rockfish	*Sebastes maliger*	F112726-te-SEMA-2	OR641533	Yang et. Al
te-SEMA	Quillback rockfish	*Sebastes maliger*	F112729-te-SEMA-5	OR641536	Yang et. Al
te-SEMA	Quillback rockfish	*Sebastes maliger*	F112728-te-SEMA-4	OR641535	Yang et. Al
te-SEME	Black rockfish	*Sebastes melanops*	134856	GCA_916701325.1	Kolora et. Al
te-SEMI	Vermillion rockfish	*Sebastes miniatus*	SEB-11	GCA_916701115.1	Kolora et. Al
te-SEMI	Vermillion rockfish	*Sebastes miniatus*	SEB-74	GCA_916701275.1	Kolora et. Al
te-SEMI	Vermillion rockfish	*Sebastes miniatus*	SEB-153	GCA_916701005.1	Kolora et. Al
te-SEMI	Vermillion rockfish	*Sebastes miniatus*	SEB-153 2	NA	Kolora et. Al
te-SEML	Blackgill rockfish	*Sebastes melanostomus*	HO-5	GCA_916701165.1	Kolora et. Al
te-SEMN	Beaked redfish	*Sebastes mentella*	NA	KUI3097	NCBI
te-SEMO	Whitespeckled rockfish	*Sebastes moseri*	SEB-5	GCA_916701145.1	Kolora et. Al
te-SEMO	Whitespeckled rockfish	*Sebastes moseri*	SEB-9	GCA_916701315.1	Kolora et. Al
te-SEMY	Blue rockfish	*Sebastes mystinus*	SEB-17	GCA_916701245.1	Kolora et. Al
te-SEMY	Blue rockfish	*Sebastes mystinus*	xSEB-27	GCA_916701235.1	Kolora et. Al
te-SENE	China rockfish	*Sebastes nebulosus*	SEB-720	GCA_916701055.1	Kolora et. Al
te-SENI	Tiger rockfish	*Sebastes nigrocintus*	NA	NC_039778.1	NCBI
te-SENI	Tiger rockfish	*Sebastes nigrocintus*	UW114045	GCA_916701185.1	Kolora et. Al
te-SENU	No common name	*Sebastes nudus*	133427	GCA_916701225.1	Kolora et. Al
te-SENV	No common name	*Sebastes nivosus*	134373	GCA_916701345.1	Kolora et. Al
te-SEOB	No common name	*Sebastes oblongus*	137157	GCA_916701095.1	Kolora et. Al

te-SEOC	Patagonian redfish	*Sebastes oculatus*	ER03	GCA_916701125.1	Kolora et. Al
te-SEOC	Patagonian redfish	*Sebastes oculatus*	IQ03	GCA_916701355.1	Kolora et. Al
te-SEOC	Patagonian redfish	*Sebastes oculatus*	PAR11	GCA_916701155.1	Kolora et. Al
te-SEOC	Patagonian redfish	*Sebastes oculatus*	PAR11 2	NA	Kolora et. Al
te-SEPA	Bocaccio rockfish	*Sebastes paucispinis*	SEB-180	GCA_916701045.1	Kolora et. Al
te-SEPA	Bocaccio rockfish	*Sebastes paucispinis*	F112743-te-SEPA-1	OR641537	Yang et. Al
te-SEPA	Bocaccio rockfish	*Sebastes paucispinis*	F112744-te-SEPA-2	OR641538	Yang et. Al
te-SEPA	Bocaccio rockfish	*Sebastes paucispinis*	F112745-te-SEPA-239	OR641539	Yang et. Al
te-SEPA	Bocaccio rockfish	*Sebastes paucispinis*	F112747-te-SEPA-238	OR641541	Yang et. Al
te-SEPA	Bocaccio rockfish	*Sebastes paucispinis*	F112746-te-SEPA-3	OR641540	Yang et. Al
te-SEPI	Canary rockfish	*Sebastes pinniger*	SEB-72	GCA_916701065.2	Kolora et. Al
te-SEPI	Canary rockfish	*Sebastes pinniger*	SEB-75	GCA_916701255.1	Kolora et. Al
te-SEPI	Canary rockfish	*Sebastes pinniger*	F112721-te-SEPI-1	OR641542	Yang et. Al
te-SEPI	Canary rockfish	*Sebastes pinniger*	F112722-te-SEPI-2	OR641543	Yang et. Al
te-SEPO	Northern rockfish	*Sebastes polyspinis*	NA	KUI2030	NCBI
te-SEPR	Redstripe rockfish	*Sebastes proriger*	NA	KUI3229	NCBI
te-SEPR	Redstripe rockfish	*Sebastes proriger*	F112764-te-SEPR-2	OR641545	Yang et. Al
te-SEPR	Redstripe rockfish	*Sebastes proriger*	F112763-te-SEPR-1	OR641544	Yang et. Al
te-SERA	Grass rockfish	*Sebastes rastrelliger*	xSEB-1	GCA_916701435.1	Kolora et. Al
te-SERB	Flag rockfish	*Sebastes rubrivinctus*	UW155382	GCA_916701395.1	Kolora et. Al
te-SERB	Flag rockfish	*Sebastes rubrivinctus*	UW156309	GCA_916701605.1	Kolora et. Al
te-SERE	Yellowmouth rockfish	*Sebastes reedi*	SEB-7	GCA_916701525.1	Kolora et. Al
te-SERE	Yellowmouth rockfish	*Sebastes reedi*	F112737-te-SERE-2	OR641547	Yang et. Al
te-SERE	Yellowmouth rockfish	*Sebastes reedi*	F112736-te-SERE-1	OR641546	Yang et. Al
te-SERO	Rosy rockfish	*Sebastes rosaceus*	SEB-73	GCA_916701555.1	Kolora et. Al
te-SERO	Rosy rockfish	*Sebastes rosaceus*	xSEB-30	GCA_916701535.1	Kolora et. Al
te-SERS	Greenblotched rockfish	*Sebastes rosenblatti*	SEB-147	GCA_916701545.1	Kolora et. Al
te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	5822396486	GCA_916701515.1	Kolora et. Al
te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	SEB-91	GCA_916701565.1	Kolora et. Al

te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	F112715-te-SERU-2	OR641548	Yang et. Al
te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	F112718-te-SERU-5	OR641549	Yang et. Al
te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	F112714-te-SERU-1	OR641496	Yang et. Al
te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	F112717-te-SERU-4	OR641498	Yang et. Al
te-SERU	Yelloweye rockfish	*Sebastes ruberrimus*	F112716-te-SERU-3	OR641497	Yang et. Al
te-SERU	Rougheye rockfish	*Sebastes aleutianus*	F112712-te-SEALE-4	OR641500	Yang et. Al
te-SESA	Stripetail rockfish	*Sebastes saxicola*	NA	KUI2167	NCBI
te-SESA	Stripetail rockfish	*Sebastes saxicola*	F112771-te-SESA-1	OR641550	Yang et. Al
te-SESA	Stripetail rockfish	*Sebastes saxicola*	F112772-te-SESA-2	OR641551	Yang et. Al
te-SESC	Korean rockfish	*Sebastes schlegelii*	139475	GCA_916701485.1	Kolora et. Al
te-SESE	Halfbanded rockfish	*Sebastes semicinctus*	xSEB-12	GCA_916701385.1	Kolora et. Al
te-SESR	Treefish	*Sebastes serriceps*	S68	GCA_916701455.1	Kolora et. Al
te-SEST	No common name	*Sebastes steindachneri*	141714	GCA_916701425.1	Kolora et. Al
te-SESY	No common name	*Sebastes scythropus*	145769	GCA_916701475.1	Kolora et. Al
te-SETA	White-edged rockfish	*Sebastes taczanowskii*	133425	GCA_916701585.1	Kolora et. Al
te-SETE	No common name	*Sebastes tertius*	139529	GCA_916701665.1	Kolora et. Al
te-SETH	Goldeye rockfish	*Sebastes thompsoni*	137678	GCA_916701625.1	Kolora et. Al
te-SETR	Threestripe rockfish	*Sebastes trivittatus*	137156	GCA_916701465.1	Kolora et. Al
te-SEUM	Honeycomb rockfish	*Sebastes umbrosus*	SEB-10	GCA_916701575.1	Kolora et. Al
te-SEVA	Light dusky rockfish	*Sebastes variabilis*	NA	KUI2038	NCBI
te-SEVR	Harlequin rockfish	*Sebastes variegatus*	NA	KUI3213	NCBI
te-SEVR	Harlequin rockfish	*Sebastes variegatus*	F112758-te-SEVA-2	OR641553	Yang et. Al
te-SEVR	Harlequin rockfish	*Sebastes variegatus*	F112757-te-SEVA-1	OR641552	Yang et. Al
te-SEVU	No common name	*Sebastes vulpes*	130347	NA	Kolora et. Al
te-SEWI	Pygmy rockfish	*Sebastes wilsoni*	NA	KUI3199	NCBI
te-SEZA	Sharpchin rockfish	*Sebastes zacentrus*	NA	KUI3201	NCBI
te-SEZA	Sharpchin rockfish	*Sebastes zacentrus*	F112767-te-SEZA-1	OR641554	Yang et. Al
te-SEZA	Sharpchin rockfish	*Sebastes zacentrus*	F112768-te-SEZA-2	OR641499	Yang et. Al
te-SEZO	No common name	*Sebastes zonatus*	130348	GCA_916701635.1	Kolora et. Al

Appendix 21. Running parameters set on *unikseq* program for each assay design

eDNA Assay	Length of K-mers	Threshold Length	% of Average ID in Ingroup Sequences	Tolerated # of consecutive K-mers in Outgroup	% Unique	Maximum Proportion of Outgroup Sequences Tolerated
eSEMA3	25	100	25	3	60	20
eSEPA9	25	100	25	1	90	10
eSERU5	25	100	25	1	90	10

Appendix 22. Tissue voucher DNA specimens used to for in situ targeted assay testing

