Assessment of remote Patagonian riverine fish using environmental DNA

by

Erika Alexandra Burton

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

Statement of Contributions

This research was conducted at the University of Waterloo under the supervision of Dr. Barb Katzenback and Dr. Mark Servos. Both supervisors contributed to the planning and design of the research, as well as intellectual input and review of material. Dr. Servos also assisted with sample collection.

Work in this thesis was a continuation of eDNA surveys conducted by Dr. Patricija Marjan in 2018, including the brown trout data collected in 2018. Dr. Marjan also developed the original eDNA protocols that were the basis for the assays applied in this thesis and further optimized by Erika Burton. Dr. Gustavo Chiang and Dr. Paulina Bahamonde assisted with site selection, site access, and sample collection.

Nathanael Harper and Michael Lynch at the University of Waterloo generated the zebrafish PCR assay for analysis of inhibition. Nathanael Harper also completed the initial species search in NCBI and assisted with puye PCR probe design. Erika Burton was responsible for activities not outlined above including conceptualizing experimental design, collecting and processing samples, and data analysis.

Abstract

Freshwater systems and fish communities face many anthropogenic threats such as climate change, pollution, and invasive species, causing a rapid loss of biodiversity. To protect freshwater fish basic knowledge of their numbers, distribution, and habitat is required, but can be difficult to obtain. Remote freshwater systems can experience human impacts but are even less understood due to lack of access and potential hazards. Patagonia (the southern tip of South America) is sparsely populated but contains many freshwater systems that can be indirectly impacted by human activities. The introduction and subsequent naturalization of several invasive salmonid species in rivers and lakes. Despite the potential for adverse effects on these ecosystems, they are generally under researched and uncharacterized. One such freshwater environment is the rivers draining from the stratovolcano Melimoyu in northern Chilean Patagonia, where limited access, high flows and considerable river debris makes traditional sampling from boats or wading impractical or dangerous.

New biomonitoring techniques such as environmental DNA (eDNA) detection can be used to sensitively and non-invasively obtain data of species presence or even community structure through analysis of water samples and may provide an avenue for obtaining data about freshwater communities in remote systems such as Melimoyu. Applying eDNA barcoding or metabarcoding techniques in remote systems may allow researchers to gain knowledge about the biota in these environments where traditional sampling may be limited or impossible. Environmental DNA barcoding was used for the detection of three different fish species: invasive brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*), and native puye (*Galaxias maculatus*) in the rivers draining Volcán Melimoyu. Brown trout eDNA was detected at seven sites across four rivers, Atlantic salmon eDNA was not detected at any sample sites, and puye eDNA was detected in one river with high certainty. At several sites eDNA detection techniques were accompanied by

backpack electrofishing. The detection of brown trout eDNA was potentially influenced by differing environmental conditions (e.g., flow) between sampling events. Puye was not always detected by eDNA despite being collected during electrofishing.

eDNA can be a powerful biomonitoring tool for detection of fish in remote systems, especially if applied with consideration of controls for contamination, transport, and storage of samples. Samples for eDNA barcoding can be collected under less-than-ideal conditions, and with an appropriate sampling regime, applied to remote systems to obtain valuable data on distributions of individual fish species. However, in future studies eDNA metabarcoding (i.e., simultaneous detection of all fish eDNA present using universal primers) may be a more powerful tool for use in remote freshwater environments to gain an understanding of entire communities. Collections of supplementary data could be used to inform occupancy models for a better understanding of the eDNA and presence of fish in these freshwater systems. This study demonstrates the potential application of eDNA for informing resource managers about fisheries resources, even in areas where traditional fisheries techniques are difficult or impractical to complete.

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Dedication

“These things [species] count, whether or not there is anybody to do the counting”

H. Rolston, 1986

Table of Contents

Author's Declaration ii
Abstract iv
Acknowledgements vi
Dedication vii
List of Figures xi
List of Tables xii
List of Abbreviations xiii
1. Introduction 1
1.1. Challenges for freshwater biomonitoring 1
1.2. Threats to Patagonian freshwater ecosystems 4
1.3. Species of interest 8
1.4. Introduction to environmental DNA (eDNA) 15
1.5. Controlling error in eDNA sampling 22
2. Objectives and hypotheses 24
3. Methods 25
3.1. Site selection 25
3.2. Variations in 2018 sampling 29
3.2.1. eDNA sample collection and processing 29
3.2.2. Brown trout PCR 30
3.3. Optimization of sample preservation methodology 31
3.4. Sampling and analysis regime for 2020 Patagonian samples 32
3.4.1. Water sample collection 32
3.4.2. Positive control sample collection 33
3.4.3. Site assessment and electrofishing 33
3.4.4. Filtration and preservation 34
3.4.5. DNA extraction 35
3.4.5.1. Extraction from filter 35
3.4.5.2. Extraction from fish tissue 36

3.4.6. Quantitative polymerase chain reaction (qPCR) 36
3.4.7. qPCR assay generation and optimization 37
3.4.7.1. Brown trout 37
3.4.7.2. Puye 38
3.4.7.3. Atlantic salmon 40
3.4.8. Assay efficiency and standards 41
3.4.9. Assay sensitivity 41
3.4.10. Sample quality assurance 42
3.4.11. qPCR for species of interest 43
3.4.11.1. Brown trout 44
3.4.11.2. Puye 45
3.4.11.3. Atlantic salmon 45
3.4.12. Data analysis 45
4. Results 47
4.1. Method development and validation 48
4.1.1. Sample desiccation 48
4.1.2. Brown trout positive control samples 52
4.2. Environmental sample quality determination 52
4.3. qPCR results 56
4.3.1. Detection probabilities 56
4.3.2. eDNA in river samples 58
4.3.3. Occupancy analysis 61
4.3.4. Brown trout 63
4.3.5. Puye 63
4.3.6. Atlantic salmon 64
5. Discussion 65
5.1. eDNA performance 65
5.2. Design considerations in eDNA barcoding assays 67
5.3. Fish occupancy 69

5.4. Analysis methods 74
5.5. Lessons for remote eDNA surveys 76
5.5.1. Improved sample preservation 76
5.5.2. Concentrate a greater volume of sample 78
5.5.3. Optimize the time and location of sampling...79
5.6. Future directions...81
5.6.1. Expand barcoding to additional species 82
5.6.2. Use metabarcoding for entire community composition...............................83
5.6.3. Incorporate supplementary information on rivers 84
6. Conclusions 85
6.1. eDNA can elucidate the distribution of fish in remote rivers.. 85
6.2. Optimizing eDNA sampling regimes could increase fidelity of eDNA data. 86
6.3. Occupancy analysis is a useful and powerful tool to interpret eDNA results if used appropriately...86
6.4. Additional barcoding and application of metabarcoding could improve fish surveys in central Patagonian rivers...87
References..88
Appendix A..98
Appendix B...101

List of Figures

Figure 1. Volcán Melimoyu and surrounding rivers. 7
Figure 2. Global distribution of puye (*Galaxias maculatus*). 13
Figure 3. Environmental DNA workflow 21
Figure 4. Map of eDNA sample collection sites surrounding Mount Melimoyu. 27
Figure 5. Concentration of DNA isolated from frozen or desiccated filtered water samples.. 50
Figure 6. ePlant assay results for frozen and desiccated eDNA filters..................... 51
Figure 7. Internal positive control for PCR inhibition indicate samples do not contain PCR inhibiting substances.. 54
Figure 8. ePlant results for eDNA samples collected in January 2020..................... 55
Figure 9. Occupancy analysis for species of interest in January 2020..................... 62
Supplementary Figure 1. Plate layout for sample PCR on a 96-well plate............... 99
Supplementary Figure 2. Marchant River flows in October 2018 and January 2020 102

List of Tables

Table 1. Summary of fish species that could occupy rivers draining from Volcán Melimoyu........10
Table 2. Description of river sites sampled in 2018 and 2020 on the Melimoyu Nature Reserve........28
Table 3. Additional rivers draining Volcán Melimoyu sampled for eDNA in 2020.....29
Table 4. Summary of primers and probes used for various qPCR assays...............37
Table 5. Water positive controls for brown trout cytochrome b eDNA detection held in river water for >1 hour........52
Table 6. Rates of false positives (FP) and detection probabilities in PCR for brown trout (*Salmo trutta*), puye (*Galaxias maculatus*), and Atlantic salmon (*Salmo salar*)........57
Table 7. Summary of qPCR results from environmental DNA samples in 2018 and 2020........59
Table 8. Comparison of electrofishing and eDNA evidence for species presence in January 2020........60
Supplementary Table 1. Summary of standard curves used to determine PCR efficiency........98
Supplementary Table 2. Summary of PCR reactions........100
Supplementary Table 3. Water chemistry measurements at sample collection sites........101

List of Abbreviations

BLAST	Basic Local Alignment Search Tool
C_t	Cycle threshold
Cytb	Cytochrome b
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
eDNA	Environmental DNA
FAM	Fluorescein amidite
IPC	Internal positive control
LOD	Limit of Detection
MERI	Melimoyu Ecosystem Research Institute
MGB	Minor groove binder
NCBI	National Center for Biotechnology Information
ND	No data
NTC	Non-template control
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
P_{PR}	Probability of presence
qPCR	Quantitative polymerase chain reaction
RFU	Relative fluorescence units
Rn	normalized reporter value
RNA	Ribonucleic acid
T_m	Melting temperature
T_a	Annealing temperature
UV	Ultraviolet

1. Introduction

Despite being the most imperiled ecosystems in the world, with population declines of many species, freshwater systems are often understudied or ignored (Reid et al., 2019). Recent advancements in freshwater monitoring, such as environmental DNA barcoding and metabarcoding, represent emerging new methods that may help to assess and monitor freshwater biodiversity (Taberlet et al., 2018). This may close the limnological knowledge gap to allow for a better understanding (and therefore management) of freshwater systems (Reid et al., 2019). The detection of environmental DNA (eDNA, Section 1.4) through barcoding may be especially useful in remote environments where traditional techniques may be difficult and/or costly, and little information is known about fish communities. The Melimoyu Nature Reserve is a protected area in Chilean Patagonia surrounding the stratovolcano Volcán Melimoyu. In this area there are several rivers draining from the volcano that are generally uncharacterized in terms of the river topography, flow, and fish communities. Due to their remote setting in central Patagonia, an area that is sparsely populated, these sites are very difficult to access and study using traditional approaches. In this thesis, eDNA detection methods are further developed and applied to detect several key fish species in several rivers draining from Volcán Melimoyu. Working at this site in Chilean Patagonia highlights the feasibility and challenges of applying eDNA detection protocols in remote systems.

1.1. Challenges for freshwater biomonitoring

Freshwater species are declining at a faster rate than marine and terrestrial species (International Union for Conservation of Nature, 2018; Reid et al., 2019; Thomsen et al., 2012), experiencing a 83% loss in freshwater vertebrate populations between 1970 and 2014 (Reid et al., 2019). This loss of biodiversity is expected to

accelerate as we see the effects of human actions leading to habitat loss, introduction of invasive species, reduced water quality, pathogen spread, and climate change (Dudgeon, 2019; Dudgeon et al., 2006; Reid et al., 2019). The cumulative effects of these anthropogenic sources of environmental stressors are leading to deteriorating freshwater habitats and communities (Cussac et al., 2016), and the impact of human activities extends to even the most remote areas of the world (Bargagli, 2008; Schindler, 1998). These secluded water bodies contain ecosystems that are critical for the sustainability of many human activities, providing natural resources and ecosystem services (Brandt et al., 2013). Unfortunately, our understanding of the decline of freshwater ecosystems is poor and understudied (Reid et al., 2019) and new approaches are needed to better inform resource management (Culp et al., 2022; Heino et al., 2020). An important component of freshwater systems are their fish communities. Not only are fish of economic importance even in remote areas of the world, such as Chilean Patagonia (Sepúlveda et al., 2013), the communities they compose can provide insight into the strength and resilience of entire ecosystems. Information such as the number of endemic species, presence of invasive species, and overall biodiversity within fish communities (as well as changes to these parameters) can provide managers with critical information regarding how to best protect the fish present. At a population level, to protect a species, it is important to understand its range and movements (Stem et al., 2005). However, the management of these systems relies on knowledge that is not easily generated, especially when the systems in question are remote or inaccessible.

Determining change in fish communities is a challenge for resource managers as traditional approaches are typically labour intensive. For example, many fish surveys rely on netting or electrofishing from a vessel, but many locations may be inaccessible by boat. Other methods may rely on wading through water to survey fish via seine net or backpack electrofishing, but this can also be difficult or even

dangerous because of flows, habitat, or access. Assessment of fish communities usually involves capturing and identifying the individuals to determine the relative distribution of the species. However, each capture method has considerable bias and success is dependent on the characteristics of each species and environmental factors, such as habitat, temperature, and water quality. Due to difficult and labour-intensive sampling regimes little is known about fish communities, especially in remote and difficult to access locations where transportation of personnel and equipment may be difficult and scientific resources scarce. In sparsely populated areas like Patagonia, there is a lack of basic information available to inform fisheries and environmental management (Pascual et al., 2007). In order to understand change, the natural variability in the fish populations and their habitat must be understood, but this requires repeated spatial and temporal sampling (Mathieu et al., 2020).

To assess changes to ecosystems and fish communities, large data sets are required over long periods of time as a single sampling event provides only a snapshot into the health of the system. Ideally, studies should not only encapsulate a large temporal range, but also include different physical habitats to ensure no species are missed or misrepresented (Mathieu et al., 2020). Despite the difficulties that may be present when sampling remote systems, it is vital that managers are able to detect and assess changes in them quickly to be able to implement protective measures. It is therefore important to look to emerging biomonitoring techniques for effective ways to more easily monitor systems that are not accessible using traditional approaches.

1.2. Threats to Patagonian freshwater ecosystems

Patagonia is a region of southern Chile (and Argentina) that, although sparsely populated, is experiencing change that threatens sensitive ecosystems (Becker et al., 2018). Despite covering over 1.4 million km^2 of Chile and Argentina, Patagonia contains less than 5% of the population of these countries combined (Pascual et al., 2007). There are a variety of freshwater habitats in Patagonia, including oligotrophic Andean lakes, large glacial fields, and several major rivers draining into the Pacific and Atlantic oceans. Rivers in west Patagonia draining into the Pacific are generally shorter than their Atlantic counterparts, flowing into fjords (Pascual et al., 2007). Although remote, Patagonian freshwater systems are experiencing human impacts. Despite the low population in the area, biota may be impacted by a variety of anthropogenic stressors. This includes long-range transport of contaminants (MERI Foundation, n.d), anthropogenic climate change (Becker et al., 2018), and introduction of invasive species (Cussac et al., 2016).

It is well established that contaminants can be globally dispersed. For example, polychlorinated biphenyls (PCBs) and other persistent organic contaminants have been found in the air and biota in areas as remote as Antarctica (Montone et al., 2003; Rudolph et al., 2016). It has also been demonstrated that brown trout in Andean Patagonia have detectable levels of PCBs, polybrominated diphenyl ethers (PBDEs), and organochlorine pesticides in their tissues and gut contents, likely originating from atmospheric transport (Ondarza et al., 2011). Despite the remote setting, contaminants such as lead can also reach the relatively pristine aquatic ecosystems of Chilean Patagonia. Lead accumulates in biota in Patagonia even though there is a lack of anthropogenic sources in the vicinity (Espejo et al., 2019). Unfortunately, there is little information about the distribution of contaminants in Patagonia, or in Chilian river systems.

Climate change may also alter fish habitat (e.g., temperature, flow, water quality) which can adversely affect fish species (Becker et al., 2018). Glaciers in Patagonia are retreating as a result of climate change which alters the local water cycle (Paul & Mölg, 2014). Projected increases in evapotranspiration and the decrease in precipitation may contribute to reduced water yield in the headwaters of northern Patagonian watersheds (Natalia et al., 2020). Changes in glacial melt and river flow may also influence water quality (Vargas et al., 2018), alter fish habitats and therefore have implications for fish communities. Overall, reductions and alterations to freshwater sources due to climate change is one of the most significant challenges facing freshwater fish communities (Reid et al., 2019).

Patagonian rivers and lakes are also deeply impacted by the presence of invasive salmonid species. Trout and salmon thrive in the cold waters of Patagonia where they have few predators (Arismendi et al., 2014; Tagliaferro et al., 2014). Throughout the 20th century, several salmonid species were introduced into Patagonia deliberately for sport fishing (e.g., brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*)) and accidentally through aquaculture (e.g., salmon). These species have now greatly expanded their range, and competition and predation are having significant impacts on native fish populations (Cussac et al., 2016). In Patagonian systems invaded by salmonids there is often an absence of native species indicating that salmonid invasions may lead to local extirpations (Soto et al., 2006). The addition of invasive trout to a freshwater system can lead to behavior changes and decreased population abundance in native fish species, as well as complete trophic cascades (Tagliaferro et al., 2014). Invasive salmonids can also act as vectors for pathogen transfer, introducing new diseases to native populations, and have synergistic negative effects when combined with other stressors (Sepúlveda et al., 2013). Invasive species can profoundly alter the communities and health of freshwater systems (Reid et al., 2019), making it vital to understand their distribution and the effects they are having on local biota.

Most water bodies in west Patagonia (i.e., Chile) have been sampled for fish infrequently, or not at all. While there is some data available for lake-dwelling fish, knowledge about fish populations in streams and rivers is scarce (Cussac et al., 2016). Due to their remoteness and inaccessibility, traditional biological surveys can be very difficult in these locations, creating knowledge gaps with respect to the state of fish populations and habitat (Pascual et al., 2007). However, understanding the distribution of freshwater species, both native and exotic, is critical for fisheries management (Stem et al., 2005).

The Melimoyu Ecosystem Research Institute (MERI) is a private foundation with the mission to strengthen research and education for the conservation and sustainable management of the terrestrial, freshwater, marine, and cultural heritage of northern Patagonia (MERI Foundation, n.d.). MERI maintains the Melimoyu Nature Reserve, a remote nature reserve surrounded by several national parks and protected areas. The Marchant River runs through the Reserve and there are several additional major rivers that drain from the Volcán Melimoyu (Figure 1). Near the mouth of the Marchant River there are several large salmonid aquaculture facilities (Figure 2). In these rivers there is overlap in the ranges of native and invasive freshwater fish and understanding their distributions and diversity would allow MERI to manage rivers through the reserve to increase survival and reproduction of native fish (MERI Foundation, n.d.). Although many species are endemic to the area, Patagonian freshwater fish communities generally have low native biodiversity (Soto et al., 2006), making native communities precious from a conservation standpoint as well as fragile due to the few redundancies in these low diversity systems (Naveh, 1994).

