Optimisation of eDNA replication and detection distance in Aotearoa New Zealand lotic systems

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

MSc (Research) in Ecology and Biodiversity

at

The University of Waikato

by

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2024

Abstract

The use of environmental DNA (eDNA) is a relatively new tool in New Zealand, and its increased use worldwide in the last decade has seen a surge in its utility and application. The information gaps in its use and effectiveness in the field of environmental science are fast being explored and answered. However, many questions remain unanswered, particularly in an Aotearoa New Zealand context. The aim of this thesis is to extend the knowledge needed for effective and efficient use of this new and exciting tool in lotic Aotearoa New Zealand conditions.

National standardisation of ecological sampling protocols between different agencies in Aotearoa New Zealand has historically been difficult to attain, creating challenges for combining datasets for national scale analyses. The introduction of new methods for biological monitoring, such as environmental DNA (eDNA), presents an opportunity to standardise aquatic sampling protocols prior to widespread adoption. The objective of this study (chapter two) was to optimise eDNA sample replication for the consistent characterisation of freshwater fish and macroinvertebrate communities in flowing waters, and ultimately, to inform the development of robust national monitoring standards. A comparison of field replication and extraction methods (pooling of preservation buffer) was also trialled as part of this high replication (n=16) eDNA study to assess any potential benefits in measuring species richness and reducing processing costs alongside replication optimisation. This involved two 'syringe' sampling methods ('standard' and 'boosted', eight each) conducted across 54 riverine sites throughout the country. No significant difference was found for species richness between the standardised (eight replicates) or boosted (16 replicates composited to eight) eDNA methods for fish and macroinvertebrates. Results indicated that six replicates were needed to consistently detect 89.5% of fish species likely to be present using field-based syringe eDNA sampling and preservation. However, an altitudinal species richness effect was observed for fish. For macroinvertebrates, six replicates were required to identify 86% of taxa identified to the NEMS (National Environmental Monitoring Standards) level used for the Macroinvertebrate Community Index (MCI: usually genera) while eight replicates were required to detect 89% of NEMS taxa. For fish and macroinvertebrate biodiversity, this study suggests that six replicates are a reasonable trade-off between effective community characterisation and cost in Aotearoa New Zealand lotic systems.

Understanding the spatial limits and the way in which eDNA behaves in lotic systems is important to understand when designing eDNA sampling programmes and interpreting results. Questions around biodiversity detection at spatiotemporal scales in relation to stream order and the influence of tributaries remain. We sought to extend our knowledge of longitudinal eDNA dispersal and detectability in wadable lotic systems by using live caged fish and a naturally occurring habitat specific fish species. An additional unexpected external molecular source was also included in the analysis. An eDNA collection method comparison between active and passive sampling techniques was also trialled alongside caged experiments in the second half of this study. A correlation between stream discharge and the distance in which eDNA travels downstream is reported. An initial drop in eDNA concentration from caged fish over the first kilometre downstream is followed by a peak 4 km downstream, is suggested to be reflective of plume dynamics. Tributary-specific fish species at low biomass and their associated eDNA signal is largely diluted and not detectable directly downstream of confluences, though there was some evidence that if sampled further downstream in the mainstem, tributary signals may be detected. The caged fish trials showed how far an eDNA signal can travel, while results from naturally occurring longitudinal signals confirm that eDNA metabarcoding can measure biodiversity at spatiotemporal scales. Active sampling in general outperformed passive sampling. Issues around field contamination and sampling strategies based on results from this study are also discussed.

Overall, this thesis provides insights and potential guidance in the use of eDNA in lotic Aotearoa New Zealand systems. It highlights the importance of eDNA field replication in the measurement of biodiversity for fish and macroinvertebrates and some terrestrial species. It helps interpret the ecology of eDNA in distance and structure. This information may assist in future eDNA sampling strategies and monitoring.

Acknowledgements

I would like to acknowledge my supervisor, Bruno David. Thanks for all your help from initial study conceptualisation to field assistance, analysis and manuscript reviews. Your extensive knowledge and ideas of all things freshwater and beyond have been instrumental in the completion of this thesis. Thanks to Nick Ling for providing support especially in the reviewing of manuscripts. Thanks to Andy Hicks for conceptualisation and support particularly for the replication section (chapter 2) of this thesis. Special thanks to Shaun Wilkinson (and the wider Wilderlab team), for your ongoing support and extensive knowledge of everything eDNA. Thanks to Alastair Suren for his input into the replication section regarding macroinvertebrates.

Thanks to Waikato Regional Council (WRC) for their ongoing support in particular Michael Pingram and Mark Hamer. Thanks also to summer students (Brandon Ruehle and Nina Hennig 2021, and Flavian Ember and Cassidy Chaytor 2022) who assisted in the SoE (State of the Environment) monitoring programme at WRC which extended into field sampling for this project. Thanks to Michael Pingram and Brandon Ruehle for their assistance in R scripting.

Thanks to all Regional Councils and unitary authorities who contributed to the high replication study (Chapter 2) by collecting samples and providing data for analysis. Thanks to Greater Wellington Regional Council for providing fishing and flow data for the Wainui stream site. Thanks to Doug Stewart (WRC) for providing flow gauging training and interpretation of the flow data.

Thanks to Fleur, Abbey and Zoe for ongoing support and patience.

Ngā mihi nui ki a koe

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Chapter 1. Introduction and literature review

1.1 Environment

Until relatively recently, water quality assessments in New Zealand relied primarily on periodical measures of water chemistry (temperature, dissolved oxygen, pH, nutrients) to establish state. However, subsequent overseas (OEPA 1989, Rosenberg & Resh 1993) and New Zealand studies (Noris and Noris, 1995, Ward and Pyle 1997, MfE 1999, Boothroyd & Stark 2000, David and Hamer 2010) have illustrated the value and importance of aquatic biological indicators for improved evaluation of water quality and health. Additional information, such as the integrity of terrestrial biodiversity and riverscape connectivity, can also be included to enhance the quality of biological assessments. One notable advantage of biological indicators over chemical water quality indicators is that some biological organisms, particularly long-lived and sensitive resident species, integrate aquatic conditions over time more effectively. That is, their presence typically reflects low levels of temporal anthropogenic impact at a site. Today, biological assessments are routinely used in many aquatic environments and can include a broad array of organisms including macroinvertebrates, fish, and benthic algae. With this increase in biological sampling, biological indices have been developed to encompass several aspects of community structure and function, rather than just a single metric to reflect conditions at a given site. Karr's (1981) Index of Biotic Integrity (IBI) for freshwater fish communities was followed by the Invertebrate Community Index (ICI) first developed by the Ohio Environmental Protection Agency (OEPA 1988) to rate or score the health of rivers and streams. These indices have been adopted in many parts of the world including New Zealand. Joy and Death (2004) developed New Zealand's first modified version of the fish IBI based on Karr's original work (1981) and subsequent papers (Karr 1987, Karr et al. 1986). New Zealand's version of the ICI, the MCI (Macroinvertebrate Community Index), was developed in 2007 by Stark and Maxted. These metrics (IBI and MCI) are now required to be reported on as compulsory attributes in the National Policy Statement for Freshwater Management (NPS-FM) amendment 2017. The NPS-FM was created in 2011 to set improved standards for water quality, quantity, and the biological health of freshwater environments.

Recommendations by The Ministry for the Environment (MfE) for standard methods to be used for invertebrate sampling were raised in 1997 and documented in an ensuing report (MfE 1999). Standard methods were needed, as many organisations and biologists were collecting and analysing stream invertebrate samples by numerous different methods. With the growing public pressure to improve the environment and curtail pollution, together with the legislative requirements and reporting required, sound scientifically defensible and comparable data was needed at a national scale. As a result, Stark et al. (2001) developed the NZ wadeable stream protocols for monitoring aquatic invertebrates. And in 2013, Joy et al. developed NZ freshwater fish sampling protocols based on earlier work by David et al. (2010) and David and Hamer (2010). Despite the establishment of national freshwater fish sampling protocols, fish surveys by many organisations remains low compared to macroinvertebrate sampling and lower still relative to water quality sampling. This appears to be largely due to the time and resources needed to establish meaningful site networks and undertake the work annually (David et al. 2021). Currently the main sampling methods for freshwater fish in wadeable streams are electric fishing, netting, and trapping, and to a lesser extent spotlighting (Joy et al. 2013). Invertebrate samples are normally collected with a D-net or Surber sampler (Stark et al. 2001). New and alternative tools in environmental science have been limited in use and the information they provide, however environmental DNA (eDNA) is a relatively new tool in New Zealand that has the potential to revolutionise monitoring of biodiversity (Pawlowski et al., 2021).

1.2 Environmental DNA (eDNA)

The number of eDNA publications has increased exponentially in the last decade (Takahashi et al. 2023) with many aquatic environments (lakes, rivers, wetlands, estuarine, marine) having been studied via eDNA. With improved methodologies and technological advancements, the costs of sample processing have reduced, making the use of eDNA feasible and in many cases more cost effective and less invasive than traditional sampling methods (Sigsgaard et al. 2015; Qu & Stewart 2019). This new tool has many potential uses and applications in gathering ecological information from organisms living both in and around waterways. Genetic material originating from organisms (as faeces, urine, mucous, scales, cells, gametes) is shed into the surrounding environment (Pilliod et al., 2013). These genetic fragments are extracted from water samples (aquatic sampling) and used to deduce community composition. Typically, water samples are filtered through a membrane (<5 µm) to concentrate these fragments (Jerde et al. 2011). eDNA water samples contain both intra-organismal DNA (whole small planktonic organisms) and extra-organismal DNA (shed cellular or extracellular material) which can be degraded to a greater or lesser extent.

eDNA persistence in water varies from a few days to a few weeks at normal water temperatures (Dejean et al. 2011, Pilliod et al. 2014), but can persist much longer in cold, dry conditions in the absence of light (Willerslev et al. 2003). Filtered samples are processed by using polymerase chain reaction (PCR) primers and next-generation sequencing (NGS) platforms (eDNA metabarcoding) resulting in rapid and accurate bioassessments of often complex aquatic communities and their terrestrial links.

Most eDNA studies use either barcoding or metabarcoding. Barcoding uses species specific primers to detect a single species within a sample (Strickland & Roberts 2019, Akamatsu et al. 2020, Kessler et al. 2020, Togaki et al. 2020), while metabarcoding is more commonly used and will use universal primers to detect as many species as possible within a sample (David et al. 2021, Alexander et al. 2020, Djurhuus et al. 2020, Yang & Zhang 2020). Barcoding analysis is often less expensive as only one species is investigated and is the preferred technique to gather information on a single species of interest (Levi et al. 2019) which is often either rare or invasive (Furlan et al. 2019). While metabarcoding (generally more expensive) can also be used for rare (Laramie et al. 2015, McKelvey et al. 2016, Spence et al. 2021 McElroy et al. 2020) and invasive species (Ficetola et al. 2008, Jerde et al. 2011, Carim et al. 2019, Wang et al. 2021), it also produces information on all species present (that are able to be sequenced), enabling an inventory of species (biodiversity) (DiBattista et al. 2019, Lyet et al. 2021), holistic health ratings (Cordier et al. 2019, Wilkinson et al. 2024), trophic interactions (Jusino et al. 2019, Mata et al. 2019), monitoring (David et al. 2021), and biosecurity incursions of both aquatic (Banks et al. (2021) and aquatically linked terrestrial organisms (Sales et al. 2020, Lyet et al. 2021) to be studied.

While some studies suggest that eDNA monitoring is more accurate and less invasive than traditional methods (Takahara et al. 2012, Rees et al. 2014, Cividae et al. 2016, Deiner et al. 2017, Pont et al. 2018), other researchers remain less convinced (e.g. Hinlo et al. 2017, Ulibarri et al. 2017). Often eDNA has the added benefits of being less expensive in relation to time, equipment, staff, and training (Biggs et al. 2015, Huver et al. 2015, Sigsgaard et al. 2015; Qu & Stewart, 2019). eDNA metabarcoding has proven to be a powerful tool for describing fish (Taberlet et al. 2012, Civade et al. 2016, Pont et al. 2018) and invertebrate communities (Deiner & Altermatt 2014; Fernández et al. 2019). However, eDNA methods can include the generation of false negatives (non-detection when present) which can occur for several reasons, but the main reason is insufficient sample replication (Ficetola et al. 2015). The analyses of filtered samples are often based on very little and degraded starting material especially for rare and patchy species, and the amplification of this DNA material in low amounts can be random, hence the need for replication. Other method failures leading to false negatives can include poor sample preservation, poor marker sensitivity (Darling and Mahon 2011), and PCR inhibition (Jane et al. 2015). PCR inhibition can also increase as the volume of water filtered increases (Matheson et al. 2014).

False positives (detection when not present) can occur, again with poor methodologies like sample contamination (one site to another, poor handling practices), low DNA species marker specificity, PCR, or sequencing errors (Ficetola et al. 2015), and detection from external sources like animal excrement and possible sewage inputs (Darling and Mahon 2011, Stoeckle et al. 2017, Pont et al. 2018). Robust eDNA methodologies will reduce the chances of false negatives and positives. Furthermore, having a good ecological understanding of sites being sampled, their location, history, condition and season can assist with highlighting any potential issues with resulting data. Without this knowledge of sampled sites, any potential false negatives/positives will be overlooked resulting in flawed data with long-term implications especially when these data are entered into databases (Berry et al. 2021).

In addition to ensuring sound methodologies are applied to the collection of field-based eDNA samples, robust protocols are also required when samples are processed within the laboratory. The use of molecular markers is an important consideration, and successful amplification of eDNA depends on primer sensitivity and specificity (Nichols et al. 2018) which can present a number of post-processing challenges (Beng and Corlett 2020). For instance, eDNA collected via filtering is a highly heterogeneous mix of many different taxa and the matching process between target sequences and primers is not always fully achieved (Ficetola et al. 2008, Stadhouders et al. 2010, Ficetola et al. 2015, Nichols et al. 2018). For example, bias may occur by the preferential amplification of common sequences over rare sequences, or a swamping effect from common non-target organisms may reduce or exclude target organisms (Rourke et al. 2021). Fragment size (length) may also lead to bias (Xia et al. 2018; Nichols et al. 2018). The use of quantitative PCR (qPCR) methods is likely to be more sensitive and reduce the likelihood of false positive reads compared with conventional PCR (cPCR) methods that can cross-amplify (Wilcox et al. 2013). Primer bias is typically only an issue for metabarcoding approaches where a broad range of organisms are being targeted using universal primers (Shu et al. 2021, Tsuji et al. 2022). Conversely, barcoding targets single species via the use of species-specific primers thus reducing identification errors (Wilcox et al. 2013, Davy et al. 2015, Cannon et al. 2016). The PCR process is also vulnerable to inhibitors which can affect the sensitivity of assays and in some cases lead to false negatives (Nichols et al. 2018, Hunter et al. 2019). Inhibitors include substances like polysaccharides (plant materials), collagen (tissues), humic acid (soil), heme (blood), urea (urine), melanin and eumelanin (hair and skin) and bile salts from faeces (Watson & Blackwell 2000, Radstrom et al. 2004, Schrader et al. 2012). Without matrix-specific procedures for preparation of nucleic acids before the PCR process (Schrader et al. 2012; Hunter et al. 2019) these inhibitors can prevent PCR amplification, block enzyme activity, and facilitate co-purification of an inhibitor and DNA (Schrader et al. 2012; Jane et al. 2015).

1.3 eDNA in lotic systems

The detection of eDNA in lotic systems is dependent on many variables (Barnes et al. 2014, Barnes & Turner 2015) like eDNA production, rate of degradation, transport, deposition, resuspension, dilution, and discharge (Carraro et al. 2018). eDNA degradation may increase with UV penetration (Ravanat et al. 2001, Pilliod et al. 2014, Strickler et al. 2015, endogenous nucleases (Willerslev and Cooper 2005) temperature (Zhu 2006, Lacoursière-Roussel et al. 2016, Kasai et al. 2020) and nutrient limitation (Salter 2018). These variables will likely change from one system/site to the next, with changes in land use, elevation, geology, substrate (Jerde et al. 2016), and flow (Jane et al. 2015, Deiner et al. 2015, Wilcox et al. 2016, Altermatt et al. 2020, Thalinger et al. 2021, Wood et al. 2021, Van Driessche et al. 2022). There will also be spatial and temporal changes in biological communities (Yamamoto et al 2017, Stoeckle et al. 2017, Byleman et al. 2018) from estuaries to headwater streams. This is especially true in New Zealand where elevation is correlated to native fish diversity because of high levels of diadromy (McDowall 1993, Jowett and Richardson 1996). The rate of detection is also likely to vary between species (Deiner et al. 2015, Mächler et al. 2016) as habitat preferences and the biology of one species may be less conducive to another in terms of eDNA detection. For example, a fish species that prefers calm backwaters with lots of cover and that biologically sheds less eDNA is likely to be less detectable compared to a species that lives in open fast-flowing water and in comparison, sheds more eDNA. Or a prey species may become more prevalent by increased levels of predation followed by excretion. As metabolic rate is mass dependent, relative rates of shedding might be greater for smaller species relative to larger fish (Tsuji et al. 2022). The life stage of a species and season (peak activity/spawning/migration) will also influence species detection (De Souza et al. 2016, Wacker et al. 2019, Thalinger et al. 2019) as will density (Carraro et al. 2021). All are important spatiotemporal dynamics that are likely to affect eDNA capture.

Currently eDNA monitoring does not have the ability to provide information about recruitment size, maturity, or condition all of which are largely possible with conventional methods. Abundance or biomass is also available and readily recorded with conventional surveys and can be attainable in some cases with eDNA (Takahara et al. 2012, Pilliod et al. 2013, Doi et al. 2015, Elbrecht and Leese 2015, Baldigo et al. 2017; Pont et al. 2018, Li et al. 2019, Di Muri et al. 2020, Rourke at al. 2021). However, the accuracy of biomass with eDNA may be difficult to achieve because of variation of species cell dispersal, the numbers of copy of per cell, and numerous other environmental factors. Nevertheless, at a temporal or spatially relative context, with sound methods and sufficient replication some researchers have demonstrated that a semi-quantitative signal for estimating biomass may be possible (Ficetola 2008, Elbrecht and Leese 2015).

The use of eDNA has many benefits and advantages compared to conventional lotic sampling techniques, in that little training is needed and the equipment required is minimal. One person can take eDNA samples compared with a minimum of two or three for electric fishing (David et al. 2010). eDNA sampling is also more time efficient with an individual sample able to be taken within 5 minutes (or 6 replicates within 30 minutes) compared to 4 to 5 hours for some larger electric fishing sites (using the New Zealand 150 m electric fishing protocols) or repeat visits when netting/trapping (leaving overnight to fish). Another advantage of eDNA monitoring is that accessing and sampling a site is relatively straight forward (Reinhardt et al 2019). That is, a broad variety of aquatic habitat types (including non-wadeable rivers) can be sampled using the same equipment. For more conventional techniques such as electrofishing or netting, capture efficiencies at repeat visit sites may be constrained by physical changes to the site over time (e.g. instream or riparian vegetation changes between sampling events), though high sediment levels within waterways can limit eDNA sample volumes in some cases (Bruno et al. 2017).

With the growing use of eDNA, studies are becoming difficult to compare because of the variety of methods employed, in collection (filter type, volume, replication, preservation) and laboratory processes (DNA purification, marker choice, sequencing, bioinformatics, taxonomic assignments). Different choices in each of these steps can alter the results obtained and highlight the importance of consistency in these controllable factors. Awareness of uncontrolled factors like differences in environmental conditions, degradation and taxa shedding rates is also needed.

Just as standard sampling protocols have been established for conventional fish and invertebrate sampling in New Zealand, a similar approach is needed for eDNA sampling. This is especially true where eDNA data is used for SoE reporting, assessments of environmental effects (AEEs), and biosecurity surveillance. Additional ecological information or bycatch data, both aquatic and terrestrially linked, can also be used for conservation and biosecurity purposes.

1.4 Thesis purpose and objectives

1.4.1 Thesis purpose

Ongoing advances in eDNA technology provide rapid biodiversity detection in many environments. In this study we focus on freshwater lotic systems and try to address requirements around field replication and the ecology of eDNA for detectability over spatial scales in a New Zealand context.

An inherent risk with the use of eDNA is that results are not representative of the environment sampled leading to false negatives (Takahashi et al. 2023). Increases in field replication as part of eDNA studies have resulted in increases in species richness (West et al, 2020, Macher et al, 2021). As the use of eDNA grows we have seen a shift from low levels of field replication in early studies to higher levels in more recent studies, however for many studies (>60%), replication remains low at three replicates or less (Takahashi et al. 2023). The level of replication will vary between systems and environments and will largely be dependent of levels of diversity and the biomass of targeted taxa (Bylemans et al. 2018, Furlan et al. 2016). Reach scale diversity of freshwater fish in New Zealand when compared to traditional methods can in some cases (based on the above variables) be attained with one single replicate (David et al. 2021). These types of result would reduce the need for higher levels of replication and reduce the cost of eDNA sampling (Beng and Corlett, 2020). However, this is not the case for all sites and in this study a more accurate assessment using eDNA rather than traditional methods was used to attain species richness via increased levels of replication.

The persistence and transport of eDNA material in lotic systems is controlled by several environmental factors that affect suspension, decay and retention. Understanding these processes in relation to biomass and discharge is important when interpreting eDNA data and designing optimal sampling strategies. Several questions remain about the “ecology” of eDNA from source, state, decay and transport (Burian et al. 2021). For example, how far can we expect an eDNA signal to travel downstream before it is affected by decay and what biotic factors influence transport and decay. If decay is rapid, then the eDNA signal can be inferred as local, but if decay is slow, depending on flow rates and biomass, the variability in signal for a particular species could be considerable (Pont 2018). Gaining a better understanding of the spatiotemporal resolution of eDNA in lotic systems will help in assessing eDNA data and designing future monitoring/research programmes.

1.4.2 Objectives and hypotheses

There were two main objectives in this study 1) Establish an appropriate level of field replication to detect fish and macroinvertebrates in New Zealand lotic systems using eDNA metabarcoding. 2) Gain a better understanding of the ecology of eDNA in New Zealand lotic system via metabarcoding in relation to biodiversity at spatiotemporal scales. Several associated questions for each of these two main questions are also addressed. For replication these include a comparison of field replication and extraction methods to assess any potential benefits in measuring species richness and reducing processing costs alongside replication optimisation. This involved two 'syringe' sampling methods ('standard' and 'boosted'). Based on the literature I hypothesised that replication levels would need to be somewhere between three and eight to provide a good level of species richness. I also hypothesised that the greater levels of filtration via the 'boosted' sampling methodology would produce better results compared to the standard method. Additionally, I predicted an altitudinal-biodiversity gradient effect in the number of replicates needed for fish due to high levels of diadromy found for New Zealand fish species.

For the second objective, again based on the literature, I hypothesised that the distance an eDNA signal will travel will be dependent on discharge and biomass. Prior to the field work for this part of the study there was limited information on the distance an eDNA signal would travel. In the time since the field work for this study and writing this material up there have been several important publications focusing on this subject. Some of these studies also highlight the effect of eDNA plume dynamics from planted fish. Though this was not initially considered in the distance part of this study, the design of the study (short distance spacing between sampling points) also highlighted the effect of plume dynamics. I hypothesised that the short distance sampling would also provide us a stochastic measure of less common species and how their signals might drop in and out of sampling points or possibly be sustained, but at a lower level to that of more common fish found throughout the study reaches. I hypothesised that the use of koaro as a natural molecular source would provide a more natural eDNA source which could be used to compare against the planted fish. Additionally, the use of koaro from the tributaries would help us understand the dilution effect and whether this signal would be detected in the mainstem. Several surprise or unplanned factors in this study have also aided in our understanding of the way eDNA behaves in lotic systems.

1.4.3 Thesis outline

Chapter 1 provides a general literature review of biological indicators that can be used to measure the health and connectivity of lotic systems in New Zealand followed by a broad introduction to eDNA, its uses, advantages and limitations. Chapter 2 provides analysis of high replication data from a broad range of lotic New Zealand systems where the intent was to optimise sample collection (field replication) to enable consistent, comparative and robust aquatic biodiversity assessment at the national scale for both fish and macroinvertebrates. An additional part of this chapter was to determine whether a “boosted” eDNA sampling method is better than the “standard” method. Chapter 3 investigates the ecology of eDNA, the distance a signal for freshwater fish at different discharges rates in wadeable lotic systems will travel based on both natural signals and those from caged fish. In the second half of this chapter, eDNA collection methods (active and passive) were also compared. Chapter 4 incorporates each chapter's key findings as an overall summary. Chapters 2 is written as a standalone paper prepared for publication; therefore, there is minor repetition between the chapters, particularly for introductory and background information.

Chapter 2. Optimum eDNA replication for standardisation in lotic systems in Aotearoa New Zealand

2.1 Introduction

Environmental DNA (eDNA) can be detected in soil, air and water and its use in aquatic science has rapidly increased since first being introduced for species detection in water in the late 2000's (Ficetola et al. 2008, Takahashi et al. 2023). The collection of eDNA is especially suited to rivers and streams as genetic material carried downstream is regularly mixed and suspended and transported in a similar manner to fine suspended sediment (Pont et al. 2018). For eDNA metabarcoding, DNA targets are usually processed by using Polymerase Chain Reaction (PCR) primers and next-generation sequencing (NGS) platforms resulting in rapid and accurate bioassessments of often complex aquatic communities (Valentini et al, 2015, Wilkinson et al, 2024). Other more targeted and often more sensitive PCR methods (qPCR and ddPCR) are also available depending on the study objectives.