Assay	Species	Common Name (Species)	Detection	# Voucher Specimens	Sample Sources/Locations
eSEMA3	te-SEMA	Quillback rockfish (*Sebastes maliger)*	Yes	5	Hecate Strait
	te-seaLA	Shortspine thornyhead (*Sebastolobus alascanus*)	No	2	Hecate Strait
	te-SEAU	Pacific Ocean perch (*Sebastes alutus)*	No	2	Hecate Strait
	te-SEBA	Redbanded rockfish (*Sebasetes babcocki*)	No	2	Hecate Strait
	te-SEBR	Silvergrey rockfish (*Sebastes brevispinus*)	No	2	Hecate Strait
	te-SECA	Copper rockfish (*Sebastes caurinus*)	No	2	Hecate Strait
	te-SECR	Darkblotched rockfish (*Sebastes crameri*)	No	2	Hecate Strait
	te-SEDI	Splitnose rockfish (*Sebastes diploproa*)	No	2	Hecate Strait
	te-SEEL	Greenstriped rockfish (*Sebastes elongatus*)	No	2	Hecate Strait
	te-SEEN	Widow rockfish (*Sebastes entomelas*)	No	2	Hecate Strait
	te-SEFL	Yellowtail rockfish (*Sebastes flavidus*)	No	2	Hecate Strait
	te-SEGO	Chilipepper rockfish (*Sebastes goodei*)	No	2	Hecate Strait
	te-SEHE	Rosethorn rockfish (*Sebastes helvomaculatus*)	No	2	Hecate Strait
	te-SEJO	Shortbelly rockfish (*Sebastes jordani*)	No	2	Hecate Strait

	te-SEPA	Bocaccio rockfish (*Sebastes paucispinus*)	No	2	Hecate Strait
	te-SEPI	Canary rockfish (*Sebastes pinnigers*)	No	2	Hecate Strait
	te-SEPR	Red stripe rockfish (*Senastes proriger*)	No	2	Hecate Strait
	te-SERE	Yellowmouth rockfish (*Sebastes reedi*)	No	2	Hecate Strait
	te-SERU	Yelloweye rockfish (*Sebastes ruberrimus*)	No	2	Hecate Strait
	te-SESA	Stripetail rockfish (*Sebastes saxicola*)	No	2	Hecate Strait
	te-SEVA	Harlequin rockfish (*Sebastes variegatus*)	No	2	Hecate Strait
	te-SEZA	Sharpchin rockfish (*Sebastes zacentrus*)	No	2	Hecate Strait
	te-ANFI	Sablefish, black cod (*Anoplopoma fimbriatum*)	No	1	Hecate Strait
	te-GAMA	Pacific cod (*Gadus macrocephalus*)	No	1	Hecate Strait
	te-ATST	Arrowtooth flounder (*Atheresthes stomias*)	No	1	Coastal British Columbia
	te-EOJO	Petrale sole (*Eopsetta jordani*)	No	1	Coastal British Columbia
	te-GACH	Walleye pollock (*Gadus chalcogrammus*)	No	1	Coastal British Columbia
	te-GLZA	Rex sole (*Glyptocephalus zachirus*)	No	1	Coastal British Columbia
	te-HIEL	Flathead sole (*Hippoglossoides elassodon*)	No	1	Coastal British Columbia
	te-LEBI	Rock sole (*Lepidopsetta bilineata*)	No	1	Coastal British Columbia
	te-MIPA	Dover sole (*Microstomus pacificus*)	No	1	Coastal British Columbia
	te-OPEL	Ling cod (*Ophiodon elongatus*)	No	1	Coastal British Columbia
	te-PAVE	English sole (*Parophrys vetulus*)	No	1	Coastal British Columbia
	te-HIST	Pacific halibut (*Hippoglussus stenolepis*)	No	1	Coastal British Columbia
	te-AMPE	Pacific sandlance (*Ammodytes personatus*)	No	1	Coastal British Columbia
	te-THPA	Eulachon (*Thaleichthys pacificus*)	No	1	Coastal British Columbia
	te-HYPR	Surf smelt (*Hypomesus pretiosus*)	No	1	Coastal British Columbia
	te-CLPA	Pacific herring (*Clupea pallasii*)	No	1	Coastal British Columbia
	te-ONMY	Rainbow (steelhead) trout (*Oncorhynchus mykiss*)	No	1	Southwestern British Columbia
	ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands
	ma-CAFA	Dog (*Canis lupus familiaris*)	No	1	Southwestern British Columbia
	ma-FECA	Cat (*Felis catus*)	No	1	Southwestern British Columbia
eSEPA9	te-SEPA	Bocaccio rockfish (*Sebastes paucispinus*)	Yes	5	Hecate Strait
	te-seaLA	Shortspine thornyhead (*Sebastolobus alascanus*)	No	2	Hecate Strait

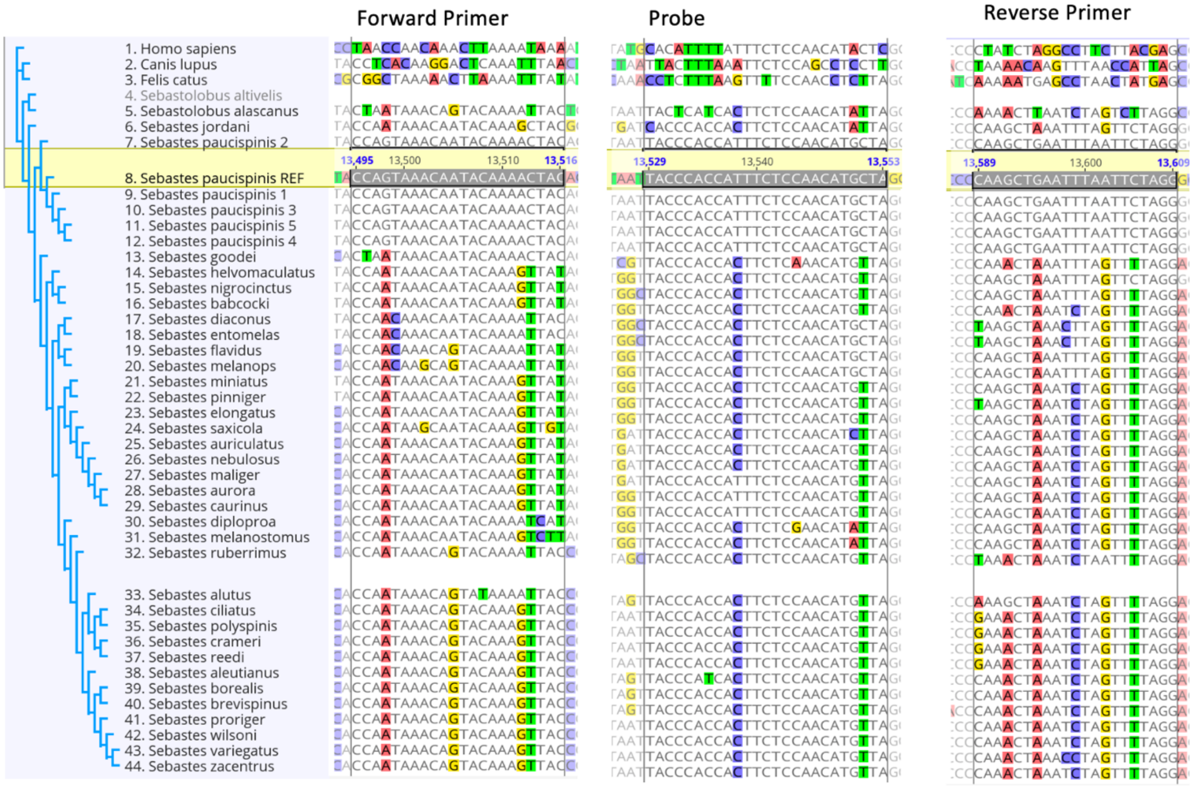
	te-SEAU	Pacific Ocean perch (*Sebastes alutus)*	No	2	Hecate Strait
	te-SEBA	Redbanded rockfish (*Sebasetes babcocki*)	No	2	Hecate Strait
	te-SEBR	Silvergrey rockfish (*Sebastes brevispinus*)	No	2	Hecate Strait
	te-SECA	Copper rockfish (*Sebastes caurinus*)	No	2	Hecate Strait
	te-SECR	Darkblotched rockfish (*Sebastes crameri*)	No	2	Hecate Strait
	te-SEDI	Splitnose rockfish (*Sebastes diploproa*)	No	2	Hecate Strait
	te-SEEL	Greenstriped rockfish (*Sebastes elongatus*)	No	2	Hecate Strait
	te-SEEN	Widow rockfish (*Sebastes entomelas*)	No	2	Hecate Strait
	te-SEFL	Yellowtail rockfish (*Sebastes flavidus*)	No	2	Hecate Strait
	te-SEGO	Chilipepper rockfish (*Sebastes goodei*)	No	2	Hecate Strait
	te-SEHE	Rosethorn rockfish (*Sebastes helvomaculatus*)	No	2	Hecate Strait
	te-SEJO	Shortbelly rockfish (*Sebastes jordani*)	No	2	Hecate Strait
	te-SEMA	Quillback rockfish (*Sebastes malinger*)	No	2	Hecate Strait
	te-SEPI	Canary rockfish (*Sebastes pinnigers*)	No	2	Hecate Strait
	te-SEPR	Red stripe rockfish (*Senastes proriger*)	No	2	Hecate Strait
	te-SERE	Yellowmouth rockfish (*Sebastes reedi*)	No	2	Hecate Strait
	te-SERU	Yelloweye rockfish (*Sebastes ruberrimus*)	No	2	Hecate Strait
	te-SESA	Stripetail rockfish (*Sebastes saxicola*)	No	2	Hecate Strait
	te-SEVA	Harlequin rockfish (*Sebastes variegatus*)	No	2	Hecate Strait
	te-SEZA	Sharpchin rockfish (*Sebastes zacentrus*)	No	2	Hecate Strait
	te-ANFI	Sablefish, black cod (*Anoplopoma fimbriatum*)	No	1	Hecate Strait
	te-GAMA	Pacific cod (*Gadus macrocephalus*)	No	1	Hecate Strait
	te-ATST	Arrowtooth flounder (*Atheresthes stomias*)	No	1	Coastal British Columbia
	te-EOJO	Petrale sole (*Eopsetta jordani*)	No	1	Coastal British Columbia
	te-GACH	Walleye pollock (*Gadus chalcogrammus*)	No	1	Coastal British Columbia
	te-GLZA	Rex sole (*Glyptocephalus zachirus*)	No	1	Coastal British Columbia
	te-HIEL	Flathead sole (*Hippoglossoides elassodon*)	No	1	Coastal British Columbia
	te-LEBI	Rock sole (*Lepidopsetta bilineata*)	No	1	Coastal British Columbia
	te-MIPA	Dover sole (*Microstomus pacificus*)	No	1	Coastal British Columbia
	te-OPEL	Ling cod (*Ophiodon elongatus*)	No	1	Coastal British Columbia