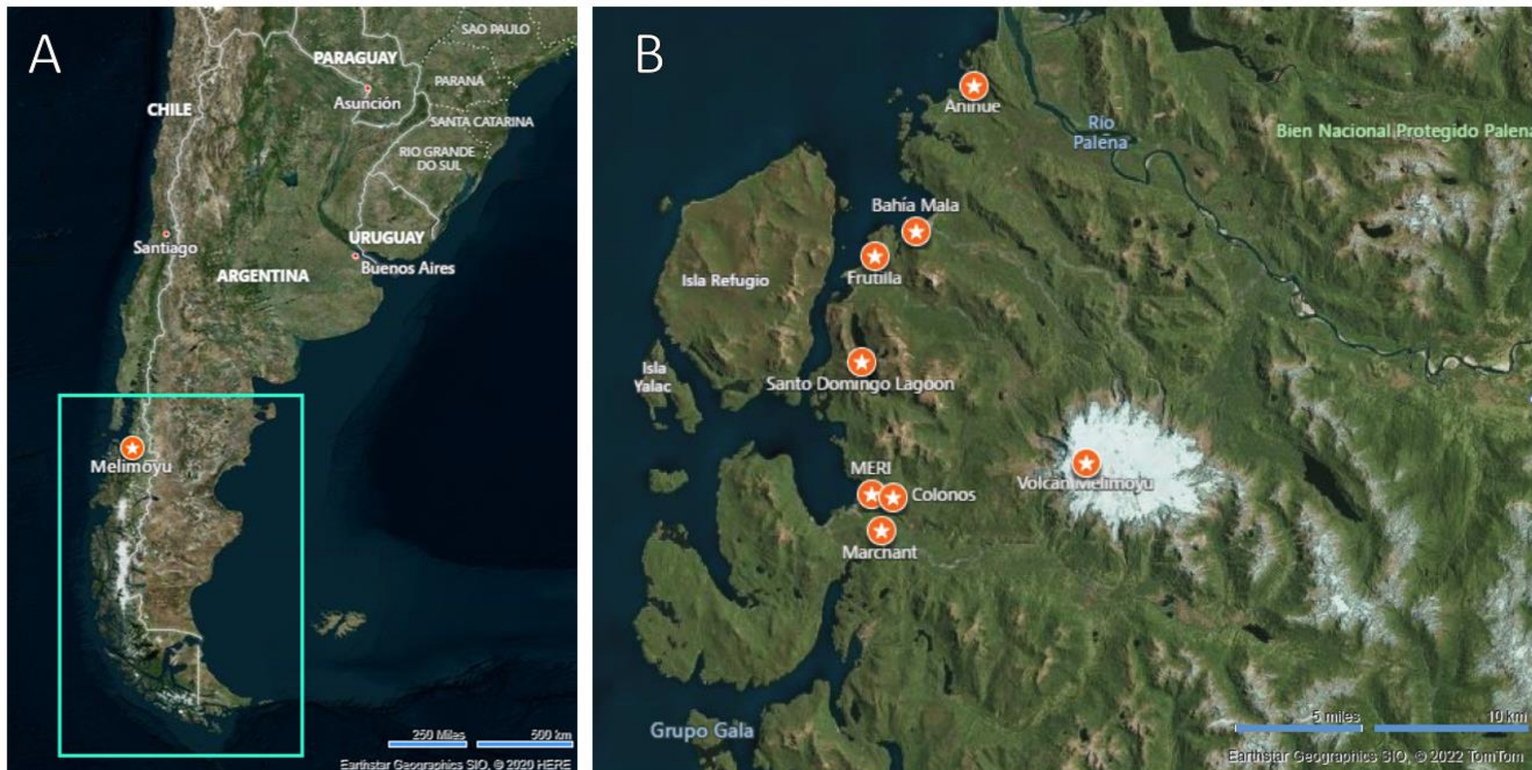

Figure 1. Volcán Melimoyu and surrounding rivers. (A) Location of the stratovolcano Volcán Melimoyu in Chilean Patagonia and (B) The mouths of 6 rivers fed from the volcano (Bing Maps, 2022)

The rivers draining from Volcán Melimoyu are generally inaccessible and very remote, therefore only minimal fish studies have been completed at the downstream stretch of the Marchant and Colonos Rivers. MERI has a desire to assess and monitor the biodiversity of these rivers as a basis to protect this unique environment. For example, if rivers or streams that have not yet been invaded by salmonids can be identified, they may also be protected. Unfortunately, the lack of access makes sampling these rivers using traditional techniques very difficult (i.e., backpack electrofishing, boat electrofishing, and laying gill nets). Due to the high flow and considerable debris, boats cannot pass through the channels to allow upstream access for boat electrofishing. The flow also prevents laying nets or wading across the river for backpack electrofishing (although backpack electrofishing can be completed nearshore). The emerging approach of capturing environmental DNA (eDNA) in water samples was identified as a potential alternative to assess the fish communities in these remote rivers where traditional methods cannot be applied.

1.3. Species of interest

Rivers draining from Volcán Melimoyu contain uncharacterized fish communities as they have been sampled infrequently or not at all. Generally, Patagonian freshwater systems have the lowest freshwater biodiversity in South America (Chalde & Llompart, 2021), and have contended with invasive salmonids for over a century (Soto et al., 2006). Salmon and trout are not native to the southern hemisphere but have been able to thrive in Chilean freshwater systems with cold water, low diversity, and little competition (Soto et al., 2006). These exotic species can pose a major threat to the biodiversity of native fish in the region, especially as there is a high degree of endemism (the state of a species being native to a single defined geographic location) in the fish native to Patagonia and 90% are considered vulnerable or in “serious danger” (Soto et al., 2006). In total there are 26 freshwater

fish species found throughout Patagonia (Pascual et al., 2007), 19 of these species (14 native species and 5 exotic salmonids (G. Chiang, personal correspondence)) may be found in the rivers draining from Volcán Melimoyu (Table 1). Both native and exotic species are of interest as there is a desire to protect native fish and this requires knowledge of the invasive species impacting them. Three species were chosen to survey with eDNA detection methods, two exotic salmonids (brown trout and Atlantic salmon) and one native species (puye).

Table 1. Summary of fish species that could occupy rivers draining from Volcán Melimoyu.

	Common Name	Scientific Name	Mitogenome Sequenced (Y/N)	Sequenced Fragments
Exotic Species	Rainbow trout	*Oncorhynchus mykiss*	Y	
	Brown trout	*Salmo trutta*	Y	
	Coho salmon	*Oncorhynchus kisutch*	Y	
	Chinook salmon	*Oncorhynchus tshawytscha*	Y	
	Atlantic salmon	*Salmo salar*	Y	
Native Species	Common galaxias	*Galaxias maculatus*	Y	
	Galaxias platei	*Galaxias platei*	Y	
	Red Jollytail	*Brachygalaxias bullocki*	N	Cytb, 12S, 16S
	Aplochiton zebra	*Aplochiton zebra*	N	COI, Cytb, 16S
	Aplochiton teniatus	*Aplochiton teniatus*	N	COI, Cytb, 16S
	Pejerrey Cauque	*Basilichthys australis*	N	Cytb
	Pouched Lamprey	*Geotria australis*	Y	
		Cheirodon australe	N	Cytb, 16S
		Cauque mauleanum	N	Cytb, ND2
		Percichthys trucha	Y	
		Diplomystes camposensis	N	Cytb, control region, ND4, ND5, D-loop
	Pencil catfish sp.	*Hatcheria macraei*	N	Cytb
	Chilean lamprey	*Mordacia lapicida*	N	COI, 12S, Cytb
		Odontesthes hatcheri	N	Cytb, COI, 12S

Sequence data from NCBI BLAST (*BLAST: Basic Local Alignment Search Tool*, n.d) is also included; whether the full mitogenome of the species was sequenced and any other fragments of the mitogenome that have been sequenced are indicated.

Brown trout (*Salmo trutta*)

Brown trout are a historic and prolific invader of Chilean freshwater systems. Trout may be able to fill empty niches, and have the potential to prey on, out-

compete, and even extirpate native species (Soto et al., 2006). When brown trout and rainbow trout were introduced by the Chilean government the potential impact on native species was not fully appreciated. Brown and rainbow trout now make up more than 80% (as high as 95%) of biomass in south Patagonian streams (Soto et al., 2006).

Brown trout tend to occupy the upper reaches of streams in higher density, but can find suitable habitat in downstream reaches as well (Tagliaferro et al., 2014). They prefer cold, clean, well oxygenated water with adequate flow to allow migration and prevent silt build up (Hendry et al., 2003). Adult brown trout preferentially occupy riverine sites that have pool refugia (e.g. due to boulders or submerged materials blocking flow), cobble substrate, and nearby suitable spawning areas (Hendry et al., 2003). Spawning sites are generally in riffle zones with riparian vegetation, high flow, and gravel substrate. Brown trout will typically occupy lotic environments with habitat diversity to accommodate different life stages (Hendry et al., 2003).

For this study brown trout were selected as a test species as they are known to inhabit the rivers in the vicinity of Volcan Melimoyu in high density (relative to other species). Brown trout also seem to have a greater distribution in this area of Patagonia compared to rainbow trout (Soto et al., 2006), making them a better target for initial eDNA analyses. It is important to understand the distribution of trout to determine the effects they may be having on local biota, and potentially identify systems that have not yet been impacted by brown trout so that they can be protected moving forward.

Puye (*Galaxias maculatus*)

Puye (also commonly referred to as Inanga) are a species of Galaxiids native to much of the Southern hemisphere (Figure 2), circumventing the south pole (Carrea et al., 2013; Soto et al., 2006). Galaxiids are one of the freshwater fish families most threatened by salmonid invasions (Minett et al., 2021).
Chilean puye can be land locked or diadromous (Macchi et al., 2007). Puye in coastal regions (such as those in rivers draining Volcan Melimoyu) typically spend their adult lives throughout rivers, but spawn and spend the first 6 months of their lives in the riparian vegetation of tidally influenced or estuarine river mouth environments (Hickford & Schiel, 2013). In rivers containing both puye and rainbow trout, puye were found in greater numbers in downstream reaches where more trout occupied the upstream, however there was considerable overlap between the occupation of both species throughout the river (Tagliaferro et al., 2014). Puye have been identified as an important prey species for both native fish (including other puye) and invasive salmonids (Pascual et al., 2007).

Puye are also a very genetically diverse species, varying up to 14.6% in their mitochondria cytochrome b sequence between geographic regions, and up to 3.8% just within South America (Waters & Burridge, 1999). Additionally, they are found to be more genetically diverse in coastal populations compared to their landlocked counterparts (Carrea et al., 2013). As an abundant and important freshwater species to the area (Soto et al., 2006), it is of management interest to conserve puye.

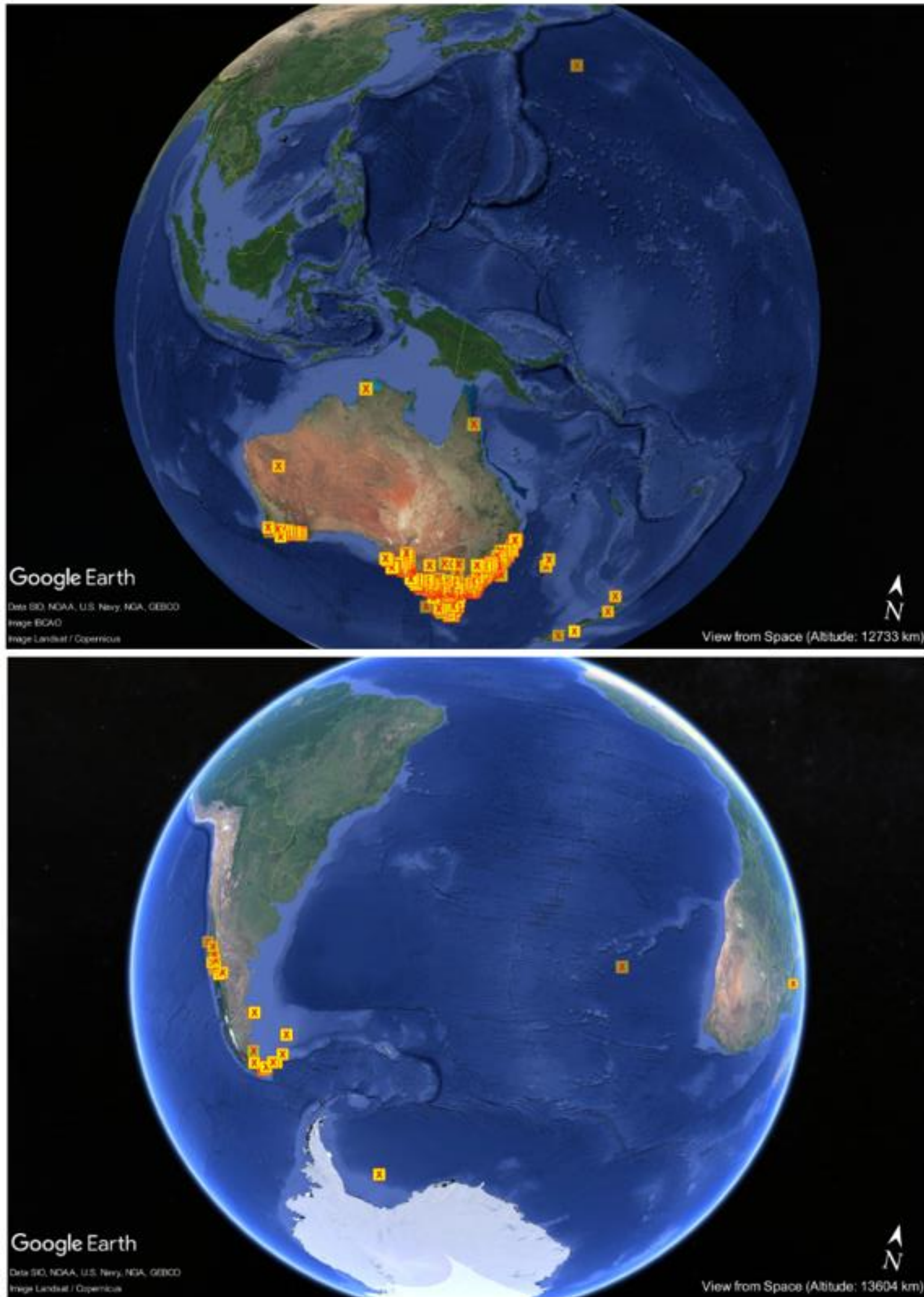

Figure 2. Global distribution of puye (*Galaxias maculatus*). Yellow boxes represent reported incidences of *Galaxias maculatus* from Fishgate (Torres & Bailly, 2021). Point map generated with Google Earth (Google Earth Pro 7.3.4.8248, 2021).

Atlantic Salmon (*Salmo salar*)

Salmonid aquaculture is a major source of revenue in Chile, and Atlantic salmon are the most frequently farmed fish in the country, comprising 60.3% of fish production nationally (Sepúlveda et al., 2013). Chile is the second largest exporter of Atlantic salmon in the world (Norway is the first) making up 13% of global production (Poblete et al., 2019). In 2014 Chile exported 346,332 tonnes of Atlantic salmon (61% of their fish exports) valued at $2,968 million USD (66% of fish export value) (Poblete et al., 2019). Much of the commercial Atlantic salmon farming occurs in the relatively pristine coastal environment of central Chile (including northern Patagonia) (Buschmann et al., 2006).

Atlantic salmon have the potential to invade rivers with suitable habitat as they escape or are intentionally released from marine aquaculture pens (Soto, 2006), although they have had less invasion success than other salmonid species in Patagonia (Arismendi et al., 2014). While there is not yet evidence of Atlantic salmon invasions in the rivers sampled in this study area (G. Chiang, personal communication), the presence of nearby Atlantic salmon pens makes them a species of interest, especially one in the vicinity of the Marchant River mouth (Figure 3). Atlantic salmon spawn in rivers and can remain in the freshwater environment for months or years if conditions are suitable (Bardonnet et al., 2011). Like brown trout, Atlantic salmon prefer to spawn in high flow riffle zones with course substrate, and this environment is also where juvenile salmon will stay for their first year before moving to rapids or runs (Bardonnet et al., 2011). Adult Atlantic salmon will occupy pools near spawning sites, and occupy rivers that allow free migration between freshwater spawning areas and estuarine or marine environments (Bardonnet et al., 2011). Both juvenile and adult salmon inhabit the bottom of the river, although juvenile salmon do this to contend with high flows and adults as a light avoidance behaviour (Bardonnet et al., 2011). Several rivers sampled in this study may provide

suitable habitat for Atlantic salmon (e.g., Marchant River, see Table 2), and although no Atlantic salmon populations have arisen yet in the area it would be prudent to try and detect a potential invasion as early as possible.

1.4. Introduction to environmental DNA (eDNA)

A method for assessing freshwater macroorganisms that has recently been gaining popularity is the detection of environmental DNA (eDNA) (Burian et al., 2021; Taberlet et al., 2018). eDNA is defined as DNA that an organism has shed as cellular or extracellular material into their environment via mucus, feces, urine, or tissue (Hänfling et al., 2016; Thomsen & Willerslev, 2015). By capturing and analyzing eDNA from an environmental sample (e.g., water) it is possible to determine the presence of an organism without sampling the organism directly, making it less invasive than many traditional aquatic research methods. It can also be more cost effective and sensitive compared to other fish survey methods (e.g., backpack electrofishing) (Thomsen & Willerslev, 2015), and while some traditional fish biomonitoring techniques are limited by seasonal access or flow conditions (Chalde & Llompart, 2021) eDNA surveys can be completed year round (Thalinger et al., 2021). This method has been proposed for detection of species that are difficult to find or occur in low numbers, (such as invading species, rare, and/or endangered species) due to the high sensitivity of environmental DNA detection (Turner et al., 2015). As eDNA sampling in rivers only requires collection of water samples in the field it could be advantageous to use this method for species detection in systems that are difficult to sample with traditional fisheries methods while maintaining good detection sensitivity.

While eDNA detection techniques were traditionally used to characterize microbial communities from environmental samples (such as water or soil) the

technique has more recently been shown to sensitively and non-invasively detect fish and other aquatic macroorganism DNA from environmental samples (Taberlet et al., 2012). For example, researchers have successfully used eDNA detection to identify the presence of fish (e.g., chinook salmon (Laramie et al., 2015)) and amphibian (e.g., American bullfrogs (Dejean et al., 2012)) species in freshwater systems without the need to capture or even see the species of interest (Nardi et al., 2020; Taberlet et al., 2018). The rapid development of this method has allowed for single species to be detected through barcoding (aka species-specific qPCR) as well as entire community compositions via metabarcoding (Taberlet et al., 2018).

There are two main approaches for detecting eDNA extracted from environmental media, barcoding and metabarcoding. For either approach the preliminary steps are the same; water samples are collected, filtered, preserved, then eDNA is extracted from the filters (outlined in Figure 3). Once all DNA is isolated from the sample the extract can be used for either the detection of a single target species with barcoding or all species of interest at once through metabarcoding (Taberlet et al., 2018). Barcoding is a species-specific assay, wherein real time quantitative polymerase chain reaction (qPCR) detection methods are used to amplify a single DNA sequence. The sequence selected should be unique to a single species and can therefore be used to determine the species presence in a water body via its DNA in collected water samples. PCR primers can be designed to analyze a sample easily and quickly since there is only a single target, and results of qPCR are easy to interpret (Taberlet et al., 2018), requiring relatively little post-processing. Barcoding is generally quicker and simpler than metabarcoding, and can be more sensitive than metabarcoding for a given single species since it is more targeted (Harper et al., 2018). While this method is reliable, it is most useful for the detection of a single (or a few) species of interest, and therefore does not provide information on the community composition of a target system (Thomsen & Willerslev, 2015).

The second approach for detecting eDNA uses metabarcoding, which involves development and use of a taxon specific set of primers to amplify DNA from all target species simultaneously and the resulting amplicons are then sequenced using high throughput sequencing (Taberlet et al., 2012, 2018; Valentini et al., 2016). Unique sequences or "barcodes" present in the sample can then be used to assign taxonomic identities to eDNA fragments detected in the water samples, provided adequate reference databases containing sequences for all the relevant species are available (although gaps in taxon coverage is a major barrier to eDNA metabarcoding (Cristescu & Hebert, 2018)). Metabarcoding provides a more complete picture of all target taxa in a system, however high throughput sequencing can be more complex and costly than a single species assay. It also requires an appropriate, short metabarcode that can be used to detect all target species (Taberlet et al., 2018; Valentini et al., 2016) containing a region that is highly variable between target species, including those that are closely related (Cristescu & Hebert, 2018), but flanked by regions that are highly conserved in all target species (Harper et al., 2018). Although metabarcoding can be very useful, the preliminary studies in this thesis are focused on the use of barcoding of three species individually.