Detection of eDNA in lotic systems is dependent on many variables (Barnes et al. 2014, Barnes & Turner 2016), including its rate of production and degradation, deposition, resuspension, dilution, transport (Carraro et al. 2018) and season (Furlan et al. 2015). eDNA degradation may increase with UV irradiance (Ravanat et al. 2001, Pilliod et al. 2014; Strickler et al. 2015), temperature (Zhu 2006, Lacoursière-Roussel et al. 2016; Kasai et al. 2020) and microbial consumption (Salter 2018). These variables will likely alter from one system/site to the next, with changes in land use, elevation, geology, substrate (Jerde et al. 2016) and flow (Shogren, et al. 2018). Spatial and temporal longitudinal changes in biological communities are also likely to occur (Yamamoto et al 2017, Stoeckle et al. 2017, Byleman et al. 2018) from estuaries to headwater streams. This is especially true in Aotearoa New Zealand (NZ) where elevation has a strong influence on native fish diversity because of high levels of diadromy (Hayes et al. 1989; McDowall 1993; Jowett and Richardson 1996).

eDNA metabarcoding in many cases has proven to be a powerful tool for describing fish (Taberlet et al. 2012; Civade et al. 2016; Yamanaka and Minamoto 2016; Nakagawa et al. 2018; Pont et al. 2018) and invertebrate (Deiner & Altermatt 2014; Fernández et al. 2019) communities. However, one limitation of the eDNA method includes the generation of false negatives (non-detection when present) which occur more frequently when insufficient sample replication is used (Ficetola et al. 2015; Roussel et al. 2015), or when insufficient water is filtered per replicate (Mächler et al. 2016; Cantera et al. 2019). eDNA in water tends to be patchily distributed and differentially degraded, and therefore sample replication and volume are important factors for reducing the potential of false negatives. However, higher replication increases study costs, and possible false positives due to more samples being collected and the potential for contamination during handling associated with these extra samples (Ficetola et al. 2015). Thus, optimising sample replication is one of the most critical steps in the design of a robust eDNA biodiversity study (Yoccoz 2012, Takahashi et al. 2023).

Prior to the development of eDNA analysis' aquatic biological indicators for assessing ecosystem health, such as the Index for Biotic Integrity (IBI) for fish (Karr, 1981) or the Macroinvertebrate Community Index (MCI), (Stark and Maxted 2001) have historically been developed using more conventional sampling methods (e.g., electric fishing, trapping, netting for fish surveys, and kick sampling for aquatic invertebrates). These indices are used to evaluate various aspects of biological community structure and function, such as for evaluating presence/absence of native or introduced species, population structure, or assessing environmental effects of a range of stressors.

eDNA offers a relatively new and promising method to monitor and assess freshwater systems and to complement conventional sampling approaches. The use of eDNA has many benefits and advantages compared to conventional lotic sampling techniques, in that little training is needed, and in many cases eDNA sampling is more cost effective (equipment, staff and time required is minimal) and less invasive (Sigsgaard et al. 2015; Qu & Stewart 2019). There is no need for taxonomists to identify specimens (Uchida et al. 2020), and there is improved detection of cryptic species (Yao et al. 2022) and a wider range of taxa (Takahara et al. 2012; Cividae et al. 2016; Deiner et al. 2017; Pont et al. 2018). Accessing and sampling a site is relatively straight forward with eDNA (Reinhardt et al. 2019) as only one access point is required for well mixed streams compared to at least 150m for conventional methods (Joy et al. 2013) in NZ which can be an issue for smaller streams with heavy riparian vegetation and/or non-wadeable systems (this "single access" sampling may require further thought for larger less mixed lotic systems). Additional ecological information or bycatch (non-target) data both aquatic, and terrestrial, can also be used for conservation and biosecurity purposes. eDNA however, does have several limitations, such as size structure, fish health and abundance cannot be obtained by eDNA but are readily obtained by traditional methods.

While standardised sampling protocols have been established for conventional fish (Joy et al 2013) and macroinvertebrate sampling (NEMS 2022), in NZ and elsewhere, a similar standardised approach is also needed for eDNA sampling. This is particularly true where eDNA data is used for national level reporting, assessments of environmental effects (AEEs), and biosecurity surveillance. A standardised national approach also helps with the development of ecosystem health indices and benchmarks. In this study standardisation mainly refers to the amount of field replication needed rather than the entire eDNA workflow used where these replication levels can be applied irrespective of downstream steps. In this paper we aim to progress the preliminary work of David et al. (2021) to standardise eDNA sampling in NZ streams and rivers. By critically analysing high replication data from a broad range of lotic systems, the intent was to optimise sample collection and pooling to enable consistent, comparative, and robust species richness assessment at the national scale.

2.2 Materials and methods

2.2.1 eDNA collection

Fourteen of sixteen NZ regional councils and two unitary authorities contributed samples to the study (Figure 2-1). Each council contributed one to six sites for sampling, with 54 sites being sampled. This nationwide approach enabled a wide range of species and sites to be sampled with differences in land use, and geology. Sites ranged, from near sea level (2.2m) to 600m in elevation and from small first order streams to NZ's longest river (The Waikato), a 7th order site and NZ's largest river by volume (The Clutha), an 8th order site. Baseline invertebrate data (kick net) was available for all but one of the sites sampled (n=53) and six sites also had electric fishing data (single pass, 150m) for comparison with the eDNA results. At each of the 54 sites (Table A-1, Appendix A) 24 replicate eDNA water samples were collected using Wilderlab V2 eDNA mini kits (each containing a 1.2-micron, 30 mm cellulose acetate syringe filter and 350 µl Zymo 2X DNA/RNA Shield for filter preservation and sample lysis).

Fieldworkers entered wadeable stream sites from a downstream direction standing parallel to each other (when in pairs or larger teams) and filtered stream water from a flowing section of the stream (normally the middle of a riffle section) rather than a backwater or slow flowing pool. For larger waterways (stream order >6) streams were again waded and sampled in flowing water but closer to the margins rather than the middle of the stream/river where depth and velocity made wading difficult.

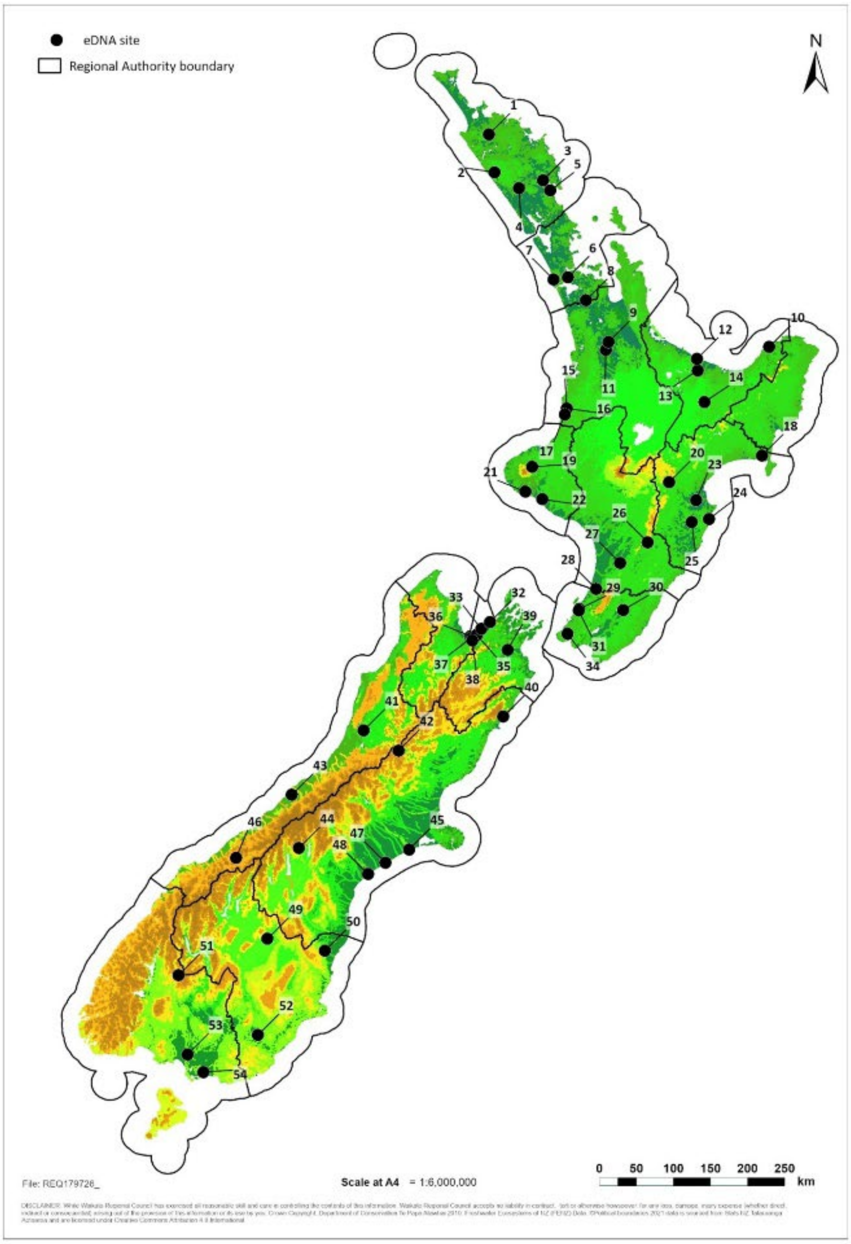

Figure 2-1. National high replicate eDNA sites (n=54) where samples were collected from December 2020 to May 2021.

All 24 field replicates were collected from that single location for each site. At each site new latex gloves were used along with a sterile 60 ml syringe to filter surface water through the encapsulated filter membrane for the collection of biological material 50mls at a time. After 1 L of water was filtered (or the filter became clogged, whichever came first), 60 ml of air was pushed through the filter using the same syringe to remove excess water from the filter. Each sample (1 L) took between 5-10 minutes to filter depending on turbidity. Eight sites out of the 54 were filtered till clogged due to higher levels of turbidity. The Shield preservative (Zymo

Research Cat. No. R1200-125) was then injected directly into the filter for metazoan cell lysis and to prevent degradation of captured DNA, and the outlet end of the filter sealed using a luer-lock syringe cap (so no liquid can spill out). This was repeated for each replicate. Eight of the replicates were randomly separated and labelled as “Standard” while the remaining 16 were labelled “Boosted”. These 16 “boosted” samples were randomly paired and combined on receipt at the Wilderlab laboratory to make eight (600 µl) “boosted” samples. Each of these eight “boosted” samples is a composite of two “standard” replicates, or the pooling of the preservation buffer of two “standard” replicates to make one “boosted” sample. The total volume filtered, and GPS co-ordinates of the location were recorded on the supplied sample kit bag. This same process was repeated for all sites and samples were kept at ambient temperature and couriered to the Wilderlab laboratory (Wellington, NZ) within one week of collection for analysis. No negative field control samples were taken in this study and in hindsight some should have been taken. However, from the 51 sites assessed in this study no fish species was found where it shouldn’t be. More recently obtained data (see chapter 3, 3.5.10) using the standard six replication method with some negative field controls did show some contamination (low sequence reads) for both fish and invertebrates for species that occurred at sampling sites. We suggest that this is likely caused by aerosolised biological molecules from the waterway entering the water used for the negative control.

On receipt of the preserved filters at the laboratory, the 8 “standard” kits were processed as normal, where the Shield buffer was drawn from the capture filter using the attached 3 cc syringe and transferred into clearly labelled individual LoBind Eppendorf tubes. The volume of Shield that can be drawn from the filter varies but is generally in the vicinity of 300ul, then 200ul that is transferred to the extraction cartridge. The Shield is drawn off the filter, rather than digesting the filter directly, since the Zymo DNA/RNA shield is a lysis buffer (https://files.zymoresearch.com/protocols/_r1100-50_r1100-250_r1200-25_r1100-125_dna_rna_shield.pdf), so target metazoan DNA is in solution by the time the lysate is extracted from the filter. The ‘boosted’ samples were treated in the same way after pooling to create 8 x 600µl samples. All samples were stored in a -20˚C freezer until further processing. The indirect extraction method compared favourably to direct filter digestion in a previous study (Banks et al. 2020) noting that the extractions were carried out in different laboratories. Filter capsules are not cracked to minimise the risk of contamination.

2.2.2 DNA quality analysis and NGS library preparation

For DNA extraction and purification, 200 µl of lysate from each sample were loaded into a Genolution GD141 DNA/RNA extraction kit cartridge and run on the Genolution Nextractor NX-48S system using the standard (default) extraction settings. The same volume of eluate for both "standard" and "boosted" samples was used in the amplification process (200 µl). DNA quality/quantity analysis, adapter-fusion, indexing, and amplification were carried out in single-step quantitative PCR (qPCR) reactions on an Applied Biosystems QuantStudio 1 qPCR instrument. DNA extracts were PCR-amplified (single-plex for each primer pair) using the Wilderlab comprehensive freshwater multi-species analysis package (see Table 2-1 for list of primers).

Table 2-1. Assays included in the Wilderlab comprehensive freshwater multi-species package, with sequences for forward and reverse primers.

Assay	Gene	Forward Primer	Reverse Primer	Ref
BE	18S-V9	CCCTGCCHTTTGTACACAC	CCTTCYGCAGGTTCACCTAC	[1]
BU	18S-V9	TTGTACACACCGCCC	CCTTCYGCAGGTTCACCTAC	[1]
CI	COI	DACWGGWTGAACWGTWTAYCCHCC	GTTGTAATAAAATTAAYDGCYCCTARAATDGA	[2]
DG	mt16S	TCTTCGGTTGGGGCGAC	GGATTGCGCTGTTATCCCT	[3]
LV	12S	TCGTGCCAGCCRCCGC	CATAGTGGGGTATCTAATCCCAGTTTG	[4]
MZ	rbcL	CTTCTTCAGGTGGAACTCCAG	GTCACCACAAACAGAGACTAAAGCAAGT	[5]
RV	12S-V5	TTAGATACCCCACTATGC	TAGAACAGGCTCCTCTAG	[6]
TP	trnL	GGGCAATCCTGAGCCAA	CCATTGAGTCTCTGCACCTATC	[7]
UM	16S-V5	GGATTAGATACCCTGGTA	CCGTCAATTCMTTTRAGTTT	[8]
WV	mt16S	GACGAGAAGACCCTWTGGAGC	CCRYGGTCGCCCCAAC	[9]
ZC	mt16S	GGACGATAAGACCCTATAAADCTT	CGCTGTTATCCCTAAAGTAAYTT	[10]

[1] Forward and reverse primers from (Amaral-Zettler, McCliment, Ducklow, & Huse, 2009); [2] Forward primer adapted from (Vamos, Elbrecht, & Leese, 2017); reverse primer developed in-house at Wilderlab; [3] Forward primer developed in-house at Wilderlab; reverse primer from (Deagle, Chiaradia, McInnes, & Jarman, 2010); [4] Forward primer developed in-house at Wilderlab; reverse primer adapted from (Miya et al., 2015); [5] Forward and reverse primers from (Bradley et al., 2007); [6] Forward primer adapted from (Riaz et al., 2011); reverse primer from (Kelly, R. P., Port, Yamahara, & Crowder, 2014); [7] Forward and reverse primers from (Taberlet et al., 2006); [8] Forward primer from (Morey et al., 2006); reverse primer from (Lane et al., 1985); [9] Forward and reverse primers adapted from (Nester et al., 2020); [10] Forward and reverse primers developed in-house at Wilderlab.

In total six assays were used to detect fish, and eight primers were used to detect NEMS (MCI) taxa (Table 2-2). Fusion-tagged forward primers were designed as follows: P5 Illumina adapter sequence + Illumina TruSeq sequencing primer + 8-bp unique index tag + locus specific forward primer from Table 2-1. Fusion-tagged reverse primers were designed as: P7

Illumina adapter sequence + 8-bp unique index tag + locus specific reverse primer from Table 2-1. All 8-bp index tags differed from each other by at least 3-bp. Each reaction contained 5 µl SensiFAST 2x LoRox SYBR Mix (Bioline), 0.25 µl forward primer (10 µM), 0.25 µl reverse primer (10 µM), 0.5 µl BSA (10 mg ml-1, Sigma Aldrich), 2 µl de-ionised water and 2 µl template DNA. qPCR cycling conditions included an initial denaturation of 3 min at 95°C; followed by 40 cycles of 5 s at 95°C, 10 s at 58°C (WV and RV assays), 45°C (CI assay) or 52°C (all other assays), and 15 s at 72°C. There was no multiplexing, and all PCRs were run separately with a single pair of primers per reaction. Aside from the annealing temperatures, cycling conditions were the same for each assay. DNA quality and quantity were assessed by checking that a sigmoidal log-amplification curve was visible with a CT value < 35. A negative control reaction containing 2 µl de-ionised water in place of the template DNA was included with each qPCR run. The blank controls are included as raw fastq files in the SRA archive PRJNA950216 (https://www.ncbi.nlm.nih.gov/bioproject/950216).

Table 2-2. eDNA primers used for fish and NEMS taxa.

MCI taxa	Fish
WV	RV
DG	LV
CI	WV
BE	DG
BU	CI
ZC	ZV
ZV	
LV	

None of the blanks contained identifiable fish sequences at levels above the sequence count threshold of 5. This threshold is set to account for PCR misreads and sequencing errors; the forward and reverse indexes are all 8bp and all differ from each other by at least 3bp, but there still exists a low sequencing misread/PCR error rate that can occasionally lead to sequences being assigned to the wrong sample during demultiplexing (tag jumping; see Schnell et al. 2015). We find that a minimum sequence count of 5 prevents false positives from PCR misreads and sequencing errors at the sequencing depth at which we operate. The low-pass sequencing method we present here was designed to minimize the cost benefit ratio for the purposes of fish monitoring by Regional Councils in NZ, and we have shown in a previous paper (David et al. 2020) that this approach can outperform traditional monitoring methods for this purpose (i.e., electrofishing). Aside from increasing the costs, which leads to lower uptake of the tool, increasing the sequencing depth also increases the number of PCR misreads, which leads to higher minimum count thresholds and other stringent controls such as minimum occupancy (i.e., a species must appear in more than one replicate to be considered a 'true positive'). The assays in this panel range from specific to generalist, and for the more specific assays it is very difficult to get the number of sequence reads above 5000 when the target organisms are at low prevalence in the sampling area. For example, the RV 'ecoprimer' assay is very specific for vertebrates and is virtually impossible to reliably get > 5000 reads at reference sites where the incidence of fish is low. Next Generation Sequencing (NGS) libraries were pooled at roughly equimolar concentration using the final normalised ΔRn fluorescence values as a guide and cleaned and double-end size selected using AMPure XP magnetic beads (0.9x and 1.2x for lower and upper size bounds, respectively). The final pooled library concentration was determined using a Qubit 4 Fluorometer (ThermoFisher Scientific) and the concentration was adjusted to 50 pM in sterile DNAse/RNAse free water. The library was then loaded onto an iSeq i1 V2 reagent cartridge with 300-cycle flow cell (Illumina) with 5% Phi X and run for 290 cycles in a single direction on an Illumina iSeq 100 instrument.

2.2.3 Sequence depth analysis

A key variable requiring optimization in metabarcoding analysis involves sequencing depth, or the number of reads per assay per sample. Too little depth/coverage can increase the false negative rate, as DNA signals from rare species may not appear in the ASV profile or fall below the minimum sequence count threshold. Excessive sequencing depth can inflate costs, requiring larger sequencing platforms with significantly more expensive reagent kits. This inevitably comes at the cost of reduced field replicates, sites to include, or the number of metabarcoding assays to run. As such, it is important to find the point of diminishing return as the number of reads at which the number of species detected in a sample, levels off, and temper sequencing depth accordingly.

Using the core fish assays (mitochondrial 12S 'RV' and 16S 'WV) and invertebrate assay (COI 'CI'), we ran species accumulation profiles within each sample where over 5000 sequence reads where available, by subsampling the sequences to 100, 200, 300, … 4900, & 5000 reads. Species accumulation plots with 95% confidence intervals were generated using the ggplot2 package in R (Wickham (2016); R Core Team (2021)), and the sequencing depth (no. of reads per sample per assay) at which 95% of the full target taxon profile was detected was interpolated from the plots. For the WV and RV assays, the target taxon profile was restricted to fish resolved to species level, and for the CI assay, the target taxon profile was restricted to insects resolved to genus or species level (since most MCI indicators require identification to genus level).

All species accumulation curves had leveled off at 5000 sequence reads, with the vertebrate-specific 12S 'RV' assay requiring only 500 reads on average to capture 95% of the fish species present in the sample. As expected, the more generalist CI assay had a flatter curve, requiring 2500 reads on average to account for 95% of the insect genera and species in each sample. The WV assay, which was designed for vertebrates but also detects several worms and other inverts, had an intermediate species accumulation curve, requiring 1000 reads to reach 95% fish species saturation (Figure 2-2). Our average read depths for the three assays covered here were well above their 95% saturation limits, with 4,444, 11,377 and 7,621 reads per sample for the RV, WV, and CI assays, respectively (see Wilkinson et al. (2024); Table 2-1).

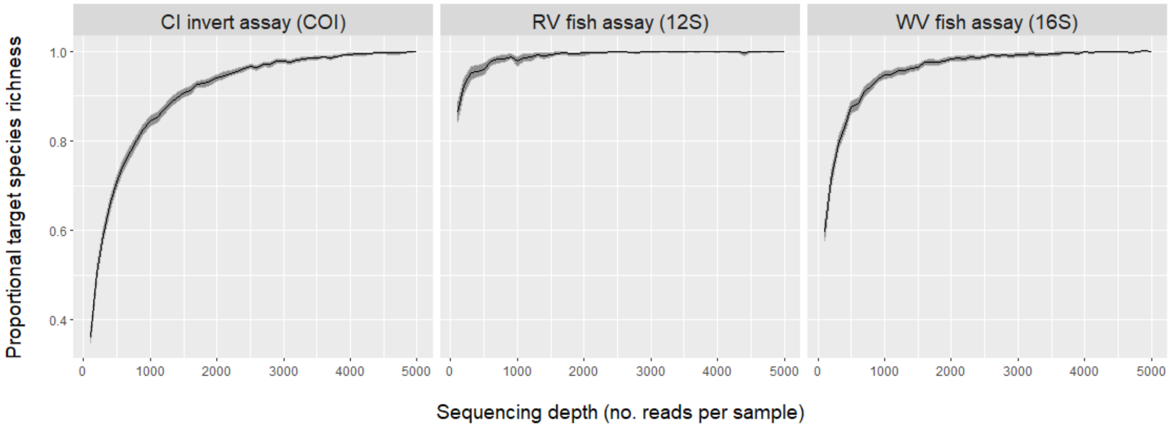

Figure 2-2. Species accumulation curves by sequencing depth for the core fish (RV, WV) and insect (CI) metabarcoding assays used in this study.

2.2.4 ASV generation and taxonomic assignment

The iSeq 100 output sequence FASTQ files were de-multiplexed in R (R Core Team, n.d.) using the insect package (v 1.4.0; (Wilkinson et al., 2018)) and trimmed sequences were filtered to produce a table of exact amplicon sequence variants (ASVs) using the DADA2 R package (Callahan et al., 2016). Sequence filtering was done using the DADA2 R package to remove PCR errors and chimeras using a standard model-based approach. The ASVs produced by this approach are considered to be the true sequences present in the sample (though a 100% error removal rate is not strictly possible). ASVs were identified to the lowest possible taxonomic rank using Wilderlab's four-step classification process. This involves: 1) exact matching against an intensively-curated database of previously detected eDNA sequences; 2) remaining (i.e. non-matched) sequences are exact-matched against a global reference sequence database primarily compiled of trimmed reference sequences downloaded from GenBank (Benson et al., 2010) and BOLD (Ratnasingham & Hebert, 2007) then matching sequences assigned at the lowest common ancestor level (LCA; i.e. assigned to genus level if matched with 100% identity to more than one species, or to family level if matched to more than one genus); 3) remaining sequences that are > 50 bp in length are matched with single indel/substitution tolerance against the same GenBank/BOLD reference database and matching sequences assigned at LCA level; and 4) remaining sequences are queried against the local GenBank/BOLD reference database using the SINTAX classification algorithm (Edgar, 2016) with a conservative assignment threshold of > 0.99 (all rank levels) and maximum genus level assignment threshold.

Verification of previously detected eDNA sequences involves initial classification using publicly available reference data (i.e., GenBank) using matching and SINTAX classification (as above) and then checking the distribution of each unique eDNA sequence against the known distribution of the taxon it has been assigned to. If an eDNA sequence that has been assigned to a species repeatedly appears outside the known range of the species, this suggests the presence of a congeneric with an identical barcode sequence that is missing from the reference database and causing an over-classification error (Edgar, 2018). In this case the taxon identification of the eDNA sequence can be manually assigned to a higher rank level, which it will continue to be called at if encountered in future eDNA surveys. This manual curation process is a means to include geographical information into the taxon assignment process, and accounts for missing reference sequences in GenBank, which can lead to over-classification and mis-classification errors. This manual curation step is carried out exhaustively for fish, since fish distribution records are available through fishbase and other repositories, and a limited number of freshwater fish species are present in New Zealand. We do this to a lesser extent for other taxa based on the availability of comprehensive distribution records. The in house reference database used for initial assignment of new eDNA sequences is predominantly comprised of GenBank data that is retrieved, trimmed (using a virtuaPCR approach with the same primers sequences used in the analysis), and clustered at 97% similarity in order to spot any spurious reference sequences that is labelled as a completely different taxon to the other members of the cluster (using the purge function of the insect R package with default parameters).