	te-PAVE	English sole (*Parophrys vetulus*)	No	1	Coastal British Columbia
	te-HIST	Pacific halibut (*Hippoglussus stenolepis*)	No	1	Coastal British Columbia
	te-AMPE	Pacific sandlance (*Ammodytes personatus*)	No	1	Coastal British Columbia
	te-THPA	Eulachon (*Thaleichthys pacificus*)	No	1	Coastal British Columbia
	te-HYPR	Surf smelt (*Hypomesus pretiosus*)	No	1	Coastal British Columbia
	te-CLPA	Pacific herring (*Clupea pallasii*)	No	1	Coastal British Columbia
	te-ONMY	Rainbow (steelhead) trout (*Oncorhynchus mykiss*)	No	1	Southwestern British Columbia
	ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands
	ma-CAFA	Dog (*Canis lupus familiaris*)	No	1	Southwestern British Columbia
	ma-FECA	Cat (*Felis catus*)	No	1	Southwestern British Columbia
eSERU5	te-SERU	Yelloweye rockfish (*Sebastes ruberrimus*)	Yes	5	Hecate Strait
	te-seaLA	Shortspine thornyhead (*Sebastolobus alascanus*)	No	2	Hecate Strait
	te-SEAU	Pacific Ocean perch (*Sebastes alutus)*	No	2	Hecate Strait
	te-SEBA	Redbanded rockfish (*Sebasetes babcocki*)	No	2	Hecate Strait
	te-SEBR	Silvergrey rockfish (*Sebastes brevispinus*)	No	2	Hecate Strait
	te-SECA	Copper rockfish (*Sebastes caurinus*)	No	2	Hecate Strait
	te-SECR	Darkblotched rockfish (*Sebastes crameri*)	No	2	Hecate Strait
	te-SEDI	Splitnose rockfish (*Sebastes diploproa*)	No	2	Hecate Strait
	te-SEEL	Greenstriped rockfish (*Sebastes elongatus*)	No	2	Hecate Strait
	te-SEEN	Widow rockfish (*Sebastes entomelas*)	No	2	Hecate Strait
	te-SEFL	Yellowtail rockfish (*Sebastes flavidus*)	No	2	Hecate Strait
	te-SEGO	Chilipepper rockfish (*Sebastes goodei*)	No	2	Hecate Strait
	te-SEHE	Rosethorn rockfish (*Sebastes helvomaculatus*)	No	2	Hecate Strait
	te-SEJO	Shortbelly rockfish (*Sebastes jordani*)	No	2	Hecate Strait
	te-SEMA	Quillback rockfish (*Sebastes malinger*)	No	2	Hecate Strait
	te-SEPA	Bocaccio rockfish (*Sebastes paucispinus*)	No	2	Hecate Strait
	te-SEPI	Canary rockfish (*Sebastes pinnigers*)	No	2	Hecate Strait
	te-SEPR	Red stripe rockfish (*Senastes proriger*)	No	2	Hecate Strait
	te-SERE	Yellowmouth rockfish (*Sebastes reedi*)	No	2	Hecate Strait
	te-SESA	Stripetail rockfish (*Sebastes saxicola*)	No	2	Hecate Strait

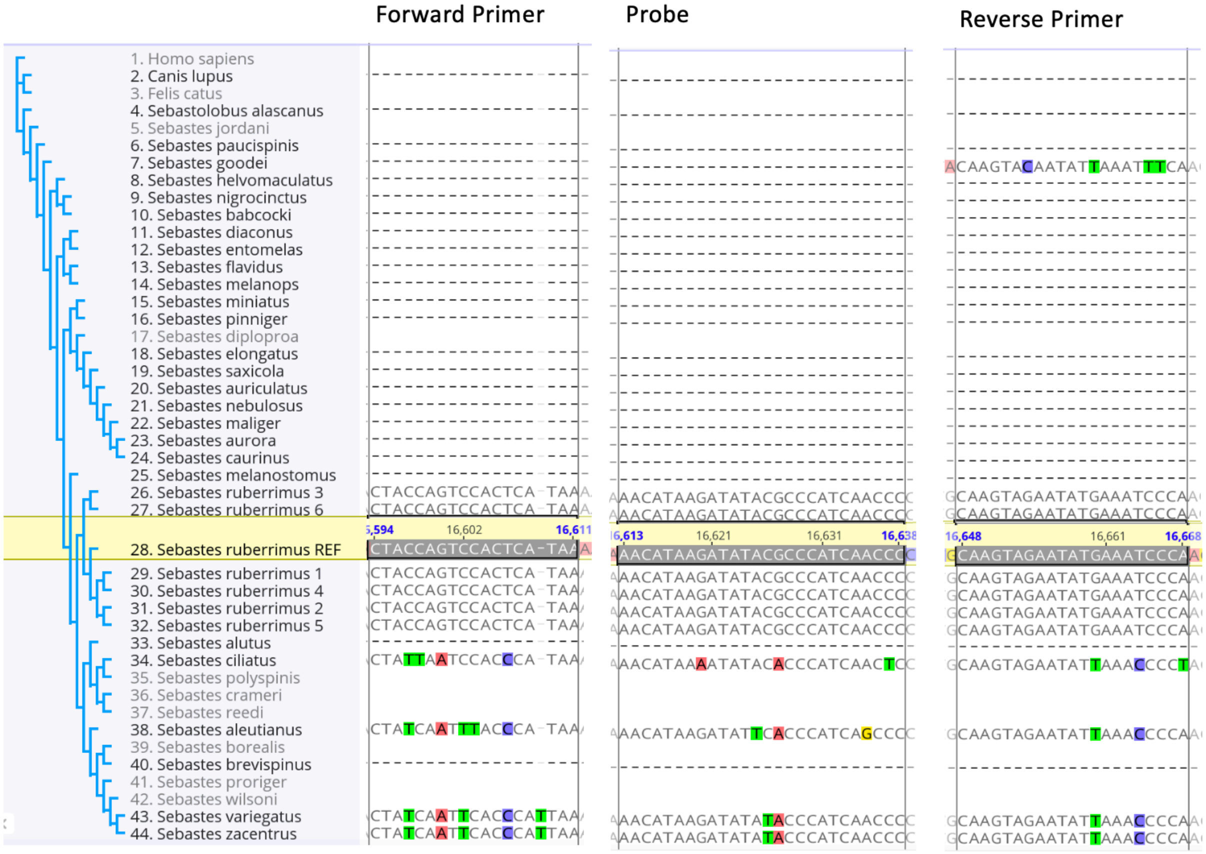
	te-SEVA	Harlequin rockfish (*Sebastes variegatus*)	No	2	Hecate Strait
	te-SEZA	Sharpchin rockfish (*Sebastes zacentrus*)	No	2	Hecate Strait
	te-ANFI	Sablefish, black cod (*Anoplopoma fimbriatum*)	No	1	Hecate Strait
	te-GAMA	Pacific cod (*Gadus macrocephalus*)	No	1	Hecate Strait
	te-ATST	Arrowtooth flounder (*Atheresthes stomias*)	No	1	Coastal British Columbia
	te-EOJO	Petrale sole (*Eopsetta jordani*)	No	1	Coastal British Columbia
	te-GACH	Walleye pollock (*Gadus chalcogrammus*)	No	1	Coastal British Columbia
	te-GLZA	Rex sole (*Glyptocephalus zachirus*)	No	1	Coastal British Columbia
	te-HIEL	Flathead sole (*Hippoglossoides elassodon*)	No	1	Coastal British Columbia
	te-LEBI	Rock sole (*Lepidopsetta bilineata*)	No	1	Coastal British Columbia
	te-MIPA	Dover sole (*Microstomus pacificus*)	No	1	Coastal British Columbia
	te-OPEL	Ling cod (*Ophiodon elongatus*)	No	1	Coastal British Columbia
	te-PAVE	English sole (*Parophrys vetulus*)	No	1	Coastal British Columbia
	te-HIST	Pacific halibut (*Hippoglussus stenolepis*)	No	1	Coastal British Columbia
	te-AMPE	Pacific sandlance (*Ammodytes personatus*)	No	1	Coastal British Columbia
	te-THPA	Eulachon (*Thaleichthys pacificus*)	No	1	Coastal British Columbia
	te-HYPR	Surf smelt (*Hypomesus pretiosus*)	No	1	Coastal British Columbia
	te-CLPA	Pacific herring (*Clupea pallasii*)	No	1	Coastal British Columbia
	te-ONMY	Rainbow (steelhead) trout (*Oncorhynchus mykiss*)	No	1	Southwestern British Columbia
	ma-HOSA	Human (*Homo sapiens*)	No	1	Netherlands
	ma-CAFA	Dog (*Canis lupus familiaris*)	No	1	Southwestern British Columbia
	ma-FECA	Cat (*Felis catus*)	No	1	Southwestern British Columbia

Appendix 23. Primer and probe regions of eSEMA3 assay showing base pair mismatches for off target species

Appendix 24. Primer and probe regions of eSEPA9 assay showing base pair mismatches for off target species.



Appendix 25. Primer and probe regions of eSERU5 assay showing base pair mismatches for off target species.