Both barcoding and metabarcoding for environmental samples generally target mitochondrial genes (Valentini et al., 2016), and require a short target sequence (usually ≤150 base pairs) since eDNA is found in various stages of degradation (Cristescu & Hebert, 2018). Since target DNA may be degraded or rare, mitochondrial genes are preferred as they are more plentiful in cells (Wilcox et al., 2013) and therefore the environment (Valentini et al., 2016). Mitochondrial genes are also highly conserved within species compared to genomic DNA making them a preferred target to identify all individuals of a species with the same locus (Valentini et al., 2016). Generally, it is prudent to target protein coding genes for eDNA

analysis (e.g. cytochrome b, COI) rather than ribosomal genes (e.g. 16s rRNA) as it is easier to detect sequencing errors in these regions (Cristescu & Hebert, 2018). eDNA analysis of Patagonian species may be complicated as there is little genetic information about many of the native species that could be found in the area (Table 1). Mitochondrial genes are used for eDNA surveys and for most native fish species their mitochondrial genome has not been sequenced in full. However, the data available (i.e., sequenced mitochondrial fragments) can be used to generate eDNA assays for several important species (see Section 3.4.6). For this study, primers and probes targeting the COI region were used for Atlantic salmon and brown trout as they had been previously validated. When generating new primers for detection of puye cytochrome b was targeted as it was the only locus sequenced for all native species in the area (Table 1) and therefore the only way to ensure there was no sequence overlap between puye and other species *in silico*.

While eDNA detection can be an effective tool with advantages over traditional sampling, there are also many knowledge gaps with regards to best practices, limitations, and efficacy (Burian et al., 2021; Cristescu & Hebert, 2018; Stoeckle et al., 2017; Tsuji et al., 2017). For example, eDNA samples can be prone to contamination, giving false positives. Successful eDNA detection in surface waters usually indicates recent species occupation since cells and DNA are usually removed from the water within 24 hours due to cell settling and dilution (Jane et al., 2015). However, DNA that is bound to sediment may persist for millennia if the environment is suitable (Cristescu & Hebert, 2018). Persistent "relic" DNA from organisms that have moved or died can also be detected in eDNA analyses if sediment is disturbed or resuspended due to high flows, potentially leading to false positive results if relic eDNA is detected in a water sample (Mathieu et al., 2020). Suspended sediment (or suspended solids generally) may also contain PCR inhibiting substances that can prevent DNA detection in PCR leading to a false negative result (Jane et al., 2015). False negatives are also possible if the assay

used is not appropriate, sensitive, or specific to the target species (Burian et al., 2021; Goldberg et al., 2016). The collection timing and location can also lead to false negatives, as eDNA probabilities can be influenced by season, flow, distance, and species biomass (Jane et al., 2015) which can all vary depending on time and location.

eDNA persistence and movement in water bodies is poorly understood, and especially complicated in lotic systems where it may be travelling or removed rapidly (Cristescu & Hebert, 2018; Jane et al., 2015). Downstream reaches of rivers receive DNA from upstream, as well as DNA carried into the river from the entire catchment. This may be beneficial to gaining an understanding of the local community structure via metabarcoding, but also means that eDNA results are not localized since there is no way to know how far it travelled before it was collected (Cristescu & Hebert, 2018). Jane et al. (2015) determined that eDNA detection probabilities in rivers can be positively influenced by species biomass and proximity, and negatively influenced by flow. However, they also determined that the effects of distance from target species were mitigated by high flow as the DNA could be carried further downstream increasing detection probabilities farther away from target fish (but decreasing detection probabilities near the fish). Generally, a multitude of physical and biological processes may affect the quality and persistence of eDNA in water, and therefore have implications for detection probabilities (Cristescu & Hebert, 2018).

If eDNA detection methods can be applied in remote areas where traditional sampling is difficult (or impossible) it could be a powerful tool for assessing the diversity of and changes to remote fish communities. However, complications that arise in typical eDNA studies may also be exacerbated when sampling in remote or understudied systems. While many literature sources have successfully completed eDNA studies under ideal circumstances there is little information available on completing these studies under less-than-ideal conditions, such as sampling remote

river systems where conditions may not be clean, and materials and access are limited. To account for any contamination during sampling and analysis and limit false negatives it is vital to ensure that controls and validation steps are incorporated into any remote eDNA sampling. The type of assay selected for the study is also an important consideration to maximize the likelihood of successfully capturing target eDNA if it is present. Several researchers have begun to implement these techniques for fish detection in south Patagonia. For example, researchers have successfully detected the presence of invasive coho salmon (*Oncorynchus kisutch*) on Tierra del Fuego Island (remote southern tip of Patagonia) using barcoding, thus demonstrating the efficacy of the technique for early detection of fish invading new areas of Patagonia (Chalde et al., 2019). In another study the previously uncharacterized distribution of pouched lamprey (*Geotria austalis*) in several rivers on Tierra del Fuego was successfully determined using eDNA techniques (Nardi et al., 2020). eDNA studies from this area are an encouraging indication that eDNA surveys are a suitable method for assessing remote Patagonian waterways.

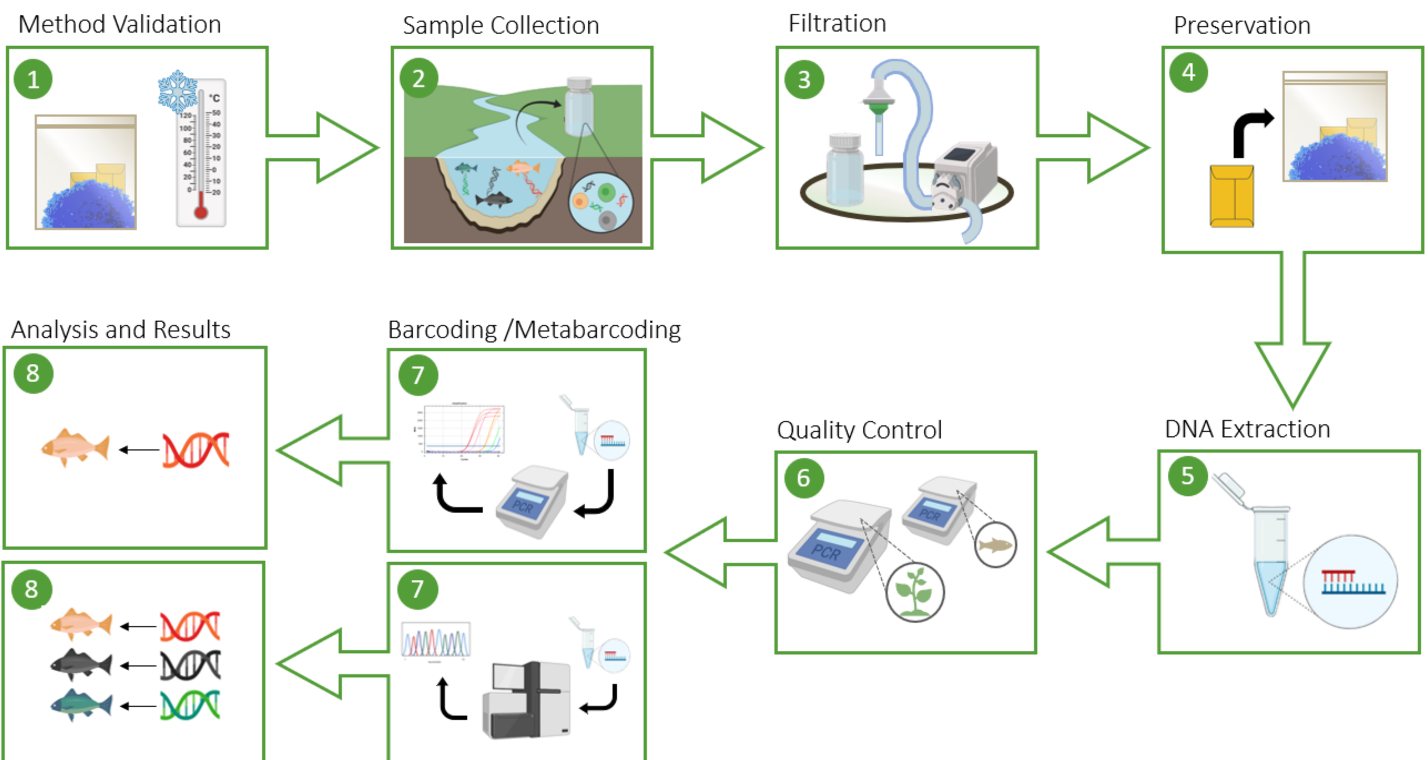

Figure 3. Environmental DNA workflow (Created with images from Biorender.com). 1) Methods and materials for sampling are chosen and validated. 2) Water samples containing environmental DNA (eDNA) from freshwater fish are collected from rivers. 3) eDNA and other solids from water samples are concentrated onto a filter 4) Filters are preserved via desiccation for transport and storage. 5) DNA is extracted and isolated from filters 6) eDNA extracts are tested for degradation and PCR inhibition. 7) PCR barcoding (or metabarcoding) is used to detect a specific DNA sequence in the eDNA sample. 9) Fish DNA sequences with positive detection in PCR are used to infer species presence in sampled river.

1.5.Controlling error in eDNA sampling

Due to the highly sensitive nature of eDNA detection the method is very useful for identifying species in low abundance. However, the high sensitivity of eDNA detection also makes the method prone to contamination. Special considerations must be made when completing eDNA surveys in remote systems to ensure results are trustworthy and any contamination is accounted for. Steps should be taken to minimize both type I (false positive) and type II (false negative) errors as both are possible in eDNA analysis, especially when sampling in settings where scientific infrastructure is limited, and the working environment may be contaminated.

eDNA detection is prone to false positive errors which need to be accounted for at multiple stages: sample collection, processing, and PCR analysis (Buxton et al., 2021). Incorporating blanks into multiple process stages allows for any contamination to be detected and quantified. If sample bottles need to be reused, they should be washed thoroughly with bleach between uses, as contamination can result if materials are not sufficiently decontaminated (Goldberg et al., 2016). Field blanks should be used to detect any contamination that may arise from reusing collection bottles and for handling in the field. These blanks should be treated exactly as samples are throughout all processing steps, and new blanks should be introduced at the DNA extraction stage to identify if contamination is introduced during extraction (Goldberg et al., 2016). False positives in PCR are also a concern but can be addressed by requiring more than 1 PCR replicate to amplify for a true positive detection (Rees et al., 2014) and having negative controls (e.g., field and extraction blanks, NTCs (non-template controls) in PCR) for reference (Goldberg et al., 2016). Using a probabilistic analysis of eDNA samples (such as Bayesian

occupancy modelling) that integrates other information about sampling sites (e.g., flow, substrate, presence of prey species) and error rates at multiple analysis stages can also help to identify false positives and provide a more holistic understanding of the likelihood of species presence (Buxton et al., 2021). By combining these techniques contamination can be accounted for, therefore reducing the chance of making wrong conclusions about species detections due to type I error.

False negatives for target DNA can occur if target species are rare or DNA is degraded (Cristescu & Hebert, 2018; Jane et al., 2015), but may be reduced by increasing detection probabilities. For example, it is prudent to collect targeted water samples for the species of interest, ensuring the location (i.e., habitat) of the sample collection and the volume of water filtered is appropriate with regards to the study and sampled system, although there is a tradeoff between collecting adequate sample volume and the ability to filter large volumes. It is also important to detect and remove any PCR inhibiting substances in a water sample, as they can lead to a false conclusion that a target sequence is not present by preventing its amplification in PCR (Goldberg et al., 2016). Striving for a low limit of detection (LOD) for qPCR assays ensures the assay is as sensitive as possible so that if even a few copies of the target sequence are present they will be amplified, and chances of a false negative can be reduced. The use of standard curves and/or positive controls for each PCR plate can ensure all plates are comparable and suitably sensitive (Goldberg et al., 2016). The use of barcoding rather than metabarcoding can also result in more sensitive detections of species of interest as it does not risk species being missed due to primer amplification bias in PCR (Harper et al., 2018). Since there is no *a priori* information for several rivers sampled in this study, sensitive eDNA protocols were essential to ensuring the chances of a species of interest being missed (i.e., a type II error) were minimized.

2. Objectives and hypotheses

The overarching objective of this thesis was to evaluate and improve the feasibility of applying eDNA detection methods as a biomonitoring tool for remote aquatic species detection and monitoring. This was completed using the rivers in the vicinity of Volcán Melimoyu as a pilot study for remote eDNA sampling. By identifying the presence of several key fish species in the rivers draining Volcán Melimoyu the efficacy of eDNA detection as a remote biomonitoring tool could be assessed, and if successful, MERI could be informed of the distribution of several key fish species in this area.

In the region surrounding the Melimoyu Nature Reserve there are many remote streams and rivers draining Volcán Melimoyu with poorly or uncharacterized fish communities. Many of these systems are suspected to contain a variety of invasive species, including brown trout and other salmonids that can adversely affect native species. This thesis therefore outlines how eDNA assays were developed and applied to begin to characterize the distribution of key fish species in rivers surrounding Volcán Melimoyu.

Knowledge of the range of invasive salmonids as well as identifying areas where they are coexisting with native species can inform how natural waterways are managed in Chile, and specifically within the Melimoyu Nature Reserve. For example, managers may be able to designate an area as protected, remove invasive fish, or put up semi-permeable fish barriers to protect native fish refugia (Minett et al., 2021). Identification of rivers that have not yet been invaded would also be vital to protection efforts as such systems are scarce (Sevulpida, 2013). Using qPCR detection of eDNA, the presence of three key species in rivers in and surrounding the Melimoyu Nature Reserve was assessed. Brown trout, as an established invader with high biomass in Patagonian waterways (Soto, 2006) are a species of concern as well as a good candidate for detection with eDNA. Filtered water samples were

also tested for puye (a native freshwater species), and Atlantic salmon (a potential invader).

Using the case study of Patagonian rivers in the vicinity of Volcán Melimoyu, this thesis provides insights into the challenges of eDNA surveys under less-than-ideal conditions and will provides recommendations to strengthen studies of this nature to ensure best practises are employed for remote eDNA studies.

Objective: Assess the challenges of and improve application of eDNA barcoding as a tool for use in remote areas using the Melimoyu Nature Reserve as a pilot study.

Hypotheses:

H_0: No qPCR amplification of brown trout, puye, or Atlantic salmon DNA will occur in samples from rivers draining from Volcán Melimoyu

H_0: qPCR detection of brown trout, puye and Atlantic salmon do not correspond to electrofishing detection of these species.

3. Methods

3.1. Site selection

Six rivers in the vicinity of Volcan Melimoyu were sampled for eDNA across two years (Figure 4). The Marchant and Colonos Rivers run through the Melimoyu Ecological Reserve. The Colonos river is spring fed and drains into the Marchant River. The Marchant River is glacially fed from Volcán Melimoyu and discharges into the ocean, and sites near the mouth are tidally influenced. In this study the Colonos was sampled at one location and the Marchant at three; the mouth, upstream of the Colonos confluence, and a third upstream site (descriptions in Table 2.). A pilot study was conducted at these sites in 2018, and in 2020 sampling was expanded to

include four additional rivers; the Añihue, Bahía Mala, Frutilla, and Santo Domingo Lagoon rivers (see Table 3).

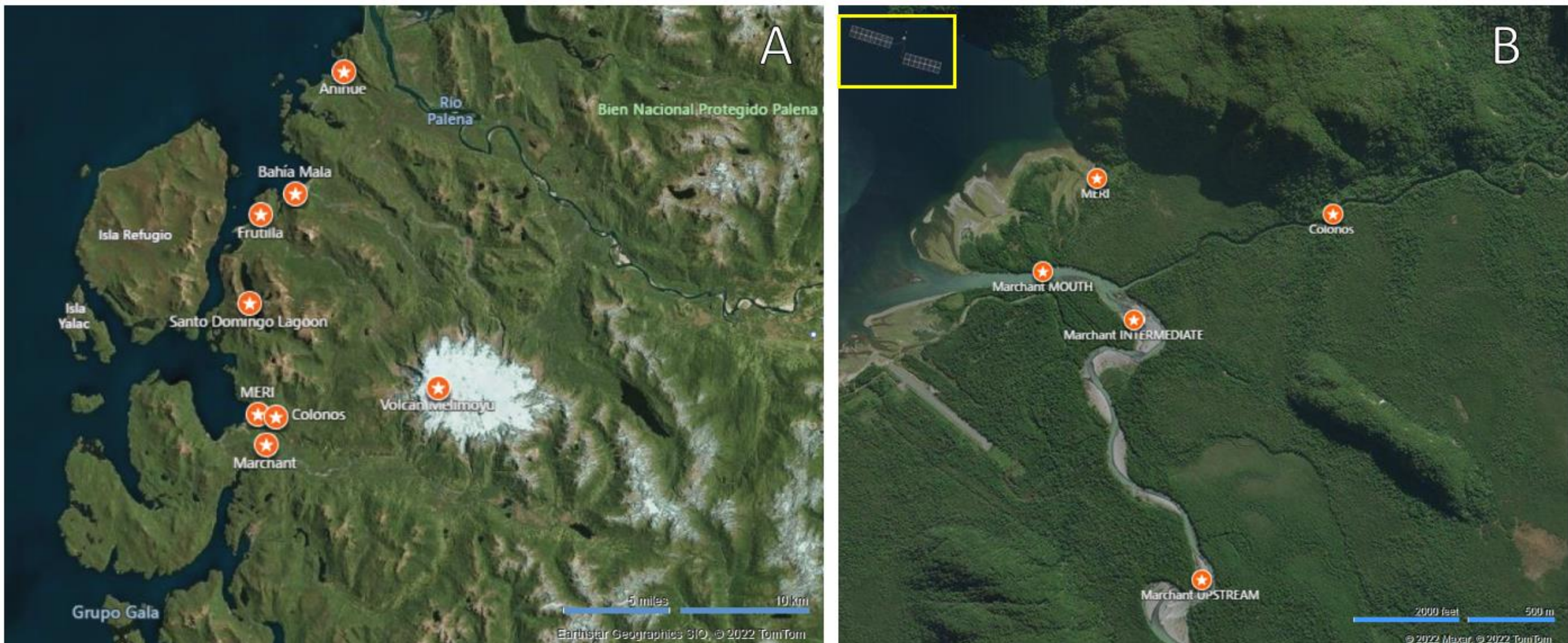

Figure 4. Map of eDNA sample collection sites surrounding Mount Melimoyu. All sampled rivers are labelled as well as the mountain itself (A). All rivers were sampled at one site, with the exception of the Marchant River where three sites were sampled (B). MERI indicates the location of the Melimoyu Nature Reserve field station. The yellow box encapsulates a nearby salmon aquaculture facility (Images from Bing Maps, 2022).

Table 2. Description of river sites sampled in 2018 and 2020 on the Melimoyu Nature Reserve.

Site	Description	Coordinates
Colonos	• Spring fed • Tree cover • River is wadable • Bed is mostly cobble with large rocks and branches providing fish habitat	S44°05.233' W073°04.024
Marchant MOUTH	• Downstream of confluence with Colonos • Heavy tidal influence and possible upwelling • River mostly gravel with little habitat heterogeneity	S44°05.418 W073°05.318
Marchant INTERMEDIATE	• Glacially fed • Tidally influenced • Approximately 500 m upstream of Marchant MOUTH site • Gravel and cobble bed • Large sandbars and debris throughout, not wadable, rapids and runs with few pools	S44°05.573 W073°04.911'
Marchant UPSTREAM	• Glacially fed • Minimal influence of tides • Approximately 2 km upstream of Marchant INTERMEDIATE site • Gravel and cobble bed • Large sandbars and debris throughout, not wadable, rapids and runs with some pools	S44°06.409' W073°04.612

Table 3. Additional rivers draining Volcán Melimoyu sampled for eDNA in 2020.