2.2.5 Data analysis

Three of the 54 sites were excluded from the data analysis due to field sampling inconsistencies (sites 29 and 44) and heavy rain prior to sampling reducing DNA on the filters (Site 13, Table A-1). Sequences identified to species level for freshwater fish and macroinvertebrates were used to generate separate species accumulation plots with quantiles using R, version 4.0.4 (R Core Team, 2021). R packages used for data analysis include readxl (Wickham and Bryan, 2022), tidyr (Wickham and Girlich 2022), dplyr (Wickham et al. 2022) and BiodiversityR (Kindt and Cole, 2005). Accumulation plots were produced using ggplot2 (Wickham, 2016) and compared for both the standard and boosted methods. To maintain randomness the order the samples were collected in the field (random) was the same order the samples were analysed for the accumulation plots. The proportion of species detected at each site from each replicate is a proportion of the total number (100%) of species detected from all 16 replicates taken (8 single and 8 boosted). This total is the total number of species/taxa detected from eDNA sampling only. Recreational fishers are known to discard marine fish frames (skeletons) into streams (often to feed the local eels), therefore any truly marine fish species detected by eDNA were excluded from the data analysis. Though rare in this study, any marine "wandering" species that inhabit both marine and freshwater systems were left in the analysis. Although eDNA allowed freshwater invertebrates to be identified to the lowest taxonomic level available in the GenBank/BOLD reference database (usually to species), this data was reassigned to the same taxonomic level as the National Environmental Monitoring Standards (NEMS) invertebrate taxa list (NEMS 2022), which recognised 197 MCI taxa. This NEMS list included 138 genera, 42 families, sub-families or tribes, and 17 higher taxonomic levels (Order, Class or Phyla). We refer to this simplified list as the "NEMS taxa list", which is the level of resolution used by regulatory monitoring agencies throughout NZ. By reducing the level of resolution of the eDNA to that used in NEMS meant that any assessment of taxonomic resolution plots made in this study should mimic the same level of taxonomic resolution used throughout the country. As such we acknowledge that we are probably underestimating the real effect of sample replication on species richness, but that this analysis has more relevance to regional council ecologists.

Single factor ANOVA was used to compare species richness between the two sampling methods (standard and boosted). Sites that were "filtered to clogged" were also checked separately as a group (8 sites) to check if less water filtered at more turbid sites may have resulted in any differences between the method used. Dominant land cover of each site 5 km upstream of the sampling site was established using the New Zealand Land Cover database (LCDB v5., Mainland, New Zealand https://lris.scinfo.org.nz/). Elevation, NZ segment, NZ reach and stream order for each site was derived from the NIWA (National Institute of Water and Atmosphere) REC2v5 (River Environment Classification NZ) dataset River Environment Classification (REC2) NZ.

Analysis of stream order for fish and NEMS taxa was achieved by sorting sites into two stream order groups of small sites (n=23), with a stream order of 3 and below and large sites (n=28), with a stream order of 4 and above Land, Air, Water Aotearoa (LAWA) . Similarly for elevation two groups were sorted based on sites with an elevation of less than 100m (n=38) and sites at elevations greater than 100m (n=13, Table 2-3). ANOVA was also used to see if invertebrate taxonomic richness differed between sampling sites when assessed using eDNA (using both the original level of identification from the eDNA results, and the eDNA data amalgamated to the NEMS level of identification), and traditional kick net samples, also identified to the NEMS level. Although the kick net samples were not collected concurrently with eDNA sampling, the median time between sampling with both methods was just over 3 months. Such a short time difference between samples was considered relatively insignificant, especially given the overall persistence of invertebrate communities in New Zealand, and lack of strong seasonal patterns (Scarsbrook 2002).

Table 2-3. Mean species/taxa proportional richness (%) for fish and NEMS taxa at one and six replicates (standard method) and by stream order and elevation. The last two columns are number of species per replicate (16 replicates) with range (minimum to maximum) and average.

Group	Standard		Stream order				Elevation				Number of species/rep	
	n=51		1-3, n-23		4-8, n=28		>100m, n=13		<100m, n=38		n=16	
	1 rep	6 reps	1 rep	6 reps	1 rep	6 reps	1 rep	6 reps	1 rep	6 reps	min-max	Av
Fish	64.0	89.5	65.3	90.6	64.1	89.5	70.4	91.6	61.9	88.8	0-8	3.2
NEMS taxa	57.0	86.0	54.8	87.1	59.5	85.6	57.0	88.6	57.5	85.1	3-17	9.9

2.3 Results and discussion

2.3.1 Summary of eDNA detected

From 51 national sites (Figure 2-1) thirty-one different taxonomic groups are represented in the sequence reads ranging from the largest “Other” to the smallest “Lizards” (Figure 2-3 and Table A-2). The “Other” grouping comprises 33.5% of the total reads of all sites and mainly consists of unidentified sequences which are a combination of organisms not currently in the reference database or are undescribed. Freshwater fish represented 11.4% of the total reads. The NEMS taxa include a variety of groups (crustaceans, worms, flatworms, insects, springtails, and molluscs) making up the NEMS taxa list (NEMS 2022) and equates to 13.9% of the total reads. Twenty-three terrestrial mammals (excluding humans and zoo species) and 48 bird species were detected in this study. Several of these species, as are species in other groups (lizards, frogs, plants, and freshwater mussels) are of interest for either conservation or biosecurity. For more information on “other species” including replication analysis for mammals and birds, please see Appendix C.

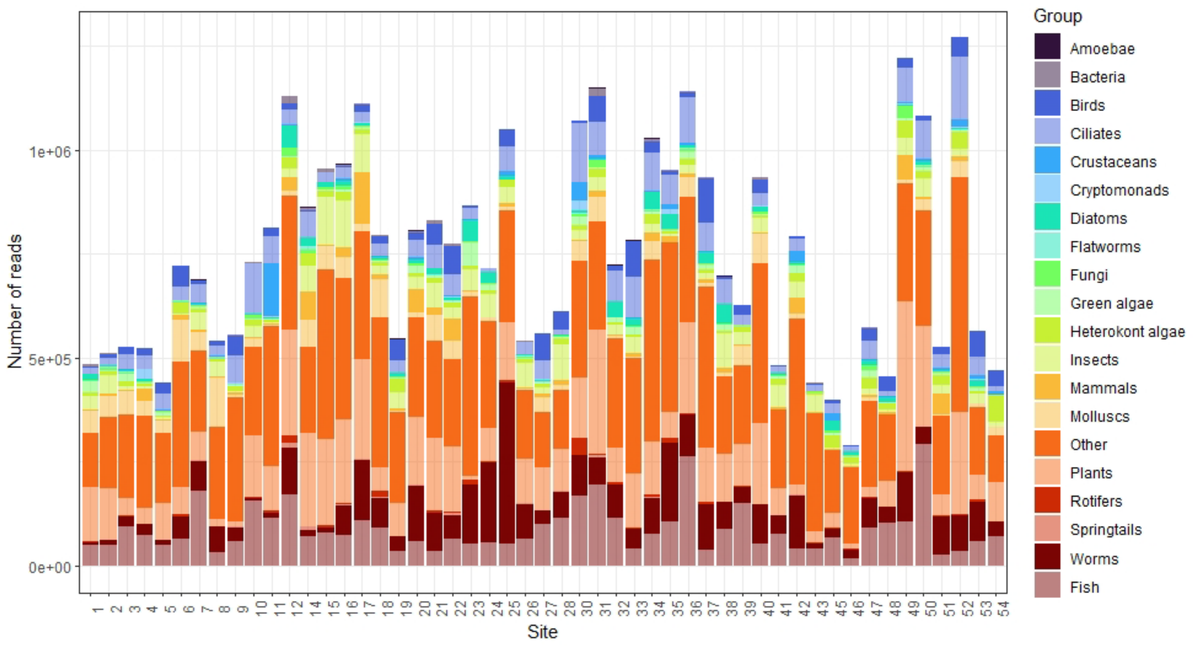

Figure 2-3. Total eDNA reads by group for all sites analysed (n=51).

2.3.2 Effects of replication

Accumulation plots were prepared for the two target groups (fish and NEMS taxa) comparing the two methods (standard and boosted, Figure 2-4). To reach 89.5% and 86% richness (standard method) for fish and NEMS taxa respectively, six replicates are needed. A further 2 replicates were required to reach 89% richness for invertebrates (Figure 4). As there was no significant difference between the two methods (standard and boosted) all 16 replicates can effectively be combined to assess typical levels of confidence (90% and 95%), for fish this is 7 and 11 replicates respectively and 9 and 11 replicates respectively for NEMS taxa. Variation at the 95th percentile for both groups is reduced with increased replication (Figure 2-4). Fish, however, have the largest reduction in variation initiating at 69.2% for one replicate which reduces to 27.7% at six replicates, a 41.5% improvement (standard method). In comparison variation for NEMS taxa reduces by 11% over six replicates indicating similar species richness between sites. As two different sampling methods were used in this study, the asymptote at eight replicates never reached 100% because of the different species detected between methods (standard and boosted). The species proportional richness range for both fish and NEMS taxa improved with each replicate sample.

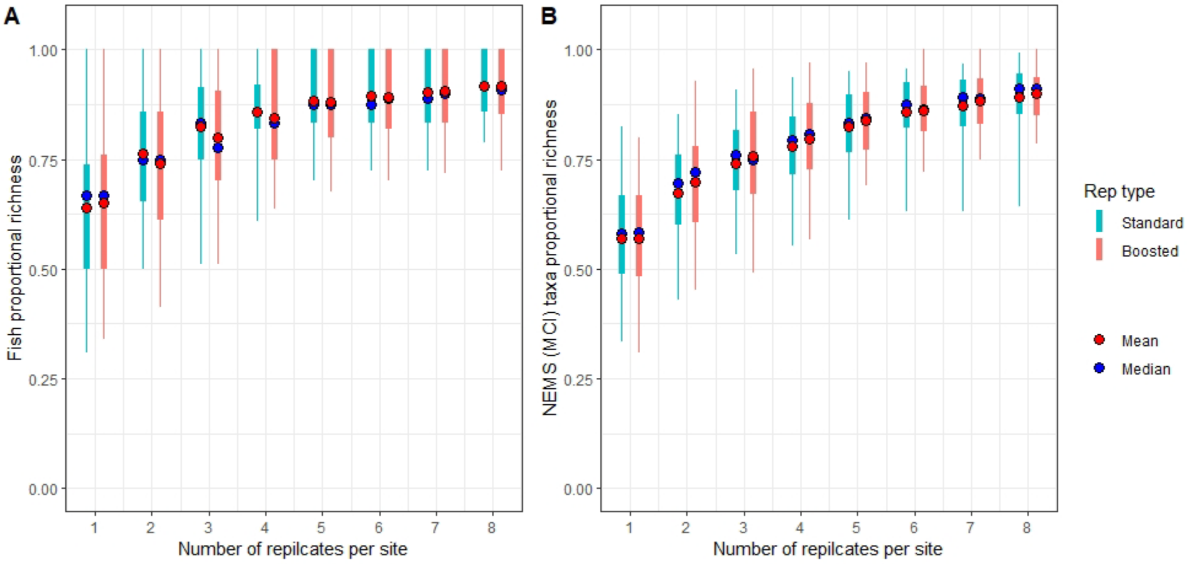

Figure 2-4. Proportion of taxa richness detected by eDNA sampling for (A) freshwater fish and (B) NEMS (MCI) taxa with standard versus boosted method. Box and whiskers are 50th and 95th percentiles. Red dot = mean, blue dot = median. Proportion of taxa richness for 16 replicates=1.0.

The accumulation plots for both were similar, other than the different starting points or first replicate, for fish at one replicate (standard method) species richness was at a mean of 64% compared to NEMS taxa (57%). After six replicates the additional increase (relative to one replicate) for fish and NEMS taxa was 25.5% and 29% respectively, resulting in 89.5 and 86.0 mean percent species richness for these two groups. For fish the greatest gains in mean species richness occurred within the first four replicates before species richness started to asymptote at around 6 replicates. Invertebrates mirrored fish but at a slightly lower starting point (57%

compared with 64%) which is maintained over the first five replicates and narrows to 3.5% after 6 replicates (86% compared to 89.5%). The replication difference for fish and NEMS taxa (species occurrence) over all 16 replicates is summarised at the end of Table 2-3.

2.3.3 Standard versus boosted method

There was no significant difference for species richness between the two methods (standard and boosted) for both fish and NEMS taxa at 6 or 8 replicates (ANOVA, df = 1, F = 0.08, n = 3264, $p = 0.769$). When this study was conceived, the 'boosted' method (pooling two samples prior to DNA extraction) was considered as a possibility to improve species detections with less laboratory processing costs. Increasing filtration volumes can improve eDNA yields and species detection rates by promoting the capture of low-concentration biological material from rare species (Hunter et al. 2019). However, filters tend to clog quickly in turbid environments, and the only options to increase filtration volumes are to either switch to a larger pore size (and risk losing more material through the pores) or increase the area of filter membrane, which then requires more preservative to inactivate DNA breakdown. In the latter case, the concentration of DNA in the preservative reaches a limit since it is countered by the dilution effects of adding more preservative. Given that only a limited amount of the preservative can be carried through to the DNA extraction step, increasing the effective filter membrane area to increase filtration volume (including pooling samples prior to extraction) is subject to diminishing returns. Both the standard and boosted samples were collected at the same time and taken from the same parcel of water, but the extra water filtered via the boosted samples provided no advantage in detecting more species. There are differences in results with species detected and sequence read counts between replicates and between the two methods at most sites, reflecting the stochastic nature of eDNA sampling (Cannon et al. 2016, Kawakami et al. 2023), but as a whole no benefit from using the boosted method was detected.

2.3.4 Turbid sites

At eight of the 51 sites (15.7%) the targeted amount of water filtered (one litre for standard and two litres for boosted) could not be achieved due to excessive turbidity. At these sites a relative doubling of water filtered from standard to boosted was still achieved (Table A-1). These sites were run separately as a group to check for any significant difference in species richness between the standard and boosted methods at 6 replicates and again none was found (ANOVA p=0.650 for fish, p=0.786 for NEMS invertebrates, df for all = 1, n for all = 128).

Filtering such large volumes of water in NZ waterways can require the use of very coarse filters, which in our experience have not yielded improved detection rates over the smaller volume, fine-membrane methods used here (Bird et al. 2023). This is likely due to the loss of target molecules through the pores of the coarse filters, and the accumulation of other debris such as sediment and phytoplankton (Stoeckle et al. 2022). Due to the varied water clarity (high sediment and/or algal loads) of many of NZ's streams, filtering large volumes of water is regarded as somewhat impractical (Bruno et al. 2017), whereas the eDNA mini kits used in this study only require small volumes of water to be filtered (up to 1 litre). For sites with higher levels of turbidity the use of a pre-filter or large filter size could be considered.

2.3.5 Fish

For the 51 sites analysed, 40 of 78 (51.3%) recognised freshwater fish species were detected using eDNA in this study (31 native species and 9 introduced). Eight fish had a conservation status of "Threatened" and 10 were "At risk" (Dunn et al. 2018). Fish species not detected were either rare, or geographically isolated to specific areas not covered in this study. For instance, within the family *Galaxiidae*, 13 of the 35 galaxiid species were accounted for in this study, mostly widely distributed species. Most of the undetected galaxiids are naturally uncommon or relict populations residing in small specific geographical areas, mainly in NZ's South Island. The situation was similar for 14 introduced species (4 *Gobiidae*, 4 *Poeciliidae*, 4 *Salmonidae* and 2 *Cyprinidae*), with all being either rare to occasional, isolated or both (McDowall, 2000). In contrast, all the widely distributed (nationwide) freshwater fish species of NZ were represented in the eDNA results of this study. These distributions varied between species and ranged from common to rare or patchy. For example, several rarer species were only detected at one site (Table A-3), whereas longfin eels (*Anguilla dieffenbachii*) were present at all sites. Longfin eel occurrence detection was 94% (standard method) after the first replicate at the sites it occurred (all but one) indicating its generality, its large size and status as the apex predator (likely elevating its chance of detection) in NZ freshwater systems and reflecting its ability to penetrate far inland, despite being a diadromous species (McDowall, 1978). Shortfin eels (*Anguilla australis*) were the second most common fish species in occurrence and were found at 43 sites. Their presence was patchy at some sites, occurring 81% of the time after two replicates and 88% after 5 replicates using standard methods. Shortfin eels are generally more common than longfin eels but tend to prefer warmer lowland waters (Jellyman, 1974) and the finer substrates at these locations. The Waikato River site at Hamilton (Site 11, Table A-1,

Figure 2-3) had the highest fish diversity with 17 species, eight of which are introduced species (the highest of any site). While this site is approximately 115km inland it remains at low elevation (<20m) and is easily accessible by diadromous species. Additionally, the warmer northern waters of the lower Waikato River are a hotspot for invasive fish (Collier and Grainger, 2015). Fish species richness ranged from 2 to 17 across all sites. On average, across all 16 replicates, fish richness was 3.2 species per replicate and ranged from 0 to 8 per replicate (Table 2-3).

Single pass electric fishing (150m) was undertaken directly after eDNA sampling at three of the sampled sites with a further three sites also compared (but not directly after sampling.) The details (methods, results, discussion) of these six sites comparing eDNA results with electric fishing are provided in Appendix B. In summary: acknowledging differences between sampling distance of eDNA and electric fishing, a 32.6% increase in fish species richness was attained with eDNA sampling compared with electric fishing. However, among these six sites, two fish species not found with eDNA (six replicates) were found via electric fishing (sites fished directly after eDNA sampling), indicating that some rare, low biomass species may not be detected by eDNA metabarcoding. All other fish species (from the six sites) caught via electric fishing (95.3%) were detected with eDNA plus an additional 17 species across the six sites that were fished. This baseline data aligns with data collected by David et al (2021) from five sites electric fished (single pass, 150m) after three eDNA replicates, where a 29.3% increase in fish richness was observed from the eDNA sampling. Similarly, two species caught via electric fishing in low biomass were not detected in the eDNA metabarcoding results, though with only three replicates taken. More recently obtained data (J. Smith unpub. data) from 44 stream sites (Waikato Region, stream order range 1 to 4) that were electric fished (single pass, 150m) directly after eDNA sampling (six replicates) showed that 35.2% more species were detected with eDNA and that 5 species or 2% were not detected via eDNA metabarcoding. Again, as with other sampling events these species detected via electric fishing and not by eDNA metabarcoding were rare with a low biomass.

2.3.6 Effect of elevation on fish eDNA replication

There was no significant difference (ANOVA, df = 1, F = 1.42, n = 51, p = 0.239) in proportional richness between small and larger waterbodies (stream order) for fish at 6 replicates, but there was a significant difference (ANOVA, df = 1, F = 8.34, n = 51, p = 0.005) between sites at low versus high elevation (>100m), with sites at higher elevation starting at

70.4% species proportional richness at one replicate compared to lowland sites at 61.9% at one replicate (Table 2-3). For fish the asymptote at these higher elevated sites is obtained faster (90.8% proportional fish richness after five replicates). In general, fish biodiversity throughout NZ decreases as elevation increases reflecting the elevation limited diadromy of NZ's native fish (McDowall, 2000). Therefore, fish diversity is often much lower and homogenous for considerable distances at higher elevation sites. Sample replication could reduce again at even higher elevation, but the final choice of the number of replicates also needs to consider other factors such as sampling consistency, waterway size, and the likely amount of introduced species present. Similar results were seen by Bylemans et al. (2018) study where replication needed for fish varied along the altitudinal and biodiversity gradient in an Australian river. Sites above 100m were limited to 13 in our study, but diadromous species often still occur at and above this elevation if the rise is gradual, and more work may be needed above elevations of around 200m to assess optimal replication at these sites for fish in NZ.

2.3.7 NEMS taxa

The eDNA analysis originally identified 561 freshwater invertebrate taxa, but this list was reduced to 129 taxa (Table A-4) when identified to the NEMS taxonomic level. The higher taxonomic richness for the NEMS invertebrates, reflects the inherently larger species pool compared to fish, resulting in a higher species/taxa difference between replicates with an average of 10 taxa per replicate and a range of 3 to 17 (Table 2-3). Not surprisingly, there was a significant difference in the average taxonomic richness between sites when identified using either eDNA or kick nets to the NEMS standard, or when using the original (mostly species) data from the eDNA results (ANOVA, df = 2, F = 139.8, n = 53, $p < 0.001$). Post-hoc tests showed no differences in the taxonomic richness between taxon richness from kick nets or from the eDNA samples when identified to the NEMS level.

The identification of 561 freshwater invertebrate taxa from the eDNA highlights the ability of this technique to identify taxa to lower levels than those obtained by traditional morphological methods, which are often limited to relatively coarse taxonomic resolutions, e.g., the NEMs protocols (NEMS 2022). Furthermore, Greenwood et al. (2015) developed an even more comprehensive list of 240 freshwater invertebrate taxa (identified to genus level where possible) that was gleaned from surveys at 1266 sites throughout NZ, and which currently represents the best information to date on invertebrate distribution patterns throughout NZ. That the eDNA survey of only 51 sites in this study identified more than double this number highlights the benefits of eDNA over traditional sampling, particularly in terms of undertaking proper biodiversity assessments. In particular, many invertebrate groups that are currently identified only to coarse taxonomic levels such as oligochaetes, were found to have a very high diversity. The eDNA sampling thus recognised 58 different taxa within the class Oligochaeta, of which 26 were identified to species, while the other 32 taxa were identified to higher taxonomic levels (genera, family). Other diverse taxonomic groups where eDNA had a clear advantage over the traditional NEMS level of identification included Cladocera (22 eDNA taxa), Copepoda (16 eDNA taxa), and Nematoda (18 eDNA taxa), where these were simply grouped into their single taxon under NEMS.

Despite the obvious increased ability of eDNA to identify taxa to lower taxonomic levels, we only assessed cumulative taxa richness plots at the same taxonomic resolution as used in the NEMS protocols. This allowed us to compare cumulative taxa richness plots collected by eDNA and traditional methods used in the NEMS protocols. Our results found that a single eDNA sample contained on average about 56% of the total taxonomic information that was found from 16 replicates, and that collecting six replicates (the number we recommend for on-going eDNA surveys) could be expected to contain 86% of taxa at a site. These results are very different to those from data collected by traditional techniques. For example, Shaver and Suren (2011) examined the effect of increased sample replication on cumulative taxa richness curves from samples collected by using either a kick net, or a Hess sampler in streams in Wellington, New Zealand. They found that cumulative richness tended to plateau between 8 and 15 replicate samples, but that the first sample contained only about 25% of the total taxa richness found in the stream, based on 20 replicates. Collecting 6 replicate samples contained only about 60 – 68% of total taxa richness from the kick, or Hess samplers respectively. A similar result was found by Suren and Lambert (2006), who found that a single kick sample contained about 39% of the total taxa richness in a small stream in New Zealand's west coast, while 6 replicates contained about 72% of total taxa richness.

These cumulative taxa curves from traditional morphological identification used in the NEMS protocols thus had a lower rate of taxa increase per replicate compared to that found in the eDNA samples. The higher initial number of taxa encountered using eDNA most likely reflects the much greater chance of DNA fragments that are lost from organisms upstream from the sampling site being collected with the syringes from the moving water (like fish). In contrast, animals collected with kick nets must be physically dislodged from the substrate in a stream and trapped in a downstream net. Given the often highly patchy nature of invertebrate distribution patterns (e.g., Winemiller et al 2010), it is not surprising that taxa accumulation curves often start out with so few taxa in the first few samples.

National state of environment (SoE) monitoring throughout NZ currently identifies invertebrates to the NEMS taxa list, which recognises only 197 taxa. Even the more comprehensive list of 240 taxa developed by Greenwood et al. (2015) is far fewer than we found in this work. A key part of this biomonitoring work is the use of biotic indices such as the MCI, and its quantitative variant, the QMCI (Stark and Maxted 2001), or the Average Score per Metric (ASPM: Collier 2008). These are now recognised as compulsory attributes for councils to monitor under the new National Policy Statement for Freshwater Management (2020), a central government initiative to help improve water quality throughout NZ. The ASPM is the average of the % of sensitive Ephemeroptera, Plecoptera and Trichoptera (EPT) abundance, EPT richness and the MCI. This means that all three biotic indices used in the NPSFM (2020) rely to a very large extent on the MCI. This index was originally developed to assess organic enrichment in streams and assigns individual taxa specific tolerance scores based on their observed distributions above and below point source pollution sources from dairy farm milking sheds (Stark 1993). An obvious weakness of the MCI is that it has published tolerance scores only for very broad taxonomic groups of many freshwater groups. For example, the class Oligochaeta has a general tolerance score of 1, indicating a tolerance to organic enrichment. However, given the large diversity of Oligochaeta identified by eDNA (including 28 identified species), this is an obvious weakness, as many of these species are likely to have a narrower tolerance to organic enrichment. The relatively rapid advancements in eDNA technology, and creation of ever-expanding genetic reference databases like GenBank/BOLD suggests that eDNA may have an important role to play in future biomonitoring programmes throughout NZ. However, a major requirement in accepting this technology in biomonitoring programmes is to create a consistent methodology for sample collection. Based on the results of the cumulative taxonomic plots, a key methodological component is the acceptance of using six replicate samples to characterise the invertebrate communities, as this number of replicates was shown to detect the vast majority of taxa expected at a site. Finally, another obvious step in using eDNA for routine biomonitoring throughout NZ would be to develop an eDNA version of the MCI, using taxa identified to the lowest possible level. This would likely perform better that the current version, where much information on the tolerances of different taxa is lost when groups are combined to Class, or higher. eDNA sampling also has additional advantages over traditional invertebrate collection methods (kick net/surber sampling) in that the quality of the data collected by traditional methods can often depend on the experience and skills of the staff involved in the collection and processing of the samples (Baird & Hajibabaei, 2012, Uchida et al. 2020). eDNA monitoring can overcome some of these issues, by simply collecting a water sample. Moreover, the site isn't disturbed, animals aren't killed to identify them, sampling bias in different mesohabitats is reduced, and both lentic and lotic invertebrates are detected (Macher et al. 2018). Finally, results would generally arrive much quicker than traditional sampling, and there is also likely to be fewer inconsistencies in identification compared with using morphological characteristics (Serrana et al. 2018).