Appendix 26. Contents of Vancouver Aquarium tanks used for verified positive environmental sample validation

Tank	Common Name	Species Name
Bella-Bella	Bat sea star	*Patiria miniata*
	Blue topsnail	*Calliostoma ligatum*
	Bowerbanks' crumb of bread sponge	*Halichondria bowerbanki*
	China rockfish	*Sebastes nebulosus*
	Chiton	*Lepidozona*
	Corraline- encased filament worm	*Dodecaceria choncharum*
	Creeping pedal sea cucumber	*Psolus chitonoides*
	Daisy brittle star	*Ophiopholis aculeata*
	Fringed filament worm	*Dodecaceria fewkess*
	Giant acorn barnacle	*Balanus nubilus*
	gumboot chiton	*Cryptochiton stelleri*
	Giant pacific oyster	*Crassostrea gigas*
	Giant plumose anemone	*Metridium farcinem*
	Rock scallop	*Crassodoma gigantea*

	California sea cucumber	*Apostichopus californicus*
	Graceful decorator crab	*Oregonia gracilis*
	Green sea urchin	*Stronglyocentrotus droebachiansis*
	Leafy hornmouth	*Ceratostoma foliatum*
	Lined chiton	*Tonicella lineata*
	Mossy chiton	*Mopalia kennerleyi*
	Mossy chiton	*Mopalia*
	Orange cup coral	*Balanophyllia elegans*
	Oregon triton	*Fusitriton oregonensis*
	Painted greenling	*Oxylebius pictus*
	Plume worm	*Serpula*
	Proliferating anemone	*Epiactis prolifera*
	Puget Sound king crab	*Lopolithodes mandtii*
	Rainbow sea star	*Orthasteria Kohleri*
	Red Irish lord	*Hemilepidotus hemilepidotus*
	Red sea sucumber	*Cucumaria miniata*
	Red turban snail	*Pomaulax gibberosa*
	Red veiled chiton	*Placiphorella rufa*
	Rock entodesma	*Entodesma neavicula*
	Rose anemone	*Urticina piscivora*
	Rose encrusting sponge	*Alpysilla glacialis*
	Rose sea star	*Crossaster papposus*
	Rough Keyhole limpet	*Diadora aspera*
	Salt and pepper sea cucumber	*Cucumaria piperata*
	Sea cucumber	*Eupentacta*
	Short plumose anemone	*Metridium senile*
	Skeleton shrimp	*Caprella*
	Smooth mopalia	*Mopalia vespertina*
	Solitary pink-mouth hydroid	*Ectopleura marina*
	Spnge	*Ophlitaspongia pennata*
	Spot prawn	*Pandalus platyceros*
	Strawberry anemone	*Cornyactis californica*
	Striped perch	*Embiotoca lateralis*
	Stubby rose anemone	*Urticina clandestina*
	Tennis ball sponge	*Craniella villosa*
	Tiger rockfish	*Sebastes nigrocinctus*
	Vermillion sea star	*Mediaster auqualis*
	White line chiton	*Tonicella insignis*
	Whitecap limpet	*Acmaea mitra*
	Wolf eel	*Anarrhichthys ocellatus*
	Worm snail	*Vermetus*
	Yelloweye rockfish	*Sebastes ruberrimus*
Pac-Can	Big skate	*Beringraja binoculata*

	Black rockfish	*Sebastes melanops*
	Bocaccio rockfish	*Sebastes paucispinis*
	Bowerbanks' crumb of bread sponge	*Halichondria bowerbanki*
	Brown rockfish	*Sebastes auriculatus*
	Canarya rockfish	*Sebastes pinniger*
	Coho salmon	*Onoryhnchus kisutch*
	Copper rockfish	*Sebastes caurinus*
	Creeping pedal sea cucumber	*Psolus chitonoides*
	Crimson anemone	*Cribrinopsis rubens*
	Dungeness crab	*Metacarcinus magister*
	gumboot chiton	*Cryptochiton stelleri*
	Giant pacific octopus	*Enteroctopus dofleini*
	Giant pink star	*Pisaster brevispinus*
	Giant plumose anemone	*Metridium farcinem*
	California sea cucumber	*Apostichopus californicus*
	Green sea urchin	*Stronglyocentrotus droebachiansis*
	Hind's mopalia	*Mopalia Hindsii*
	Kelp greenling	*Hexagrammos decagrammus*
	Leather star	*Dermasterias imbricata*
	Lined chiton	*Tonicella lineata*
	Ling cod	*Ophiodon elongatus*
	Many-ribbed punturella	*Puncturella multistriata*
	Morning sun star	*Solaster dawsoni*
	Mottled sea star	*Evasterias troschelii*
	Orange cup coral	*Balanophyllia elegans*
	Pacific halibut	*Hippoglossus stenolepsis*
	Painte anemone	*Urticina crassicornis*
	Pile perch	*Rhacochilus vacca*
	Plume worm	*Serpula*
	Pygmy rock crab	*Glebocarcinuc oregonensis*
	Quillback rockfish	*Sebastes maliger*
	Painted sea star	*Orthasteria Kohleri*
	Red Irish lord	*Hemilepidotus hemilepidotus*
	Red rock crab	*Cancer productus*
	Red sea urchin	*Mesocentrotus fransiscanus*
	Rose encrusting sponge	*Aplysilla*
	Rough Keyhole limpet	*Diadora aspera*
	Sablefish	*Anoplopoma fimbria*
	Short plumose anemone	*Metridium senile*
	Spot prawn	*Pandalus platyceros*

	Starry flounder	*Platychthys stellatus*
	Strawberry anemone	*Corynactic californica*
	Striped perch	*Embiotoca lateralis*
	Striped sun star	*Solaster stimpsoni*
	Seunflower star	*Pycnopodia helianthoides*
	Tiger rockfish	*Sebastes nigrocinctus*
	Velcro star	*Stylasterias forreri*
	Vermillion rockfish	*Sebastes miniatus*
	White line chiton	*Tonicella insignis*
	White sturgeon	*Acipenser transmontanus*
	Whitespotted greenling	*Hexagrammos stelleri*
	White-spotted rose anemone	*Urticina eques*
	Wolf eel	*Anarrhichthys ocellatus*
	Woody chiton	*Mopalia linosa*
	Yelloweye rockfish	*Sebastes ruberrimus*
	Yellowtail rockfish	*Sebastes flavidus*

Appendix 27. Dive Site Metadata

Site Label	Location	Year	Site Code	Geography	Protection	Depth	Transect Number	Date	Tide Height	Tide Stage	Current	Depth	Visibility
A	Finlayson_Head	2021	FH_OD	Inside	non-RCA	Deep	1	15/9/2021	2.71	falling	0	31	10
A	Finlayson_Head	2021	FH_OM	Inside	non-RCA	Mid	2				0	16	10
A	Finlayson_Head	2021	FH_OS	Inside	non-RCA	Shallow	3				0	5	5
B	Hells_Gate	2021	HG_OD	Inside	non-RCA	Deep	1	19/9/2021	4.39	rising	0	30	15
B	Hells_Gate	2021	HG_OM	Inside	non-RCA	Mid	2				1	16	10
B	Hells_Gate	2021	HG_OS	Inside	non-RCA	Shallow	3				1	6	8
C	Jorkins	2021	J_OD	Outside	non-RCA	Deep	1	16/9/2021	4.06	high slack	0	32	8
C	Jorkins	2021	J_OM	Outside	non-RCA	Mid	2				1	17	5
C	Jorkins	2021	J_OS	Outside	non-RCA	Shallow	3	Transect not done because of no 5m substrate					
D	Laredo_RCA_1	2021	L1_ID	Outside	RCA	Deep	1	18/9/2021	4.39	high slack	0	31	20
D	Laredo_RCA_1	2021	L1_IM	Outside	RCA	Mid	2				0	16	15
D	Laredo_RCA_1	2021	L1_IS	Outside	RCA	Shallow	3				0	5	7
E	Laredo_RCA_2	2021	L2_ID	Outside	RCA	Deep	1	18/9/2021	4.28	falling	0	29	15
E	Laredo_RCA_2	2021	L2_IM	Outside	RCA	Mid	2				0	16	15
E	Laredo_RCA_2	2021	L2_IS	Outside	RCA	Shallow	3				1	6	10
F	South_Aristozabal	2021	SA_OD	Outside	RCA	Deep	1	14/9/2021	3.65	falling	0	30	8
F	South_Aristozabal	2021	SA_OM	Outside	RCA	Mid	2				0	18	5
F	South_Aristozabal	2021	SA_OS	Outside	RCA	Shallow	3	Transect not done because of no 5m substrate					
G	Swindle_Tip	2021	ST_ID	Outside	non-RCA	Deep	1	21/9/2021	4.5	high slack	0	27	10
G	Swindle_Tip	2021	ST_IM	Outside	non-RCA	Mid	2				0	15	15
G	Swindle_Tip	2021	ST_IS	Outside	non-RCA	Shallow	3				1	5	10
H	West_Moore_Island	2022	D_WM	Outside	RCA	Deep	1	21/9/2022	3.3	rising	0	30	8
H	West_Moore_Island	2022	M_WM	Outside	RCA	Mid	2				0	18	5
H	West_Moore_Island	2022	S_WM	Outside	RCA	Shallow	3				0	6	5

I	Steel_Francis_Rock	2022	D_SI	Outside	RCA	Deep	1	21/9/2022	2.7	falling	0	31	15
I	Steel_Francis_Rock	2022	M_SI	Outside	RCA	Mid	2				0	18	4
I	Steel_Francis_Rock	2022	S_SI	Outside	RCA	Shallow	3				0	6	4
J	Oz_Pinnacle	2022	D_OP	Outside	RCA	Deep	1	22/9/2022	2.9	rising	0	30	15
J	Oz_Pinnacle	2022	M_OP	Outside	RCA	Mid	2				0	18	10
J	Oz_Pinnacle	2022	S_OP	Outside	RCA	Shallow	3				0	5	4
K	Goby_Palace	2022	D_GP	Outside	RCA	Deep	1	22/9/2022	3.5	falling	0	30	20
K	Goby_Palace	2022	M_GP	Outside	RCA	Mid	2				0	18	15
K	Goby_Palace	2022	S_GP	Outside	RCA	Shallow	3				0	5	5
L	Hole_In_The_Wall	2022	D_HW	Inside	non-RCA	Deep	1	24/9/2022	4.06	high slack	0	26	20
L	Hole_In_The_Wall	2022	M_HW	Inside	non-RCA	Mid	2				0	18	15
L	Hole_In_The_Wall	2022	S_HW	Inside	non-RCA	Shallow	3				0	4	10
M	Crickety_Crack_Lil_Drizzy	2022	D_CC	Inside	non-RCA	Deep	1	24/9/2022	3.99	high slack	0	32	15
M	Crickety_Crack_Lil_Drizzy	2022	M_CC	Inside	non-RCA	Mid	2				0	15	15
M	Crickety_Crack_Lil_Drizzy	2022	S_CC	Inside	non-RCA	Shallow	3				0	5	10
N	Larkin	2022	D_LK	Outside	RCA	Deep	1	25/9/2022	2.8	falling	0	32	10
N	Larkin	2022	M_LK	Outside	RCA	Mid	2				0	18	10
N	Larkin	2022	S_LK	Outside	RCA	Shallow	3				0	5	5
O	Trahey	2022	D_TK	Outside	RCA	Deep	1	25/9/2022	3.2	rising	0	25	12
O	Trahey	2022	M_TK	Outside	RCA	Mid	2				0	18	10
O	Trahey	2023	S_TK	Outside	RCA	Shallow	3	Transect not done because of no 5m substrate					
P	Dallain	2022	D_DK	Outside	RCA	Deep	1	25/9/2022	1.9	rising	0	20	10
P	Dallain	2022	M_DK	Outside	RCA	Mid	2				0	18	10
P	Dallain	2022	S_DK	Outside	RCA	Shallow	3				1	6	10