River	Description	Site Coordinates
Añihue	• Gravel substrate with some fish habitat • Emergent vegetation	S43°50.437' W072°59.967
Bahía Mala	• Cobble substrate • Tidal influence	S43°55.689 W073°02.843'
Frutilla	• Minimal submerged habitat • Cobble substrate • Many sand bars	S43°56.572' W073°04.904'
Santo Domingo Lagoon	• Feeds into a saltwater lagoon	44°0'23.42"S 73°5'33.00"W

3.2. Variations in 2018 sampling

Several modifications to sampling regime were made for 2020 sampling (Section 3.4). Section 3.2 outlines the protocols used to obtain samples in 2018 as well as PCR data for brown trout from these samples. This work was conducted as a pilot study by P. Marjan, and any details not described here were the same as those used in 2020 and are described in subsequent sections.

3.2.1. eDNA sample collection and processing

In October of 2018, two rivers were sampled for eDNA, the Colonos and the Marchant Rivers (led by P. Marjan). The Colonos river was electrofished two days before eDNA sample collection occurred, and the Marchant Upstream site shortly before sample collection. Two sites on the Colonos River and three on the Marchant River were sampled in triplicate using 1 L acid washed glass bottles (bottles were sterilized with 10-20% bleach with 20 minutes of contact time between uses). A field blank containing 1 L distilled water was also taken to each site and treated as the

sample bottles. Following electrofishing at the Marchant River six trout were added to a bucket containing 12 L of river water for 1 hour, then a 1 L water sample was collected for use as a positive control.

Samples were filtered using a 0.47 μM cellulose nitrate filter (changed to a 1 μM filter in 2020 due to slow filtration). Each filtered water sample was rolled using a new pair of nitrile gloves, placed into a separate sterile 5 mL tube, and frozen at -20°C. Filters were then transported to Santiago in a cooler with ice for storage at -20°C prior to transport to the University of Waterloo on dry ice via courier. Upon arrival, samples were stored at -20°C before being extracted using the DNeasy Blood and Tissue Kit (Qiagen, Cat. # 69504).

3.2.2. Brown trout PCR

The oligonucleotides used for brown trout eDNA detection in 2018 was sourced from Gustavsen et al (2015) but the protocol was modified for a 20 μL reaction volume. PCR was conducted using a TaqMan based assay with a FAM-labelled probe (Table 4.). Samples were plated along with a standard curve (10-fold serial dilution from 10 ng/ μL). Each well contained: 10 μL TaqPath ProAmp MasterMix (ThermoFisher, Cat # A30865), 0.18 μL (900 nM final concentration) forward and reverse primer, 0.05 μL probe (450 nM), 5.77 μL ultrapure water, and 4 μL eDNA extract (or brown trout DNA extract for standards). PCR was completed on a Biorad CFX instrument with the following cycling conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds then 60°C for 60 seconds.

3.3. Optimization of sample preservation methodology

A method for preserving eDNA filters via desiccation was validated prior to use for sampling in Patagonia in 2020. The method used for preservation in 2018 (freezing filters at -20°C) was compared to filter desiccation using silica desiccant beads across 4 time points (1, 2, 4, and 8 weeks post filtration). To compare these methods 24 x 1 L water samples were collected back-to-back from the bank of Washington Creek (a first order stream in Ontario, Canada). Six field blanks containing only ultrapure water were taken into the field and transported in coolers with sample bottles as well as handled and opened (for ~ 1 minute each) to detect any contamination introduced at the collection stage. Samples were then transported to the laboratory on ice in decontaminated coolers (20% bleach with 20-minute contact time).

Samples were filtered in the lab in a decontaminated fume hood less than two hours after sample collection using a peristaltic pump. The fume hood and materials were sterilized internally and externally with a 20% bleach solution (contact time ~20 minutes). Pump tubing was internally rinsed being rinsed with 500 mL deionized water before sample filtration. Filters were handled and cut in half using decontaminated forceps and scissors (soaked in 20% bleach for 20 minutes then flamed), then each filter half was randomly assigned to a treatment group. eDNA filters were either frozen at -20°C or desiccated in a coin envelope placed in a Ziploc bag containing self-indicating silica desiccant (ThermoFisher, Cat. # AA44389A1). Desiccated samples were subsequently added to an air-tight container and stored in the dark at ambient temperature (20 - 25°C). For each preservation method a subset of four samples and one blank were left for 1, 2, 4, and 8 weeks prior to DNA extraction from the filters using the DNeasy Blood and Tissue Kit (Qiagen, Cat. # 69504), although two different lots were used for DNA isolation. Once extractions

were complete the integrity of the samples was compared using the ePlant assay (see Section 4.2) and their DNA concentration was determined using a Qubit instrument and High Sensitivity dsDNA assay (ThermoFisher, Cat. # Q32851).

3.4. Sampling and analysis regime for 2020 Patagonian samples

3.4.1. Water sample collection

Water samples were collected during low tide prior to other research activities from approximately the middle of the water column. Each river site was sampled in triplicate back-to-back (within ~5 minutes) moving from downstream to upstream and covering a range of habitats. One litre samples were collected in one litre polypropylene bottles due to their low affinity for binding DNA (Hobbs et al., 2017). Before sample collection bottles were rinsed with river water. Bottles needing to be reused were sterilized between collections by filling with approximately 250 mL of a 30% bleach solution (P. Marjan used 10-20% in 2018 and found no contamination, the concentration was increased for the sake of caution) and inverted and shaken intermittently for 20 minutes, then rinsed three times with tap water and once with distilled water. At the Melimoyu station tap water comes from a spring that is unlikely to contain fish eDNA. To account for any contamination that could occur in the sample collection a field blank containing only distilled water was transported with the samples and was handled and opened once at the collection site for approximately one minute. Each site and sampling date had an independent field blank. Water samples were kept as cool as possible by placing on ice, if available, and/or in a cool location and were transported back to MERI field station in a cooler that had been sterilized with a 30% bleach solution. All samples were filtered (Section 3.4.4) within 8 hours of collection.

3.4.2. Positive control sample collection

Positive control samples for brown trout were collected to verify the efficacy of PCR methods on environmental samples confirmed to have contacted brown trout. In 2018, this was accomplished by filling a 20 L bucket with river water and stocked with 6 young-of-the-year trout. In 2020, two different positive control water samples were collected. The first from a cooler (45 L) filled with 10 brown trout after they were captured and transported. The second positive control sample was collected from a ~150 L tank filled with 7 adult and 3 juvenile brown trout. All water samples were collected from the vessels then processed (Sections 3.4.4 through 3.4.11) and analyzed (Section 3.4.12) using the same methods as river samples.

3.4.3. Site assessment and electrofishing

Water chemistry measurements were taken using a YSI multimeter probe immediately after water sample collection. Parameters recorded were pH, dissolved oxygen, temperature, conductivity, and salinity (see Appendix A). In particular, salinity was assessed to ensure fresh water was being sampled. GPS coordinates were also recorded at all sample collection sites (using a Garmin etrex 30 GPS) as well as observed information about river and habitat characteristics (e.g., substrate, tree cover, debris).

After water samples were collected, backpacking electrofishing was completed along the same stretch of river at two Marchant sites (2069.9 shocking seconds at Intermediate and an additional 3612.5 between Intermediate and Upstream), the Colonos site (1450.32 shocking seconds), and briefly in the Añihue (1028.3 shocking seconds). Electrofishing teams were required to stay near shore

due to high flow and were therefore unable to assess fish from all river habitats. Teams included a backpacker and two individuals with nets to collect shocked fish. Captured fish were measured and counted, and a subset of captured puye and brown trout (from the Colonos and Marchant Rivers) were retained and transported back to MERI in coolers containing river water. Captured fish were then measured, dissected, and preserved for other studies.

3.4.4. Filtration and preservation

Water samples were filtered directly from collection bottles using Smith-Root eDNA Filter Packs comprising of decontaminated housing and an internal filter with a pore size of 1 μm. A peristaltic pump was used to concentrate the water samples onto the filter. Prior to sample filtration all equipment including the table, holders, and peristaltic pump were cleaned with 30% bleach and tap water. All pump tubing was bleached externally and 500 mL of 30% bleach followed by 500 mL of distilled water were pumped for internal sterilization. Samples were inverted once approximately halfway through the filtration process to ensure all material was suspended evenly throughout the sample. Approximately 900 mL of each 1 L was filtered by the Smith Root filter assembly as the filter would sometimes clog or lose suction for the final 100 mL.

After the water samples were filtered, filters were folded in half using decontaminated forceps included in the Smith-Root filter set and inserted into a clean coin envelope (Staples® Envelopes Kraft Coin #1, Cat. # STP530164). All filters from a given site (including the blank) were then added to the same Ziploc® sandwich bag which was approximately 25% full of self-indicating silica desiccant (ThermoFisher Cat. # AA44389A1). More desiccant was added if more than half the desiccant present began to visually indicate it was saturated. Preserved samples in

Ziploc® bags were stored in in airtight containers. Several days after sampling was completed the filters were transported to Waterloo (Ontario, Canada) in personal checked luggage. They were then stored in a dark drawer at room temperature in airtight containers until DNA extraction (Section 3.4.5.1). Due to the COVID-19 pandemic, the preserved filters were stored as described for 157-178 days prior to extraction.

3.4.5. DNA extraction

3.4.5.1. Extraction from filter

All DNA extractions were completed in a decontaminated laminar flow hood, physically separated from where PCR amplification occurred. The laminar flow hood and all materials were wiped with 70% ethanol and left under a UV light for a minimum of 10 minutes. All forceps and scissors used to handle sample filters were sterilized with minimum 20% bleach for 20 minutes, rinsed with deionized water, subjected to UV sterilization, and flamed immediately before use.

Samples collected and processed by P. Marjan in 2018 were extracted using the DNeasy Blood and Tissue DNA extraction kit (Qiagen, Cat. # 69504). However, when this method was tested on several 2020 samples they were found to contain PCR inhibiting substances not removed during DNA extraction (samples amplified more than 1 C_t later than positive controls on average during inhibition tests (see Section 3.4.10)). Instead, the Sox Soil DNA Extraction Kit was used (Metagenom Bio, Cat. # 18011_S0). For both extraction methods filters were introduced to the kit by cutting half of the preserved filter into 10-12 small pieces then adding it to the first step of the extraction. For the rest of the extraction the standard instructions from the kits were followed (Metagenom Bio, n.d) resulting in 100 µL of DNA extract. The

final extract was aliquoted into 20 µL subsamples to avoid repeated thawing and stored at -20°C. Quantification of DNA in samples was attempted using a Qubit High Sensitivity dsDNA Assay, however samples were too dilute and fell below the limit of quantification (0.02 ng/µL).

3.4.5.2. Extraction from fish tissue

Fish DNA from voucher specimens was required for method development and standards. DNA was extracted from the following tissues: brown trout and Atlantic salmon muscle, puye tails (from individuals captured in the Marchant River), and zebrafish heads. Extractions were completed with the DNeasy Blood and Tissue DNA extraction kit following the standard kit instructions, and final extract was split into 20 µL aliquots and stored at -80°C.

3.4.6. Quantitative polymerase chain reaction (qPCR)

Assays that target mitochondrial DNA were used for detection of fish eDNA apart from the ePlant assay which targets chloroplast ribosomal RNA (Veldhoen et al., 2016). Assays for quality assurance were previously validated and optimized for eDNA analyses, as well as assays for brown trout and Atlantic salmon. An eDNA assay for puye could not be found and was therefore designed and validated in house (see Section 3.4.7.2). Summary of assay targets and sources can be found in Table 4. Custom oligonucleotides (TaqMan based qPCR assays using probes labelled with FAM dye on the 5' end and MGB on the 3') were sourced from ThermoFisher Scientific (Cat. # 4316034 and 4304970) except for the puye assay (5` FAM and 3`BHQ-1) which was ordered through Millipore Sigma (Cat. #

OLIGO). Supplementary information about PCR standard curves, plate layout, and reactions can be found in Appendix A.

Table 4. Summary of primers and probes used for various qPCR assays.

Assay	Locus	Amp. Length	Assay Sequences	LOD	% Eff.	Source
ePlant	Chloroplast 23S rRNA	147 bp	F: 5'-TCTAGGGATAACAGGCTGAT -3' R: 5'-TGAACCCAGCTCACGTAC -3' P: 5'-TTTGGCACCTCGATGTCGG-3'			Veldhoen et al., 2016
Internal Positive Control (Zebrafish /*Danio rerio*)	Cytochrome b (*cytb*)	298 bp	F: 5'-TGCGAAAAACACACCCAG-3' R: 5'-GGCAGATGAAGAAGAAGGAAG-3' P: 5'-CAATACACTACACCTCAGACATCT CAACAGCA-3'		99%	In house (Michael Lynch, Nathanael Harper)
Brown trout (*Salmo trutta*)	Cytochrome oxidase (*coI*)	61 bp	F: 5′-TTTTGTTTGGGCCGTGTTAGT-3′ R: 5′-TGCTAAAACAGGGAGGGAGAGT-3′ P: 5′-ACCGCCGTCCTCT-3′	10 copies	90.5%	Gustavson et al., 2015
Puye (*Galaxias maculatus*)	Cytochrome b (*cytb*)	86 bp	F: 5'-GCTTGGCCAGTCAGATCCTT-3' R: 5'-GTGGGTAACCGAGGAGAACG-3' P: 5'-ACGGGACTGTTCCTTG-3'	5 copies	104%	Present work
Atlantic salmon (*Salmo salar*)	Cytochrome oxidase (*coI*)	74 bp	F: 5'-CGCCCTAAGTCTCTTGATTCG A-3' R: 5'-CGTTATAAATTTGGTCATCTCCCA GA-3' P: 5'-AGA ACT CAG CCA GCC TG-3'	5 copies	98%	Atkinson et al., 2018

Amp. Length refers to length of PCR amplicon from a given assay. The approximate LOD (Limit of detection) represents the lowest number of copies where amplification occurred in all three technical replicates. % Eff. refers to PCR efficiency, derived from standard curves of whole DNA extract from fish tissue. F = Forward, R = Reverse, P = Probe.

3.4.7. qPCR assay generation and optimization

3.4.7.1. Brown trout

The assay used to detect brown trout in 2018 was sourced from a paper from Gustavson et al. (2015) but was optimized for a smaller reaction volume (15 µL

rather than 20 µL used in 2018 and 30 µL used in the original paper) for the 2020 samples to increase PCR replicates (from 3 used in 2018 to 7) while using less sample volume in each reaction. A comparison was completed for the 30 µL assay and the new 15 µL assay at two different concentrations (1000 and 10 DNA copies per well), a two tailed t-test showed no difference in amplification between the two reactions (no significant difference in C_t between the assays at 1000 ($p = 0.94$) and 10 ($p = 0.08$) copies per well). In 2018 the brown trout primers were tested on brook trout (*Salvelinus fontinalis*) and rainbow trout DNA (by P. Marjan) to ensure DNA of closely related species did not amplify and were further tested against Atlantic salmon and puye in 2020. To optimize the assay an array of primer and probe concentrations were evaluated. Maintaining the primer concentrations from the original source, but slightly decreasing probe concentrations (from 200 nM to 150 nM) was found to yield better PCR efficiency (90%) with a 10-point standard curve (Section 3.4.8). A gradient of PCR annealing temperatures (T_a) were also assessed, the highest efficiency was at T_a = 60°C, as indicated by Gustavsen (2015) (see 3.9.3 for final reaction).

3.4.7.2. Puye

Since no suitable primers targeting short sequences of mitochondrial DNA could be found in the literature for detecting puye eDNA new oligos were designed in-house. The mitochondrial cytochrome b locus was used since a survey of Genbank sequences indicated it was the only available sequence for all native and invasive fish that could occur in the sampling area (Table 1) and therefore the only way to validate the specificity of the primers and probe *in silico*. Although an Genbank reference sequence (refseq) is available for the *Galaxias maculatus* mitochondrial genome (NC_004594.1) it was not usable for Patagonian sampling since it varied from the cytochrome b of fish sampled and sequenced in Chilean

Patagonia (12.7% mismatch in cytochrome b). The source of this refseq sequence did not specify where the voucher fish was sourced (Ishiguro et al., 2003). Puye are one of the most widespread freshwater species in the world, occurring throughout the southern hemisphere (Figure 2). Waters (1999) indicated that there is up to 14% mismatch between individuals from different regions.

To generate primers a cytochrome b sequence was used from a puye individual found in Laguna Saval, Chile (NCBI: AF007026.1, Waters 1999). Five species specific primer sets were designed using NCBI Primer BLAST based on NCBI sequence AF007026.1. Selected primers were tested *in silico* to ensure their specificity using NCBI BLAST. The generated primers where subject to a BLAST search and returned no hits for any of the local Patagonian fish species or other galaxiids, only one species showed a match with both the forward and reverse primer (*Chanos chanos* with an 85% similarity from the chosen primer set, however, the range of the species does not extend to South America (Luna, 2021)).

Once the primers were designed, they were tested using a 5-point standard curve (10x serial dilution with 4 down to $4 \cdot 10^{-4}$ ng/µL puye tissue DNA extract) in the lab using a SYBR chemistry (PowerUp SYBR Green, ThermoFisher, Cat. # A25741) to determine both efficiency and specificity. The reaction contained 7.5 µL PowerUp SYBR Green MasterMix, 0.5 µL (333 nM) forward and reverse primer, 2.5 µL ultrapure water, 4 µL puye DNA extract. Cycling conditions were 95°C for 3 minutes then 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, followed by melt curve analysis (65°C to 95°C at 0.5°C/ 5 second increments). The primer pair with the efficiency closest to 100% (at 99.5%) and the highest relative fluorescence units (RFUs) were selected. The melt curve analysis revealed only one peak (at 85.5°C) for the primers, and they were determined to be amplifying only a single target of DNA.

Once primers were tested a probe was generated for the cytochrome b target using PrimerExpress software. The probe was designed to align with the beginning of the target sequence on the forward strand (starting on the second base pair). It was designed to have a T_m of 68°C, approximately 4°C higher than the primers. When the probe sequence was queried through NCBI BLAST it returned none of the fish species found in the region and did not match with *Chanos chanos* as the primers did. No species was found matching all three oligos *in silico*, this indicates that the assay is likely specific to *Galaxias maculatus,* but *in vitro* testing of other fish tissues would be required to be certain.

Like the brown trout assay (Section 3.4.7.1), the assay was optimized for the highest efficiency by using a standard curve and a range of primer and probe concentrations. Primer concentrations were optimized first and found to yield the best PCR efficiency at 300 nM. This concentration was then used with a range of probe concentrations. The best efficiency and highest ΔRn for the reaction occurred at a 400 nM probe concentration. An assay with a T_a of 60°C yielded acceptable efficiency (104%) and was consistent with the assays for brown trout and Atlantic salmon. The final assay was tested with brown trout and Atlantic salmon extract to ensure its specificity by subjecting DNA from the salmonid species to the puye primers and probe with the final assay outlined in Section 3.4.11.2. The limit of detection for the puye assay was determined as outlined in Section 3.4.9.

3.4.7.3. Atlantic salmon

The Atlantic salmon assay was derived from Atkinson et al. (2018) but slightly altered for a smaller reaction volume (15 µL) than the original source. The first trial of the 15 µL assay on the QuantStudio 5 instrument using the primer and probe concentrations from the source paper and cycling conditions of the brown trout

assay yielded high efficiency (98%) and no further trials were completed (see Section 3.4.11.3. for final assay). The assay was tested against brown trout and puye DNA and showed no amplification. The limit of detection was determined as outlined in Section 3.4.9.