2.3.8 Replication optimisation

This study as well as others (Machler et al. 2014, Evans et al. 2017, Beentjes et al. 2019, Cantera et al. 2019, Macher et al. 2021, David et al. 2021) showed a positive relationship between increased replication and/or water volume and increases in species richness. However, the extra work and cost involved collecting and processing these samples needs to be optimised, thus finding a balance between cost and comprehensive biodiversity assessment. Work by Macher et al (2021), found fish species richness increased by 24.8% with 18 one litre water replicates from 4 sites along a 2km stretch of the Mulde River in Dessau Germany. This is comparable to 25.4% for fish over six replicates or 36.0% for all 16 replicates in this study but with many more sites. Macher et al. (2021) also exhibited a similar starting point for species richness after 1 replicate and recommended 8 to 10 replicates for fish fauna, with no substantial richness increase above these numbers. This high starting point for fish after one replicate was also seen by Cantera et al (2019) for fish in tropical Guianese rivers and streams, where after one replicate (34 litres per replicate) average species richness was 67% in rivers and 87% in streams. This compares to 64% for fish in this study, where only one litre of water was filtered. After two replicates (or 68 litres of water filtered) Cantera et al. (2019) found little additional species richness, leading to their conclusion that filtering 34 to 68 litres of water was sufficient for inventorying the fish fauna in tropical streams.

Cantera et al. (2019) along with others (Pont et al. 2022) also suggested that more replicates may be needed for larger flowing waterbodies to describe the typically greater species diversity found in these larger systems. However, this does not appear to be the case in this study and in earlier work by David et al. (2021), with little difference being found between small streams (order 1 to 3) and larger streams and rivers (order 4 to 8). Another difficulty with using findings from other countries about defining the optimum volume of water to be filtered is that fish diversity is relatively low in NZ compared to many other countries, so less replication may in fact be required to adequately characterise the fish community, especially in larger water bodies. The same may also apply to the volume of water filtered, and the boosted method may show a different result in more diverse systems.

Differences between taxa groups based on diversity within a given system can also influence the number of replicates that might be needed to assess a community, especially if the focus is two or more taxa groups (Furlan et al. 2016). In this study, fish and the NEMS taxa are the main taxa of interest and there is a diversity difference, with fewer species for fish, 40 in total compared to 129 invertebrate taxa identified or <10 fish species per site compared to 35+ invertebrate taxa. As a result, fish maintain slightly a higher level of proportional detection relative to NEMS taxa. However, this cannot entirely be attributed to diversity, and several other factors like biomass and shedding rates (habitat, physiology, behaviour, predation) will also account for differences between fish and invertebrate apparent richness (Allen et al. 2021, Deiner et al. 2017). Despite these differences, the level of replication needed to describe these two groups adequately in this study is similar, which is a useful outcome when designing sampling programs.

This study highlights the need for replication in the detection of rare or patchy species (Lopes et al. 2017, Cantera et al, 2019, Machler et al. 2021, Takahashi et al, 2023). However, replication for abundant/common species or near field species (species present just upstream of the sampling location), is less of an issue as they are easily detected compared to rarer species at the location sampled. Most sites in this study had some fish species present across all 8 replicates with high sequence reads. These species at the electric fishing sites were considered common to abundant at this reach level and clearly near field when eDNA samples were taken. Detection of fish species between replicates in this study was stochastic particularly for rare species and those with patchy distributions. It should also be noted that this analysis is based on species/taxa being detected from the 16 replicates from each site and it is possible that some species were entirely missed by eDNA sampling due to either being very rare or a species having low DNA shedding rates compared with other species (see Appendix B). The best results for eDNA as with more traditional sampling methods will likely be during the warmer months (December-April) in NZ (Joy et al. 2013), when fish are more active, migrations have/are occurring, and water flows are generally lower and more stable (Toshiaki et al. 2021, Buxton et al. 2018). More replication could be required during colder months for effective community characterisation (Furlan et al. 2105). Also, seasonality maybe more species specific in terms of detection with eDNA when spawning occurs (Hayami et al 2020, Jo et al 2021).

When trying to detect or locate very rare or newly invasive fish species more than six replicates may be required as several rare/patchily distributed species only occurred in the 7th or 8th replicate at some sites in this study. Metabarcoding can be used as a cost-effective means to gain a perspective on the various taxa present for broad biodiversity monitoring. It should be noted that targeted approaches such as qPCR and ddPCR can offer increased sensitivity for individual species when the risk tolerance for type II errors/false negatives is low (Wood et al. 2019). In these situations, such as biosecurity incursion responses and threatened species mapping, a combined general metabarcoding and targeted assay approach may be preferable.

After discussion with stake holders involved in this study, six replicates were recommended as the “A” standard for eDNA sampling for freshwater fish and NEMS taxa in NZ. Any fish species undetected with six replicates are likely to be either rare or located a considerable distance upstream of the sampling point (dependant on flow).

2.4 Conclusion

The benefits of eDNA sampling by agencies throughout NZ to describe the overall species composition in waterways are obvious. However, the ultimate use of eDNA is likely to be site specific and depend on the questions being asked. Undertaking biodiversity assessments using eDNA will, in most cases, be easier with eDNA sampling compared to more labour-intensive traditional methods when presences/absence data is required. A core thrust of this work was consequently to develop a standardised protocol for the collection of eDNA throughout NZ that regional councils and other agencies can use. Although we have also suggested that there may be instances where six replicates may be considered unnecessary (for example to document fish diversity in high elevation inland waterways), it is also considered important that we support a standardised monitoring program throughout the country. Collecting the full set of 6 replicates at all sites is likely to provide more valuable community information, for aquatic communities and, also, some terrestrial species, as well as allowing us to better describe overall stream health using novel approaches such as the Taxon-Independent Community Index (TICI), (Wilkinson et al. 2024), or future prevalence indicators based on sequence reads that can be generated from 6-replicate data. Although there are now established biotic metrics to assess fish community health (Fish IBI: Joy et al. 2013) and invertebrate community health (MCI: Stark and Maxted 2001), these metrics (especially for macroinvertebrates) are unlikely to be applicable to the full suite of taxonomic data collected by eDNA. An obvious next step in enhancing the interpretative power of eDNA is to establish an eDNA Fish-IBI (Index of Biological Integrity) and an eDNA MCI (Macroinvertebrate Community Index) based on NEMS taxa for environmental reporting throughout NZ. Although eDNA has an obvious advantage in obtaining species composition data, and to a lesser extent, relative abundance data using the “read counts”, it does have its limitations. For example, traditional methods can measure fish and macroinvertebrate abundance and provide fish size structure. It is unlikely that eDNA will be able to provide these types of information. However, eDNA is a rapidly evolving field, and the current methods used in this study are likely to evolve into yet new and more efficient methods that may be able to solve some of these issues. Despite the undoubtful huge progress made to date with eDNA technology, there are several uncontrollable factors in the various stages of eDNA collection and analysis that need to be minimised to obtain the best results. This can only be achieved by adopting sound standard practices both in the field and the laboratory, which is why we recommend collecting 6 replicate syringe samples for ongoing eDNA surveys throughout NZ.

Data archiving statement: The raw data from this study has been uploaded to SRA with the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA950216.
Standard Bioinformatic processing script is available with the following link: Laulala333/High Replication paper Bioinformatics.R at main · Laulala333/Laulala333 (github.com)

Chapter 3. Spatial scale insights for freshwater fish in wadeable streams using environmental DNA metabarcoding.

3.1 Introduction

A common theme globally is the degradation of freshwater environments (Dudgeon et al, 2006). In New Zealand, 76% of indigenous freshwater fish are classified as threatened (Dunn et al. 2018). Growing pressures from land use change (agriculture, silviculture, urban development) have led to the degeneration of habitat, water quality and riverscape connectivity for New Zealand's native fish (Larned et al. 2020, Franklin et al. 2022). Additional pressures from introduced and invasive fish species compound these effects (McIntosh et al. 2010, Collier and Grainger, 2015).

The use of environmental DNA (eDNA) as a biomonitoring tool in assessing freshwater systems has grown exponentially in the last 20 years (Takahashi et al. 2023). The increased use is largely driven by time efficiency and sensitivity gains relative to more traditional methods (Sigsgaard et al. 2015, Cividae et al. 2016; Deiner et al. 2017; Qu & Stewart 2019, Beng and Corlett, 2020, Lee et al. 2024). Traditional methods used in New Zealand lotic systems (Joy et al. 2013) have largely revolved around electric fishing, netting/trapping and spotlighting techniques that can be invasive, effort-intense and range limited.

eDNA genetic material originating from organisms (as faeces, urine, mucous, scales, cells, gametes) is constantly being shed into the surrounding environment (Deiner et al, 2015). The collection of eDNA is especially suited to rivers and streams as genetic material carried downstream is regularly mixed and suspended and transported in a similar manner to fine suspended sediment (Pont 2018).

Filtered water samples with genetic fragments are processed by using polymerase chain reaction (PCR) assays, either via DNA barcoding or eDNA metabarcoding. Barcoding uses species-specific primers to detect a single species within a sample (Strickland & Roberts 2019, Akamatsu et al. 2020, Kessler et al. 2020, Togaki et al. 2020), while metabarcoding uses universal primers and next-generation sequencing (NGS) platforms to detect as many species as possible within a sample (David et al. 2021, Alexander et al. 2020, Djurhuus et al. 2020, Yang & Zhang 2020), producing rapid and accurate bioassessments of often complex aquatic communities (Deiner et al, 2017). eDNA bioassessments can detect rare (Laramie et al. 2015;

McKelvey et al. 2016; McKelvey et al. 2016; Sutter & Kinziger, 2019; Spence et al. 2021) and invasive species (Ficetola et al. 2008; Jerde et al. 2011; Carim et al. 2019). Metabarcoding will produce information on all species able to be sequenced within a sample enabling species (biodiversity) inventorying (DiBattista et al. 2019, Lyet et al. 2021), holistic ecosystem health assessment (Cordier et al. 2019, Wilkinson et al. 2023) and the evaluation of biosecurity incursions of both aquatic (Banks et al. 2021), and aquatically linked terrestrial organisms (Sales et al. 2020, Lyet et al. 2021). Metabarcoding has also been used for monitoring (David et al. 2021) and for describing trophic interactions (Jusino et al. 2019, Mata et al. 2019).

In lotic environments, the detection and interpretation of eDNA can be challenging due to the dilution and movement of the molecular source material. As this source material moves downstream it mixes with new fresh material from both new communities and those also found further upstream. This distribution and mixing of source material complicates the interpretation of results relative to those in lentic environments (Van Driessche. 2022).

The amount of source material available is dependent on the abundance or biomass of species present and each species can be expected to have their own eDNA shedding profile (Horiuchi et al. 2019; Murakami et al. 2021, Zhao et al. 2021). This profile will be dependent on the biology of a particular species i.e., physical traits, habitat preferences, size (allometric scaling), feeding behaviour, spawning locations, and migrations. Each of these profiles will also have different levels of eDNA degradation based on environmental conditions found at a given location, which can be influenced by sediment (Fremier et al. 2019), water temperature, acidity, turbidity, UV and nutrients (Harrison et al, 2019). Information on the effects of eDNA in different environmental conditions is currently lacking and requires further work (Wood et al. 2021).

eDNA shedding profiles are also influenced by the hydrology/discharge at a given location that will mix, and dilute eDNA (Pont et al. 2018, Wood et al. 2021). The distribution of eDNA as it moves downstream is further complicated by the distribution profile or plume as it moves downstream. This plume effect is the result of eDNA being continuously broken down and homogenised, starting from the source as larger fragments and gradually breaking down into smaller fragments that are laterally mixing and dispersing, making eDNA concentrations higher at a location downstream than just downstream of the initial source location. (Laporte et al., 2020, Wood et al, 2021, Van Driessche et al. 2022, Van Driessche et al. 2023). Furthermore, this initial concentration from the source material will be transported midstream before dispersing further downstream into stream margins as it breaks down into smaller fragments and disperses wider (Wood et al, 2021, Van Driessche et al, 2022).

The distance this plume moves downstream is also dependent on the discharge of a given system (Van Driessche et al., 2022, Van Driessche et al., 2023). The plume profile of a given species will likely not be uniform based on the eDNA shedding profile of each species. The reported travelling distances of fish eDNA in riverine environments largely corelates to the rate of discharge (Van Driessche et al, 2022) and is anywhere from just over 200 meters (Jane et al, 2015, Wilcox et al, 2016) to over 100 kilometres (Pont et al, 2018) from their original source.

It is unclear whether the greater eDNA travel distance relationship to higher discharge is due to higher flows or whether there is just more eDNA in bigger systems that typically provide more habitat and support larger fish populations (Van Driessche et al, 2022). A large system, however, is also expected to have a greater dilution effect on eDNA concentrations which may limit the detection of less abundant or rarer fish species, and sampling at higher-than-normal flows will likely reduce detectability through dilution (Curtis et al. 2020).

The use of eDNA in lotic systems for biomonitoring strategies requires an improved understanding of the expected distance within which organisms can be expected to be detected. Growing evidence from international studies suggest this range is influenced by discharge and biomass (Van Driessche et al, 2023, Van Driessche et al, 2022). In this study we aimed to test and improve our knowledge on detection distance in New Zealand streams via eDNA metabarcoding using both caged fish and the natural signal of a habitat-specific native fish species in wadable stream systems.

3.2 Study overview

3.2.1 Background

As with most lotic systems there are spatial and temporal changes in biological communities (Yamamoto et al 2017, Stoeckle et al. 2017, Byleman et al. 2018) from estuaries to headwater streams. This is especially true in New Zealand where elevation and distance inland are correlated to native fish diversity because of high levels of diadromy (Hayes et al. 1989; McDowall 1993; Jowett and Richardson 1996). We used this community dynamic as part of the experimental design which was conducted over two consecutive early autumn seasons (2021-22) at two sites close to the west coast of New Zealand's North Island in the Waikato region (Figure 3-1).

Many of New Zealand's diadromous fish species can be divided into three categories "climbers", "partial climbers" and "swimmers", and barriers, either natural (i.e., waterfalls, large rapids) or anthropogenic (i.e., culverts, weirs), will prevent these swimming species accessing upstream habitat, while the juveniles of many climbing species are able to climb these types of barriers provided, they are not over-hung or perched (Franklin et al. 2018). Partial climbers can negotiate smaller barriers. Two "swimming" species, inanga (*Galaxias maculatus*) and common bully (*Gobiomorphus cotidianus*), one partial climber torrentfish (*Cheimarrichthys fosteri*), and one "climbing" species (banded kokopu *Galaxias fasciatus*) were relocated upstream from below a small barrier within the same catchment (Manganui River) in this study. By sourcing these "caged" fish from the same catchment, any possible escapees would be contained within the same catchment and would be able to move back downstream to preferred habitat, reducing translocation risks.

The natural eDNA signal of a local, habitat-specific fish species, koaro (*Galaxias brevipinnis*), that resides in steeper tributaries of the Manganui River and the upper reaches of the Paparahia Stream (2nd site) was also used to establish travel distance. This diadromous species moves into freshwater from the ocean as a juvenile (c. 45-50 mm) in late winter to early summer (August to December), and as juveniles they are very good climbers. Their preferred habitats are in higher gradient areas of the riverscape with coarse substrates and swifter flows (McDowall, 1978 and 2000) often above significant rapids and waterfalls. During late summer/early autumn, this upstream migration period has finished and is prior to spawning (Smith, 2014), which in turn makes this time of year a good time to use koaro as a natural molecular source as movement from preferred habitat will be reduced.

3.2.2 Site A

The Manganui River at the field site location (A. Figure 3-1) is a 4th order (Strahler) stream at an elevation of 80 m above mean sea level (lower end of field site). It flows north to south for approximately 16 (km) before it converges with the Awakino River which flows east to west before entering the ocean a further 6 km downstream. The reach of river section used in this study (10 km) sits just above a small gorge that has several small natural falls (<0.5m) that prevent "swimming" species passage to upstream habitat. This barrier also largely prevents access to torrentfish a partial climbing species. This 10 km of water has a gradual drop in elevation of 80 m with no additional fish barriers from the top of the site ("Cage") to the bottom site (9.5 km) just above the gorge. The study reach flows through native bush for the first 4 km and then through dry stock (sheep and beef) farmland thereafter. There is also some sheep and beef farming and forestry upstream of the site reach in addition to native bush. Within the study reach the stream is a pool, run, riffle, cobble/gravel stream. Caged inanga and torrentfish were used at this site in both 2021 and 2022.

The Manganui River is electric fished annually as part of the Waikato Regional Council's State of the Environment Monitoring (SOE) at the upper site (410_10, past 14 years, ongoing) and every third year at SOE site (410_8) 2 km below the gorge or 2.2 km below the 9.5 km site. The sites had been eDNA sampled prior to the field trials at three locations (410_8, just above the gorge (9.5 km) and just upstream of the cage position at 410_10). These data were used to establish that the fish species used as a molecular source were unlikely to be resident in the mainstem Manganui River above the gorge (none caught by electric fishing and limited detections from eDNA samples at six replicates per site). A small detection in the eDNA sample just above the gorge did detect torrentfish indicating that a small percentage of torrentfish may be able to pass through the gorge. Koaro were detected with eDNA at the upper site, however this was during early summer when the upstream migration season for this species was still occurring (Table 1). Although banded kokopu is a climbing species, it hasn't previously been detected above the gorge (in pre-2021sampling) indicating that habitat for this species is limited.

3.2.3 Site B

The Paparahia Stream (B. Figure 3-1) is a 3rd order stream that flows 4km from the start of the study area (waterfall, upstream) at an elevation of 42 m to the coast. Below the waterfall it maintains a gradual gradient with no further fish barriers downstream. It flows in a north-west direction. The first km from the waterfall downstream largely flows through native bush, with the remaining 3 km being a mix of dry stock (sheep and beef) farmland and scrubland. It is a pool, run, riffle, gravel/cobble stream. A natural population of koaro is present above the 24 m high waterfall and their eDNA signal was used at the site as a natural molecular source. The steeper, swifter-flowing water conditions, together with a coarser substrate and native bush catchment found above the waterfall, is the preferred habitat of this species. As per the Manganui River, 14 years of annual electric fishing at this site together with eDNA sampling the year prior to the field trials, were used to establish that koaro were resident above the waterfall in preferred habitat during early autumn. No koaro have ever been found below the waterfall during annual late SOE summer monitoring.

3.2.4 Site C

Site C is a subset of the Manganui River using two unnamed tributaries that flow into the Manganui River (Figure 3-1). The first tributary (Upper tributary) is a 3rd order boulder/cobble stream that is steeper than the mainstem Manganui. Likewise, the second tributary (Lower tributary) is a similar stream, though slightly smaller (2nd order). Both these tributaries flow in a westerly direction and have resident populations of koaro, that during late summer, early autumn, aren’t found in the mainstem Manganui River. As per Site B (above the waterfall), koaro habitat found at these tributary sites is excellent. The natural koaro eDNA signal was utilised in both 2021 and 2022 field trials. Additionally, in 2022, two caged species, banded kokopu (upper trib.) and common bully (lower trib.), were employed as additional molecular sources within the tributaries themselves and in the mainstem to test the dilution effect on these tributary signals.

The results from eDNA in the upper tributary during the 2021 field trials did indicate that banded kokopu may be present (small detection, one out of six replicates) in this tributary. Therefore, caged banded kokopu used in 2022 at this site were added to boost this species eDNA signal.

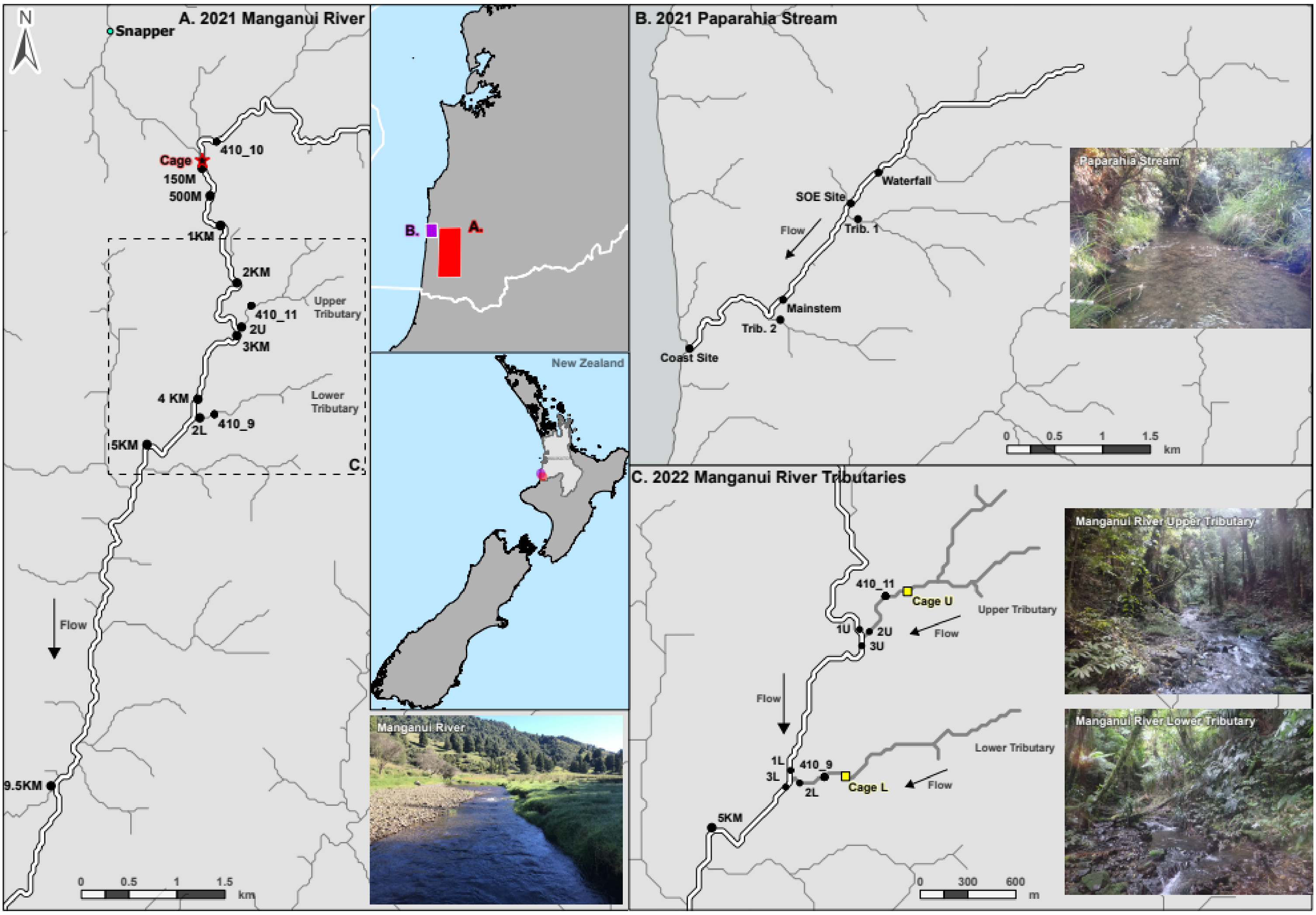

Figure 3-1. Field site locations. A) Manganui River sampling locations 2021, B) Paparahia Stream sampling locations 2021, and C) Manganui River and tributary sampling locations 2022.

3.3 Material and methods

3.3.1 (A) Manganui River 2021

On the 6th of April 2021, "Pre" (pre) eDNA sampling (before caged fish were added) was carried out on the Manganui River. Six replicates (1000 ml per replicate) were taken moving in an upstream direction from the lower site (9.5 km) to the upper site (0.150 km) just below the planned "caged" location (see the eDNA methods for more details). Two pre-eDNA samples were also taken at the two tributary sites (upper tributary (2U) and lower tributary (2L)) just before they entered the mainstem. These samples were taken to measure the eDNA signal of koaro and possibly shortjaw kokopu (*Galaxias postvectis,* another habitat specific native fish species) before it enters the mainstem. Water quality parameters were also taken at each sample location (see Appendix D, Table D-1. Manganui River 2021 sampling. Locations and timing of samplings with water quality measurements (Pre, Post and after fish removal). Species: in = inanga, tor = torrentfish. 2U = upper tributary and 2L = lower tributary. Sampling after fish out *denotes eDNA samples analysed.**)**. After the last pre sample was taken (0.150 km site), two closed, pre-cleaned (10% bleach solution - sodium hypochlorite) 4 mm mesh fyke nets (stored in clean/new large plastic bags) were then set up in the river at the "caged" location to soak overnight. These fykes have zipped openings on top of each net that enable quick transfer of captured fish. The "caged" location was a small pool/run that had a steady and even flow and deep enough (600mm) to fully submerge the fykes.