Appendix 28. Relevant metadata for eDNA sample collection and processing

Site	Year	Month	Day	CCH station code	CCH sample replicate	Sample volume (L)	Passed IntegritE	Samples Passed miSebastes Sequence QC	Comments
South_Aristozabal	2021	September	14	SA_OD	A	0.4	Y	Y	
South_Aristozabal	2021	September	14	SA_OD	B	0.075	Y	Y	
South_Aristozabal	2021	September	14	SA_OD	C	0.45	Y	Y	
South_Aristozabal	2021	September	14	SA_OM	A	0.8	Y	Y	
South_Aristozabal	2021	September	14	SA_OM	B	0.7	Y	Y	
South_Aristozabal	2021	September	14	SA_OM	C	0.7	Y	Y	
South_Aristozabal	2021	September	14	SA_OS	A	0.6	Y	Y	
South_Aristozabal	2021	September	14	SA_OS	B	0.6	Y	N	
South_Aristozabal	2021	September	14	SA_OS	C	NA	NA	NA	Bag floated away
Field Blank	2021	September	14	NC_BW	1	0.5	Y	Y	
Finlayson_Head	2021	September	15	FH_OD	A	0.6	Y	Y	
Finlayson_Head	2021	September	15	FH_OD	B	0.4	Y	Y	
Finlayson_Head	2021	September	15	FH_OD	C	0.9	Y	Y	
Finlayson_Head	2021	September	15	FH_OM	A	0.65	Y	Y	
Finlayson_Head	2021	September	15	FH_OM	B	0.625	Y	Y	
Finlayson_Head	2021	September	15	FH_OM	C	0.65	Y	Y	
Finlayson_Head	2021	September	15	FH_OS	A	0.8	Y	Y	
Finlayson_Head	2021	September	15	FH_OS	B	0.8	Y	Y	
Finlayson_Head	2021	September	15	FH_OS	C	0.8	Y	Y	
Field Blank	2021	September	15	NC_BW	2	0.5	Y	Y	
Jorkins	2021	September	16	J_OD	A	0.8	Y	Y	
Jorkins	2021	September	16	J_OD	B	0.75	Y	Y	
Jorkins	2021	September	16	J_OD	C	0.8	Y	Y	

Jorkins	2021	September	16	J_OM	A	0.825	Y	Y	
Jorkins	2021	September	16	J_OM	B	0.9	Y	Y	
Jorkins	2021	September	16	J_OM	C	0.85	Y	Y	
Jorkins	2021	September	16	J_OS	A	0.825	Y	Y	
Jorkins	2021	September	16	J_OS	B	0.4	Y	Y	
Jorkins	2021	September	16	J_OS	C	0.8	Y	Y	
Field Blank	2021	September	16	NC_BW	3	0.5	Y	Y	Sample Cleaned and IntegritE Cts ~32 so samples progressed
Laredo_RCA_1	2021	September	18	L1_ID	A	0.5	Y	Y	
Laredo_RCA_1	2021	September	18	L1_ID	B	0.45	Y	Y	
Laredo_RCA_1	2021	September	18	L1_ID	C	0.8	Y	Y	
Laredo_RCA_1	2021	September	18	L1_IM	A	0.65	Y	Y	
Laredo_RCA_1	2021	September	18	L1_IM	B	0.8	Y	Y	
Laredo_RCA_1	2021	September	18	L1_IM	C	0.65	Y	Y	
Laredo_RCA_1	2021	September	18	L1_IS	A	0.6	Y	Y	
Laredo_RCA_1	2021	September	18	L1_IS	B	0.55	Y	Y	
Laredo_RCA_1	2021	September	18	L1_IS	C	0.7	Y	Y	
Laredo_RCA_2	2021	September	18	L2_ID	A	0.7	Y	Y	
Laredo_RCA_2	2021	September	18	L2_ID	B	0.6	Y	Y	
Laredo_RCA_2	2021	September	18	L2_ID	C	0.6	Y	Y	
Laredo_RCA_2	2021	September	18	L2_IM	A	0.55	Y	Y	
Laredo_RCA_2	2021	September	18	L2_IM	B	0.575	Y	Y	
Laredo_RCA_2	2021	September	18	L2_IM	C	0.575	Y	Y	
Laredo_RCA_2	2021	September	18	L2_IS	A	0.9	Y	Y	
Laredo_RCA_2	2021	September	18	L2_IS	B	0.7	Y	Y	
Laredo_RCA_2	2021	September	18	L2_IS	C	0.7	Y	Y	
Field Blank	2021	September	18	NC_BW	4	0.5	Y	Y	
Hells_Gate	2021	September	19	HG_OD	A	0.8	Y	Y	
Hells_Gate	2021	September	19	HG_OD	B	0.875	Y	Y	
Hells_Gate	2021	September	19	HG_OD	C	0.9	Y	Y	
Hells_Gate	2021	September	19	HG_OM	A	0.975	Y	Y	

Hells_Gate	2021	September	19	HG_OM	B	0.775	Y	Y	
Hells_Gate	2021	September	19	HG_OM	C	0.65	Y	Y	
Hells_Gate	2021	September	19	HG_OS	A	0.65	Y	Y	
Hells_Gate	2021	September	19	HG_OS	B	0.85	Y	Y	
Hells_Gate	2021	September	19	HG_OS	C	0.7	Y	Y	
Field Blank	2021	September	19	NC_BW	5	0.5	Y	Y	
Swindle_Tip	2021	September	21	ST_ID	A	0.85	Y	Y	
Swindle_Tip	2021	September	21	ST_ID	B	0.95	Y	Y	
Swindle_Tip	2021	September	21	ST_ID	C	1.2	Y	Y	
Swindle_Tip	2021	September	21	ST_IM	A	0.9	Y	Y	
Swindle_Tip	2021	September	21	ST_IM	B	1.2	Y	Y	
Swindle_Tip	2021	September	21	ST_IM	C	0.9	Y	Y	
Swindle_Tip	2021	September	21	ST_IS	A	0.95	Y	Y	
Swindle_Tip	2021	September	21	ST_IS	B	0.85	Y	Y	
Swindle_Tip	2021	September	21	ST_IS	C	0.95	Y	Y	
Field Blank	2021	September	21	NC_BW	6	0.5	Y	Y	Sample Cleaned and IntegritE Cts ~32 so samples progressed
Suit Control	2021	September	21	NC_SW	A	0.8	Y	Y	
Suit Control	2021	September	21	NC_SW	B	0.8	Y	Y	
Suit Control	2021	September	21	NC_SW	C	0.8	Y	Y	
West_Moore_Island	2022	September	21	D_WM	A	0.80	Y	Y	
West_Moore_Island	2022	September	21	D_WM	B	0.80	Y	Y	
West_Moore_Island	2022	September	21	D_WM	C	0.75	Y	Y	
West_Moore_Island	2022	September	21	M_WM	A	0.75	Y	Y	
West_Moore_Island	2022	September	21	M_WM	B	0.80	Y	Y	
West_Moore_Island	2022	September	21	M_WM	C	0.80	Y	Y	
West_Moore_Island	2022	September	21	S_WM	A	0.80	Y	Y	
West_Moore_Island	2022	September	21	S_WM	B	0.80	Y	Y	
West_Moore_Island	2022	September	21	S_WM	C	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	D_SI	A	0.80	Y	Y	

Steel_Francis_Rock	2022	September	21	D_SI	B	0.60	Y	Y	
Steel_Francis_Rock	2022	September	21	D_SI	C	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	M_SI	A	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	M_SI	B	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	M_SI	C	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	S_SI	A	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	S_SI	B	0.80	Y	Y	
Steel_Francis_Rock	2022	September	21	S_SI	C	0.80	Y	Y	
Field Blank	2022	September	21	FB	1	0.50	Y	Y	
Oz_Pinnacle	2022	September	22	D_OP	A	0.80	Y	Y	
Oz_Pinnacle	2022	September	22	D_OP	B	1.00	Y	Y	
Oz_Pinnacle	2022	September	22	D_OP	C	0.90	Y	Y	
Oz_Pinnacle	2022	September	22	M_OP	A	1.00	Y	Y	
Oz_Pinnacle	2022	September	22	M_OP	B	0.95	Y	Y	
Oz_Pinnacle	2022	September	22	M_OP	C	0.95	Y	Y	
Oz_Pinnacle	2022	September	22	S_OP	A	1.00	Y	Y	
Oz_Pinnacle	2022	September	22	S_OP	B	0.90	Y	Y	
Oz_Pinnacle	2022	September	22	S_OP	C	1.00	Y	Y	
Goby_Palace	2022	September	22	D_GP	A	0.53	Y	Y	
Goby_Palace	2022	September	22	D_GP	B	0.78	Y	Y	
Goby_Palace	2022	September	22	D_GP	C	1.00	Y	Y	
Goby_Palace	2022	September	22	M_GP	A	0.98	Y	Y	
Goby_Palace	2022	September	22	M_GP	B	1.00	Y	Y	
Goby_Palace	2022	September	22	M_GP	C	0.90	Y	Y	
Goby_Palace	2022	September	22	S_GP	A	0.85	Y	Y	Sample Cleaned and IntegritE Passed
Goby_Palace	2022	September	22	S_GP	B	0.98	Y	Y	Sample Cleaned and IntegritE Passed
Goby_Palace	2022	September	22	S_GP	C	0.90	Y	Y	Sample Cleaned and IntegritE Passed
Field Blank	2022	September	22	FB	2	0.50	Y	Y	
Hole_In_The_Wall	2022	September	24	D_HW	A	0.95	Y	Y	
Hole_In_The_Wall	2022	September	24	D_HW	B	0.25	Y	Y	Sample Cleaned and IntegritE Passed