3.4.8. Assay efficiency and standards

qPCR assay efficiencies for the three target species were calculated with the ThermoFisher Connect PCR Design and Analysis software based upon DNA concentrations plotted against their cycle of amplification. For all three fish assays a seven-point standard curve with a 10-fold serial dilution was used to assess efficiency starting with a quantified (Qubit High Sensitivity dsDNA Assay) standard of extracted fish DNA. Samples were run in triplicate with the reaction conditions outlined in Section 3.4.11. The target for efficiency was 100% but efficiencies between 90-110% were considered acceptable. Once an assay with acceptable efficiency was identified two standards were chosen to include on sample plates, one that amplified at approximately C_t 28, and one at C_t 32. Standard curve data can be found in Appendix A.

3.4.9. Assay sensitivity

To determine the sensitivity of fish PCR assays an approximate limit of detection (LOD) for each assay. Amplicon from a standard curve of fish DNA (Section 3.4.5.2) was retained, and its concentration quantified using a a Qubit High Sensitivity dsDNA Assay. The copy number in the amplicon was calculated using the

following equation, where X is the amount of amplicon in ng and N is the length of the amplicon (Prediger, 2017):

$$\text{number of copies (molecules)} = \frac{X \text{ ng} * 6.0221 \times 10^{23} \text{ molecules/mole}}{(N * 660 \text{ g/mole})^{\dagger} * 1 \times 10^{9} \text{ ng/g}} \quad \text{[Equ. 1]}$$

Amplicon standards were then diluted via a serial dilution from the highly concentrated amplicon to a 10^3 copies/µL working solution. The working solution was then further diluted to make standards, which were then again subject to qPCR in triplicate. The limit of detection for a qPCR assay is the concentration where amplification occurs 95% of the time or more (The dMIQE Group, 2020), since samples were run in triplicate the approximate LOD for these assays was the lowest concentration where all wells amplified. For example, for the brown trout assay the estimated LOD was determined to be 10 copies per well, although standards containing 5 and 1 copies per well did amplify, they only reliably did so in one or two technical replicates.

3.4.10. Sample quality assurance

Once samples were extracted, their quality was evaluated by testing for DNA degradation and PCR inhibition. Both assays are TaqMan based qPCR assays using probes labelled with FAM dye on the 5' end and MGB on the 3' (See Table 4 for sequences). The ePlant assay (Veldhoen et al., 2016) was used to determine the integrity of an eDNA sample using primers for genes coding for chloroplast ribosomal RNA. If the target sequence is successfully amplified before the 30th cycle, then the sample can be considered intact. Reactions for the ePlant assay contained 7.5 µL Environmental Mastermix 2.0 (ThermoFisher, Cat. # 4396838), 0.9 µL (900

nM) forward and reverse primer, 0.025 µL (250 nM) probe, 5.295 µL ultrapure water, and 2 µL of sample, yielding a total reaction volume of 15 µL.

To determine whether a sample contains PCR inhibitors, a sample aliquot from each collection site was pooled, then spiked with 0.2 ng of zebrafish (*Danio rerio*) DNA, a fish species not found in the sampled rivers (see Table 4 for oligonucleotides). Each well for the inhibition assay contained a 15 µL reaction: 7.5 µL TaqMan Environmental Mastermix 2.0 (ThermoFisher, Cat. # 4396838), 1.35 µL (900 nM) forward and reverse primer, 0.375 µL (250 nM) probe, 0.425 µL ultrapure water, and 2 µL of sample. Samples were run two ways on the same plate, 1) in triplicate spiked with 2 µL zebrafish DNA and 2) in duplicate with an extra 2 µL of ultrapure water to ensure there were no background levels of the target DNA. Spiked samples were run on the same plate as positive control samples containing only ultrapure water and the zebrafish DNA internal positive control (IPC) for later comparison. To be considered uninhibited samples containing the zebrafish IPC in an environmental sample were required to amplify no later than 1 C_t after the positive controls in ultrapure water. If the samples did amplify more than 1 C_t later than the controls, they were considered PCR inhibited (i.e., essential activities by endonucleases and polymerases in the PCR reaction were prevented from occurring (Tebbe & Vahjen, 1993)). ePlant and zebrafish assays were both run with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The quality assurance assays were completed using a Bio-Rad CFX instrument.

3.4.11. qPCR for species of interest

Fish PCR was completed using a QuantStudio 5 instrument. Each plate was run with 12 NTCs, and two standards in triplicate (aside from brown trout which

included four standards). Samples were run with seven technical replicates, and each plate included eight samples representing two sampling events (i.e., three biological replicates from a site and their respective field blank). To avoid contamination, samples and eight NTCs were plated and covered before standards and the remaining four NTCs were plated. Process blanks from DNA extractions were run in the same manner as environmental samples if amplification was observed in field blanks, however no process blanks amplified.

3.4.11.1. Brown trout

The final reaction for the brown trout assay contained 7.5 µL Environmental Mastermix 2.0, 0.3 µL (200 nM) forward and reverse primer, 0.225 µL (150 nM) probe, 4.675 ultrapure water, and 2 µL sample in each well (for a total reaction volume of 15 µL). PCR was completed with the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Two of the standards contained whole DNA extract from brown trout tissue (0.045 and 0.0045 ng/ µL), and two standards contained only copies of the target sequence from amplified tissue extract (10^4 and 10^3 copies). The standards were compared across plates and there was a smaller range in C_t values for the whole brown trout extract (range of 2.1 and 2.5 C_t respectively) than in the standards containing only the target sequence (range of 2.9 and 3.4 C_t respectively). As the amplicon standards were less reliable (and likely not representative of the condition of eDNA) only extract standards were used for puye and Atlantic salmon.

3.4.11.2. Puye

The qPCR assay for puye contained 7.5 Environmental Mastermix 2.0, 0.45 µL (300 nM) forward and reverse primer, 0.6 µL (400 nM) probe, 4 µL ultrapure water, and 2 µL sample in each well. PCR was completed using a QuantStudio 5 instrument with the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standards contained 0.015 and 0.0015 ng/µL of extracted puye DNA.

3.4.11.3. Atlantic salmon

The reaction for the Atlantic salmon assay contained 7.5 µL Environmental Mastermix 2.0, 0.3 µL (200 nM) forward and reverse primer, 0.225 µL (150 nM) probe, 4.675 ultrapure water, and 2 µL sample in each well (for a total reaction volume of 15 µL). PCR was completed using a QuantStudio 5 instrument with the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Two standards containing whole DNA extract from Atlantic salmon tissue were included on each plate (0.002 and 0.0002 ng/ µL).

3.4.12. Data analysis

Assay efficiencies and sample data were analyzed using the Thermo Fisher Connect Realtime qPCR software. C_t values and standard curve data were then exported for further analysis of species detections, DNA quantification, and assessment of contamination in Microsoft Excel, and occupancy analysis was

completed using R (R Core Team, 2021). Prior to data analysis a dichotomous criterion was determined for analyzing a positive “hit” in a sample where 4 out of 7 PCR replicates must amplify for a successful positive result. If at least one water sample out of three had successful amplification the site would be considered occupied. This analysis is a simple and conservative tool to determine whether there is strong evidence for species eDNA captured in a water sample. The 4/7 criteria was chosen based on the following probability calculation for non-mutually exclusive events (Cheung, 2022):

$$P(A \text{ or } B) = P(A) + P(B) - P(A \text{ and } B) \quad \text{[Equ. 2]}$$

Calculations were based upon the rationale outlined in Griffin et al. (2020) and generated from observed rates of false positives in field blanks (P(A)) and NTCs (P(B)) for each species. Based on these calculations it was possible to determine the probability of a result being a false positive based on the number of wells that amplify in PCR (Table 6). For brown trout and puye 4/7 wells amplifying in PCR provided a >99.9% probability of a true detection. Although this method is conservative, it is limited as it does not account for PCR hits across all samples from a site or other data that could influence the likelihood of eDNA being present in a sample (for example, whether a species of interest was previously observed at a site). Since this method of analysis is highly conservative with respect to limiting false positives, it could be prone to false negatives since it is unable to incorporate all data that could inform whether a species was truly detected in a sample.

To reduce false negatives while incorporating the rate of potential false positives a Bayesian occupancy modelling application was employed as well as the dichotomous evaluation. The model, based upon Griffin et al. (2020) and developed by Diana et al. (2020) specifically for eDNA analysis, uses a Markov Chain Monte Carlo method. The rShiny application generates posterior probabilities of species

occupancy at a site by incorporating rates of false positives at both the collection and PCR stage. It also allows for the inclusion of other predictor variables, such as habitat information or whether the species of interest had previously been documented at the site. The output of the application is a site-specific probability of species presence with a 95% credible interval. The analyses for the 2020 Patagonia samples employed 46000 iterations with 20 burn in iterations and 20 thinned iterations. The model incorporates rates of false positives at both the collection and PCR stages of eDNA analysis. The recommended model settings for both are set at a probability of 0.1, this was found to be more conservative than the rates observed in actual samples (i.e., amplification in field blanks and PCR NTCs) and were therefore kept for the analysis. Electrofishing capture data for the species of interest was also incorporated into the model as a binary value of either 0 or 1 depending on if the species were observed. Due to large credible intervals for some sites, the main determination of species presence at a site should be from the dichotomous evaluation outlined above, with the occupancy analysis serving to reinforce the findings and provide avenues for further research.

4. Results

Although site access was limited and some samples were found to be degraded, brown trout eDNA was successfully detected at seven sites across four rivers, and puye eDNA was detected with high certainty in one river. Puye and brown trout were also the main species captured with backpack electrofishing. Brown trout eDNA was detected at the most sites, including two sites where they were not electrofished, demonstrating the efficacy of the assay for species detection in remote systems. However, the lack of high certainty of puye detections at sites where they were found with electrofishing demonstrates a need for further improvements to sampling protocols to continue to control error in remote eDNA

surveys. Atlantic salmon were not found at any sites via electrofishing or eDNA analysis.

4.1. Method development and validation

4.1.1. Sample desiccation

Due to the relative instability of eDNA, filtered water samples need to be processed immediately or be preserved for later eDNA analysis. Since sampling occurred in a remote area of Patagonia, a preservation method was required that did not require dry ice or freezing, as it would not be readily available. Shipping frozen samples from remote Chile to Canada has been problematic in the past due to shipping restrictions such as availability of freezers and dry ice. As an alternative, several researchers (Bakker et al., 2017; Hobbs et al., 2017; Sales et al., 2019) have used desiccation methods instead of freezing to preserve eDNA samples prior to DNA extraction. A method for preserving eDNA filters via desiccation rather than freezing was therefore tested.

To evaluate the suitability of desiccation as a means to preserve eDNA integrity during transport and storage, water samples were collected from Washington Creek (a tributary of the Grand River in Ontario), then filtered samples were either frozen at -20°C or added to a coin envelope and stored in self-indicating silica desiccant then left for up to 8 weeks in storage. Desiccated filters were found to have equal (week 1 and 4) or greater eDNA yield (week 8) than frozen samples after being preserved (Figure 5), although some variation was observed, possibly due to changes in kit lot between extraction dates. The ePlant PCR assay was used to assess eDNA integrity (Veldhoen et al., 2016) of environmental samples from Washington Creek by testing them for relative levels of chloroplast ribosomal RNA.

Chloroplasts are ubiquitous in freshwater systems and chloroplast ribosomal RNA should be detectable in qPCR within the first 30 cycles if a sample is intact (Veldhoen et al., 2016). At 1, 2, 4, and 8 weeks after filtration, desiccated samples amplified at the same time or sooner than their frozen counterparts. All frozen and desiccated samples were considered intact as they amplified before $C_t = 30$, while blanks and NTCs amplified after the C_t 30 cutoff, indicating a low background level of chloroplast ribosomal RNA (Figure 6). Generally, results of the preservation test demonstrated that filter desiccation at ambient temperature was equally or more effective than freezing (up to 2 months) and therefore silica desiccant was used to preserve eDNA samples from Melimoyu in 2020 collections. The university closing due to Covid-19 prevented further analysis, such as the quantification of samples from week 2 and potential retesting of samples.

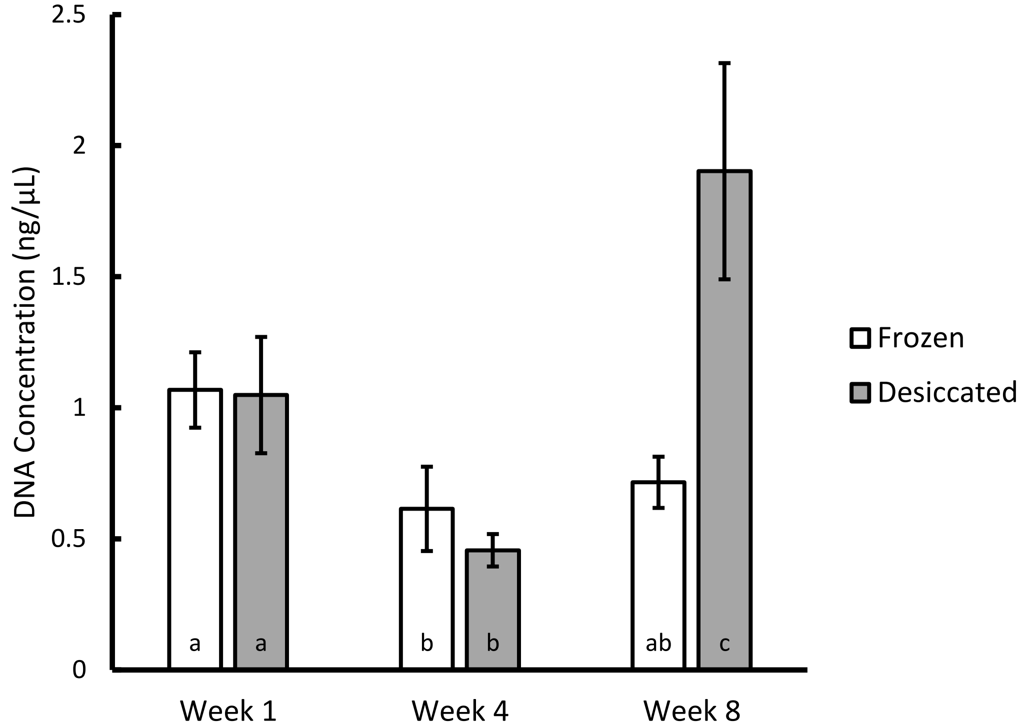

Figure 5. Concentration of DNA isolated from frozen or desiccated filtered water samples. Extracted DNA was quantified using a high sensitivity Qubit assay. Bars represent average DNA concentration (N= 4 processed filters); error bars show standard deviation. All blanks were below the LOD (0.2 ng/uL) and thus not included. A two-way ANOVA with a Holm-Sidak post-hoc was completed (significance of $P < 0.05$), which indicated statistically significant differences due to time ($P < 0.001$) and treatment ($P < 0.001$), as well as significant interaction between time and treatment ($P < 0.001$), letter labels differ between statistically distinct treatments.

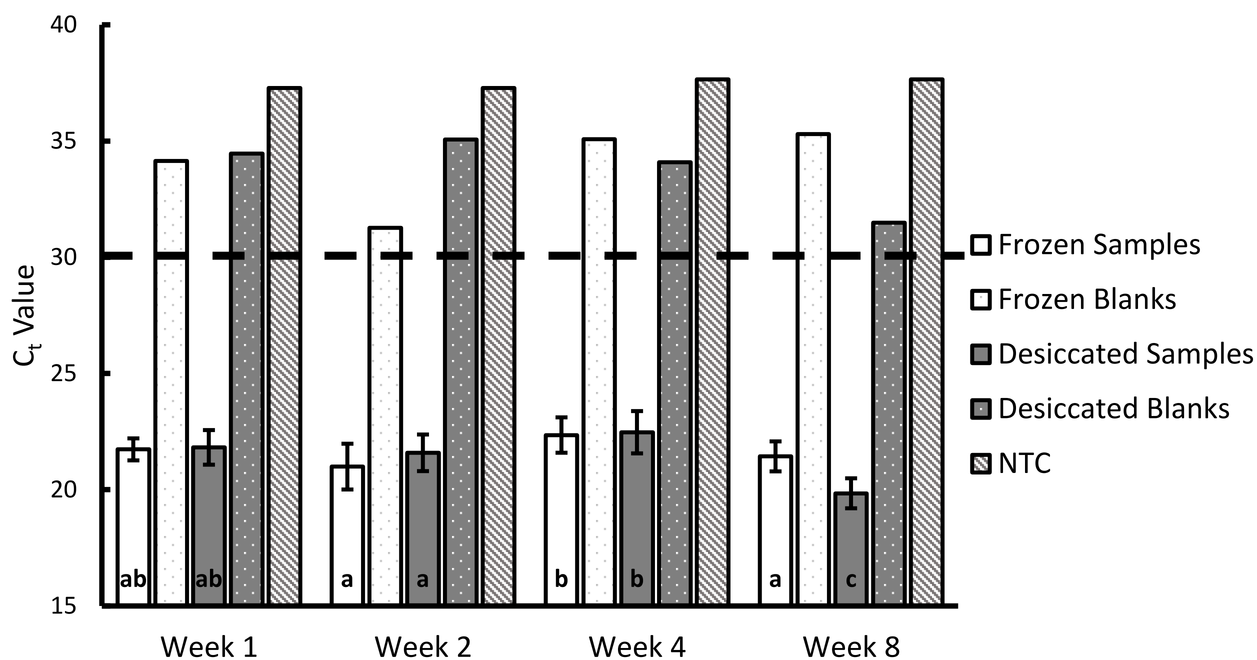

Figure 6. ePlant assay results for frozen and desiccated eDNA filters. Bars represent the average cycle of amplification (C_t) in PCR for 4 filter replicates with 3 technical replicates (error bars are standard deviation of the 4 filter replicates). Blank and NTC bars represent the average of 3 technical replicates. A two-way ANOVA with a Holm-Sidak post-hoc was completed on sample Cts (significance of $P < 0.05$), there were significant differences due to time ($P <0.001$) but not treatment ($P = 0.207$), as well as significant interaction between time and treatment ($P <0.001$). Dashed line shows the threshold (at C_t 30), samples amplifying sooner than C_t 30 are intact (i.e., all samples). Letter labels indicate statistically distinct groups.

4.1.2. Brown trout positive control samples

To verify the brown trout PCR assay on environmental samples known to contain the target DNA, brown trout were collected from the Marchant River, placed in a bucket, cooler, or tank, and after 1-1.5 hours water samples taken for analysis. Brown trout mitochondrial eDNA from positive control samples amplified in the chosen qPCR assay (Table 5) demonstrating that the assay was suitable for environmental samples.

Table 5. Water positive controls for brown trout cytochrome b eDNA detection held in river water for >1 hour.

Sample Source	Year	Water Volume	Trout Density	Average Ct	Standard Deviation
Bucket	2018	12 L	6	23.23	0.1
Tank	2020	~150 L	10 (3)	30.26	0.17
Cooler	2020	~30 L	10 (3)	26.31	0.2

For 2018 positive controls three PCR replicates were used per sample, and in 2020 seven PCR replicates were used per sample. Trout density represents the total number of individuals in the control and brackets indicate how many were juvenile.

4.2. Environmental sample quality determination

Prior to analyzing samples for the fish species of interest, the quality of the DNA extract was ascertained to avoid a false negative result for any samples that may be degraded or prevented from amplifying in qPCR due to the presence of PCR

inhibiting substances (e.g., humic acids, melanin (Tebbe & Vahjen, 1993)) or DNA degradation (Goldberg et al., 2016). DNA quantification was also attempted using the Qubit high-sensitivity dsDNA assay; however, all samples fell below the limit of detection (0.2 ng/µL). This indicates that the samples were dilute but does not necessarily signify that PCR would be unsuccessful.

Samples were tested for qPCR inhibition using an internal positive control of *Danio rerio* DNA. Only two PCR technical replicates amplified later than 1 C_t compared to positive controls (1/9 PCR technical replicates in pooled eDNA samples from the Marchant Mouth and Bahía Mala sites), however as the average and standard deviation fall below the 1 C_t mark the eDNA extract from these samples overall (as well as all other samples) were considered free of PCR inhibiting substances (Figure 7).