While this pre-sampling was taking place, another field team electro-fished a section of the Manganui River below the gorge to collect torrentfish. Captured torrentfish were held overnight in a closed fyke net in the river close to where they were caught. Further downstream, seven fine-mesh (4 mm) fykes nets and 14 Gee minnow traps (4 mm mesh) were set in the late afternoon/evening and left overnight targeting inanga. The following morning these fykes and minnow traps were retrieved and captured inanga were pooled and held in live nets in the river. Additional inanga were also seine netted in the lower Manganui River the same morning to boost numbers.

Table 3-1. Manganui River fish species below the gorge (natural barrier), directly above the gorge and 400m above the caged fish location. Electric fishing (EF) for SOE site 410_8 (2km below the gorge) is average number of species from four samplings (December 2013, 2016, 2019, 2022, 150m). Electric fishing for SOE site 410_10 (400m above caged location) is average number of species from 14 samplings (December 2010-2023, 150m). EF1 is electric fishing (300m, presences/absence only) directly above the gorge barrier. eDNA is total reads with the number of replicates detection occurs (up to 6). Green is caged fished species used in field trials and blue is koaro used a natural molecular source. The * denotes December sampling for koaro when juveniles of this species will be migrating upstream, hence mainstem signal.

Fish Species		Below gorge		Directly above the gorge		400m above caged location	
Scientific name	Common name	EF (410_8)	eDNA (410_8)	EF (1)	eDNA (1)	EF (410_10)	eDNA (410_10)
Cheimarrichthys fosteri	Torrentfish	18.25	18.25	n	14/1	0	0/0
Galaxias maculatus	Inanga	234.5	234.5	n	0/0	0	0/0
Gobiomorphus cotidianus	Common bully	0.25	0.25	n	0/0	0	0/0
Galaxias fasciatus	Banded kokopu	0	0	n	0/0	0	0/0
Galaxias brevipinnis	Koaro	0*	0*	n	0/0	0*	91/3*
Anguilla dieffenbachii	Longfin eel	230	230	y	3213/6	24.35	7434/6
Gobiomorphus huttoni	Redfin bully	292.5	292.5	y	343/6	32.71	8377/6
Anguilla australis	Shortfin eel	217.75	217.75	y	521/6	7.75	807/4
Oncorhynchus mykiss	Rainbow trout	6	6	y	401/5	0.43	194/2
Geotria australis	Pouched lamprey	13	13	y	6/1	5.28	65/3
Salmo trutta	Brown trout	0.75	0.75	n	0/0	1.07	0/0
Galaxias postvectis	Shortjaw kokopu	0	0	n	0/0	0.21	188/2
Gobiomorphus hubbsi	Bluegill bully	0	0	n	0/0	0.07	0/0
Retropinna retropinna	Common smelt	0.75	0.75	n	0/0	0	0/0

Captured torrentfish and inanga were wet weighed live with minimal handling so that a similar caged biomass could be established. This weight was 368.8 grams for inanga and 368.7 for torrentfish and equated to 98 inanga and 25 torrentfish. These fish were added into the waiting fyke nets at the "caged" location. After 21 h, "Post" (post) eDNA sampling began in the same order as the pre-sampling. The top site (0.150 km) was the last site sampled and at the conclusion of sampling the caged fish had been held for a total of 25 h and 30 min (Appendix D, Table D-1. Manganui River 2021 sampling. Locations and timing of samplings with water quality measurements (Pre, Post and after fish removal). Species: in = inanga, tor = torrentfish. 2U = upper tributary and 2L = lower tributary. Sampling after fish out *denotes eDNA samples analysed.). More accurate weights as well as lengths were collected after the last eDNA sample, this weight differed from the wet weights, with torrentfish having a slightly higher biomass (Table 3-2). All fish were then transported back downstream and released back to where they had been captured prior. eDNA samples were also taken the following day (after "caged" fish had been removed) with only one (6 replicate) site sample (0.150 km) being analysed due to a limited budget. This sample was taken 22.32 h after the caged fish were removed (Appendix D, Table D-1. Manganui River 2021 sampling. Locations and timing of samplings with water quality measurements (Pre, Post and after fish removal). Species: in = inanga, tor = torrentfish. 2U = upper tributary and 2L = lower tributary. Sampling after fish out *denotes eDNA samples analysed.).

Table 3-2. Caged fish species per year (numbers of individuals, size range and average size (mm), weight range, average weight, and total biomass (grams).

Parameter	Mainstem Manganui River				Manganui River tributaries	
	Inanga (*Galaxias maculatus*)		Torrentfish (*Cheimarrichthys fosteri*)		Banded kokopu (*Galaxias fasciatus*)	Common bully (*Gobiomorphus cotidianus*)
Year	2021	2022	2021	2022	2022	2022
Number	98	76	25	13	3	20
Size range (mm)	58 - 109	48 - 96	57 - 132	59-141	64 - 181	51 - 104
Av. Size (mm)	84.82	70.38	101.8	87.61	139.66	75.85
Weight range (g)	0.9 - 7.1	0.5 - 5.4	2.2 - 36.2	2.1- 33.8	2.2 - 66.1	1.1 - 12.0
Av. Weight (g)	3.73	2.03	14.62	10.03	42.2	5.11
Total biomass (g)	335.8	154.8	365.7	130.49	126.6	102.3

3.3.2 (B) Paparahia Stream 2021

On the 22-23 March 2021, eDNA sampling was carried out on the Paparahia Stream. Six replicates (1000ml per replicate) were taken moving in an upstream direction from the lower site (Coast) to the upper site (Waterfall, see the eDNA methods for more details). eDNA samples were also taken at the two tributary sites (Trib. 1, Trib. 2) just before they entered the mainstem. These samples were taken to measure the possible eDNA signal of koaro and before it enters the mainstem. Water quality parameters were also taken at each sample location (see Appendix D, Table D-2. Paparahia Stream 2021 sampling. Locations, distance, and timing of sampling with water quality measurements.**).** Just over 500m of water was also electric fished in each of these tributaries targeting koaro and none were found. Annual monitoring (150m) using standard protocols for electric fishing (Joy et al. 2013) at the Paparahia SOE site was conducted after eDNA sampling on the second day (23rd), and an additional 320m was fished from the top of the SOE site to the base of the waterfall. One juvenile koaro was caught indicating that some koaro may be present below the waterfall but at low densities. An eDNA sample was then taken just above the waterfall and then was electric fished for 100m with eight koaro caught (Table 3-3). Previous annual SOE monitoring (March/April) at the Paparahia site (14 years) has never recorded koaro.

Table 3-3. Paparahia Stream single pass electric fishing koaro results March 2021, plus all SOE samplings (March/April, 150m, 14 years).

Parameter	Koaro (*Galaxias brevipinnis*)				
	Above the waterfall (100m)	Waterfall to below SOE (470m)	SOE site all years (2009-2024, 150m)	Trib 1. (500m +)	Trib 2. (500m +)
Number	8 (adult)	1 (juvenile)	0	0	0
Size range (mm)	75 - 135	59	0	0	0
Weight range (g)	5.40 - 37.68	0	0	0	0
Av weight (g)	25.2	0	0	0	0
Total biomass (g)	201.67	0	0	0	0

3.3.3 (C) Manganui River tributaries and Manganui River 2022

For the 2022 Manganui River and tributary sampling, both passive and syringe (standard method) eDNA sampling was conducted in tandem to compare these methods of eDNA collection. The “active” syringe sampling was undertaken in the same manner the previous Manganui sampling in 2021 with six replicates and 1000ml of water filtered per site.

The passive collection method utilizes 'pod' samplers’. These pod samplers are equipped with a pre-filter to block out larger debris followed by qualitative dacron filter (Aqua One Micro Pad; relatively coarser than the 1.2µm filter in the syringe). They were set midstream just off the stream bottom in aluminium manifold housings (secured with small rebar stakes) which hold the six individual plastic pods (giving six replicates, Figure 3-2). Passive samplers were left in situ for at least 21 h before being retrieved.

The use of the passive samplers in the 2022 study elevates the risk of contamination for both passive and syringe sampling, as either setting from downstream to upstream or upstream to downstream involves being in the stream itself. Any potential contamination signal will flow downstream to any passives samplers already set or about to be set depending on the logistics used. Because of this elevated risk of contamination, care was taken to decontaminate field footwear between sites using a 10% bleach solution. Fresh sterile latex gloves were also used at each location prior to sampling being conducted.

For the passive sampler deployments, the river was divided into upper half (including upper tributary) and lower half (including lower tributary), and two field teams deployed the samplers at the same time taking the upper and lower half of the river (see Appendix D, Table D-3. Manganui River Tributary sampling. Locations, distance, and timing of samplings with water quality measurements (Pre and Post). Species: bk = banded kokopu, cb = common bully. Ds = downstream, us = upstream and conflu. = confluence.) for the timing of both passive and syringe sampling).

On the 28th of March 2022, pre eDNA samples (six replicates, passive and syringe) were taken. The cleaned (bleached) metal “passive” manifolds were set instream and after five minutes of stream water running through them the pods were attached. For the pre sampling the syringe samples were taken as the passive samplers were set. Samples were taken at the SOE site below the gorge (410_8), 9.5 km, 5 km and 0.150 km below the “caged” location on the mainstem Manganui River. Pre tributary samples were taken, 0.150 km below (3U, 3L) and 10 m above (1U, 1L) each of the inflowing tributaries (upper 410_11 and lower 410_9) on the mainstem. The 1L site is 100 m below the original 4 km site location and effectively remains as the 4 km site, and the 3U site is the same location as 3 km. Samples were also taken in the tributaries 10m upstream of the mainstem confluence (2U, 2L) and at the start (downstream end) of the SOE site locations (410_11 and 410_9) upstream (Figure 3-1, Appendix D, Table D-3. Manganui River Tributary sampling. Locations, distance, and timing of samplings with water quality measurements (Pre and Post). Species: bk = banded kokopu, cb = common bully. Ds = downstream, us = upstream and conflu. = confluence.).

Figure 3-2. Passive manifold housing with six plastic pods with Dacron filter within each pod.

Inanga and torrentfish were used again as per the 2021 sampling, using the same "caged" location. The same (2021) methods to catch and hold these species were repeated. However, the "caged" method for holding the fish was changed at short notice due to issues around nuisance eel predation and the soft-meshed fykes nets (see more detail in discussion). As a result, the holding fyke nets were swapped out for two 60 l chilly bins. Holes were drilled into the chilly bins on site to allow water to flow through the bins. Torrentfish were held in one bin and inanga in the other. Due to the size difference between these two species smaller (4mm) holes were drill in the "inanga" bin and larger (8 mm) holes were drilled into the "torrentfish" bin. This resulted in different flow rates between the two bins (0.65 l/s for the "inanga" bin and 1.65 l/s for the "torrentfish" bin). Both inanga and torrentfish were weighed and measured after the field trials (Table 3-2) before being released back to their capture location.

Five fine-mesh (4mm) fyke nets and 10 Gee minnow traps were set in the lower Manganui River and left overnight to target common bullies. The following morning these fykes and minnow traps were retrieved and captured common bullies were pooled and held in live nets in the river. For approximately half of the common bullies caught, dorsal fin rays were counted to confirm they were common bully rather than giant bully (common bully have 7-8 dorsal rays while giant bully will have 6). A small bush tributary in the lower catchment was electric fished targeting banded kokopu, three were caught and were also held in live nets overnight. Banded kokopu and common bully were weighed and measured after the field trials (Table 3-2) before being returned to their original capture location.

Clean fyke nets were originally planned to be used as the holding "cages" for both banded kokopu and common bully, but as per above these were swapped for buckets at short notice. Two single 20 l buckets that each had 54, 8 mm holes drilled into them were then cleaned with a 10% bleach solution and rinsed with site stream water and set in the stream with the lids on, 150 m above the downstream end of both SOE sites (410_11 and 410_9). The buckets were set in the stream to a level just above the highest line of holes (150 mm from the bottom of the bucket). Both buckets had an average flow rate of 2.43 l/s when filled.

For post-sampling, once the "caged fish" had been introduced, there was a two-hour and 40-minute delay, then fresh passive pods were inserted into the already deployed manifolds, working downstream starting at the sites closest to the planted fish. After a minimum of 21 hours these passive samplers were collected. The post syringe samples were taken at a minimum 21 h after the introduction of the fish, with the last sample taken 25 hours and 10 minutes post-introduction (Table D-3. Manganui River Tributary sampling. Locations, distance, and timing of samplings with water quality measurements (Pre and Post). Species: bk = banded kokopu, cb = common bully. Ds = downstream, us = upstream and conflu. = confluence., Appendix D).

3.3.4 Equipment

All equipment used to transfer fish upstream was soaked in a 10% bleach solution for one hour to prevent contamination. Water from the "caged locations" was used to transfer the fish upstream. All eDNA sampling other than the 2022 sampling was done in an upstream direction to avoid any potential contamination. Neoprene booties were used by fieldworkers when collecting samples or moving through water to the next site for the 2022 sampling. After each site, new booties were used, and a 10% bleach solution was used soak any used booties for at least one hour before they were used at a new site.

3.3.5 Ethics

The capture and use of live fish in these field trials was approved by the University of Waikato animal ethics committee (Protocol Number 1142). The capture and transfer of these fish species from the lower Manganui/Awakino catchment to the upper Manganui River field location was also approved by Fisheries New Zealand (File Ref: NFT 351 and 377).

3.3.6 Electric fishing and biomass

Electrofishing at SOE sites followed standardised protocols, ‘button time’ effort and machine settings (Joy et al. 2013). Fishing was conducted in an upstream direction and captured fish were measured for length and identified to species level at the completion of every 15 m sub-reach. Lengths (mm) from individually measured fish (total fish length) were used to estimate a relative one-pass biomass for each species using established species-specific quadratic equations (Jellyman et al. 2013). All fish data were entered into standardised electronic data capture forms (fish template) and were later assessed for data quality prior to their upload to corporate databases (KiEco, KISTERS, North America Inc.) and the New Zealand Freshwater Fish Database (NZFFD). See Appendix D, Table D-5. SOE site fish parameters.for fish counts, lengths and calculated biomass for SOE electric fishing sites associated with this study.

3.3.7 Negative controls

Six negative control sites were randomly chosen and sampled (syringe) alongside both pre- and post-field samples (three each) using the same methods as for all other sites. Ultra-clean water housed in sterilised plastic bottles was sourced from Waikato University and syringed directly from the bottle on site (beside the stream) in between field replicate sampling. Fresh latex gloves were used for all the negative control sites.

3.3.8 Stream flow

Stream flow gauging was undertaken at five sites (0.150 km, 2 km, 4 km, 5 km, 9.5 km) for the Manganui 2021 mainstem sampling and three sites for the Manganui mainstem 2022 sampling (0.150 km, 5 km, 9.5 km). Both Manganui tributaries (410_11 and 410_9) were also gauged just before they entered the mainstem in both sampling years (2021-22). The Paparahia Stream was gauged (2021) at five sites (“SOE” (3009_1), “upper tributary”, “Mainstem”, “lower tributary” and “Coast”) All gauging apart from the “lower tributary” (Paparahia) was undertaken using a Sontek Flowtracker (Serial Number P1700) with flows calculated using the on-board software. The “lower tributary” Paparahia flow measurement was done using a 20-litre bucket by timing the volume of water (averaged from 5 replicates) from a perched culvert just prior to this tributary entering the mainstem.

The downstream gauging at the “Coast” site for the Paparahia stream wasn’t used, as errors associated with this gauging site (inconsistent cross section due to sand substrate) resulted in a gauged flow less than flows found upstream (mainstem site). As a result, the “Coast” site was modelled using sites that were gauged upstream and Specific Discharge (SpQ). SpQ is calculated by a known flow (m^3/s) divided by its Catchment Area (km^2) to give Specific discharge ($m^3/s/km^2$). This can then be applied to another location on the stream by multiplying the SpQ by the Catchment Area of the new location. This method can be used for ungauged catchments and is a useful way to identify erroneous readings.

Travel times were estimated for all sites based on mean velocity (ms^{-1}) between two consecutive gauging sites or just based on one single velocity for the Manganui tributaries (at the gauging site). The travel time was then calculated by dividing the distance (m) by this mean velocity and then divided by 60 to get minutes. The mean velocities are dependent on the cross-section chosen at each site (‘deeper and slower’ vs ‘shallower and faster’) and will vary more than the flow. How well the method works is dependent on how representative the measured sections cover the total stream geometry which for all sites is limited. As a result, these “travel times” should be considered a rough estimate.

3.3.9 Water quality

Water quality samples were collected from four sites (0.150 km and 9.5 km on the mainstem Manganui and from both tributaries (410_11 and 410_9). They were tested for turbidity and total suspended solids (TSS) along with indicators for nitrogen, phosphorus, and ammonia. Table D-6. Water quality testing and methods (Hill laboratories), 31 March 2022 at four sites (Upper mainstem 0.150 km, Lower tributary downstream (410_9), Upper tributary downstream (410_11) and 9.5 km). in Appendix D provides results and laboratory methods.

3.3.10 eDNA collection and analysis

Please see Chapter 2 (Replication) for syringe collection and eDNA analysis methods, see below (Passive sampling) for passive steps that differ to the syringe method. In total five assays were used to detect fish species across both sampling years. The “DG” Assay was used in 2022 but not in the 2021 sampling and ZV was used in 2021 but not 2022 (Table 3-4), see Appendix D, Table D-8. Fish molecular targets for all eDNA study sites (2021-22) with totals., for all fish targets for each site and associated sequence reads.

Table 3-4. eDNA primers used for fish 2021 (Manganui and Paparahia) and 2022 (Manganui).

2021	2022
CI	CI
LV	LV
RZ	RV
WV	WV
ZV	DG

3.3.11 Passive sampling

At each site six passive pods were inserted into pre deployed metal housing manifolds using new latex gloves. Fieldworkers stood downstream of the manifold while inserting the pods. After at minimum of 21 h the pods were retrieved from the manifolds using a fresh pair of latex gloves again working downstream of the manifold. The Dacron filter was removed from the small opening at the back end of each of the pods, in doing this excess water is removed from the Dacron filter. The filter is then placed into a small plastic jar that is part of the eDNA sampling kit (six jars per kit) and 1 ml of 2x DNA/RNA Shield preservation and lysis buffer (Zymo Research Cat. No. R1200-125) was then injected directly into the filter to prevent degradation of captured DNA, the lid was secured, and the jar was shaken briefly to ensure the preservative was mixed throughout the filter. This was repeated for each replicate. The GPS co-ordinates of the location were recorded on the supplied sample kit bag. This same process was repeated for all sites, and samples were kept at ambient temperature and couriered to the Wilderlab laboratory (Wellington, NZ) within one week of collection for analysis.

Note that the DNA/RNA Shield product acts as both a preservation and lysis buffer (see https://files.zymoresearch.com/protocols/_r1100-50_r1100-250_r1200-25_r1100-125_dna_rna_shield.pdf), so filter digestion occurs during sample transport, and target metazoan DNA is in solution by the time the lysate is drawn from the filter container.

3.4 Results

3.4.1 General eDNA

Snapper (*Pagrus auratus*), a common New Zealand marine fish dominates the fish sequence reads for most sites sampled for the pre-sampling 2021 Manganui sampling (>50%, Figure 3-3), especially for the three upper sites (0.150 km, 0.500 km, 1 km, Figure 3-4, Figure 3-5). Recreational marine fisherman commonly discard snapper fish frames along with other common recreational marine fish species, i.e., kahawai (*Arripis trutta*) and gurnard (*Chelidonichthys kumu*), into freshwater streams after filleting in New Zealand to feed the local eels. After reviewing the results this was confirmed with the local farmer who lives 2.2 km upstream from the mainstem beside the Mangakahikikatea Stream, a major tributary of the Manganui River that joins the mainstem 100 m upstream of the "cage" location. Unfortunately, we were unable to establish the number/biomass of fish that were discarded or the frequency of this activity. No marine species were detected in the 2022 Manganui sampling.

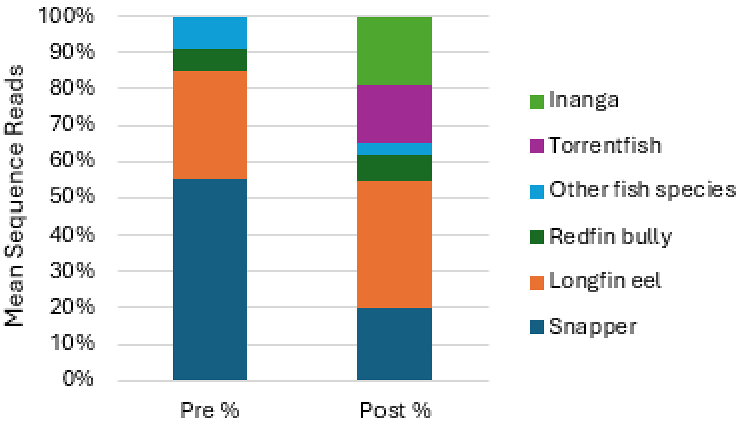

Figure 3-3. Percentage of mean sequence reads per sampling pre and post for main fish species in the Manganui 2021 sampling.

Snapper was also detected at the coastal site for the Paparahia stream, and this is likely influenced by tidal flows rather than discarded fish at this site. Apart from snapper at the upper sites for the Manganui River, longfin eel dominated the eDNA detections for fish across all sites in this study (Figure 3-5). Longfin eel is the apex predator in New Zealand freshwater systems and dominated the biomass of all fished SOE sites in this study (Manganui above the cage site (410_10), Manganui upper tributary (410_11), Manganui lower tributary (410_9), 410_8 below the gorge and the Paparahia stream site (3009_1), (Appendix D, Table D-5. SOE site fish parameters.). The average size of longfins caught at these sites is 234 mm but fish as large as 1180 mm have been caught. Redfin bullies are also common at all sites in this study, but at an average of 64mm and caught in numbers similar to longfin eels at the three upper Manganui sites (410_10, 410_11, 410_9), their biomass is much lower but still second to that of longfin eels (Appendix D, Table D-5. SOE site fish parameters.). Fish species naturally detected via eDNA for all study sites are presented in Figure 3-5.

eDNA sequence reads were variable between all sites and years for both the Manganui mainstem and tributary sites (Figure 3-4). The higher fish, mean sequence reads for the post-sampling (37134) to that of the pre-sampling (28795), for the mainstem Manganui 2022 shows no significant difference (t-Test, p=0.08, df=7) overall. However, a significant difference is observed from the 0.150 km site down to the 4 km site (t-Test, p=0.014, df=5). This result can be attributed to both the addition of the “caged” fish (inanga and torrentfish) and the presence of snapper which accounts for >50% of the fish reads in the pre-sampling for all mainstem sites (Figure 3-3).

Fish make up 21.6% of the total mean sequence reads for the seven main taxa groupings for the Manganui 2021 sampling and 10.1% of mean sequence reads for the Manganui 2022 sampling (Figure 3-4). And again, this difference can be attributed to the presence of snapper and the planted fish for the 2021 sampling. Though fish were planted for the 2022 sampling, their detection is limited compared with 2021 due to method changes. The “Other” grouping (Figure 3-4 and Figure 3-6) mainly consists of unidentified sequences which are a combination of organisms not currently in the reference database or are undescribed. The higher levels of fish sequence reads in the 2021 sampling is effectively due to one less target marker, with the “DG” primer not used the 2021 sampling. This primer accounted for 29.6% of all fish sequence reads in the 2022 sampling compared to 0.12% for the “ZV” primer which was the fifth primer used in the 2021 sampling for fish rather than the “DG” primer (see eDNA collection and analysis).