Hole_In_The_Wall	2022	September	24	D_HW	C	0.90	Y	Y	
Hole_In_The_Wall	2022	September	24	M_HW	A	0.97	Y	Y	
Hole_In_The_Wall	2022	September	24	M_HW	B	0.90	Y	Y	
Hole_In_The_Wall	2022	September	24	M_HW	C	1.00	Y	Y	
Hole_In_The_Wall	2022	September	24	S_HW	A	0.90	Y	Y	
Hole_In_The_Wall	2022	September	24	S_HW	B	0.90	Y	Y	
Hole_In_The_Wall	2022	September	24	S_HW	C	1.00	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	D_CC	A	0.28	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	D_CC	B	0.45	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	M_CC	A	0.80	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	M_CC	B	0.20	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	M_CC	C	0.70	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	S_CC	A	0.50	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	S_CC	B	0.65	Y	Y	
Crickety_Crack_Lil_Drizzy	2022	September	24	S_CC	C	0.25	Y	Y	
Field Blank	2022	September	25	FB	3	0.50	Y	Y	
Larkin	2022	September	25	D_LK	A	0.85	Y	Y	
Larkin	2022	September	25	D_LK	B	0.75	Y	Y	
Larkin	2022	September	25	D_LK	C	0.90	Y	Y	
Larkin	2022	September	25	M_LK	A	1.00	Y	Y	
Larkin	2022	September	25	M_LK	B	1.00	Y	Y	
Larkin	2022	September	25	M_LK	C	1.00	Y	Y	
Larkin	2022	September	25	S_LK	A	0.80	Y	Y	Sample Cleaned and IntegritE Passed
Larkin	2022	September	25	S_LK	B	1.00	Y	Y	Sample Cleaned and IntegritE Passed
Larkin	2022	September	25	S_LK	C	1.00	Y	Y	Sample Cleaned and IntegritE Passed
Trahey	2022	September	25	D_TK	A	0.78	Y	Y	No Shallow transect

Trahey	2022	September	25	D_TK	B	0.75	Y	Y	No Shallow transect
Trahey	2022	September	25	D_TK	C	0.78	Y	Y	No Shallow transect
Trahey	2022	September	25	M_TK	A	0.95	Y	Y	No Shallow transect
Trahey	2022	September	25	M_TK	B	1.00	Y	Y	No Shallow transect
Trahey	2022	September	25	M_TK	C	0.90	Y	Y	No Shallow transect
Dallain	2022	September	25	D_DK	A	1.00	Y	Y	
Dallain	2022	September	25	D_DK	B	1.00	Y	Y	
Dallain	2022	September	25	D_DK	C	1.00	Y	Y	
Dallain	2022	September	25	M_DK	A	1.00	Y	Y	Sample Cleaned and IntegritE Passed
Dallain	2022	September	25	M_DK	B	0.98	Y	Y	
Dallain	2022	September	25	M_DK	C	1.00	Y	Y	
Dallain	2022	September	25	S_DK	A	1.00	Y	Y	Sample Cleaned and IntegritE Passed (1 Ct ~34)
Dallain	2022	September	25	S_DK	B	1.00	Y	Y	Sample Cleaned and IntegritE Passed
Dallain	2022	September	25	S_DK	C	0.98	Y	Y	Sample Cleaned and IntegritE Passed
Suit Control	2022	September	25	SC	A	1.00	Y	Y	
Suit Control	2022	September	25	SC	B	1.00	Y	Y	Sample Cleaned and IntegritE Passed
Suit Control	2022	September	25	SC	C	0.78	Y	Y	Sample Cleaned and IntegritE Passed

Appendix 29. Target species sequence origin information

Species	**Common Name (Species)**	**Sequence DI**	**Name in Figure Alignments**	**Tissue Source**	**Sample Sources/Locations**	**Tissues sequenced internally**
Sebastes maliger	**Quillback Rockfish**	**KUI3233**	***Sebastes maliger* REF**	1	NA	N
		F112725-te-SEMA-1	*Sebastes maliger* 1	DFO	Hecate Strait	Y
		F112726-te-SEMA-2	*Sebastes maliger* 2	DFO	Hecate Strait	Y
		F112727-te-SEMA-3	*Sebastes maliger* 3	DFO	Hecate Strait	Y
		F112728-te-SEMA-4	*Sebastes maliger* 4	DFO	Hecate Strait	Y
		F112729-te-SEMA-5	*Sebastes maliger* 5	DFO	Hecate Strait	Y
		SEB-721	*Sebastes maliger* 6	1	NA	N
Sebastes paucispinis	**Boccaccio Rockfish**	**SEB-180**	***Sebastes paucispinis* REF**	1	NA	N
		F112743-te-SEPA-1	*Sebastes paucispinis* 1	DFO	Hecate Strait (122)	Y
		F112744-te-SEPA-2	*Sebastes paucispinis* 2	DFO	Hecate Strait (122)	Y
		F112746-te-SEPA-3	*Sebastes paucispinis* 3	DFO	Hecate Strait (122)	Y
		F112747-te-SEPA-238	*Sebastes paucispinis* 4	DFO	Hecate Strait (122)	Y
		F112745-te-SEPA-239	*Sebastes paucispinis* 5	DFO	Hecate Strait (122)	Y
Sebastes ruberrimus	**Yelloweye Rockfish**	**5822396486**	***Sebastes ruberrimus* REF**	1	NA	N
		F112714-te-SERU-1	*Sebastes ruberrimus* 1	DFO	Hecate Strait	Y
		F112715-te-SERU-2	*Sebastes ruberrimus* 2	DFO	Hecate Strait	Y
		F112716-te-SERU-3	*Sebastes ruberrimus* 3	DFO	Hecate Strait	Y
		F112717-te-SERU-4	*Sebastes ruberrimus* 4	DFO	Hecate Strait	Y
		F112718-te-SERU-5	*Sebastes ruberrimus* 5	DFO	Hecate Strait	Y
		SEB-91	*Sebastes ruberrimus* 6	1	NA	N

Appendix 30. eDNA field validation data for targeted eDNA assays. Frequency columns indicate the number of qPCR technical replicates amplified past the pre-set threshold. The frequency of successful amplification is used to calculate the estimated DNA copy number.

Location Name	**Sample Collection Date**	**IntegritE-DNA™ Frequency (/4)**	**Target Assay Frequency (/8)**	**Estimated Total Copies (±SE)**
Vancouver Aquarium (Pac-Can)	Jun 21 2022	4	8	5970.29 (±994.87)
		4	8	11350.14 (±1147.73)
		4	8	11135.29 (±1422.93)
		4	8	28518.31(±2691.47)
		4	8	7057.84 (±865.65)
		4	8	25917.46 (±2658.08)
		4	7	393.00 (±195.00)
		4	8	889.84 (±202.97)
		4	8	530.04 (±102.63)
		4	8	601.12 (±122.89)
		4	8	340.28 (±53.58)
		4	8	1076.75 (±270.99)
Vancouver Aquarium (Bella Bella)	May 23 2023	4	8	44002.55 (±13267.16)
		4	8	36735.67 (±9502.43)
		4	3	93.00 (±57.00)
		4	8	13592.08 (±4035.02)
		4	8	12281.71 (±2335.69)

Appendix 31. miSebastes primer sequences and sequence mismatches

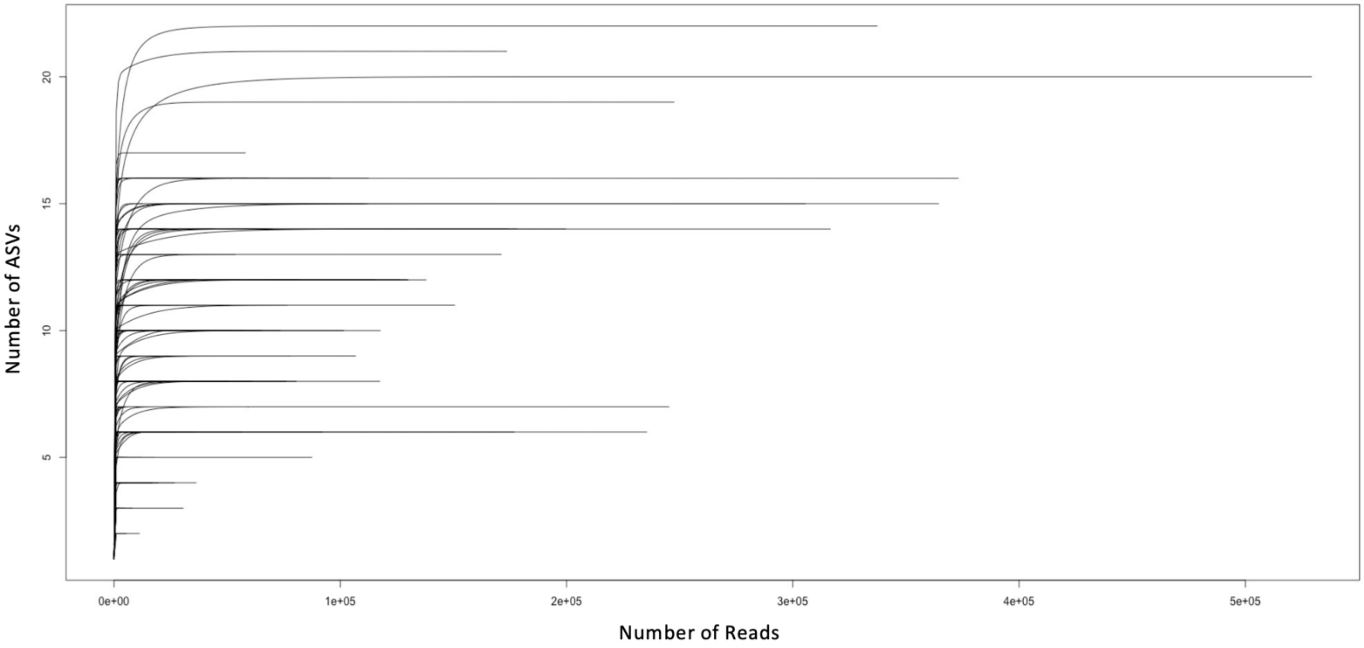
Base Pair Differences	**Forward**	**Reverse**
	GACCACTTACACAATTCT	**AAGCTCATTCAAGTGCTT**
*	GACCACTCACACAATTCT	
**	GACCACTTACACAATTCC	
***	GGCCACTCACACAATTCT	
*****	GGCCACTTACACAATTCT	
******	GACCACTTACACAACTCT	
******	GACCACTCACACAATTCC	
*******	GTCCCCTAACACAGTTCC	
********	GTCCCCTTACACAGTTCC	
*********	GACCACTCACACAATTTT	
**********	GTCCCCTAACACAGTTTC	