The ePlant assay was used for each environmental sample to determine DNA integrity. The ePlant PCR results (Figure 8) indicated that most samples were intact as they amplified before C_t = 30, however, samples from Marchant Upstream from January 27th 2020 and Frutilla from January 25th 2020 were found to be degraded as the average amplification from these samples (average C_t of 30.9 and 30.7 respectively) was later than C_t 30. Additionally, the samples from Colonos on January 23rd and Añihue on January 25th had an average C_t below 30 however some replicates amplified later than 30 indicating that the samples may have degraded but to a lesser degree. Degraded samples were still tested for fish eDNA, with the caveat that a negative result could be false negative. It is likely that some degradation of the samples occurred over time while held in the lab. Samples from Marchant Upstream (January 26) and Colonos (January 27) amplified earlier when they were extracted and analyzed March 2020 compared to July 2020 with a C_t difference of 6.2 and 4.6, respectively.

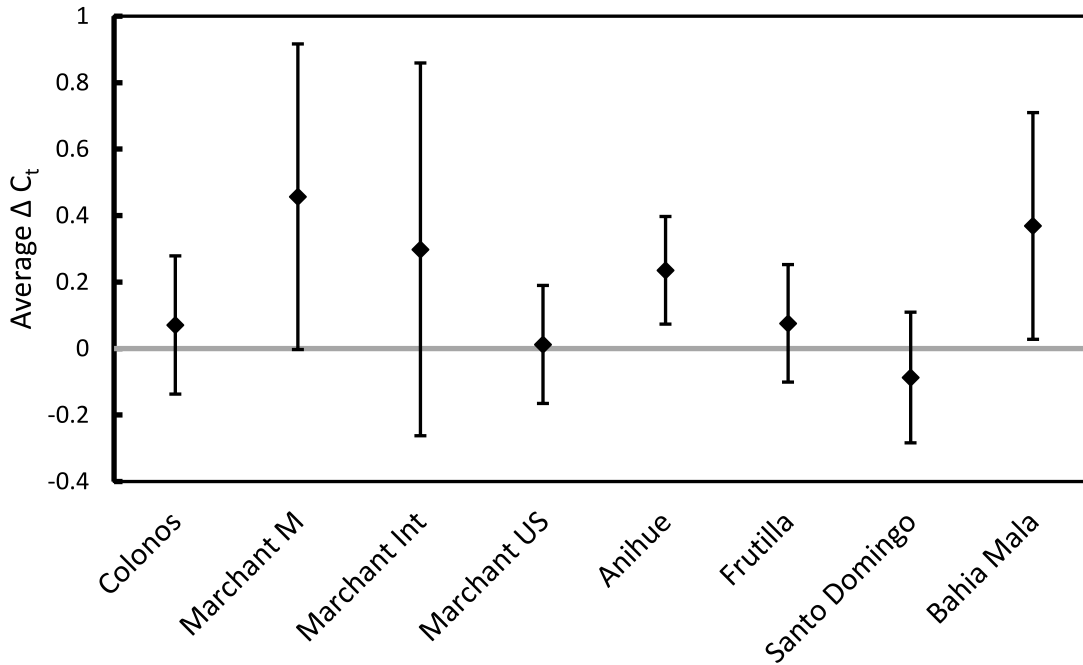

Figure 7. Internal positive control for PCR inhibition indicate samples do not contain PCR inhibiting substances. This figure displays the difference in average C_t between environmental samples spiked with an internal positive control and positive controls (ultrapure water) spiked with *Danio rerio* DNA. All samples from a given site were pooled prior to a single analysis where they were run in triplicate (aside from Marchant Mouth and Bahía Mala which were run separately with 9 PCR replicates). Error bars represent standard deviation.

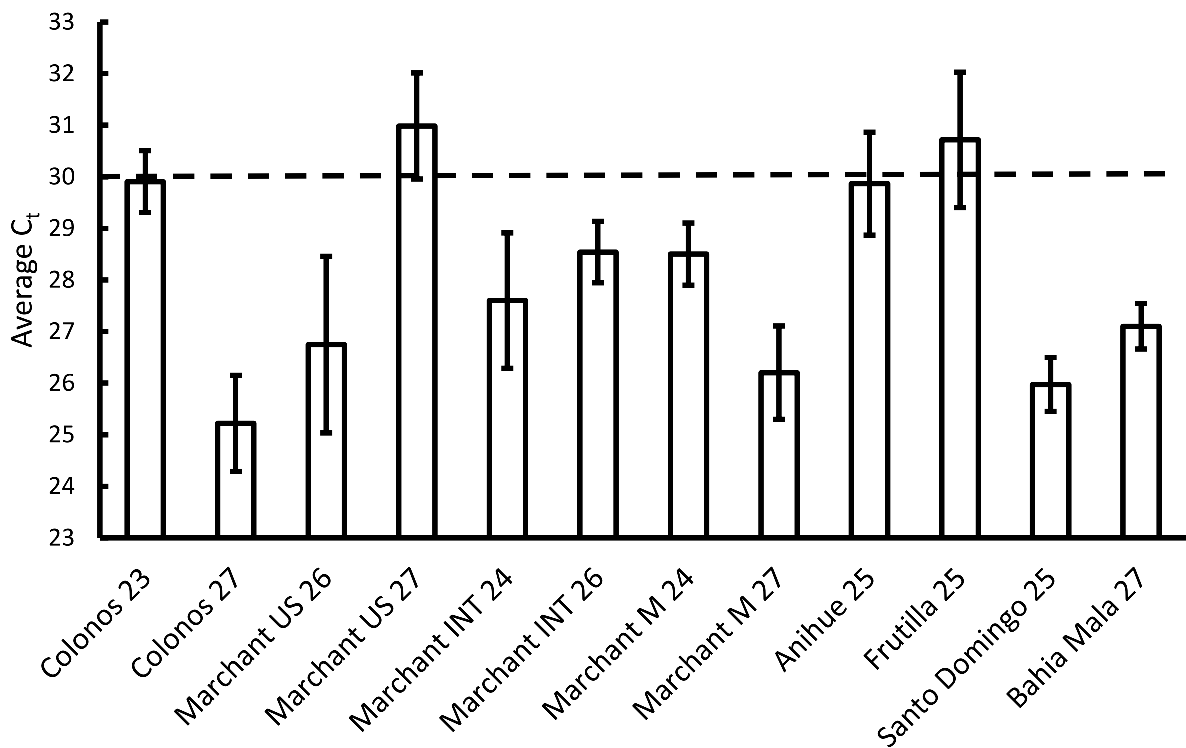

Figure 8. ePlant results for eDNA samples collected in January 2020. Site names are accompanied by the date of sample collection in January 2020. Bars represent average C_t for all samples from a collection (N= 3 water samples per site with 3 averaged PCR replicates per sample) ± standard deviation. The dashed line indicates the degradation threshold of C_t 30, samples amplifying sooner than C_t 30 contain intact DNA and samples later than C_t 30 are likely degraded.

4.3. qPCR results

4.3.1. Detection probabilities

True detection probabilities were calculated for brown trout and puye qPCR assays to quantify and account for the rates of false positives observed at both the collection and PCR stages of eDNA analysis. The selected threshold of 4/7 PCR replicates for a true hit is shown to be conservative for both the brown trout and puye assays with over a 99.99% chance of a true detection for both species at this threshold. Samples that had two or three qPCR replicates amplify out of seven are also reliable at slightly lower probabilities of a true detection (over 99% and 99.9% respectively). Rates of true detection could not be calculated for Atlantic salmon as no amplification was observed in field blanks or NTCs (Table 6).

Table 6. Rates of false positives (FP) and detection probabilities in PCR for brown trout (*Salmo trutta*), puye (*Galaxias maculatus*), and Atlantic salmon (*Salmo salar*).

			Brown trout	**Puye**	**Atlantic salmon**
Observed rates of FPs	Average Blank Amplification	Per sample (/7)	0.41	0.33	0
		%	6	4.7	0
	Average NTC Amplification	Per plate (/12)	0.5	0.33	0
		%	4.1	2.8	0
Detection probabilities	1 PCR Rep Amplifies	P (False positive)	0.098 (9.8%)	0.074 (7.4%)	
		P (True detection)	0.902 (90.2%)	0.926 (92.6%)	
	2 PCR Reps Amplify	P (False positive)	0.0095 (0.95%)	0.0054 (0.54%)	
		P (True detection)	0.9905 (99.05%)	0.9946 (99.46%)	
	3 PCR Reps Amplify	P (False positive)	0.00093 (0.093%)	0.00042 (0.042%)	
		P (True detection)	0.99907 (99.91%)	0.99958 (99.96%)	
	4 PCR Reps Amplify	P (False positive)	0.000091 (0.0091%)	0.00003 (0.003%)	
		P (True detection)	0.999909 (99.99%)	0.99997 (99.997%)	

This table summarizes the rate of amplification in field blanks and PCR non-template controls (NTCs). These values were used to calculate the overall probability of a false positive result (and true positive result) at different rates of sample amplification. False positive rates could not be calculated for Atlantic salmon as there was no observed false positive results.

4.3.2. eDNA in river samples

The analysis of brown trout, puye and Atlantic salmon eDNA in river water was completed to evaluate their presence in rivers draining from Volcán Melimoyu in 2018 and 2020 (Table 7). Overall, brown trout were successfully detected at six sites with a high degree of certainty including two sites where they were not previously captured with electrofishing (Table 8), and puye at one site with high certainty and five additional sites with a lower degree of certainty. eDNA from Atlantic salmon eDNA was not detected at any sites (Table 7). eDNA results for each species are outlined in Sections 4.3.4 – 4.3.6

Table 7. Summary of qPCR results from environmental DNA samples in 2018 and 2020.

Sample		Brown trout			Puye			Atlantic salmon		
River Site	**Collection Date (M/D/Y)**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Colonos Upstream	10/19/18	9 (/9)			ND			ND		
Colonos	10/19/18	9 (/9)			6			0		
	1/23/20	0	4	4	1	3	3	0	0	0
	1/27/20	0	1	4	2	6	5	0	0	0
Marchant Mouth	10/21/18	9 (/9)			1			0		
	1/24/20	2	0	2	1	0	1	0	0	0
	1/27/20	4	0	1	2	1	0	0 (/5)	0 (/5)	0 (/5)
Marchant Intermediate	10/20/18	9 (/9)			2			0		
	1/24/20	1	2	4	2	0	1	0	0	0
	1/26/20	2	2	3	1	1	1	0	0	0
Marchant Upstream	10/20/18	9 (/9)			3			0		
	1/26/20	1	1	0	0	0	1	0	1	0
	1/27/20	0	0	0	2	0	0	0	0	0
Añihue	1/25/20	4	2	1	2	2	1	0	0	0
Santo Domingo Lagoon	1/25/20	0	0	0	0	0	0	0	0	0
Frutilla	1/25/20	0	0	0	0	1	2	0	0	0
Bahía Mala	1/27/20	1	2	4	0	1	1	0 (/5)	0 (/5)	0 (/5)

eDNA barcoding was completed for brown trout (*Salmo trutta*), puye (*Galaxias maculatus*), and Atlantic salmon (*Salmo salar*). Table values represent the number of PCR replicates that amplified (out of 7 unless otherwise specified). Samples highlighted in blue have 4 or more PCR replicates with successful amplification and should be considered a positive detection. Samples highlighted in yellow or green have evidence for species presence (2-3 successful PCR replicates out of 7). Letters refer to the sample replicate from each site. Samples from 2018 were pooled for analysis for puye.

Table 8. Comparison of electrofishing and eDNA evidence for species presence in sites sampled in January of 2020.

River Site	Brown trout		Puye		Atlantic salmon	
	Electrofish	**eDNA**	**Electrofish**	**eDNA**	**Electrofish**	**eDNA**
Colonos	✓	✓	✓	✓	**X**	**X**
Marchant Mouth	**ND****	✓	✓	**X**	**ND**	**X**
Marchant Intermediate	✓	✓	✓	**X**	**X**	**X**
Marchant Upstream	✓	**X***	✓	**X***	**X**	**X***
Añihue	**X**	✓	✓	**X**	**X**	**X**
Santo Domingo Lagoon	**ND**	**X**	**ND**	**X**	**ND**	**X**
Frutilla	**ND**	**X***	**ND**	**X***	**ND**	**X***
Bahía Mala	**ND**	✓	**ND**	**X**	**ND**	**X**

Positive PCR results are compared to electrofishing capture data for sites sampled in January 2020. Several sites had detections with both electrofishing and eDNA (dark blue shading), and in some cases there was eDNA evidence for species presence, but they were not collected by electrofishing (light blue shading). At several sites fish were captured but not identified with high confidence in eDNA analysis (red shading). Yellow shaded boxes indicate situations where no eDNA was found and species presence was not otherwise verified with electrofishing. *Samples were degraded and could represent a false negative for eDNA results. **Site was not directly electro-fished but upstream sites on the same river were.

4.3.3. Occupancy analysis

Probability of species presence at each sampled site was calculated using a Bayesian occupancy model. Estimates generated by the model correspond to electrofishing and eDNA data, however the credible interval for most predictions was between a probability of 0 and 1 (Figure 9). Despite the high error in some combinations of sites and species the model identified four sites where brown trout are likely present (all three Marchant sites and the Colonos), and five sites where puye are likely present (all three Marchant sites, Colonos, and the Añihue).

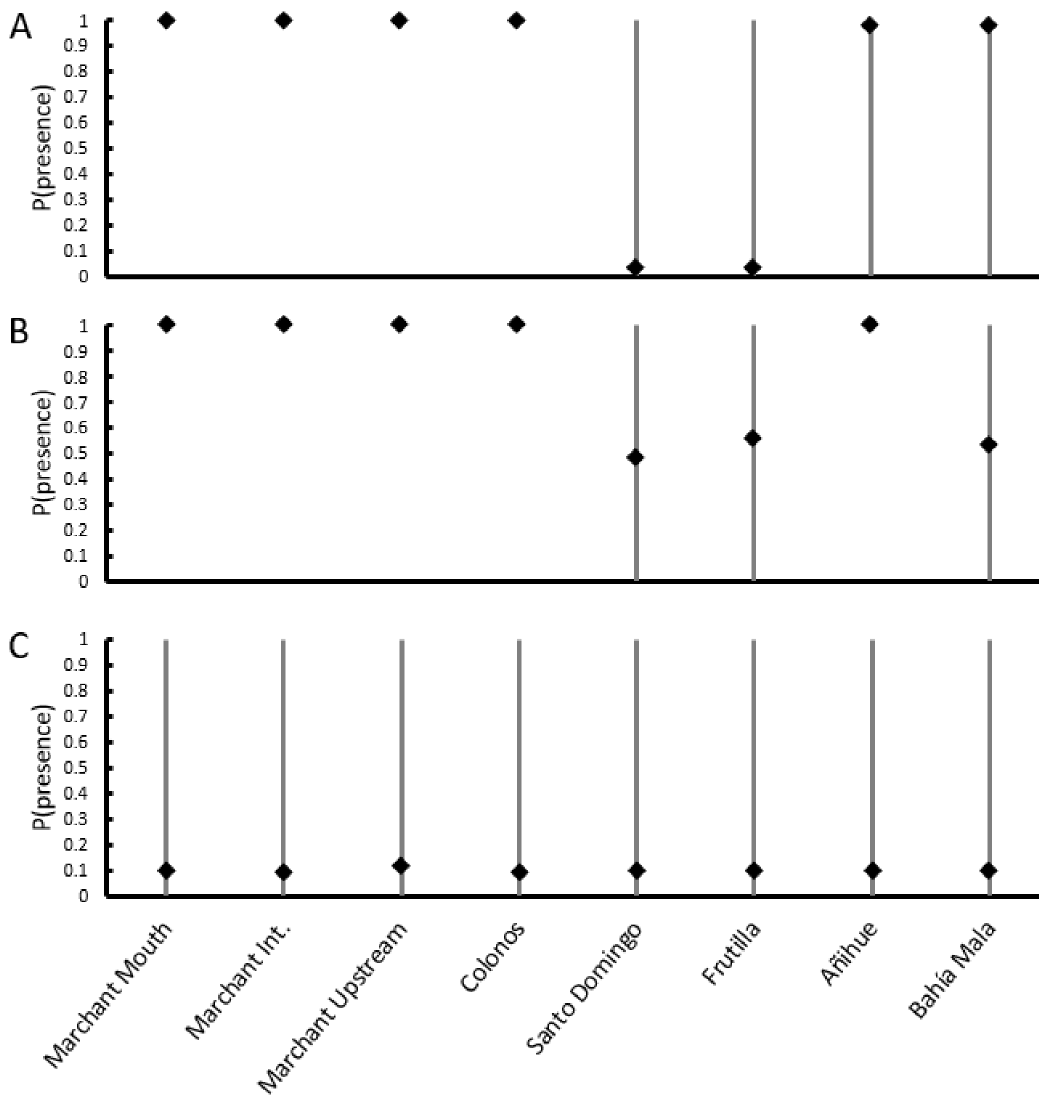

Figure 9. Occupancy analysis for species of interest in January 2020. Figure points represent average posterior probabilities of species presence for (A) brown trout (*Salmo trutta)*, (B) puye (*Galaxias maculatus)*, and (C) Atlantic salmon (*Salmo salar)* in six rivers draining Volcán Melimoyu (Chile). Grey bars represent the 95% credible interval. Posterior probabilities (P) were generated with a Markov chain Monte Carlo with 46,000 iterations, 20 burn in iterations, 1 chain, and 20 thinned iterations. Probability of false positives was set at 0.1 (as recommended in the model). Analysis was completed using the eDNAShiny application presented in Diana et al. (2020).

4.3.4. Brown trout

The samples from the Colonos and Marchant Rivers collected in 2018 and 2020 show eDNA evidence for brown trout presence. eDNA detection for brown trout was successful at all sites in 2018 (Table 7). In 2020 sampling, there were positive detections of brown trout in the Colonos and two sites along the Marchant (Mouth and Intermediate sites, see Table 7). Marchant Upstream had little to no brown trout amplification despite brown trout being captured at the site via electrofishing (Table 8), possibly because several samples from the site were found to be degraded (Figure 8). Despite the lack of eDNA amplification, the occupancy analysis model indicated a mean posterior probability (P_{Pr}) of 1 for presence of brown trout at this site in 2020 (Figure 9), likely due to the model incorporating visual observations of the species at the site as well as eDNA detection in the analysis.

Of the additional sites sampled in January 2020 there were positive results for brown trout in the Añihue and Bahía Mala rivers (further supported by the occupancy analysis with $P_{Pr} = 0.98$ for both). There was no brown trout amplification in the Frutilla or Santo Domingo Lagoon samples ($P_{Pr} = 0.03$ for both), which is expected as the Frutilla samples were found to be degraded and the Santo Domingo sample was collected in brackish water instead of fresh water (i.e., in the lagoon not the river), making eDNA detection of freshwater trout unlikely.

4.3.5. Puye

In both 2018 and 2020 puye DNA was detected in water samples from the Colonos but not the Marchant River although both rivers have puye populations. Puye were successfully collected using electrofishing at two sites on the

Marchant (Intermediate and Upstream), as well as the the Colonos, and the Añihue in 2020. However, only the Colonos had positive PCR detection of puye DNA in collected water samples based on the binary criterion of 4/7 positive PCR replicates (Table 7). Despite few PCR hits for puye, occupancy analysis for 2020 determined they were likely present in the Colonos, Marchant, and Añihue (likely due to incorporation of electrofishing data, $P_{Pr} = 1$ for all). Despite few or no successful amplifications, occupancy analysis determined that there was possible puye presence in the Santo Domingo Lagoon ($P_{Pr} = 0.48$), Frutilla ($P_{Pr} = 0.56$), and Bahía Mala ($P_{Pr} = 0.54$) rivers, but with a high degree of error.