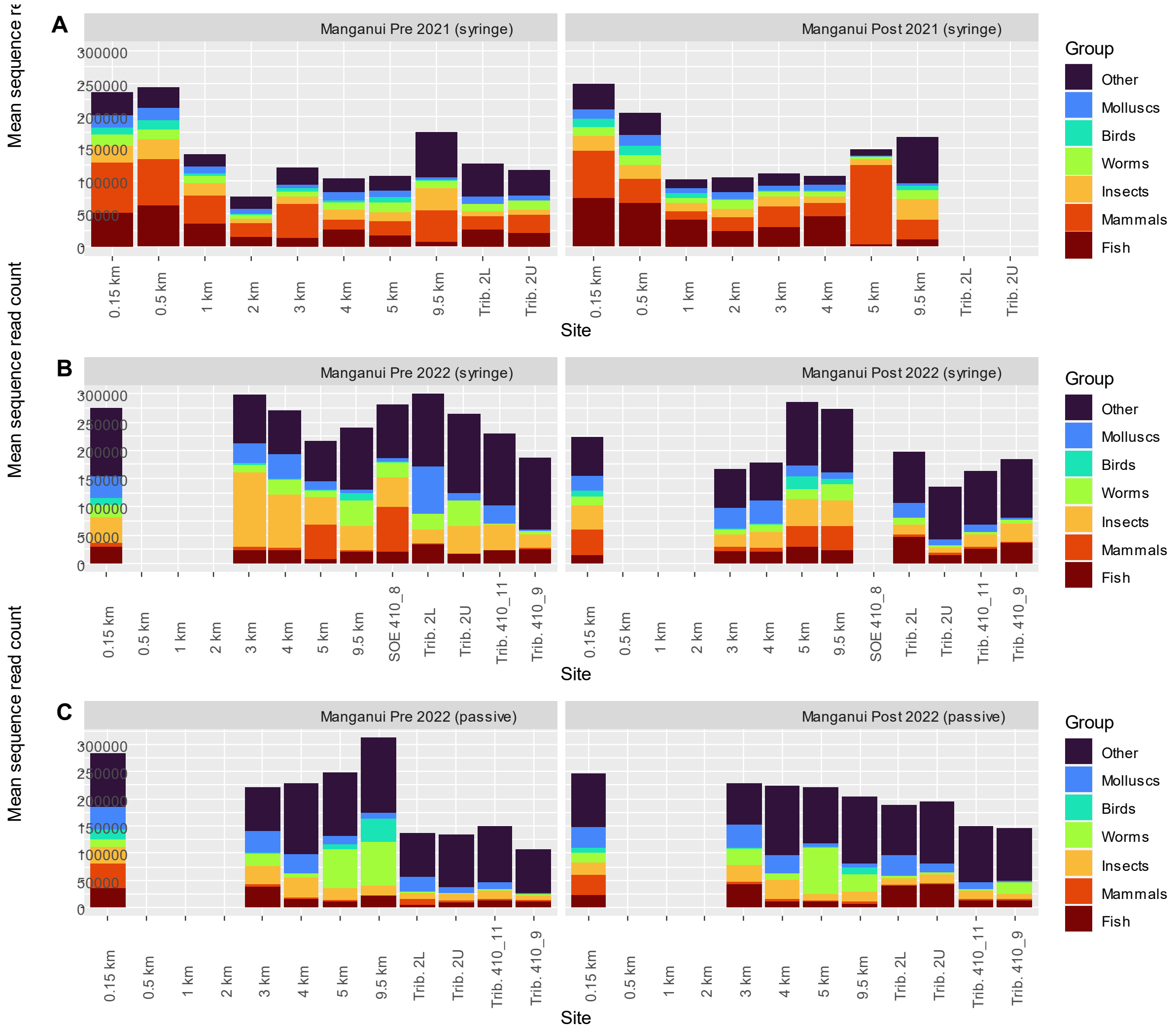

Figure 3-4. All Manganui mainstem and tributary eDNA sites for 2021 (A, Pre and Post sampling) and 2022 (B, Pre and Post sampling syringe and C, Pre and Post sampling passive) in relation to mean eDNA sequence reads for seven main groupings.

The 2022 sampling has higher reads for insects (p=0.002, df 17) which isn't affected by the "DG" primer. There are fewer mammals for the 2022 samplings despite the "DG" primer being used but this isn't significant. Some of these differences can be attributed to the fact the samples are taken a year apart. Though samples are taken at the same time of year, environmental conditions and the timing of the sampling (am/pm) together with other influences like farming practices (stock movement), will result in differences year to year or even day to day with pre- and post-sampling.

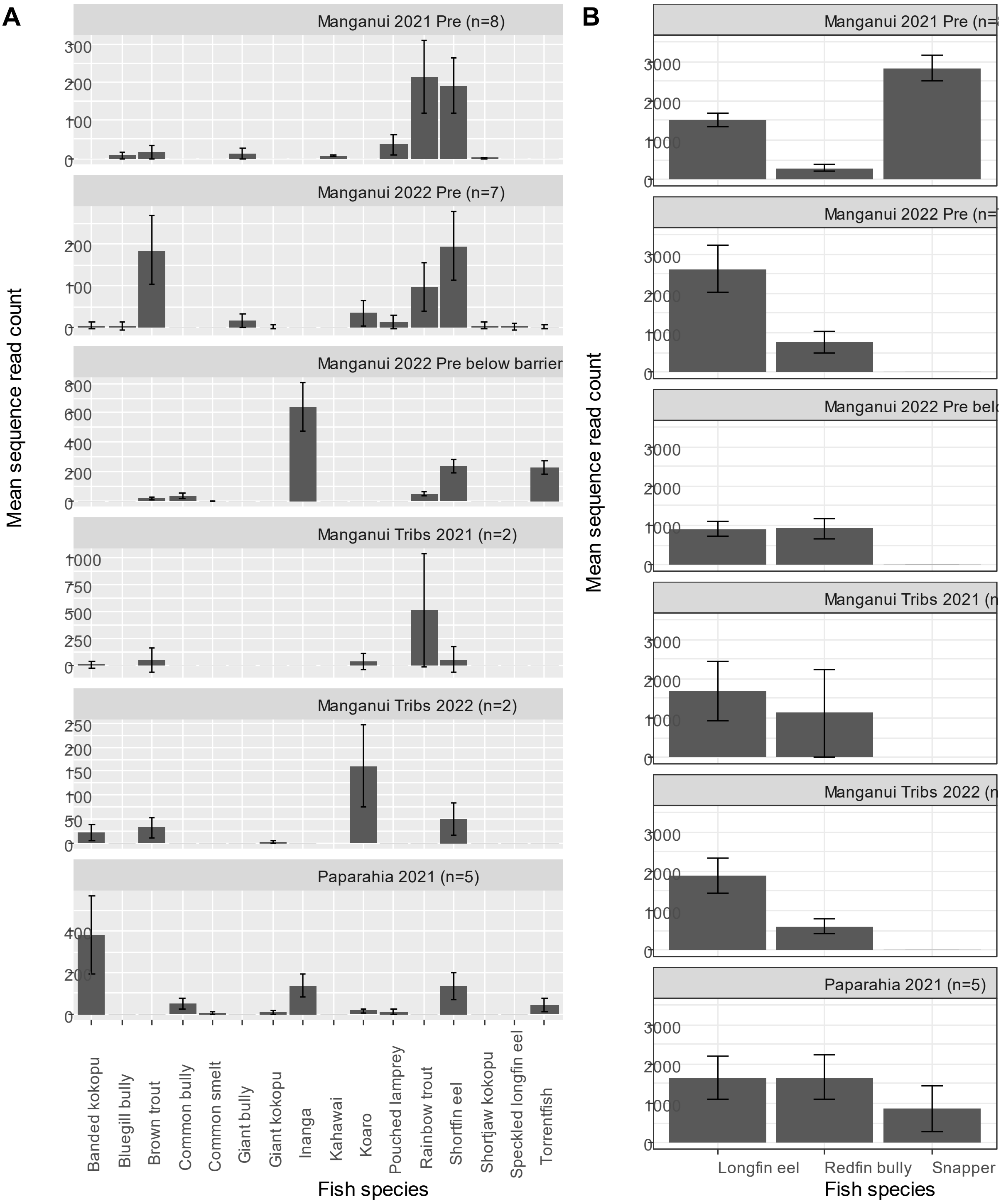

Figure 3-5. Fish species detected with eDNA sampling (mean sequence reads with standard error) across all study sites (Pre sampling for Manganui 2021 and 2022). A) less dominant species including kahawai a discarded marine species. B) dominant fish species including snapper a discarded marine species.

A single group can dominate the sequence reads, as is evident for the Manganui 5 km post-2021 sampling (Figure 3-4) where mammals make up 81% of the total sequence reads, of which cattle (*Bos taurus*) make up 98% of the mammal reads. This compares to an average of 24% for mammals at the other Manganui 2021 sites. Proportionally fish are still represented at the

5 km post-site when compared to the 5 km pre-sampling two days prior, with 8 fish species compared to 9 pre-. However, both replicate ‘hits’ and mean sequence reads (t-Test p=0.0001, df 101) dropped significantly to 22 and 24.5 compared to 31 and 150, respectively. This indicates that a swamping effect from a dominate signal can reduce replicate detection and sequence reads, but with sufficient replication species can still be detected (Wood et al. 2021). The swamping effect at this site occurred because cattle were standing in the stream at the time of sampling about 300 m upstream and likely defecating, boosting their eDNA signal significantly/disproportionately.

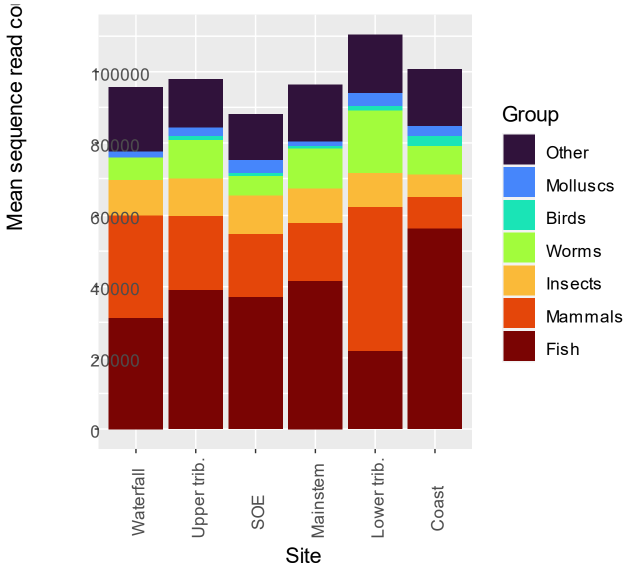

Figure 3-6. All Paparahia mainstem and tributary eDNA sites for 2021 in relation to mean eDNA sequence reads for seven main groupings.

Relative to the Manganui sites there wasn’t much difference between sites and taxa groupings for the Paparahia stream (Figure 3-6) apart from more mammals in the pasture dominant lower tributary and an increase in fish reads at the coast, where marine species like snapper appear. Fish overall make up 38.5% of the total sequenec reads at this site.

3.4.2 Manganui 2021 “Cage”

Results from the 2021 Manganui “Caged” trial (Figure 3-7) show no signal for inanga at any the sites in the pre-sampling. A small detection (35 sequence reads, or 5.8 mean sequence reads) in one replicate at the 3 km pre-site indicates that torrentfish may be above the barrier in very low numbers but was not detected at any other sites including 9.5 km. Both inanga and torrentfish showed an initial decline in mean sequence reads from the 0.150 km site to the 1 km site, but then reads increased to the 4 km site before dropping off. This pattern is the plume effect and appears to be a similar phenomenon to that reported in a number of other studies (Wood et al, 2021, Van Driessche et al., 2022, Van Driessche et al., 2023). For snapper we may also be seeing the tail end of a plume at the 0.500 km and 1 km sites which would match with the distances we see at the tail end of the plume for inanga and torrentfish (4km), with the snapper source signal being just over 3 km upstream from the 1 km site. The snapper mean sequence reads dropped off significantly after 1 km similar to that observed for the caged fish at 4 km. The snapper signal was still detectable at the 9.5 km site. Both inanga and torrentfish were detected at 5 km downstream in all six replicates for inanga and three for torrentfish but with low sequence reads. It is likely that these sequence reads would have been higher at this site had the signal not been swamped by the cattle reads. It is possible that, had extra sites been established at the 6 and 7 km mark, caged fish may have been detected further downstream. Both naturally occurring fish species (longfin eel and redfin bully) provided a consistent level of signal control to compare both the snapper and caged fish against. The single sample processed from the 0.150 km site 25 hours after the caged fish had been removed had low sequence reads for both species and in three replicates each. It is unlikely that any additional snapper source material had been dumped into the mainstem Manganui as there are no additional crossings (bridges) until below the gorge.

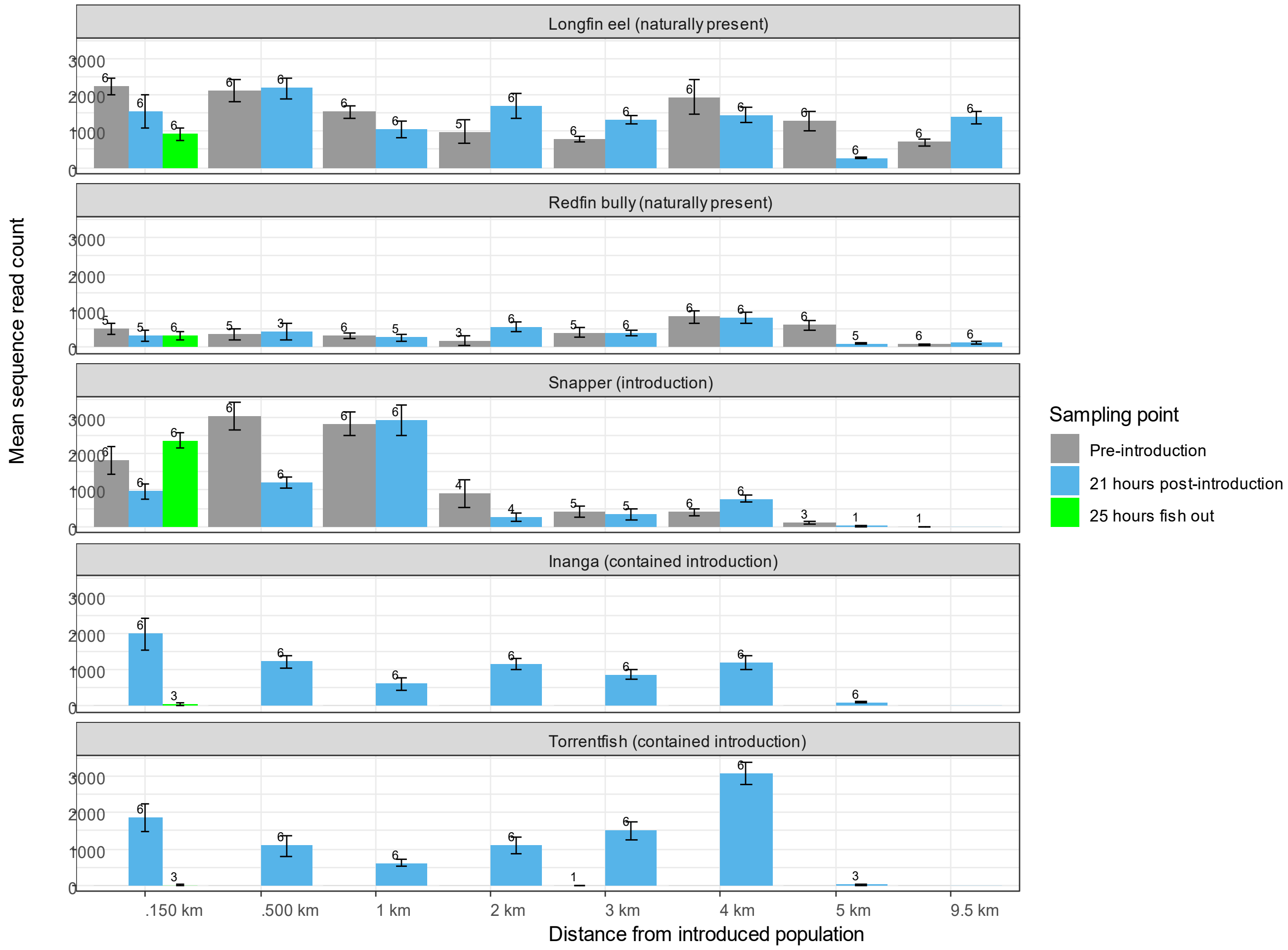

Figure 3-7. Manganui River "Caged" fish results 2021, mean sequence reads with standard error. Top two panels (longfin eel and redfin bully) are naturally occurring fish species. The middle panel is snapper an unplanned introduction. The bottom two panels are the planted fish (inanga and torrentfish). Samplings are pre, post and 25 hours after fish were removed (one site only). Numbers associated with each bar represents the number of replicates that had a positive result (1-6).

3.4.3 Manganui mainstem 2022 "Cage"

Results from the 2022 Manganui mainstem "Caged" trial (Figure 3-8) show very different results to that of the 2021 mainstem Manganui sampling. It is proposed that these differences were likely to be related to the change in holding methods (fyke nets to chilly bins) at the caged location. The mean sequence reads, and replicate hits are minimal for both species across both sampling types (passive and syringe). Background naturally occurring species in 2022 (longfin and redfin bully) had higher mean sequence reads compared to the 2021 sampling across most sites (see tables 3-11 and 3-12 in discussion) and were not used to compare with the caged fish (2022) results because of the very low sequence reads. The plume pattern we see in the 2021 results are not obvious for the 2022 results. Inanga are not present in pre sampling and are present in post sampling at the 0.150 km site only for syringe sampling and very low reads (two replicates) at the 3 km site (3U) for the passive sampling. Torrentfish have slightly higher sequence reads compared to inanga and were detected in both methods down to the 5 km site and in very low reads (1 replicate) at the 9.5 km site for the syringe sampling. Torrentfish are however present in the pre sampling at the 2.84 km (1U) site for both methods and are additionally detected at the 3 km (3U) and 4 km sites in the pre passive sampling.

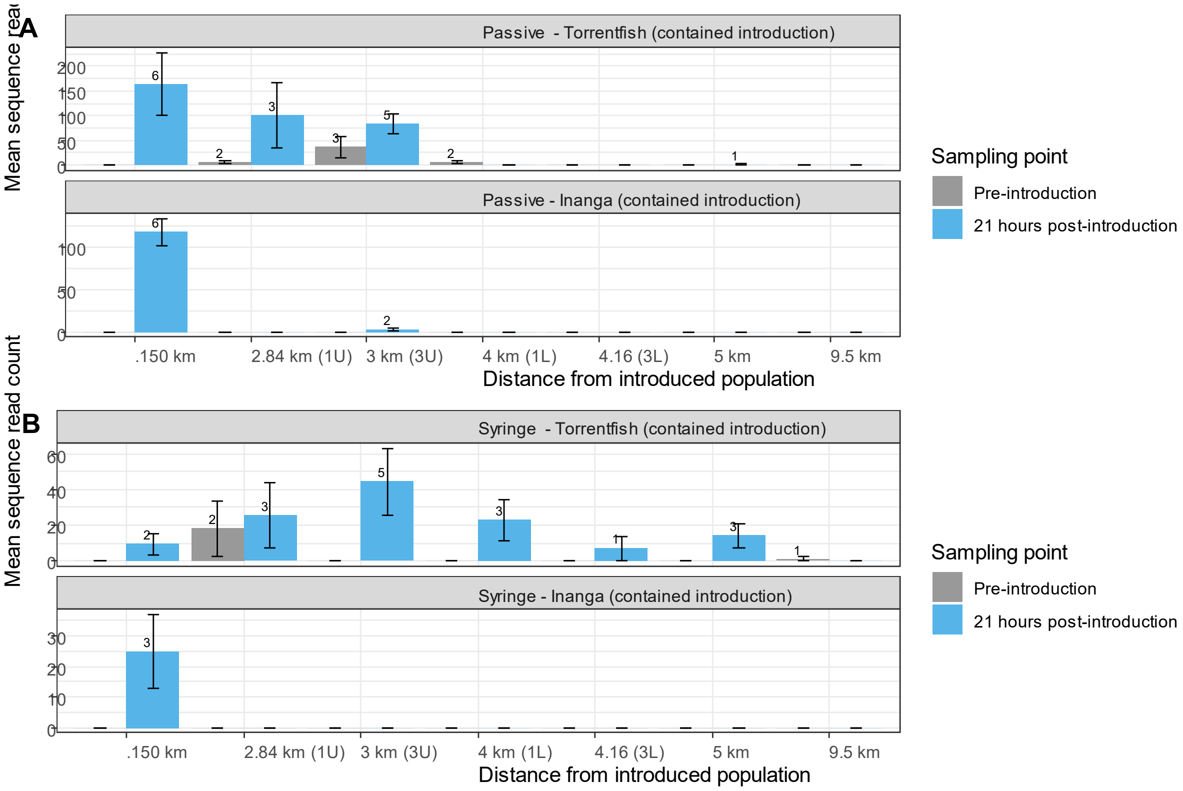

Figure 3-8. Manganui River "Caged" fish results 2022, mean sequence reads with standard error. A) Passive sampling results for inanga and torrentfish (Pre and Post) and B) syringe sampling results for inanga and torrentfish (Pre and Post). Numbers associated with each bar is the number of replicates that had a positive result (1-6).

3.4.4 Manganui tributary 2022

Mean sequence reads for all target species (koaro, banded kokopu and common bully) at the tributary sites was low (<500, Figure 3-9). There was no detection of any of the target species in the mainstem upstream of each tributary confluence. ("Main upstream" sites - 1U (upper trib.) and 2U (lower trib.)). For the syringe sampling within both tributaries, there appeared to be a downstream decline in the detectability of koaro eDNA (natural) from the upper sampling locations (“Trib. upstream” 410_11 and 410_9) to the lower locations (“Trib. downstream” 2U, 2L) prior to their confluence with the mainstem Manganui River. This reduction in downstream detectability over the 250 m between sampling points was apparent in both the pre and post samples collected at each of these sites. Additionally, koaro eDNA was not detected in the mainstem sites 150 m below either tributary confluence (“Main downstream” 3U, 3L) but a small detection was reported at one mainstem site located 1km below the lower tributary confluence (“1 km downstream” or 5 km site).

Similarly in the syringe sampling for banded kokopu, at the upper tributary site (410_11) there is no pre sampling signal at the upstream location and a small detection (41 mean sequence reads, but in all 6 replicates) at the downstream location. This prepositive detection suggests that banded kokopu may be present in low numbers below the upper sampling site, as was also supported by the eDNA data collected at this locality in 2021. The post sampling, now with a boosted signal from the planted banded kokopu shows a significant difference from the top site (506 mean sequence reads and 6 replicates) to that of the lower site 250 m downstream (6.33 mean sequence reads and in 1 replicate). The banded kokopu signal did not transfer down into the mainstem sites at 150 m or 1 km below.

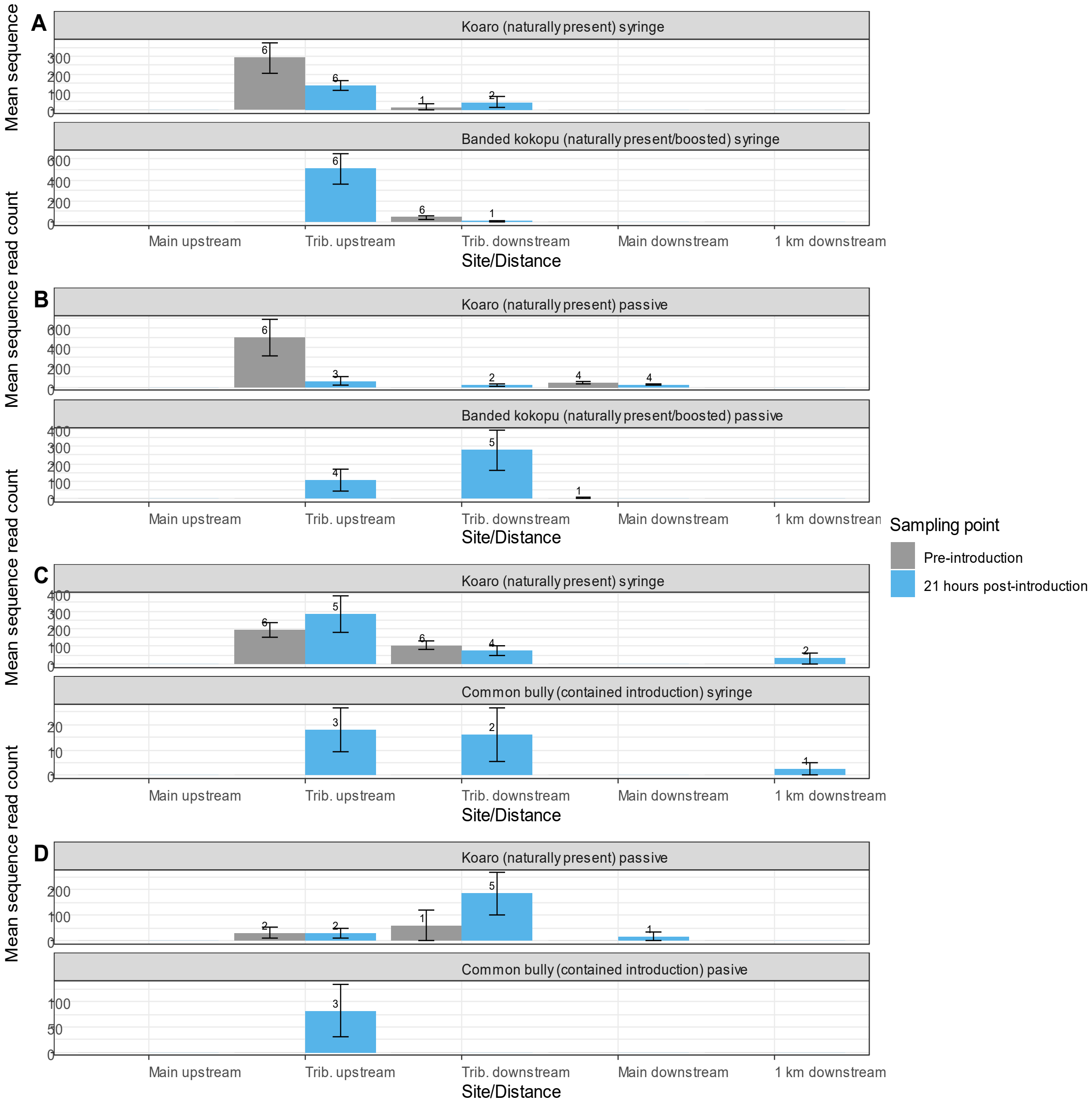

Figure 3-9. Manganui tributary "Caged" and natural signal fish results 2022, mean sequence reads with standard error. A) Upper tributary (410_11) syringe for koaro (natural) and banded kokopu (boosted). B) Upper tributary (410_11) passive for koaro (natural) and banded kokopu (boosted). C) Lower tributary (410_9) syringe for koaro (natural) and common bully (introduced). D) Lower tributary (410_9) passive for koaro (natural) and common bully (introduced).

The mean sequence reads from the syringe post samples for common bully in the lower tributary was weak at both sampling locations within the tributary (18.16 Trib. upstream with 3 replicates and 16.16 Trib. downstream with 2 replicates). Despite this weak signal a small detection (2.5 mean sequence reads and 1 replicate) occurred at the 1 km downstream site on the mainstem. Unlike the other species (koaro and banded kokopu) there was no significant drop in signal from the upper trib. site to the lower and may be a result of the weak signal for this species compared to koaro and banded kokopu.