Appendix 32. miSebastes amplicon sequences for British Columbia rockfish species

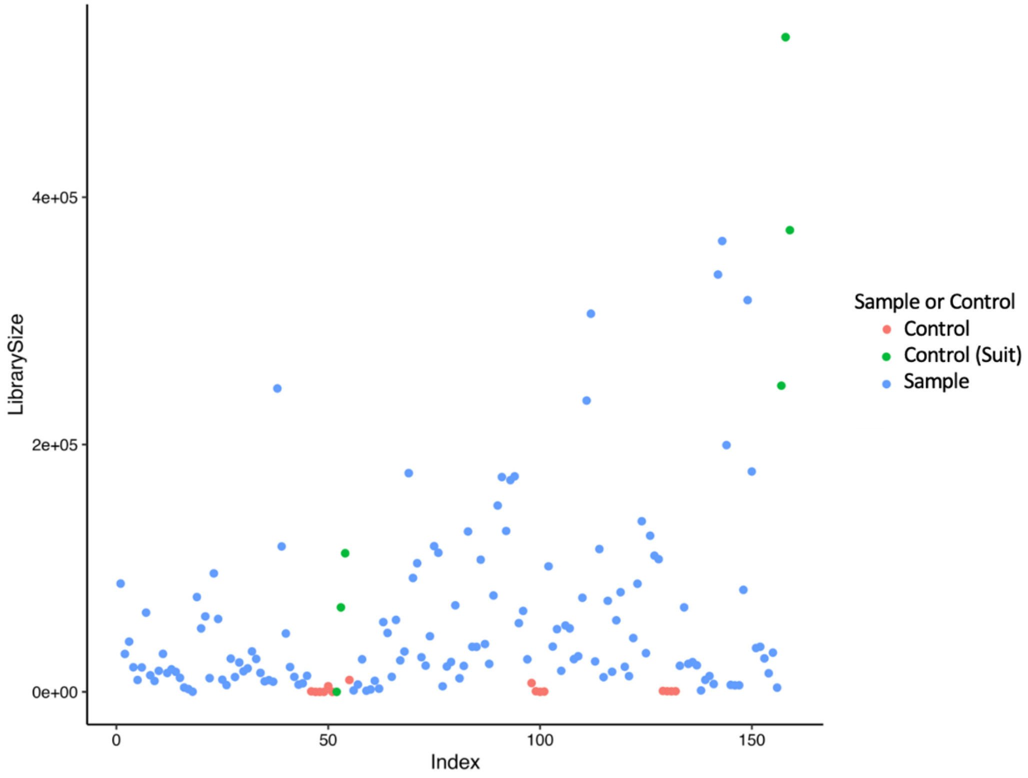
Forward Primer	Reverse Primer	Species	Base Pair Differences	Amplicon
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes aleutianus*		GACCACTCACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTCATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes alutus*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATTGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGATAAAGCCCTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes atrovirens***	*	GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTCATTATTCTCACTTGAATTGGAGGAATACCTGTATCACACCCGTTCGTTATTATTGGACAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCATTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes auriculatus*		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATTGGGCAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes aurora*		GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTCATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTCATTATTGGGCAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCATTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			
GACCACT**C**ACACAATT**T**T	AAGCTCATTCAAGTGCTT	*Sebastes caurinus*	*********	GACCACTCACACAATTTTTGTTTTGAACCCTAATCGCAGACGTCATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTCATTATTGGGCAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCATTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes babcocki*		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTCATCATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes baramenuke***		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTTACCTGAATCGGGGGAATACCTGTATCGCACCCGTTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes borealis*		GACCACTCACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes brevispinis*		GACCACTTACACAATTCTTATTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGAGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTTTTTCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes carnatus***	*	GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTCATTATTCTCACTTGAATCGGAGGAATACCTGTATCACACCCGTTCGTTATTATTGGGCAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes cheni***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGATGTCATCATTCTCACCTGAATCGGAGGAATGCCTGTATCTCACCCATTCGTCATTATCGGGCAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTAACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes ciliatus*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCGGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes polyspinis*		
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes variabilis*		
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes crameri*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTCACACCACTAGCAGGCTATGCGGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes reedi*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCATTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTCACACCACTAGCAGGCTATGCGGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes dalli***		GACCACTTACACAATTCTTGTTTTGGACCCTAATCGCGGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCGTTCGTTATTATTGGACAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTTCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes diaconus*		GACCACTTACACAATTCCTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGGATACCTGTATCACACCCATTCGTCATTATTGGACAAATCGCGTCCTTCTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes diploproa*		GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGAATGCCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes elongatus*		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATCATCGGACAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			GACCACTTACACAATTCCTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGGATACCTGTATCACACCCATTCGTCATTATTGGACAAGTCGCGTCCTTCTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTATGCAGAAGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTC**C**	AAGCTCATTCAAGTGCTT		**	GACCACTTACACAATTCCTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGGATACCTGTATCACACCCATTCGTCATTATTGGACAAGTCGCGTCCTTCTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes entomelas*	*	GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATCCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes exsul***	*	GACCACTCACACAATTCTTGTTCTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes fasciatus***		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTAACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes flavidus*		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAATCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTACGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes melanops*		
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes glaucus***		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTCATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes goodei*		GACCACTCACACAATTCTTGTTTTGAACCCTAATTGCAGACGTTATTATTCTCACCTGAATCGGAGGAATGCCTGTATCACACCCATTCGTTATTATTGGACAAATCGCATCCTTTTTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGATAAAGCACTTGAATGAGCTT
G**G**CCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes hopkinsi***	***	GGCCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATCGGACAAATCGCGTCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes inermis***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGATGTCATCATTCTCACCTGAATCGGAGGAATGCCTGTATCTCACCCATTCGTCATTATCGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTAACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes iracundus***	*	GACCACTCACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes itinus***	*	GACCACTCACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCCGTATCACACCCATTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCGGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes umbrosus***		GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAGTTGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes helvomaculatus*	*	GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes chlorosticus***	*	
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes constellatus***	*	
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes oculatus***	*	
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes rosenblatti***	*	
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCTGACGTCATTATTCTCACTTGAATCGGAGGAATGCCTGTATCACACCCGTTCGTCATTATTGGACAAATCGCATCCTTTTTATACTTCTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes jordani*		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCTGACGTCATTATTCTCACTTGAATCGGAGGAATGCCTGTGTCACACCCGTTCGTCATTATTGGACAAATCGCATCCTTTTTATACTTCTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes joyneri***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATCATTCTCACCTGAATCGGCGGAATACCTGTATCTCACCCATTCGTCATTATCGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTGACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes kiyomatsui***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACCTGAATTGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCATTAGCAGGTTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes koreanus***		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATTGGGGGAATACCTGTGTCACACCCGTTCGTTATTATCGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGTTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes levis***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTTACTTGAATCGGAGGGATACCCGTATCACACCCATTCGTTATTATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTTCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes matsubarae***		GACCACTTACACAATTCTTGTTTTGAACCCTAATTGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCATTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes melanostomus*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACTTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATCGGACAAGTCGCATCCTTTTTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes mentella***		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTAACCTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATCATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTGACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes maliger*		GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTCATTATTCTCACTTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATTGGACAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCATTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes miniatus*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACTTGAATTGGGGGAATGCCTGTATCACACCCGTTCGTTATCATTGGACAAATCGCATCCTTTTTATACTTTTTCCTTTTCTTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes moseri***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATCGGACAAATCGCGTCCTTCTTATACTTCTTCCTCTTCCTAGTCCTAACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			GACCACTTACACAATTCCTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGGATACCTGTATCACACCCATTCGTCATTATTGGACAAGTCGCGTCCTTCTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTC**C**	AAGCTCATTCAAGTGCTT	*Sebastes mystinus*	**	GACCACTTACACAATTCCTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGGATACCTGTATCACACCCATTCGTCATTATTGGACAAATCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes nebulosus*		GACCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGGGGAATACCTGTATCACACCCGTTCGTTATTATTGGGCAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes nigrocinctus*		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAATCGCATCTTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes nivosus***		GACCACTTACACAATTCTTGTTTTGAACCTTAATCGCAGACGTTATCATTCTCACCTGAATTGGGGGAATACCTGTATCACACCCGTTTGTCATCATTGGACAAATTGCATCCTTCTTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGTTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes nudus***		GGCCACTCACACAATTCTTGTTTTGAACCCTGATTGCAGATGTTATTATTCTCACCTGAATTGGGGGTATACCTGTGTCACACCCGTTCGTTATTATTGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTCACACCACTAGCAGGTTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes oblongus***		GACCACTTACACAATTCTTGTTCTGAACCCTAATCGCAGACGTTATTATTCTCACCTGAATTGGGGGAATACCTGTATCACACCCATTCGTTATTATTGGACAAATTGCATCCTTTTTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGTTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes paucispinis*		GACCACTCACCCAATTCCTATTTTGAACCCTAATCGCAGACGTTATTATTCTTACCTGAATCGGAGGAATGCCTGTATCACACCCCTTCGTCATTATTGGACAAATCGCGTCCTTCTTATACTTTTTCCTCTTCCTAGTCCTCACACCACTAGCGGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes pinniger*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACTTGAATTGGGGGAATACCTGTATCACATCCGTTCGTTATCATTGGACAAATCGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes proriger*		GACCACTTACACAATTCTTGTTTTGAACCTTAATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTATCACACCCATTCGTTATTATTGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes rastrelliger***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTGTCACACCCGTTCGTTATTATTGGACAAGTAGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes rosaceous***	*	GACCACTCACACAATTCTTGTTTTGAACCTTAATCGCAGACGTTATTATTCTCACTTGAATCGGAGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGAGGAATACCGGTATCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes ruberrimus*		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGAGGAATACCGGTATCACACCCGTTCGTTATTATCGGGCAAATCGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes rubrivincturs***	*	GACCACTTACACAATTCTTGTTTTGAACCCTTATTGCAGACGTTATTATTCTCACTTGAATCGGGGGGATACCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCATCCTTTTTATACTTCTTCCTTTTCCTAGTCCTCACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes saxicola*		GGCCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATTGGGGGAATGCCTGTATCACACCCATTCGTTGTTATTGGACAAGTAGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes schlegelii***		GACCACTTACACAATTCTTGTTTTGAACCTTAATCGCAGACGTTATTATTCTCACCTGAATTGGAGGGATGCCTGTGTCGCACCCATTCGTTATTATTGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTCACGCCACTAGCAGGCTATGCAGAAGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes scythropus***		GACCACTTACACAATTCTTATTTTGAACGTTGATCGCAGACGTTATTATTCTCACTTGAATTGGGGGAATACCTGTATCACACCCCTTCGTTATTATCGGACAAGTAGCATCCTTTTTATACTTTTTCCTCTTCCTAGTACTTACACCACTAGCAGGCTATGCGGAGGACAAAGCACTTGAATGAGCTT
G**G**CCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes semicinctus***	*****	GGCCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACTTGAATCGGGGGAATACCTGTATCACACCCATTCGTTATTATCGGGCAAGTAGCATCGTTTTTATACTTTTTCCTCTTCCTAGTCCTAACACCACTAGCGGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAA**C**TCT	AAGCTCATTCAAGTGCTT	***Sebastes serriceps***	******	GACCACTTACACAACTCTTGTTTTGAACCCTAATCGCAGACGTTATTATCCTCACTTGAATCGGGGGAATGCCTGTATCACACCCATTCGTTATTATTGGACAAGTCGCGTCCTTTTTATACTTTTTCCTTTTCCTAGTCCTTACACCACTAGCAGGCTACGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes steindachneri***	*	GACCACTCACACAATTCTTGTTTTGAACCCTGATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATGCCCGTATCGCACCCGTTCGTCATTATCGGACAAGTCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTGCACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes taczanowskii***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACTTGAATTGGAGGAATGCCCGTGTCACACCCATTCGTCATTATTGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACGCCACTAGCAGGCTATGCAGAAGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes thompsoni***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATCATTCTCACCTGAATCGGAGGAATACCTGTATCTCACCCATTCGTCATTATCGGACAAATTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTAACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes trivittatus***		GACCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTCATTATTCTTACCTGAATTGGTGGAATACCTGTGTCACACCCGTTCGTTATTATTGGACAAGTTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
G**G**CCACT**C**ACACAATTCT	AAGCTCATTCAAGTGCTT		***	GGCCACTCACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTGTCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
G**G**CCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes variegatus*	*****	GGCCACTTACACAATTCTTGTTTTGAACCCTAATCGCAGACGTTATTATTCTCACCTGAATCGGGGGAATACCTGTGTCACACCCGTTCGTTATTATCGGACAAATCGCATCCTTTTTATACTTTTTCCTCTTTCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
G**G**CCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes wilsoni*	*****	
G**G**CCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastes zacentrus*	*****	
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes vulpes***		
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	***Sebastes zonatus***		GACCACTTACACAATTCTTGTTTTGAACCCTGATCGCAGACGTCATTATTCTTACCTGAATTGGTGGAATACCCGTGTCACACCCGTTCGTTATTATTGGACAAGTTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTATGCAGAGGATAAAGCACTTGAATGAGCTT
GACCACTTACACAATTC**C**	AAGCTCATTCAAGTGCTT	***ebastiscus albofasciatu***	**	GACCACTTACACAATTCCTATTTTGGACTCTCATTGCAGACGTCCTCATTCTCACTTGAATCGGGGGTATACCTGTTACACACCCATTCGTCATTATTGGACAAGTTGCATCCTTTTTATACTTTTTCCTTTTCCTAGTCCTGACACCATTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAA**C**TCT	AAGCTCATTCAAGTGCTT	***Sebastiscus tertius***	******	GACCACTCACACAATTCCTATTTTGAACTCTCATTGCAGACGTCCTCATTCTAACTTGAATTGGGGGTATACCTGTCACACACCCATTCGTCATTATTGGACAAGTTGCATCCTTTTTATACTTTTTCCTCTTCCTAGTCTTAACACCATTAGCAGGCTATGCAGAGGACAAAGCACTTGAATGAGCTT
G**T**CC**C**CT**A**ACACA**G**TTC**C**	AAGCTCATTCAAGTGCTT		*******	GTCCCCTAACACAGTTCCTATTCTGAGCTCTTATCGCAGACGTCGTTATCCTCACTTGAATTGGAGGTATGCCTGTATCCCACCCCTTCGTTATCATCGGCCAAGTCGCATCCGTCCTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTACGCCGAGGACAAAGCACTTGAATGAGCTT
G**T**CC**C**CT**A**ACACA**G**TTC**C**	AAGCTCATTCAAGTGCTT		*******	GTCCCCTAACACAGTTCCTATTCTGAGCTCTTATCGCAGACGTCGTTATCCTCACTTGAATTGGAGGTATGCCTGTTTCCCACCCCTTCGTTATCATCGGCCAAGTCGCATCCGTCCTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTACGCCGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT			GTCCCCTAACACAGTTTCTATTCTGAGCTCTTATCGCAGACGTCGTTATCCTCACTTGAATTGGAGGCATGCCTGTATCCCACCCCTTCGTTATCATCGGCCAAGTCGCATCCGTCCTATACTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTACGCCGAGGACAAAGCACTTGAATGAGCTT
GACCACTTACACAATTCT	AAGCTCATTCAAGTGCTT	*Sebastolobus alascanus*		GTCCCCTAACACAGTTCCTATTCTGAGCTCTTATCGCAGACGTCGTTATCCTCACTTGAATTGGAGGTATGCCTGTATCCCACCCCTTCGTTATCATCGGCCAAGTCGCATCCGTCCTATACTTCTTCCTCTTCCTAGTTCTTACACCACTAGCAGGCTACGCCGAGGACAAAGCACTTGAATGAGCTT
G**T**CC**C**CTTACACA**G**TTC**C**	AAGCTCATTCAAGTGCTT	*Sebastolobus altivelis*	********	GTCCCCTTACACAGTTCCTATTCTGAGCTCTTATCGCAGACGTCGTTATCCTCACTTGAATTGGAGGCATGCCTGTATCCCACCCCTTCGTTATTATCGGCCAAGTCGCGTCCGTCCTATATTTCTTCCTCTTCCTAGTCCTTACACCACTAGCAGGCTACGCCGAGGACAAAGCACTTGAATGAGCTT