4.3.6. Atlantic salmon

Atlantic salmon were not collected by electrofishing at any sites in 2018 or 2020 (Table 8). There was only one successful PCR amplification in a Marchant Upstream sample from 2020, this sample was also found to be degraded meaning the source of the Atlantic salmon DNA was unlikely to be the river water and more probably from contamination during PCR. The occupancy analysis for Atlantic salmon indicated that the species likely does not occupy the sampled rivers ($0.095 \le P_{Pr} \le 0.11$ for all sites, see Figure 9), although the credible intervals for the Atlantic salmon analysis encapsulate 0 and 1 at all sites.

5. Discussion

The results of this study serve to demonstrate the potential of eDNA surveys in remote system as they provide evidence for brown trout and puye in previously unsampled rivers in Chilean Patagonia. However, the eDNA detection of the three species of interest was not equal and best practices must be identified to reduce uncertainty and improve sensitivity for these eDNA assays. Brown trout were successfully detected with high certainty using eDNA in four rivers (seven sites), two of which (Añihue and Bahía Mala) were not previously documented to be occupied by the species. For the native puye there was less success with eDNA analysis as the species was detected with high certainty in only one river despite having been captured at several other sites by electrofishing. Lastly, Atlantic salmon DNA was not detected in any of the sampled rivers. Although the detection success for the species differs, they each provide insight into how eDNA analysis for these species, and generally in remote areas such as Melimoyu, could be improved.

5.1. eDNA performance

For eDNA surveys to be useful in monitoring aquatic systems they must be equally or more sensitive and feasible compared to current methodologies (e.g., backpack electrofishing). When comparing eDNA results to electrofishing success it appears that at times eDNA analysis was effective at determining species presence, either detecting a species with high certainty when it was not captured with electrofishing (e.g., brown trout in the Añihue) or detecting a species where electrofishing could not be completed (e.g., brown trout in the Bahía Mala). However, in other instances fish were captured with electrofishing but eDNA detection was unsuccessful or uncertain (e.g., puye in the Marchant). Similarly,

Minett et al., (2021) were able to detect brown trout in Falkland Islands rivers where they had not previously been sampled but failed to detect brown trout and the less abundant zebra trout in several rivers they were known to previously occupy. In remote rivers such as those in Patagonia it may be possible to limit and quantify false positives with respect to these sampled systems, however since the communities of several sampled rivers are unstudied it is difficult to know when a false negative has arisen. Although a positive detection with eDNA does provide valuable information for the fisheries management and inform the design of future studies, a negative result is harder to interpret.

If several of the potential causes of false negatives in this study are addressed (e.g., sample volume, sample location), then a more robust eDNA survey of these rivers would be possible. For example, Minett et al. (2021) attributed their false negative results to low filtration volume. Increasing the volume of water filtered could increase the chances of capturing DNA that is present in low abundance and increase the concentration of the sample overall resulting in a better chance of amplification in PCR analysis. Similarly, increasing the number of biological replicates collected at each site to cover a greater variety of habitat would likely increase the possibility of having a positive sample if the species is present (Goldberg et al., 2016). It is important to recognize that there is always a tradeoff between the benefits of increased replication and the effort and cost of collecting and processing more samples (Buxton et al., 2021). An informed sampling design is critical to finding the balance between costs and scientific rigor.

Another possible source of false negatives is degradation that can occur during the preparation, transport, and storage of the samples before analysis. The desiccation method for preservation was validated for storage up to 2 months after sampling. Unfortunately, because of the onset of the Covid-19 pandemic the samples were left in the lab for 5-6 months before being extracted (i.e., the university

labs were not accessible during the lockdown period). Although such delays are not likely a concern for future studies, an improved method of preserving samples in the field should be investigated to ensure that researchers who are unable to process their samples quickly could avoid sample degradation. Improving this preservation method, using an alternative method (e.g., freezing, chemical), or completing extractions swiftly after sampling would mitigate potential degradation and therefore reduce the chances of a false negative result (see Section 5.5.1).

5.2. Design considerations in eDNA barcoding assays

Several aspects of the barcoding assays used in the Patagonian eDNA surveys were vital to quantifying contamination and controlling error. For example, ensuring that there are blanks incorporated at all steps in the eDNA pipeline (i.e., sample collection, DNA extraction, PCR) can aid in identifying the level and source of any contamination in the eDNA samples. Field blanks should always be taken and handled while samples are being collected. In this study, one blank was used per site to account for contamination during collection of samples. PCR amplification was observed in several field blanks, and while unfortunate, can allow for the identification of where contaminant DNA was introduced to samples as well as provides an estimate of any background levels of the target DNA. This was vital when completing remote sampling as the conditions were not guaranteed to be sterile or free of fish DNA. Similarly, for each DNA extraction that was completed an extraction blank was also included, although no contamination was found at the extraction stage. Amplification in field blanks indicated rates of false positives at the collection stage of the eDNA surveys which was used to determine the appropriate thresholds for positive eDNA detection. A similar approach was used in qPCR where there were 12 NTCs (non-template controls) included on each sample plate to pick up any contamination introduced during the plating process. This was essential to

determining the rate at which false positives emerged in the PCR process. Another strategy for PCR was to maximize the chances of species detection while preserving as much sample as possible by optimizing PCR assays to require less sample (2 μL) to allow for increased PCR replication without sacrificing limited eDNA extract or assay sensitivity. Overall, the inclusion of additional NTCs in PCR analysis informs rates of false positives and increasing sample replicates reduces chances of false negatives.

An important consideration that arose during this study was the importance of understanding genetic diversity that may occur in target species. As puye are widespread and genetically diverse (Torres & Bailly, 2021; Waters & Burridge, 1999) complications arose when attempting to design primers and probes for the species based on mitochondrial sequences not originating in Patagonia. Ideally, species-specific primers should only amplify the target species but also be general enough to amplify all populations of the species (Wilcox et al., 2015). This can usually be achieved by targeting mitochondrial genes which are highly conserved within species (Valentini et al., 2016), however since puye populations vary in their mitogenome and behaviour (i.e., diadromous puye can be distinct from land-locked populations (Carrea et al., 2013)) across their range even within South America (Carrea et al., 2013; Macchi et al., 2007; Waters & Burridge, 1999) Although the estimated limit of detection for the puye PCR assay is low (5 copies per well), it is possible that the primers and probe selected contain one or several mismatches with the puye at the study sites. Puye vary considerably in their cytochrome b between regions (up to 3.8% within South America (Waters & Burridge, 1999)). Although primers and probes were selected based on a sequence sourced from Laguna Saval, Chile (NCBI: AF007026.1, Waters 1999), it is possible that even within Chile there is variation in the puye cytochrome b gene sequence. This could be investigated by sequencing the mitochondrial DNA of individuals captured in the vicinity of Volcán Melimoyu. By ensuring there are no mismatches between the PCR

oligos and the puye population found at Melimoyu the assay could reduce the chances of missing puye eDNA even if it is present as lack of primer and probe specificity can contribute to false negative results (Burian et al., 2021). To generate an assay that is appropriate for all puye haplotypes within Patagonia (or globally) more individuals would need to be captured and their mitochondrial genome sequenced. An understanding of puye distribution and genetic diversity was essential to generating a functional eDNA assay, and these factors should be considered when developing assays for future eDNA studies.

5.3. Fish occupancy

Understanding the distribution of native and invasive fish species such as brown trout can allow managers and conservationists to make educated decisions about how to best protect freshwater systems. Despite non-optimal sampling and analysis regimes, eDNA barcoding was shown to effectively detect brown trout at seven sites and puye at one site with a high degree of certainty. Generally, PCR detection for brown trout was more successful than that for puye, despite puye being captured with electrofishing at several sites where eDNA amplification for the species was unsuccessful. This can likely be attributed to the relatively low biomass of the small bodied puye in systems where brown trout have invaded (trout can make up to 95% of biomass in Patagonian freshwater systems (Soto et al., 2006)). Combined with the dilute nature of the collected water samples, the lower detection of puye with eDNA may be due to low amount of DNA released into the environment (e.g., water). Understanding factors that influence eDNA detection success can further improve these methods for use in biomonitoring.

eDNA detection success for puye and brown trout varied both spatially and temporally. Generally, there were more successful PCR amplifications from samples

collected in 2018 compared to 2020. This could be a result of seasonal differences. Although no flow measurements were taken, based on visual observations it was evident that river flows were higher in January of 2020 than October of 2018. This could contribute to the dilute nature of 2020 samples, explaining in part why there was less success detecting eDNA from brown trout in these samples compared to the 2018 ones taken from the same sites. The water was also milky in appearance in 2020, likely as a result of fine particles (glacial flour or rock flour) in the water during the summer when the Melimoyu glacier is contributing meltwater to rivers (such as the Marchant, Appendix B). Glacial flour results from cycles of freezing and erosion causing the weathering of bedrock and transport of materials (e.g., iron, silicon, organic carbon, various colloidal nanoparticles) downstream to the ocean (Hopwood et al., 2014; Pryer et al., 2020; Vargas et al., 2018). The suspended glacial flower is typically carried downstream to low salinity estuarine water where it settles out from the water column (Hopwood et al., 2014). Glacier flower in Patagonia is largely composed of soluble iron, silicic acid, and various colloidal nanoparticles that may also contain iron, silica, or aluminum (Pryer et al., 2020). Depending on the surface charge of a metal nanoparticle it may adsorb proteins (Pfeiffer et al., 2014) or oligonucleotides (Abbasian et al., 2014). Silicic acid and aluminosilicates are both materials found in Patagonian glacial flour (Pryer et al., 2020) that carry a surface charge (Perrott, 1977) and therefore have the potential to adsorb oligonucleotides as nanoparticles with a small negative charge have been shown to have an affinity for bonding oligonucleotides (although the length and base content of the nucleotide also influences adsorption)(Abbasian et al., 2014). If there are higher amounts of suspended solids (particularly charged ones) in the column in the spring and summer there is the potential for free oligonucleotides in the water column to adsorb to the suspended particles and be swiftly removed from the water column with the glacial flour, thus contributing to lower amounts of eDNA in the water column and therefore collected water samples. Therefore, the presence of glacial flour or other

suspended solids combined with increased flows may have contributed to the dilute nature of the 2020 eDNA samples.

Both environmental and methodological differences may have influenced PCR success. Differences in sampling protocol such as filters used to concentrate water samples, sample bottles used, and PCR reaction volumes may also partially explain the differences between 2018 and 2020 eDNA results, however, differences in PCR amplification of brown trout were also observed within the 2020 samples between sampling dates and replicates from the same sites. For example, at the Marchant Mouth site there was a positive detection of brown trout DNA in a water sample collected on January 27th, but not earlier on the 24th. Since the same method was used for both sampling events the differing success can likely be attributed to environmental variations rather than methodological ones. Of the January 27th water subsamples, one had amplification in four PCR replicates, while the other two water sample replicates had only one and two successful amplifications. Inconsistencies in PCR replication are to be expected in samples with low concentrations of eDNA (Goldberg et al., 2016), however it also demonstrates the need for increased spatial and temporal sampling for eDNA studies to limit possibility of a false negative (Mathieu et al., 2020) as well as ensuring the appropriate amount of water is filtered to capture the target DNA since river processes can cause eDNA to be removed from the water swiftly due to dilution, degradation, transport, and deposition (Thalinger et al., 2021). When target eDNA is rare it can be easy to miss when sampling and increasing the volume of water sampled reduces chances of a false negative result.

Understanding the life cycle of a target species is also important to limit false negatives due to inappropriate sample locations. For example, in the samples collected from Santo Domingo Lagoon we were unable to reach fresh water and samples were collected in brackish water. Therefore, when no brown trout or puye

were detected in the sample it is difficult to discern whether there were indeed no trout in the river, when it is more likely that the water sample collected was taken at an inappropriate location to capture trout eDNA and has thus resulted in a potential false negative. Although trout DNA from upstream may be captured at any point downstream, if flow is high the chances of capturing DNA successfully may be lower depending on proximity to the target fish (Jane et al., 2015). Four of the rivers were only sampled at the mouth (near where it drains into the Pacific Ocean) and are therefore influenced by the tides which could contribute to sample dilution. These would also not be ideal habitat for the trout who prefer upstream reaches (Penaluna et al., 2009) and the DNA captured would likely be arising from further upstream. However, as the upstream reaches are inaccessible, the eDNA collected at the mouth, during low tide can suggest the presence of the target species upstream as eDNA can travel considerable distances when flows are high (Jane et al., 2015). For example, researchers were able to detect *Daphnia longispina* using eDNA 20 km downstream of the lake they were inhabiting (Cristescu & Hebert, 2018), and brown trout 6 km downstream of an aquaculture facility (Deutschmann et al., 2019). While eDNA from upstream reaches may be detected by sampling only the river mouth, there are many factors that may have removed the eDNA from the water (Thalinger et al., 2021), and it would therefore be prudent to sample a variety of habitats suitable to the target species to avoid potential false negatives due to inappropriate collection sites.

The numerous environmental factors influencing detection of fish eDNA in these systems makes it challenging to definitively determine the distribution of the three target species. Mitigating factors such as flow, sampling regime, and degradation make it difficult to determine whether lack of an eDNA amplification means that the area is indeed unoccupied by the target species, or if the DNA was just not successfully captured and amplified if present. Looking holistically at the

habitat as well as all presence data from both eDNA studies and electrofishing should inform whether a site is determined to be occupied.

In the case of Atlantic salmon, although there was a single PCR replicate that amplified at the Marchant upstream site, the weight of evidence indicates that the sampled sites do not contain Atlantic salmon, although puye eDNA was also not detected at all sites where puye were collected by electrofishing which indicates false negative results are possible. However, Atlantic salmon have not been collected from any of the study sites in the current or past studies of Melimoyu (G. Chiang, personal correspondence). Taking positive control samples near the aquaculture pen in the bay may have helped to verify the detection method could detect the target DNA in environmental samples to improve certainty of the negative PCR results from river samples. Atlantic salmon are expected to travel upstream to reproduce in the fall (Bardonnet et al., 2011; US Fish and Wildlife Service, n.d.). Since samples were collected in the spring (October 2018) and summer (January 2020) adult salmon would be spending their time in saltwater at the times the rivers were sampled. MERI was concerned that possible adult escapees from aquaculture facilities may be moving into the rivers, however, the low abundance of these adults would likely be difficult to detect. Unless there was a resident population of juvenile Atlantic salmon living in the sampled rivers, success with eDNA sampling would be unlikely, especially during the non-spawning season. However, the lack of any positive PCR detections (with a low LOD at 5 copies), combined with lack of success capturing Atlantic salmon during any of the studies of the Marchant and Colonos rivers, provides some evidence that these rivers may not have been invaded by Atlantic salmon, or at least do not contain a resident population.

The sites selected in the current study were greatly limited by access to the upper reaches of the rivers. Although it is well documented that eDNA can move considerable distances downstream it does dissipate as it is diluted and degraded by

environmental processes (Cristescu & Hebert, 2018; Jane et al., 2015). Most of the sites that were accessible during this study were close to the mouths of the rivers where habitat may not have been optimal for the species of interest. Although there was an intention to follow up the preliminary studies and move further upstream and sample in additional sites and seasons, the Covid-19 pandemic eliminated all travel to the site. Future studies should include consideration of fish life cycle, habitat, and assess both spatial and temporal variability to understand species distribution. In addition, consideration of season/flow and ability to collect fish, as well as eDNA (i.e., water) samples, reliably should be included. Collections during low flow may be beneficial for eDNA detection, but logistical considerations in remote locations limit what can be realistically done.

5.4. Analysis methods

Although there is no universally accepted criteria for a positive eDNA result (Goldberg et al., 2016) in many eDNA analyses a site is considered occupied or positive if as low as one PCR replicate amplifies (Goldberg et al., 2016; Minamoto et al., 2012), although most require at least two or more PCR detections for a positive result (Ficetola et al., 2015). However, there are potential sources of error or uncertainty that must be considered when determining an appropriate threshold. Since samples for this study were collected and filtered in potentially contaminated conditions it was important to be conservative with the chosen threshold and ensure that potential sources of false positives (i.e., contamination) were captured and quantified using blanks at all stages of analysis. The rate of amplification in negative controls informed the criteria for a positive detection. Four of seven PCR replicates amplifying for a given sample provides a high degree of certainty (99.99%) that a sample is a true positive and not due to contamination. However, samples with 2 or 3 successful amplifications should not be considered negative as they are likely

positive as well, just with a lower degree of certainty (see Table 7). Again, the inclusion of good controls for both negative (i.e., blanks) and positive (i.e., known positive samples) detections is critical in these studies.

Interpretation of eDNA results requires careful consideration of possible sources of error in eDNA sampling and analysis. This is one of the reasons many eDNA researchers are beginning to use modelling techniques such as Bayesian occupancy analysis as it accounts for imperfect eDNA detection (Schmidt et al., 2013). Although occupancy analysis of eDNA results for Melimoyu was attempted using the eDNAShiny application (Diana et al., 2020), the main metric used to determine whether a site had a “hit” was a dichotomous threshold of four PCR replicates amplifying out of seven. The lack of supplementary information and low number of replicates to add to the occupancy model resulted in very large credible intervals for the probability of species presence at most sites. Generally, for Bayesian occupancy analyses eDNA data should be just one part of the model and should be informed by supplementary data (Goldberg et al., 2016; Griffin et al., 2020) as a lack of covariates can lead to imprecise estimates of occupancy and error (Buxton et al., 2021). Measuring and incorporating other supplementary information, such as historical presence data, habitat information (e.g., substrate, average flow, dissolved oxygen levels), or presence of prey species (e.g., puye) could better inform the model for more precise occupancy predictions for a given species. Certainty in occupancy modelling may also be improved by increasing sites and replication at the collection stage, as increasing just PCR replicates is not typically beneficial in modelling beyond six replicates (Buxton et al., 2021). Despite the limited data in the current study, occupancy models appear to be a good approach for future studies, especially if additional information can be collected.

5.5. Lessons for remote eDNA surveys

Optimizing the sampling regime used for the species of interest could result in more detections of rare eDNA in water samples (Goldberg et al., 2016). Based on the eDNA surveys at Melimoyu it is worth investigating ways to improve the sensitivity of the surveys and reduce false negative or positive detections. There are three main alterations to the sampling regime that could improve survey results: 1) an improved sample preservation method, 2) concentrating a greater volume of sample, and 3) optimize the time and location of sampling. Investigating and improving survey methods will inform best practises for sampling species of interest in remote areas such as Melimoyu.

5.5.1. Improved sample preservation

Effectively preserving eDNA filters is important to avoid type II errors and allow retroactive tests for additional species of interest down the line (Hobbs et al., 2017). Desiccating eDNA filters via self-indicating silica desiccant is a newer preservation method still requiring additional validation (Hobbs et al., 2017), but has been employed in eDNA studies as an effective standalone method or combined with freezing (Bakker et al., 2017; Hobbs et al., 2017). Although in the current study the desiccation method of preservation for eDNA filters was tested for up to 2 months, some of the eDNA on the filters sampled from Melimoyu was found to be degraded after being left for 5.5-6 months before extraction. Since the final DNA extract of several samples contained degraded DNA, it is clear that the preservation method was not suitable for the unplanned (i.e., COVID-19 lockdown) extended time before extraction and therefore improvements or alternatives should be investigated.