The passive samples from both tributaries were stochastic in nature with no real trend between them apart from koaro in the upper tributary. Here koaro exhibited significantly higher mean sequence reads at the upstream site for both pre, and post samplings compared to the lower tributary site (Figure 3-10). Koaro for both the pre and post samples were detected 150m downstream in the mainstem (Main downstream - 3U), but not at the 1 km downstream site. Koaro (passive) at the downstream tributary displayed higher mean sequence reads at the downstream tributary site for the post sampling compared to the upper site and similar reads for the pre sampling. A weak detection occurred 150 m downstream on the mainstem.

Banded kokopu at the upper tributary site were not detected in any of the pre samples, but a weak signal is found 150 m downstream on the mainstem. For the post sampling the detection for banded kokopu is slightly higher at the lower end of the tributary compared with the upper with no detection in the mainstem. At the lower tributary site common bully is only detected in the post sampling at the tributary upstream location with low mean sequence reads and in three replicates.

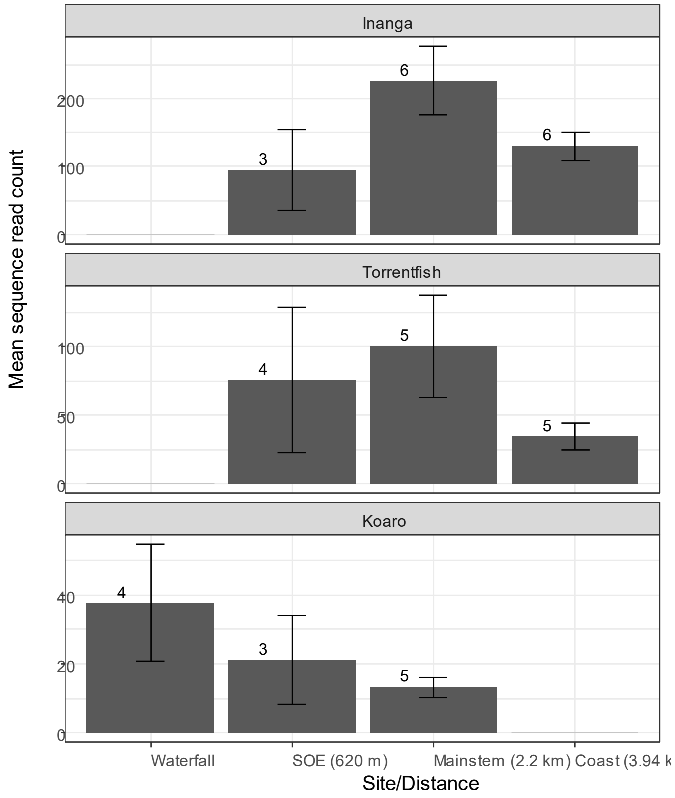

Figure 3-10. The koaro signal (mean sequence reads, with standard error) from the waterfall to the Coast for the Paparahia stream in comparison with two additional naturally occurring fish species (inanga and torrentfish). Numbers associated with each bar is the number of replicates that had a positive result (1-6).

3.4.5 Paparahia 2021

The mean sequence reads for all fish species apart from dominant species (longfin eel, redfin bully and snapper) were low (<400, Figure 3-5) in the Paparahia stream. Despite being found in good numbers (8 fish) and the majority being adult fish (117 mm average length) and a biomass of 201.67 g from 100 m of stream fished or 260 m^2 (average stream width of 2.6 m) mean sequence reads for koaro above the waterfall were low (<40) and only in 4 of the 6 replicates. The koaro signal continued to drop steadily from the waterfall and wasn't detected at the coast site. No koaro were detected in any of the inflowing tributaries prior to entering the Paparahia mainstem.

3.4.6 Methodology comparison (passive and syringe)

Overall, more fish were detected with the "active" syringe method for both sequence reads and replicate hits (Table 3-5). The significance of this result is largely due to longfin eels which make up the bulk of the sequence reads for both methods (63% passive and 69% syringe), but the syringe method outperforms the passive method significantly for sequence reads. However, there is no significant difference for replicate hits for longfin eels between methods, but there is for all fish species.

More replicate hits were found using the syringe method for all fish species other than the planted fish in the mainstem, inanga and torrentfish. The mean sequence reads were also significantly higher for these two planted species for passives. The only other species to significantly favour passives (mean sequence reads) is brown trout. If longfin eels are excluded from the analysis, then there is no statistical difference between the methods for mean sequence reads, but a difference remains for replicate hits in favour of the syringe method.

Table 3-5. Manganui River and tributary 2022 sampling methodology comparison (passive and syringe) for sequence reads and replication hits. Statisical test use - t-Test: paired two sample for means. Levels of significance * <0.001, ** <0.01, * <0.05. Sequence reads refers to the number of genetic detections for a particular species.**

Parameter	% of sequence reads		Sequence reads				Replicate hits	
	Passive	Syringe	Passive mean	Syringe mean	df	P(T<=t) two-tail	Passive	Syringe
All Fish	46.28	53.71	264.99	307.56	1716	0.019*	566	648
All Fish (excluding LF)	50.51	49.48	105.41	103.26	1583	0.800	437	516
Tribs. only (all fish)	37.39	62.6	196.222	320.721	597	0.0008***	152	196
Tribs. only (all excluding LF)	47.62	52.37	91.011	97.439	550	0.672	106	150
Banded kokopu	0.51	0.68	17.47	27.11	131	0.480	10	16
Bluegill bully	0.02	0.19	0.70	7.72	131	0.020*	4	12
Brown trout	7.78	3.86	268.02	154.28	131	0.020*	82	92
Common bully	0.11	0.04	3.73	1.67	131	0.442	3	6
Giant kokopu	0.007	0.02	0.006	0.30	131	0.849	2	1
Inanga	0.23	0.03	7.80	1.13	131	0.044*	9	3
Koaro	1.28	1.36	43.96	54.45	131	0.550	30	40
Longfin eel (LF)	63.28	69.01	2179.96	2759.19	131	0.006**	129	132
Pouched lamprey	0.16	0.17	5.65	6.95	131	0.698	25	26
Rainbow trout	2.61	2.28	89.96	91.08	131	0.950	54	66
Redfin bully	18.62	17.89	641.48	715.41	131	0.345	115	129
Shortfin eel	4.86	4.25	167.51	169.97	131	0.938	80	100
Shortjaw kokopu	0.01	0.07	0.46	2.82	131	0.071	3	6
Torrentfish	0.53	0.16	18.14	6.51	131	0.038*	22	20
	% of replicate hits		Replicate hits				Replicate hits	
	Passive	Syringe	Passive mean	Syringe mean	df	P(T<=t) two-tail	Passive	Syringe
All Fish (Hits)	32.98	37.76	43.54	49.85	12.00	0.007**	566	648
All Fish (Hits) Tribs. Only	43.67	56.32	21.714	28	6	0.026*	152	196

3.4.7 Water quality

Water quality (single point in time) results for the Managanui mainstem and tributaries show levels expected for nitrogen, phosphorus and ammonia for this type of stream (limited anthropogenic effects). And as expected the tributaries had slightly lower levels of turbidity (0.45 mean NTU) to that of the mainstem (0.74 mean NTU). See Table D-6. Water quality testing and methods (Hill laboratories), 31 March 2022 at four sites (Upper mainstem 0.150 km, Lower tributary downstream (410_9), Upper tributary downstream (410_11) and 9.5 km). in Appendix D for full results.

3.4.8 Flows

The flow from the caged location down to the 5 km site remains relatively stable between sampling years with an average flow of 0.131 m s^{-1} (2021) and 0.132.5 m s^{-1} (2022). The two main tributaries in this study are the main inflows to the system above the 5 km mark. Below the 5 km site several larger tributaries enter the mainstem boosting to flows to 0.268 m s^{-1} and 0.271 m s^{-1} at the 9.5 km mark for the two years (2021-22) respectively (Table 3-6). There was a difference seen in flows between sampling years for the two study tributaries with less flow for the 2022 sampling (Table 3-7). The flows for the upper and lower tributary in 2021 were 0.015 and 0.013 m s^{-1} and 0.008 and 0.007 m s^{-1} respectively for 2022. Due to difficulties gauging these sites (small streams) the two gauging's for each of the tributaries were averaged giving a discharge of 0.0115 and 0.010 m s^{-1} for the upper and lower tributaries respectively.

Table 3-6. Gauged flows and estimated downstream travel times for the Manganui mainstem (2021 and 2022) at locations downstream of the caged fish.

2021	Site (km)	0.15	0.5	1	2	3	4	5	9.5
	Flow (m^3 s^{-1})	0.114	-	-	0.120	-	0.137	0.148	0.268
	Mean velocity (m s^{-1})	0.32	-	-	0.582	-	0.475	0.38	0.502
	Est. travel time (min)	-	-	-	68	-	65	39	170
	Est. total travel time (hr)	-	-	-	1.08	-	2.13	2.52	5.42
2022	Site (km)	0.15	0.5	1	2	3	4	5	9.5
	Flow (m^3 s^{-1})	0.122	-	-	-	-	-	0.143	0.271
	Mean velocity (m s^{-1})	0.354	-	-	-	-	-	0.325	0.331

Table 3-7. Gauged flows and estimated downstream travel times for the Manganui tributaries (2021 and 2022) at locations downstream of SOE sites and caged fish for the 2022 sampling.

2021	Site	Upper trib. (410_11)	Lower trib. (410_9)
	Distance to confluence (m)	250	250
	Flow (m^3 s^{-1})	0.015	0.013
	Mean velocity (m s^{-1})	0.194	0.133
	Est. travel time (min)	22	32
	Est. total travel time (hr)	0.22	0.32
2022	Flow (m^3 s^{-1})	0.008	0.007
	Mean velocity (m s^{-1})	0.091	0.089

The daily mean flow for the neighbouring Awakino at Rauroa Farm site (Waikato Regional Council automated hydrology site) was 1.779 m s^{-1} on 29 March 2022. For context the Mean Annual Low Flow at Rauroa Farm is 1.62 m s^{-1}. Rivers and stream during this time of year in New Zealand (early autumn) are generally at stable and or low flow conditions (Collins, 2020), which was the case for all the field locations for both years (2021-22). Only the major tributaries at sites were flowing during the field trials, smaller 1st order tributaries were dry for all study sites in both years.

The same stable conditions were also seen at the Paparahia site (Table 3-8). The two main inflow tributaries were gauged along with the mainstem and the average flow from the SOE site to the Coast is 0.059 m s^{-1}.

Table 3-8. Gauged flows and estimated downstream travel times for the Paparahia stream (2021) at locations downstream of the SOE site (* sites not gauged, ** modelled using upstream sites and SpQ).

Site (km)	SOE	Upper trib.	D/s upper trib.	Mainstem	Lower trib.	D/s lower trib.	Coast
Distance (m)	0	90	100	1600	1610	1620	3400
Flow (m^3 s^{-1})	0.031	0.03	0.061*	0.067	0.006	0.073*	0.077**
Mean velocity (m s^{-1})	0.143	-	-	0.091	-	-	0.747
Est. travel time (min)	-	13	-	215	-	-	72
Est. total travel time (hr)	-	0.13	-	3.48	-	-	5

3.4.9 Wainui Stream Greater Wellington Regional Council

The Wainui stream just north of Paekakariki (1765040E 5462290N) on the west coast of New Zealand's North Island in the Greater Wellington region was one of the streams sampled as part of the high replication study (Chapter 2). Results from the eDNA sampling at this site (6 standard replicates and 6 boosted replicates, 4th April 2021) showed no presence of koaro despite their known presence further upstream in steeper native bush conditions. Extensive traditional sampling (single pass spotlighting – 5632 m^2 and single pass electric fishing – 587 m^2) after eDNA sampling over several days in April recorded good numbers of mainly adult koaro (>100 mm, 29 spotlighting and 26 electric fishing) at a combined biomass (using calculations outlined in methods above - Electric fishing and Biomass) of 1181 grams. Koaro were found starting 1.1 km upstream of the eDNA sampling point where the mainstem splits into two main feeder tributaries (Te Paka stream and the upper Wainui stream) it is at this point that the streams become steeper and enters native bush and from here upstream in both tributaries koaro were found in good numbers.

From stream gauging's by the Greater Wellington hydrologists (summer of 2023-24) at both feeding tributaries (Te Paka stream and the upper Wainui stream) and on the mainstem at the point of the eDNA sampling (campground) a discharge was established. These gauging results were correlated with a long-term recorder site a bit further up the coast (Wharemauku Stream) to estimate the discharge at the time when the eDNA sample was collected (Wainui stream at eDNA sampling point 0.022 m s^{-1}, Te Puke stream 0.012 m s^{-1}, upper Wainui stream 0.010 m s^{-1}).

3.4.10 Negative controls

Two of the random negative control sites had no detections for fish, however the other four all had some level of fish detections and all but one (<10 sequence reads) had significant levels of sequence reads (Table 3-9). Apart from the detection for torrentfish (5 km, post), all detections can be associated with fish species known to be present at each of the negative control sites that were sampled. Though torrentfish were one of the caged species and were detected in 3 field replicates (i.e. non-negative control replicates) at this site (post replicate field sampling) the highest sequence reads out of the three samples was 38 compared with 730 in the negative control. Additionally, the reads for longfin eel in the negative control at the upper tributary site (410_11, Pre) are higher than three (half) of the six field replicates taken at this site. In comparison to the average reads from the field samples (six replicates) taken alongside the negative controls, the amount of sequence reads found in the negative controls was as high as 8.7% and an average of 4.1% for all taxa. Four detections found (genus and above) in the field negative controls for all taxa were not found in any other eDNA samples indicating that the ultra-clean water may have had some level on contamination. See Table D-7 in Appendix D for the full list detections found in the negative controls.

Table 3-9. Negative control results for fish for the Manganui River and tributary 2022 sampling (six random sites). Numbers presented are the number of genetic detections (reads) for that species. % comparison refers to total reads from the negative control in comparison with the average total reads (six replicates) of the samples collected alongside the negative controls.

Species	Upper mainstem (pre, 150 m)	Upper trib. (pre, 410_11)	410_8 (pre below the gorge)	Upper trib. (post, 410_11)	5 km (post)	9.5 km (post)
Salmon/trout						106
Longfin eel		393		1804		
Rainbow trout						238
Torrentfish					730	6
% comparison (all taxa)	2.1	1.4	5.0	8.7	3.2	4.3

3.4.11 Contamination (field samples)

The 2022 Manganui sampling added a level of contamination risk by being in the stream above sampling points and unfortunately was unavoidable while using the passive methodology. As a result, and despite best efforts to decontaminate equipment several unexpected detections for fish occurred and for some of these, contamination appears to be the only plausible explanation (Table 3-10). The main species for which contamination in the results appears more likely are for inanga, common bully and possibly giant bully. The occurrence of torrentfish and banded kokopu are potentially real as both species have previously been found in eDNA samples at some of these sites and have been included in the table for reference. Giant bully was detected (minor) at a similar location in the 2021 pre-Manganui sampling and in 3 sites in the 2022 sampling indicating that this species could be naturally present (real) in low biomass above the gorge However, based on other results presented in Table 3-10 where contamination is likely these "real" detections cannot be discounted as contamination. Detections for "giant or shortjaw kokopu" occurred in 2021 and 2022 but the 2022 sampling is the first time giant kokopu have been detected on their own. Previous detections for "giant or shortjaw kokopu" have always been assumed as shortjaw kokopu as these species have been caught via electric fishing and detected with eDNA sampling. It is possible that giant kokopu are present naturally as they are good climbers as juveniles and are regularly found in the neighbouring Paparahia stream or detection is contamination or a mixture of both. The increased detection for giant kokopu in 2022 may have also been assisted with the introduction of the "DG" primer.

Table 3-10. Potential contamination Manganui River and tributary 2022 sampling. Method identification * Pre passive, ** Pre syringe, * Post syringe, **** Post passive. "Reads" refers to the total number of genetic detections for a particular species and "Hits" refers to the number of replicates a signal was observed. Orange fill depicts contamination likely; no fill depicts possible contamination.**

Site	Inanga		Torrentfish		Banded kokopu		Common bully		Giant bully		Giant kokopu	
	Hits	Reads	Hits	Reads	Hits	Reads	Hits	Reads	Hits	Reads	Hits	Reads
.150 km					1**	37**	1***	15***			1****	13****
.150 km											1*	10*
2.86 km			2*	36*								
2.86 km			2**	108**								
3 km					1*	2*			1*	96*		
4 km			2*	36*					1***	87***	1**	80**
4.65 km			3*	161*								
5 km												
9.5 km			1**	7**								
410_11 d/s									2****	231****		
410_11 u/s												
410_9 d/s												
410_9 u/s	1*	36*			2**	215**						

3.4.12 Biomass

Biomass data was available from all planted fish but only the 2021 data (inanga and torrentfish) was used to compare with mean sequence reads due to issues with the 2022 method change to house the planted fish (Figure 3-11). An estimate of biomass (relative one pass 150 m electric fishing) was available for naturally present fish species from some of the SOE sites close to where eDNA samples were taken. Only the Paparahia stream was electric fished the same day as eDNA sampling as this is the time of year that the annual SOE sampling takes place (late March-early April). For both Manganui tributary sites 2022, fish biomass was established from December 2021 SOE electric fishing which was 110 days prior to eDNA sampling (late March). For both these sites the eDNA samples were taken at the starting point (downstream) of the electric fished SOE reach (410_11 and 410_9). The 410_10 SOE site on the mainstem Manganui was sampled in December 2020, 116 days prior to eDNA post sampling (early April). The 410_10 SOE site is 530m upstream of the 0.150 km eDNA sampling site, additionally 250 m upstream of the 0.150 km mainstem sampling site the Mangakahikatea stream enters the Manganui mainstem on the true right side. For the Manganui sites though, electric fishing wasn't done on the same day as eDNA sampling, general species composition and biomass isn't expected to change by much from December (electric fishing) to March-April (eDNA sampling) or year to year as seen in long-term SOE data (Table D-5)

Figure 3-11. Biomass (total grams - relative one pass electric fishing (150m) for longfin eel, redfin bully and koaro) and caged biomass (total grams) for inanga and torrentfish) and Mean Sequence Reads comparison. A) Paparahia stream (longfin and redfin bully data from the SOE site and koaro data from waterfall site. B) Manganui Lower tributary (410_9) pre sampling. C) Manganui Upper tributary (410_11) pre sampling. D Manganui mainstem (0.150 km) post sampling longfin and redfin bully biomass data from SOE site 410_10.

Longfin eel and to a lesser extent redfin bullies dominate the biomass for all streams in this study and their combined signal from water extending several kilometres upstream should in theory be significantly higher than the 2021 planted fish species on the mainstem. At all sites down to the 4 km site for the mainstem 2021 "caged" study (post sampling), torrentfish (26.6%) and inanga (20.2%) made up 46.8% of the total fish reads for the five main species (torrentfish, inanga, longfin eel, redfin bully and snapper). While longfin eel, redfin bully and snapper made up 26.6, 7.8, and 18.6% respectively. This dominance of the sequence reads indicates that the planted fish with biomass at one single location created a strong and consistent an eDNA signal which may have been higher had snapper not been present swamping sequence reads. This "caged" signal was stronger than that of the natural signal of the native fish present, that had a biomass much greater than that of the planted fish since these natural fish occur throughout the study reach and several kilometres upstream providing numerous widely dispersed sources for eDNA generation. Inanga and torrentfish were also detected in all replicates as far as the 4 km site (72) while longfin eel and redfin bully were detected in 67 replicates sampled at the same locations, with redfin bullies missing from five replicates.

As a result of these time delays (not same day sampling between electric fishing and eDNA sampling), the influence of a major tributary for the 0.150 km site, together with the likelihood that molecular source material will extend well beyond the 150 m section of water electric fished at the SOE sites, the biomasses of the resident fish can only be a conservative estimate of the actual biomass. For three sites (Paparahia, and the two Manganui tributary sites - 410_11 and 410_9) looking at only natural signals we see a similar trend with biomass and mean sequence reads with mean sequence reads being double or greater to that of biomass (except for koaro at the Paparahia site), this trend is also seen with the planted fish (torrentfish and inanga) on the mainstem Manganui site (Figure 3-11). This pattern flips for the natural signal for longfin eel and to a lesser extent redfin bully at the mainstem site and may be a result of fish reads being dominated by snapper together with the planted fish. The koaro result for the Paparahia stream could be associated with one less high performing primer being used for fish species in the 2021 sampling compared to the 2022 sampling with the "DG" marker not being used in 2021 sampling. This "DG marker accounted for 30% of koaro reads for 2022 sampling and a similar percentage for longfin eel (30%) and redfin bully (34.4%, Table D-8. Fish molecular targets for all eDNA study sites (2021-22) with totals., Appendix D). Budget constraints meant the older (2021) samples could not be rerun with the newer primer set (see discussion, 3.5.5, last paragraph for more).

3.5 Discussion

3.5.1 eDNA plume

Our results for the Manganui 2021 sampling are similar to the findings in other caged fish studies (Wood et al. 2021, Van Driessche et al. 2022, Van Driessche et al. 2023), where an eDNA plume is evident and relatively localised. Initially, moving downstream from the caged fish, the plume is concentrated with larger eDNA fragments (Wilcox et al., 2015), that near to the source can be variable with higher concentration in the midstream (Wood et al, 2021). Mean sequence reads for the planted fish initially drop from the source down to the 1 km site and then rise again to the 4 km site especially for torrentfish. Source fish are detected in all six replicates to the 4 km site and then drop for torrentfish to three replicates at the 5 km site, while inanga was still detected in all six replicates but at a much lower rate at this point. As we move downstream away from the source the eDNA mixes and becomes more evenly dispersed as these larger fragments break-up into smaller more evenly dispersed particles. This "break-up" is influenced by environmental conditions of which temperature is thought to be the main factor (Jo et al. 2020, Mauvisseau et al. 2022) and is also dependant on fine suspended particles and biofilms which eDNA adsorbs to, which protects them from degradation (Barnes et al. 2014, Shogren et al. 2018). Shortly after the "plume" at the 4 km site the detection reduces dramatically. A possible explanation for this finding may relate to eDNA dilution and degradation as suggested by Jerde et al. (2016) and Barnes et al. (2014). The pool, run, riffle hydrology of the Manganui River will also influence the integrity of eDNA particles as they move downstream, a turbulent flow will be more effective at keeping DNA particles suspended and moving further downstream while a slower more laminar flow will see these particles settle (Turner et al. 2015, Shogren et al. 2017). The hydraulic conditions found from site to site have also been shown to influence eDNA transport as other studies both natural and experimental have shown (Pont at al. 2018, Jo and Yamanaka, 2022). Once this breakup and mixing occurs further downstream these finer DNA materials are more likely to found near the stream edge rather than midstream (Wood et al. 2021).

For introduced snapper we may be seeing the "fine particle plume" at the 0.500 km - 1 km site similar to what we see for the planted fish at the 4 km site. Followed by a level of increased degradation and dilution after the 1 km mark. This lower level of detection which is similar to that of the naturally occurring redfin bully continues down to the 4 km site before fading, but a remnant detection does occur in one replicate in the pre sample at the 9.5 km site. This fine particle plume is 3.3 km from the snapper source. The level of mean sequence reads for snapper which is well above the planted fish and longfin eels at the 0.500 km - 1 km site together with the result that snapper was detected down to the 9.5 km site (11.8 km in total) indicates that a substantial amount of snapper frames was discarded. But without knowing the actual biomass of the snapper and having eDNA collection sites closer to the source, the possible conclusions drawn here are speculative. Additionally, there would also be a dilution effect (Curtis et al. 2021) as the snapper source tributary (Mangakahikatea stream), which is estimated to be a quarter of the flow of the mainstem Manganui enters the mainstem. It is possible that the level of snapper signal was boosted via eel predation and excretion of the snapper frames with the potential of eels to move up and downstream for feeding, though the recorded upper level for these movements in New Zealand is 687m (Jellyman and Sykes, 2003) but may extend beyond this in larger systems and when an external food source is introduced.

Resampling 25 h after the removal of the "caged" fish at the 0.150 km site showed only minor detections compared with the mean post sampling at the same site (low sequence reads and in three replicates each) for both inanga (2.8% mean sequence reads) and torrentfish (0.6% mean sequence reads). The time remnant eDNA material will remain present and detectable has been shown to be influenced by environmental conditions and hydrological processes leading to decay and dilution (Jo & Minamoto, 2021, Jerde et al. 2016). There are conflicting studies regarding the rate of breakdown and persistence of eDNA in relation to the size of the DNA fragment. Several studies (Jo et al. 2017, Bista et al. 2017, Shogren et al. 2018) say smaller sized particles take longer to decay, while Zhao et al. (2021) stipulate that shorter DNA fragments degrade quicker than longer fragments. Bird et al. (2024) showed that foreign DNA in wetland experiments was detectable for up to 1 week following release within a 10 m radius from the source, and for only 2 days post-release in an in-situ experiment. In contrast a caged experiment conducted in a marine environment resulted in zero detections (caged fish) after just 2 h of removal in the marine environment (Murakami et al. 2019).

3.5.2 Biomass

Planted fish were not acclimated at the "caged" site but came from the same watercourse just lower in the catchment. Nevertheless, they would have been under stress from capture and relocation together with likely interest from local eels overnight (as evident from 2022 sampling) which likely resulted in increased shedding rates, and likely elevating their signal beyond what is normally produced as has been reported in other studies (Maruyamaet al. 2014, Sassoubre et al. 2016, Nevers et al., 2018).