* Bolded species are not found in BC

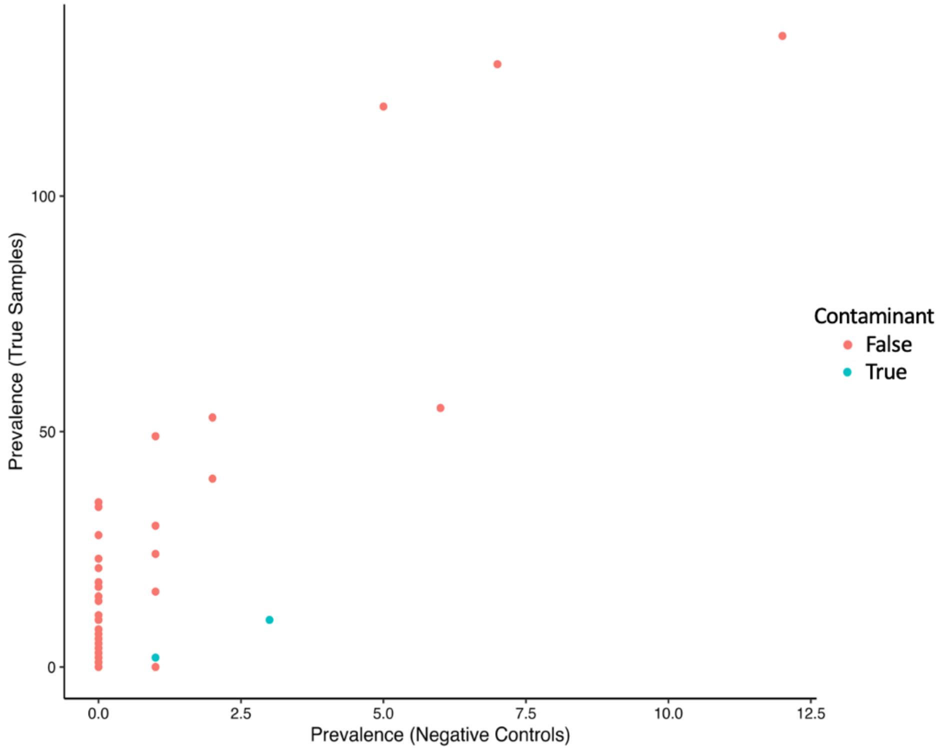
Appendix 33. Rarefaction curves generated for miSebastes to indicate whether sequencing depth was adequate for species detection. (Generated with Vegan package (R v3.6.3)



Appendix 34. Number of *Sebastes* reads for samples or controls (both negative and suit). (Generated with Decontam library (R v4.1.3))



Appendix 35. ASVs that are likely affected by contaminants based off prevalence in negative controls. These ASVs and reads associated with them are removed for subsequent analysis. (Generated with Decontam library (R v4.1.3))



Appendix 36. Percent agreement of detection between eDNA methods and dive method.

Detection methods comparison	**Target Species**	**Detection Method**	
		qPCR Amplification Low LOD	**miSebastes**
Percent Agreement	*Sebastes maliger*	70	81
	Sebastes paucispinis	98	96
	Sebastes ruberrimus	62	38
	Sebastes ruberrimus (2021 Only)	86	71
Percent Detected Just by Diving	*Sebastes maliger*	21	0
	Sebastes paucispinis	2	4
	Sebastes ruberrimus	9	4
	Sebastes ruberrimus (2021 Only)	14	10
Percent Detected by DNA Methods Only	*Sebastes maliger*	9	19
	Sebastes paucispinis	0	0
	Sebastes ruberrimus	30	57
	Sebastes ruberrimus (2021 Only)	0	19

Appendix 37. Visualization of the amplicon sequencing results per sample aggregated by location, with the number of reads normalized to proportions. The colors show the relative proportion of rockfish species detected at each depth by miSebastes and via dive surveys.