The 2018 eDNA samples from Melimoyu were preserved via freezing, and although it is difficult to compare the two methods directly as the time frame and sampling regime differed, there was greater eDNA yield in the 2018 samples. In 2020, samples were desiccated instead of frozen since it was deemed risky to transport and ship frozen samples when access to freezers and dry ice were limited during sampling and shipping. It may have helped if the desiccated samples had been held in the freezer (when possible) rather than room temperature, but this needs to be tested. If freezing samples is not feasible and desiccation ineffective over longer time periods, it may be prudent to preserve filtered samples in 95-100% ethanol after sampling (Hobbs et al., 2017), however, ethanol could also be problematic to use due to dangerous goods regulations for transportation and potential evaporation (Cooper et al., 2021). An alternative chemical preservation method using Longmire's solution was proposed by Cooper et al. (2021) as Longmire's is not considered hazardous for transport, potentially making it useful for remote work. It was also found to result in a better eDNA yield than ethanol preserved samples, although, Cooper et al. also indicated that more investigation is required into how the solution may interact with DNA extraction kits. Another alternative could be to preserve water samples prior to filtration either by freezing or by adding a cationic surfactant preservative (Sales et al., 2019), but this would require transport of large volumes of water rather than filters creating different complications. As there is not yet a best practise for filter preservation in difficult-to-access sampling locations, the most prudent solution may be to continue the use of silica desiccant (or desiccation then freezing), but ensure DNA extractions are completed as soon as possible after sampling as extracted DNA can remain stable for years if frozen (Hobbs et al., 2017).

5.5.2. Concentrate a greater volume of sample

Improved eDNA detection in remote Patagonian systems could possibly be achieved by increasing the volume of water filtered to capture eDNA. The sample volume should be chosen based on the sampled water body and species of interest (Goldberg et al., 2016). In sampling at Melimoyu, 1 L water samples were taken and filtered in 2018 and the volume allowed for reasonable detection of brown trout, although in 2020 the samples were less concentrated (resulting in low DNA concentrations and therefore higher C_t values). This was likely due to the higher flows experienced in spring/summer due to snow and glacial melt (Pascual et al., 2007). Increasing the volume of water filtered would allow more eDNA to be captured on the filter, potentially reducing the risk of false negatives. However, while this amendment appears simple it would not necessarily be easy to implement. Filtering even 1 L samples took considerable time due to clogging of the filters, so there is a major trade-off.

Due to the remote nature of the rivers at Melimoyu samples were often transported by backpack or portaging, and even the 4 L of water sampled (3 replicates and a blank) was difficult at times to transport and keep cool before it was filtered at MERI. Increasing the volume of water taken (and potentially sampling farther upstream) would make a difficult task even harder, therefore alternatives to transporting water should be evaluated. For example, it is possible to filter river water directly with a portable battery powered pump (followed by immediate preservation) rather than transporting water for later filtration. Although transporting a pump could still be difficult it may be easier than transporting large volumes of water that also need to be kept cool. However, if the sample is difficult to filter it may not be feasible to filter the samples at the site (i.e., time, logistics, safety, etc.).

Optimizing the sample volume is another example of trade offs required in remote eDNA studies between logistics and feasibility concerns and the quality of eDNA samples.

5.5.3. Optimize the time and location of sampling

The temporal and spatial design of an eDNA survey is vital to determining eDNA barcoding success. If the design is not appropriate for the aims of the survey, bias and uncertainty can be introduced (Goldberg et al., 2016; Mathieu et al., 2020). For eDNA barcoding the timing of sample collection should be specific to the species of interest to ensure the greatest chance of capturing its DNA if present. This is an important consideration for diadromous fish (such as coastal puye (Macchi et al., 2007) and Atlantic salmon (US Fish and Wildlife Service, n.d)), which may spend part of their life cycle in the sea rather than the rivers of interest. Detections could be missed altogether by eDNA surveys if the rivers are sampled at the wrong location or time. For both barcoding and metabarcoding (Section 5.6.2) increasing the temporal distribution of samples reduces the risk of missing the true presence or diversity of species of interest (Mathieu et al., 2020). With respect to sampling at Melimoyu it would be prudent to complete additional sampling in different seasons to detect changes in populations and ensure that no species of interest are missed due to experimental design. Similarly, species could be missed in eDNA surveys if the sample is taken in an inappropriate location. Although river water will carry suspended cells and free DNA downstream, the processes affecting eDNA residence time and suspension in the water is not well understood (Cristescu & Hebert, 2018; Jane et al., 2015). This means that sampling at a river mouth does not guarantee that species upstream will be detected. For example, brown trout were detected at the mouth of the Añihue and Bahía Mala Rivers, but not at the mouth of the Frutilla or Santo Domingo Lagoon Rivers. In this case it is not certain that the

Frutilla and Santo Domingo Lagoon Rivers do not contain brown trout in their upstream reaches, just that no eDNA was captured in the water samples taken.

To avoid the potential for false negatives in eDNA barcoding the sample should be taken at or slightly downstream of a location that would be likely to contain the target species (i.e., appropriate location and habitat within the river) (Goldberg et al., 2016). If multiple species are being surveyed through barcoding or metabarcoding it would be ideal to sample many locations throughout the river encapsulating different habitats to maximize the potential to capture rare eDNA. In Patagonia native species can occur with very low biomass in rivers (Soto et al., 2006), and if they are the target of future eDNA surveys the sampling should be augmented to ensure none of their eDNA is not missed during sampling as it may be rare in the river water. The methods used to collect fish for traditional surveys are habitat, species and life-stage dependent (Portt et al., 2006), and this logic should be applied when completing eDNA surveys as well.

Sample collections at the mouth of a river may be influenced by the interactions between the river and ocean salt water. Marine studies for eDNA are generally more difficult due to dilution and salinity (Cristescu & Hebert, 2018). Complications due to dilution and salinity may also arise in estuarine zones such as at mouth of a river that is tidally influenced. Although in this study salinity was measured to ensure samples were taken from fresh water, samples could still be influenced by tidal movements. Increased salinity can also increase the removal of suspended solids (Hopwood et al., 2014). eDNA can adsorb to suspended solids or sediment (Cristescu & Hebert, 2018) and is therefore potentially removed from the water column in estuarine areas (i.e., the river mouth). This could make the river mouth a repository for environmental DNA, potentially containing relic DNA, and if the sediment is disturbed by the tides (i.e., upwelling) there is potential for eDNA

collected in tidally influenced locations to contain relic DNA from the river and catchment (Cristescu & Hebert, 2018), and/or DNA from marine species.

In the current study, the sites were sampled during low tide to maximize the potential that eDNA from upstream freshwater habitats were included in the sample. However, the location of the upstream fish habitat and fate of the potential eDNA was unknown. Logistical considerations in the current study limited the sampling access to the upstream reaches of the rivers. Despite these limitations brown trout and puye eDNA were detected in several sites near the river mouth and suggests that eDNA detection may be viable tool for detection of various fish species in these rivers. Future studies should carefully consider the habitats of target species and try to overcome the limitations of upstream access. However, these sites are extremely remote with essentially no available access via boat or vehicles. Even access to the river mouths was difficult and took considerable logistic support.

5.6.Future directions

Beyond the recommended amendments to the existing eDNA sampling protocols used at Melimoyu there are many avenues of research that could be pursued to gain further understanding of the fish populations in the rivers draining from Volcán Melimoyu. There are three main avenues that would improve the utility and quality of eDNA data in this study system: 1) expand barcoding to test for additional species of interest, 2) use eDNA metabarcoding for entire community composition, and 3) collect and incorporate supplementary information for the rivers of interest to improve occupancy analysis.

5.6.1. Expand barcoding to additional species

For this study three target species were chosen to barcode that were of interest in the vicinity of Volcán Melimoyu; brown trout, puye, and Atlantic salmon. However, Patagonian rivers also contain many imperiled and endemic species that would also be of interest to managers, as well as several additional exotic salmonid species (Dudgeon et al., 2006; Soto et al., 2006). Based on currently available sequencing data (Table 1) it would be feasible to identify or generate an eDNA barcoding assay for any of the species in Patagonian rivers (such as was completed for puye in this study). Expanding surveys to additional species one at a time would be a time-consuming endeavor, however if only a few more species are of immediate interest, it could be a practical way to identify them in hard to access rivers. Assays for new species could also be retroactively applied to preserved eDNA extract from previous sampling occasions (Hobbs et al., 2017). One species that may be of management interest are rainbow trout as they are a prolific invader in north Patagonia and they have a high potential to cause harm by establishing naturalized populations (Sepúlveda et al., 2013). Like brown trout, rainbow trout have the potential to have impacts on native fish communities through predation, competition, and pathogen transfer, and their presence can also exacerbate the negative effects of other stressors on native fish (Cussac et al., 2016; Sepúlveda et al., 2013). As brown trout are not the only potential invader in these rivers, the presence of other likely invasive species should be determined to effectively manage and protect the native fish communities in Patagonian rivers.

5.6.2. Use metabarcoding for entire community composition

Rather than individually detecting each fish species with barcoding, eDNA metabarcoding could be used to determine the community composition within the rivers draining from Volcán Melimoyu. Metabarcoding would require the identification (or development) of universal fish primers that would allow DNA from all present fish species to amplify for identification via high-throughput sequencing (Taberlet et al., 2018; Valentini et al., 2016). Metabarcoding could be a useful tool to identify community compositions within uncharacterized rivers as no *a priori* information is required (Taberlet et al., 2012). However, a metabarcoding approach requires a suitable reference library of sequences in order to match eDNA sequences from water samples to known species (Taberlet et al., 2018). Of the fish species found in central Patagonia (Table 1) very few have their complete mitochondrial genome sequenced. With only the cytochrome b locus available for all relevant species it could be difficult to identify a suitable primer set for eDNA metabarcoding. If more species had their complete mitochondrial genome sequenced metabarcoding the entire river fish communities may be more efficient than individually targeting each species of interest. Ideally, local individuals should be captured and sequenced for this purpose as there may be genetic differences in the mitochondria of individuals sourced from other locations (e.g., puye (Waters & Burridge, 1999)). As it stands currently, all invasive salmonid species have their mitochondrial genome fully sequenced, and universal primers could be used to identify the distribution of exotic species in the area with metabarcoding.

5.6.3. Incorporate supplementary information on rivers

In remote systems, sample and data collection can be difficult, however the inclusion of any supplementary information about a system could improve occupancy modelling for species of interest. Bayesian occupancy analysis allows for the incorporation of any relevant categorical or continuous covariate parameters that may influence the likelihood of a species being present at a site (Griffin et al., 2020). For example, habitat and water quality data could be incorporated into the model along with presence/absence data (e.g., from eDNA or electrofishing surveys) to holistically determine the probability of a species inhabiting a given river. Occupancy models account for sources of potential error in their estimates (Schmidt et al., 2013) which would make them a powerful tool for assessing remote areas where results may be uncertain and there is a lack of prior data. Ideally, analysis should be improved by increasing the number of eDNA samples and incorporating environmental covariates (Schmidt et al., 2013).

For logistical reasons discussed previously, it may not be feasible to collect detailed supplemental information from remote river sites, however, it may be possible to collect some basic supplementary data that would inform the occupancy modelling of species of interest. For example, Griffin et al. (2020) found that pond depth and length, as well as the presence of macrophytes and fish were all useful covariates that informed the occupancy analysis of great crested newt in English ponds. Similar parameters could be used to inform occupancy modelling of Patagonian fish, for example, native freshwater fish species are more likely to occupy streams with fine sediment, high conductivity, and low brown trout density (Soto et al., 2006), while trout prefer streams with course sediment, habitat heterogeneity, and holding pools (Hendry et al., 2003). Chalde & Llompart (2021) found that presence of woody debris in the Lapataia River (Tierra del Fuego) was a predictor of brown trout presence, and related puye presence to riparian

macrophytes. Visual observations of riparian vegetation and submerged debris could be easily recorded when collecting eDNA water samples and included in the occupancy analysis of target species. Even traditional fish surveys, such as electrofishing surveys, can result in both false positive (e.g., a captured fish is misidentified) and false negative (e.g., a fish species is present but not captured) errors, and planning for and incorporating occupancy modelling could minimize these sources of error when providing presence estimates (Buxton et al., 2021).

6. Conclusions

Although this study was exploratory in nature, it outlines how eDNA detection methods could be an effective tool for studying remote and difficult to sample systems. If fine-tuned and applied appropriately considerable information could be made available that could inform environmental/resource managers. There are several major findings and recommendations to improve the methods used here for future studies in remote areas such as Melimoyu.

6.1. eDNA methods can elucidate the distribution of fish in remote rivers.

Although the collected samples were imperfect, it was still possible to detect native (puye) and invasive (brown trout) fish species from environmental samples, providing new information about previously uncharacterized rivers and fish populations in Rivers draining from Volcán Melimoyu.

6.2. Optimizing eDNA sampling regimes could increase fidelity of eDNA data.

Several steps could be taken at the collection stage of eDNA analysis that would result in a higher quality DNA extract, and thus a more accurate survey. Concentrating (i.e., filtering) more water per sample would increase the mass of DNA in the final sample and reduce the risks of false negatives. Avoiding sample degradation with an improved or alternate preservation technique would leave more intact DNA for analysis. Increasing the temporal (e.g., multiple seasons) and spatial (e.g., various river habitats) distribution of sampling (although potentially difficult) could also improve eDNA detection. However, each of these approaches have analytical and logistical limitations. Increasing understanding of how different environmental factors influence shedding, transport, and persistence of eDNA in rivers and therefore its detection in water samples would allow for less ambiguous interpretation of eDNA data.

6.3. Occupancy analysis is a useful and powerful tool to interpret eDNA results if used appropriately.

eDNA researchers are increasingly exploring Bayesian occupancy analyses as a tool for interpreting eDNA results. This should be considered at the onset of future studies to ensure the appropriate number of replicates are taken, and supplementary data can be collected and added to the model. Increasing covariates in occupancy analysis can increase precision and allows for a holistic but objective method of determining the likelihood of species presence in a water body.

6.4. Additional barcoding and application of metabarcoding could improve fish surveys in Patagonian rivers.

Many of the rivers draining from Volcán Melimoyu are uncharacterized, and while the confirmed presence of several species may offer insights, it would be more helpful to managers if the entire fish community could be ascertained. Understanding the composition and diversity of the community could allow for more effective environmental management. Future eDNA studies at Melimoyu should aim to use a metabarcoding approach to capture the full diversity of fish in these rivers. This would require ensuring that an appropriate quantity of water was filtered so that the resultant extract contains enough DNA for metabarcode analysis. It would also require an appropriate reference library be assembled as not all species native to the area have their mitochondrial genome completely sequenced.

6.5. eDNA could be a powerful bioassessment tool for remote areas

Understanding of the fish communities in remote rivers in Patagonia and other remote regions of the globe is critical for environmental protection and management. The Melimoyu Nature Reserve and surrounding national park land needs to be protected as stressors such as pollution, climate change, and invasive species represent significant risks to the local freshwater ecology, but basic information about the rivers and the fish populations is lacking. Traditional approaches to monitoring of fish communities are very difficult in remote and/or inaccessible ecosystems. Emerging tools such as eDNA detection methods, once optimized and validated may provide a tool for obtaining basic information about fish communities to support environmental protection.

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Appendix A
Supplementary Data

Supplementary Table 1. Summary of standard curves used to determine PCR efficiency.

Assay	↑ Std Conc	Slope	Y-Intercept	R^2	Efficiency
Brown trout	31.3 ng/µL	-3.572	17.027	0.999	90.5%
Puye	1.5 ng/µL	-3.34	22.265	0.997	104%
Atlantic Salmon	22 ng/µL	-3.369	19.541	0.999	98%

Each seven-point standard curve contained a 10-fold serial dilution beginning with the concentration listed as ↑ Std Conc.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="3">Standard 1</td><td rowspan="3">Standard 2</td><td></td><td></td><td colspan="7">Sample 1 A</td><td rowspan="8">NTC</td></tr>
<tr><td>B</td><td></td><td></td><td colspan="7">Sample 1 B</td></tr>
<tr><td>C</td><td></td><td></td><td colspan="7">Sample 1 C</td></tr>
<tr><td>D</td><td colspan="2" rowspan="2">NTC</td><td></td><td></td><td colspan="7">Sample 1 Field Blank</td></tr>
<tr><td>E</td><td></td><td></td><td colspan="7">Sample 2 A</td></tr>
<tr><td>F</td><td rowspan="3">Standard 3*</td><td rowspan="3">Standard 4*</td><td></td><td></td><td colspan="7">Sample 2 B</td></tr>
<tr><td>G</td><td></td><td></td><td colspan="7">Sample 2 C</td></tr>
<tr><td>H</td><td></td><td></td><td colspan="7">Sample 2 Field Blank</td></tr>
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Supplementary Figure 1. Plate layout for sample PCR on a 96-well plate. Grey boxes indicate wells that remained empty to provide a buffer between samples and standards. NTC (non-template control) wells were used as a negative control. *Only applies for brown trout

Supplementary Table 2. Summary of PCR

		PCR Assay					
		2018 Brown trout	**2020 Brown trout**	**Puye**	**Atlantic Salmon**	**IPC /Zebrafish**	**ePlant**
Mastermix	Type	TaqPath ProAmp Mastermix	Environmental Mastermix 2.0	Environmental Mastermix 2.0	Environmental Mastermix 2.0	Environmental Mastermix 2.0	Environmental Mastermix 2.0
	ThermoFisher Cat #	A30865	4396838	4396838	4396838	4396838	4396838
Reaction Composition (µL per well)	F +R Primers	0.18 (900 nM)	0.3 (200 nM)	0.45 (300 nM)	0.3 (200 nM)	1.35 (900 nM)	0.9 (900 nM)
	Probe	0.05 (450 nM)	0.225 (150 nM)	0.6 (400 nM)	0.225 (150 nM)	0.375 (250 nM)	0.025 (250 nM)
	Ultrapure water	5.77	4.675	4	4.675	0.425*	5.295
	Mastermix	10	7.5	7.5	7.5	7.5	7.5
	Sample	4	2	2	2	2	2
	Total volume	20	15	15	15	15	15
PCR Reaction	Tm (°C)	60	60	60	60	60	60
	# Cycles	40	45	45	45	40	40
Oligo Source		Gustavsen et al., 2015	Gustavsen et al., 2015	In house (E. Burton)	Atkinson et al., 2018	In house (N. Harper, M. Lynch)	Veldhoen et al., 2016

Final primer and probe concentrations in each well presented in brackets. *An additional 2 µL of zebrafish IPC or ultrapure water also included.

Supplementary Table 3. Water chemistry measurements at sample collection sites.

Site	N	Average Water Parameter Values					
		Temperature (°C)	Dissolved Oxygen (mg/L)	Specific Conductivity (µS/cm)	Turbidity (g/L)	Salinity (ppt)	pH
Colonos	6*	14.15	7.36	14.30	0.01	0.01	8.16
Marchant US	3	11.27	11.30	20.40	0.01	0.01	7.16
Marchant INT	6	10.43	8.49	21.85	0.01	0.01	7.42
Marchant M	3	10.63	3.66	55.59	0.28	0.06	7.65
Añihue	2	11.85	2.66	11.50	0.01	0.00	6.67
Frutilla	3	10.80	2.28	13.00	0.01	0.00	6.58
Bahía Mala	3	10.67	11.24	11.30	0.08	0.00	7.47
Santo Domingo Lagoon	1	17.20	3.26	550.20	0.36	0.27	4.74

This table presents a summary of water quality data collected via YSI multi-meter probe between January 24th and 27th 2020 at 8 river sites in Chilean Patagonia. N represents the number of measurements taken and averaged. Samples were collected in freshwater apart from the Santo Domingo Lagoon (brackish water). *N=3 for pH.

Appendix B

Supplementary Images

Supplementary Figure 2. Marchant River flows in October 2018 (A) and January 2020 (B). Pictures taken at the same site (provided by M. Servos) display differences in water depth (i.e., flow) and suspended solids (e.g., glacial flour) between sampling seasons (spring and summer respectively).