Though torrentfish had higher mean sequence reads than inanga based off a similar biomass, their detectability (replicate hits) and plume profile was generally similar indicating that in general they may have similar shedding profiles (though in an artificial setting) despite physiological and behavioural differences. It is likely that had additional species been used in this study we may have observed more differences in shedding profiles and resulting distances of downstream detection as observed in other studies (Van Driessche et al. 2022, Van Driessche et al. 2023). An additional aspect of species-specific shedding rates is that eDNA particle size can vary with species (Zhao et al. 2021).

In this study and others (Wood et al. 2021, Rourke et al. 2021, Van Driessche et al. 2022, Van Driessche et al. 2023) the distance an eDNA signal will travel downstream has been shown to be directly related to the abundance/biomass of the fish species present. The distance that an eDNA signal travels downstream will be considerably shorter for rare or patchy species relative to dominant species or species with higher biomasses (Pont, 2024). Logically as we generally observed in this study, the further eDNA moves away from its source, the more likely detection becomes variable (Jane et al. 2015). However, with sufficient replication this pattern can also be observed in rare species with a low biomass that are close to the sampling point. (Takahashi et al. 2023), see sampling strategies below for more.

3.5.3 Flow

Based on the differences seen between the sites in this study, stream flows and discharge are likely facilitating the mixing and transport of eDNA particles with higher discharges resulting in eDNA travelling further downstream (Cantera et al. 2022; Van Driessche et al. 2022). The distance this signal travels is also dependant on the biomass of the source material (Jane et al. 2015, Van Driessche et al. 2022, Van Driessche et al. 2023, Porto-Hannes et al. 2023). Though not known the biomass for snapper was likely considerably higher compared with "caged" inanga and torrentfish in the 2021 sampling resulting in detections beyond that of these "caged" fish. This likely higher snapper biomass may also be influenced by increased shedding rates of dead fish compared to live specimens (Tillotson et al, 2018).

For snapper in this study the end point or the distance travelled seems relatively clear at the 9.5 km site with only one detection and low reads. For torrentfish and inanga (2021) this end point is unclear because there are no sites in between 5 and 9.5 km. Mean sequence reads are low at the 5 km mark, but this is significantly influenced by cattle swamping the majority of sequence reads at this site. Previous metabarcoding studies have shown that a high abundance of one species can negatively influence the detection of another species (Bylemans et al. 2018, Rourke 2021).

It is possible had there been more sites at 6, 7, and 8 km we would see a pattern similar to that of snapper where there is a fading of the signal after the fine particle plume rather than an abrupt end. This same logic would also apply to the Paparahia site for koaro and the Manganui tributaries had they been slightly longer. Figure 3-12 displays the actual end points (A) and also an adjusted end point (B) based on re calculated flows with distance and the rate at which eDNA detectability declines. This clear trend of distance to discharge and eDNA detection with the limited number of sites in this study aligns with other studies (Jane et al. 2015, Deiner et al. 2015, Wilcox et al. 2016, Altermatt et al. 2020, Thalinger et al. 2021, Wood et al. 2021, Van Driessche et al. 2022, Van Driessche et al. 2023, Porto-Hannes et al. 2023).

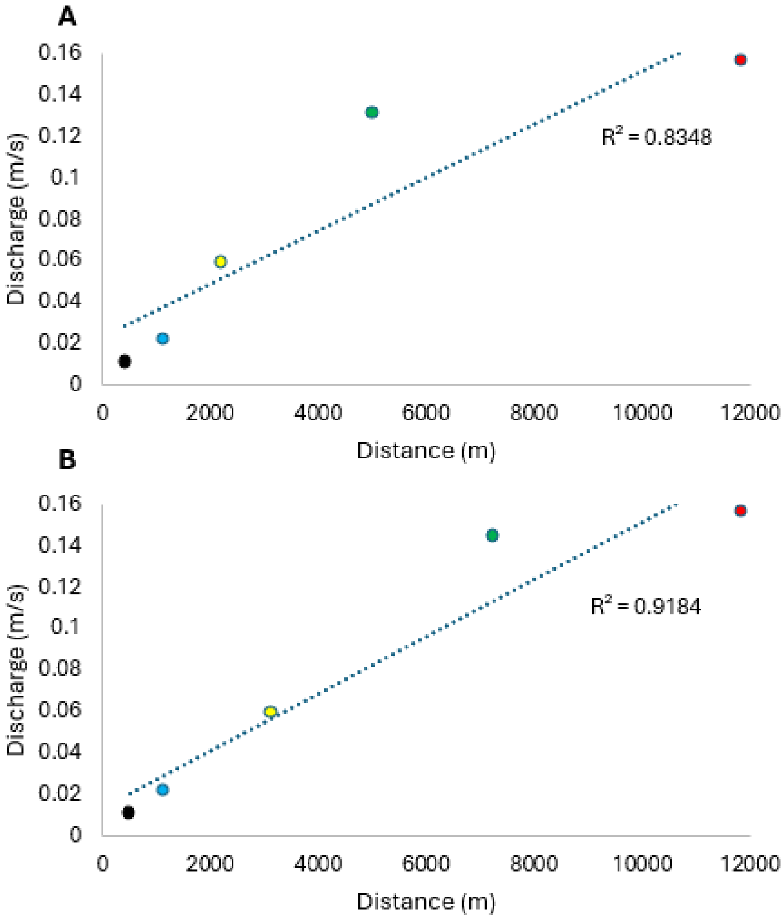

Figure 3-12. Distances the eDNA signal travelled in relation to discharge. A) Based on actual end point or position of lowest sampling point. Red = snapper, green = inanga and torrentfish 2021, yellow = Paparahia koaro, blue = Wainui koaro, black = Manganui tribs. 2022. B) Based on likely end points rather than actual (green, yellow, and black adjusted).

3.5.4 Transport in lotic systems

The discharge relationship to distance is likely influenced by a streambed or substrate to water surface ratio. The shallow nature of the tributaries (<200 mm average depth) in this study means that lower ordered streams will have a higher water surface to substrate ratio compared to deeper and faster flowing high order streams. eDNA particles will bind to the stream biofilms, substrate, and sediment via electrostatic interactions (Hou et al. 2014) in turn fish eDNA material can be more concentrated in sediments rather than surface water (Turner et al. 2015). This increased water to substrate ratio will increase the depositional rate or retention of eDNA material. This increased water surface contact with the stream substrate relative to higher order streams may help to explain why we see a rapid drop in the eDNA signal in these smaller streams (more below in 3.5.5 Koaro). Discharge effectively serves as a proxy, to this substrate to water surface ratio that effects the transport of eDNA downstream and highlights the importance of hydrological factors in interrupting eDNA data (Harrison et al. 2019, Pont et al. 2018, Shogren et al. 2018). Jane et al. (2015) found at low flows eDNA abundance was high close to the source but quickly faded over distance (239.5 m) downstream. However, there will be exceptions to the relationship where equal discharges may differ between systems i.e., a wide shallow, braided river compared to a narrow deep river or other environmental factors (i.e., heavy instream macrophytes, deep hyporheic zones). Additional metadata (stream slope, depth, width and roughness) will assist in interpretation of results (Fremier et al. 2019). The influence of retention for many studies (Harrison et al. 2019, Wilcox et al. 2016, Shogren et al. 2018, Shogren et al. 2019) appears to be more important than decay in limiting downstream eDNA transport. Sediment bound eDNA decays at slower rates to that of suspended eDNA (Romanowski et al. 1991, Hou et al. 2014) which may help explain persistence in certain situations (Harrison et al. 2019, Tillotson et al, 2018) and may have an influence on results after rainfall events (Staley et al. 2018).

3.5.5 Koaro

At the Paparahia stream site eDNA detectability for koaro steadily declines from the waterfall to the mainstem site (2.2 km downstream) and disappears at the coastal site. There is little evidence of a plume at this site for koaro other than being detected in 5 replicates at the 2.2 km mark compared with three and four (heading back upstream). This is likely the result of limited sampling sites, a lower discharge rate, the influence of a major inflowing tributary relative to the size of the stream and limited sequence reads which were very low for koaro and for other fish species at this site other than for the dominant species (longfin eel and redfin bully). The lack of a third primer ("DG") for koaro at this site limits results, compared to the 2022 sampling in the Manganui system with this primer when comparing the natural signal from koaro. Based on the signal at this site trending down it is likely that the signal for koaro disappears not too far downstream, especially with the addition of the lower tributary entering just below the 2.2 km sampling point.

Samples taken from the two Manganui tributaries just before they enter the mainstem during the pre-2021 sampling showed low levels of detection for koaro with 28 and 52 mean sequence reads and in two replicates (out of six) for the lower (410_9) and upper (410_11) tributaries respectively. The high replicate sampling (Chapter 2) for the upper tributary 114 days earlier (14th December 2020), 250 m upstream at the start of the SOE site had 604 mean sequence reads and was detected in all six standard replicates. Though not same day sampling there is a significant drop in detection over this 250 m distance. The 2022 same day sampling (syringe) for the same upper tributary site for both pre and post samplings showed a similar result with significant declines in mean sequence reads and replicate hits. The same result also occurred for "caged" banded kokopu (syringe) in the post sampling at this site. The mean sequence reads and replicate hits for common bully were very low at the lower tributary site for post syringe sampling and showed no significant drop between the upper and lower sampling sites. This result for common bully is likely the result of very low sequence reads and primer bias (Shu et al. 2021, Tsuji et al. 2022) with only one primer for this species (DG).

The lower tributary site showed a similar trend for koaro with significant drops in mean sequence reads over the 250 m to the lower sampling site but were still detected in six and four replicates respectively for pre and post sampling. Using the natural signal for koaro at these tributary sites relies on koaro being found in the "better" koaro habitat, found upstream near the start of the SOE sites and further upstream again as the streams become steeper. The water between the eDNA (lower and upper) sampling points was not electric fished and it is possible that koaro were present in this water. Based on this (the possibility of koaro being present between sampling points) the results for these tributary sites are conservative and may be the reason why the results for the lower tributary site aren't as decisive (though still significant) as the upper tributary site. For both tributary sites we see the detectability drop by half over 100 m as we move downstream and for the upper tributary this is closer to half every 50 m, a result similar to other studies in a similar sized streams (Jane et al. 2015, Wilcox et al. 2016).

The mean sequence reads for redfin bully at the two Manganui tributary sites (upstream, 2U and 2L) was on average three times higher to that of koaro despite having a similar biomass at these sites just upstream (relative single pass electric fishing) and each being found in all six replicates. Similarly, koaro in the Paparahia stream above the waterfall were common but had low sequence reads, these results for koaro may be a function of a lower shedding profile and primer bias.

There were no detections for koaro or planted fish species 150 m below the confluence of the tributaries in the mainstem (syringe) indicating that the already low detectability for these species at the lower end of the tributaries was diluted and lost in the mainstem (Pilliod et al. 2014, Jane et al. 2015, Pont et al. 2018). However, small detections did occur 1 km downstream at the lower tributary site for both koaro and common bully in the post sampling and may be the result of a limited fine particle plume for these species.

Advances in new and better primers for the detection of species continues to evolve. For example, the ‘YG’ primer has been added to Wilderlab’s sample panel since the analysis of the samples in this study, replacing the “DG” primer set for better detection of galaxiid species. This change of primer set saw more than a 2-fold improvement in the detection of shortjaw kokopu (*Galaxias postvectis*) in a study targeting this endangered species (Orchard, 2023). It is possible that an improved detection for koaro at the Paparahia and Manganui sites (2021) in both replicate hits (detection within replication) and sequence reads if samples were re-run using this updated primer set or even just replacing the “ZV” marker with the “DG” marker as per the 2022 sampling. These ongoing improvements with eDNA methodologies highlight the benefit of being able to retrospectively re-run stored eDNA samples (Wilkinson et al. 2024).

At the conception of this study, it was assumed that the primers used would remain consistent between years and sites so comparisons could be made. However, due to this method (eDNA) evolving quickly and the use of a commercial laboratory to process the samples the new “DG” primer was used in 2022 which increased detection of fish species but made comparisons slightly more complicated between sampling years. Unfortunately, the budget did not enable the old (2021) samples to be rerun with the updated primers.

3.5.6 Manganui sampling 2022

Captured fish that were held in closed soft mesh holding fyke nets overnight in the lower river below the gorge were predated on by resident eels. A hole was found in one of the holding fykes that had been created by a eel/s and other fykes also exhibited signs of interference with, without being holed. This resulted in several deaths for both inanga and torrentfish. To avoid this situation happening at the “caged” site above the gorge the with the possibility of further deaths and fish escaping, the method was changed at short notice. Hard barrier holding containers were utilised using what we had available (chilly bins and buckets). Eels are known to predate on fish caught within a fyke net (Joy et al. 2013), but this is the first time I am aware of eels attacking from the outside, though this is an issue overseas with seals (Königson et al. 2007). However, this behaviour in a stream at low flow conditions at a period when food resources will be low for eels coupled with an aggregation of housed live prey fish may have heightened the likelihood of this issue occurring.

Unfortunately, the result of this method change limited the flow of DNA material from the housed fish as was evident in the results. The biomass of inanga and torrentfish in 2022 was just under half of that used in 2021 but mean sequences reads in 2022 were just 0.4% and 2%

compared to inanga and torrentfish respectively (at equal sites) when compared to the mean sequence reads for these species in 2021. Inanga were only recorded at the 0.150 km site for syringe sampling while torrentfish were detected as far down as 5 km. This difference in both detection and sequence reads is likely due to the larger 8mm holes drilled into the torrentfish holding chilly bin compared to 4mm for the inanga bin resulting in an almost three-fold difference in flow through the bins.

The same pattern was seen at the lower tributary site where buckets were used instead of the planned closed fykes resulting in very low sequence reads for common bully. Banded kokopu however did show higher levels of sequence reads which were significantly higher than koaro at the upper site in post sampling. This difference between banded kokopu and common bully at a similar biomass may be due to primer bias in favour of banded kokopu (four to one) and possible differences in shedding profiles.

3.5.7 eDNA method comparison (passive versus syringe)

Within the Manganui system the active syringe method significantly outperformed the passive method for both sequence reads and replicate hits. Longfin eels dominated the biomass and as a result the sequence reads, when longfin eels are excluded from the analysis there is no significance. For the coarser passive filters, the relatively clear water within the catchment may have resulted in some eDNA material passing through them particularly at the tributary sites where lower turbidity to that of the mainstem occurred (Figure 3-13). It is estimated that more than 70% of eDNA particles are smaller than 10 microns (Pont 2024, Barnes et al. 2021, Brandao-Dias et al. 2023). Additionally, the relative abundance of larger eDNA particles is positively and significantly affected by the abundance of seston (Barnes et al. 2021). The tributary sites showed the highest significance levels for sequence reads and had a 22.5% increase in detections (replicate hits) with syringe method compared to passives, compared with 8.4% in favour of the syringe method over passives in the mainstem. This result may be different in other systems with lower water quality where fine sediment particles and algal loads build up in the passive samplers effectively making the filter size smaller and would need to be tested. Passive sampling in certain environments and depending on the questions being asked will be more suitable than active sampling (Bessey et al. 2021).

Figure 3-13. Clean passive filter after a 21 hour and 40-minute deployment at the upper tributary site (410_11), Manganui system.

The passive samplers did however have significantly higher sequence reads for the “caged” inanga and torrentfish in 2022 with slightly higher replicate hits indicating that the longer deployment with the passives helped in capturing eDNA material from these planted fish. The sequence reads were very low for these planted fish and for torrentfish the sequence reads steadily declined from the 0.150 km site to the 3 km mark and for inanga there were only two detections, the main one at the 0.150 km site and then a minor detection at the 3 km site. This decline from the start to the 3 km site could be the result of early plume dynamics with the larger DNA fragments being captured in the coarse passive filters and as fragments break up into finer particles they are passing through the filters. There was one minor detection at the 5 km site for torrentfish for the passive sampler, but the syringe method outperformed the passives at these lower sites beyond the 3 km mark for torrentfish indicating that the finer particles can possibly be collected better with the syringe method with its much finer filter.

The shorter distance (250 m) between sampling points for the tributary sites shows no real pattern for the passive samplers for both naturally occurring koaro and planted fish and as with the mainstem samples the sequence reads were low. The passive samplers do however show minor detections at the mainstem sites 150 m downstream of the tributaries for koaro at both tributary sites and banded kokopu at the upper tributary site. This may be the result of the of larger fragments being captured at this site as only 550 m from the source at these locations, but this is speculative and based off very low sequence reads.

3.5.8 Negative controls and contamination

Compared to traditional methods eDNA sampling has greater risks, that can create false positives (Jerde, 2021). This can lead to an erosion of confidence in results in which managers base decisions on. Methods have been developed and continue to develop to improve confidence both within the laboratory and in the field (Sepulveda et al. 2020). The use of negative field controls is growing as the general use of eDNA grows and education around potential contamination becomes clearer. However, only 49% of studies reviewed by Takahashi (et al. 2023) had some level of field control.

Best practices have been developed along with single use eDNA supplies including enclosed filters to minimise potential contamination, despite this, contamination is not uncommon (Maruyama et al. 2014, Serrao et al., 2018, Sepulveda et al, 2019). There is no specific guidance around field controls and reported methods can range from a minimum of 10% of samples being field controls to assessing field contamination as only being a concern if a species is found that shouldn't be present.

For this study the latter is true for fish, no fish species occurred in field samples or the field negative controls that weren't present within the overall study site. However, the level of detection within the negative control samples is a concern and not just for fish. In this study negative controls amounted to 2.2% of the total samples taken (2022). It should, however, be noted that there is a magnification effect in the negative control samples where small amounts of contamination relative to other targets, amplifies the results. Care was taken at all sites to manage contamination, and it is difficult to understand how contamination can occur especially for the field negative controls and highlights the how extremely sensitive this tool can be. We suggest however that this is likely caused by aerosolised biological molecules from the waterway entering the water used for the negative control. A better method could potentially be developed here to reduce the influence of aerosolised molecules in these negative control samples however, this would not provide a true reflection of contamination in the actual field samples, and it could be argued that this aerosolised "contamination" is just part of eDNA sampling and unless "new" DNA sources (not found in normal sample replicates) are found then can we call it contamination. Other more enclosed collection systems i.e., the Smith-Root eDNA sampler reduce exposure to the surrounding environment which may result in less "outside" influences.

It should be noted that contamination of samples including negative controls can occur within the laboratory. Metabarcoding has recognized several contamination problems that could lead to the wrong identification of sequences, such as the occurrence of incorrect tag pairings in the sequencing results and contamination of amplicon samples (Schnell et al. 2015, Ballenghien et al. 2017). However, based on procedures adhered to by the laboratory processing the samples in this study it is unlikely contamination occurred here. Metabarcoding studies compared with other eDNA techniques have a much higher occurrence of negative control amplification and there is a general acceptance that low-level contamination is unavoidable (Sepulveda et al. 2020).

There is possibly some contamination of the field samples as a result of accessing the sites upstream of sampling points. Two sites in particular are possibly the result of contamination whereas for serval other sites the detections could be real. One detection for torrentfish at low levels in a pre-sample did occur in 2021 and it is possible that more fish have accessed the site in the year since due to increased flooding, or a change in the gorge structure that may allow more partial climbing species through. Additionally banded kokopu have also been previously detected by eDNA and detections of "giant or shortjaw kokopu" were assumed to be shortjaw kokopu but there is potential for this species to be found above the gorge. Additionally, the 2021 samplings and sampling previous did not include the "DG" primer which accounts for 38%, 22%, and 16% of sequence reads for torrentfish, banded kokopu and giant kokopu respectively in the 2022 sampling. This makes these species more likely to be detected in 2022 compared to 2021, however contamination cannot be dismissed. The detections for giant bully are unusual at sites above the gorge but can't be discounted as real as this species was detected in the 2021 eDNA pre sampling at a similar location (3 km site) and in more recent work just above the 0.150 km site (2023, 410_10 SOE eDNA sampling at 6 replicates, J. Smith unpub. data). Additionally giant bully was detected in both eDNA samples and electric fishing collected below the gorge, which is only 9 km downstream. It is unusual to find this species this far inland and at this elevation as this species is normally found at low elevations and close to the coast. However, records from the New Zealand Freshwater fish database have recorded this species further inland and at higher elevations than recorded here so can't be discounted as real.

The two sites that contamination is possible include inanga (pre sample) at the upper tributary site (410_11) and common bully at the 0.150 km site. Both these detections were very low (36 and 15 reads respectively) and in only one replicate. For the common bully (post) detection it is possible that prior to capture one species predated upon another species i.e., torrentfish consumed a common bully and the resulting waste triggered a detection. Though this scenario is possible (West et al. 2005, Sagar and Eldon, 1983) it is unlikely. Additionally, the introduction of the discarded snapper frames could also possibly be linked with this common bully detection as circumstances around collection and discard of the snapper frames are unknown.

Precautions were taken in this study to eliminate contamination between sites but at some sites contamination may have occurred. As discussed above eDNA tools are extremely sensitive and care must be taken when sampling multiple sites not to transfer source material from one site to another. In most situations sampling would not occur above another sampling point that is downstream but given our study design this was unavoidable and some of these results possibly highlight why this is a contamination risk.

3.5.9 Sampling strategies

eDNA plume dynamics advance our understanding of how eDNA behaves in a lotic system and may help in certain sampling strategies and in the targeting of certain species of interest. It does however further complicate the interpretation of eDNA results, but in some situations, it may improve our understanding. It also further complicates aspirations to achieve more accurate quantitative abundance estimates together with the fact that more work is also required around species-specific eDNA shedding rates and decay profiles (e.g., metabolism rates, life stages and allometries) and environmental covariates (Barnes et al. 2014, Pont 2024).

Certain fish species in New Zealand dependant on geographic location can have patchy distributions (e.g. shortjaw kokopu, koaro, giant kokopu, bluegill bully). This is the case for these species at both sites (Manganui and Paparahia) where they occur in this study and in general across the wider Waikato region and for shortjaw kokopu, nationally (McDowall, 1978). The preferred habitat for species like shortjaw kokopu and koaro is often associated with lower ordered, elevated streams (McDowall, 2000). Based on these preferences and findings in this study and others (Wilcox et al. 2015, Jane et al. 2015, Van Driessche et al. 2022, Van Driessche et al. 2023) that corelates discharge and distance of detection via eDNA, alternative sampling strategies could be employed to increase detection and limit false negatives. For example, this could involve sampling from a composite water sample (Cornman et al. 2018, Kamoroff and Goldberg, 2018, Mena et al. 2021) taken from several points longitudinally up a stream at a good level of replication (six replicates). At the Manganui tributary sites, we see a significant drop in koaro detection over 250 m in these 3rd and 2nd order streams, composite water samples taken every 100-200 m over the length or certain section of the stream would likely increase detection of rare or patchy species.

Low detections for both bluegill bully and shortjaw kokopu in the mainstem Manganui at only the 0.150 km mark and the 4 km mark in the 2022 sampling indicates that these species are likely close to the sampling points based on the fact these species are found in low numbers/biomass in this system (Table D-5. SOE site fish parameters., Appendix D). Viewing these results from a plume dynamics perspective may help narrow the actual source location of these nested assemblages, for example the higher hits and reads for bluegill bully at the 4 km mark (4/385) compared to the 0.150km mark (3/74) may be a result of a fine particle plume at the 4 km mark with larger fragments being closer to the source at the 0.150 km site.

The results from this study highlight some differences between rarer species or species with a lower biomass compared to more common species particularly in the Manganui River. We see a consistent signal at all sites for these common species like longfin eel, redfin bully and rainbow trout as they are consistently present at all sites while for less common species, we see less consistent to occasional signals (Table 3-11, Table 3-12).

Table 3-11. Manganui River pre 2021 sampling. Mean sequence reads by distance (upstream 0.150 km to downstream 9.5 km) and fish species.

Fish Species	0.15 km	0.5 km	1 km	2 km	3 km	4 km	5 km	9.5 km
Longfin eel	2222.7	2107.5	1527.2	987.7	784.3	1943.3	1277.5	693.3
Redfin bully	509.0	346.5	297.7	172.2	389.0	823.3	597.7	72.5
Rainbow trout	32.2	103.7	292.7	110.3	159.3	481.5	284.8	331.0
Shortfin eel	421.7	250.0	190.8	0.8	22.3	62.5	167.8	105.2
Brown trout	0.0	9.8	16.3	0.0	0.0	36.0	92.8	0.0
Pouched lamprey	34.3	17.8	35.8	55.2	0.0	76.5	3.0	7.8
Bluegill bully	0.0	0.0	8.2	0.0	0.0	0.0	24.3	0.0
Giant or shortjaw kokopu	0.0	0.0	0.0	0.0	0.0	0.0	7.7	0.0
Giant bully	0.0	0.0	0.0	0.0	13.0	0.0	0.0	0.0
Shortjaw kokopu	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0
Torrentfish	0.0	0.0	0.0	0.0	5.8	0.0	0.0	0.0

Table 12. Manganui River pre 2022 sampling (syringe). Mean sequence reads by distance (upstream 0.150 km to downstream 9.5 km) and fish species.

Fish Species	0.15	2.84	3 km	4 km	4.165 km	5 km	9.5 km
Longfin eel	3840.0	3272.0	2392.2	2626.8	2008.3	930.8	155.1
Redfin bully	320.8	1604.8	576.7	1158.0	836.3	106.5	17.8
Rainbow trout	77.2	105.2	113.8	269.8	181.5	19.8	3.3
Shortfin eel	261.7	168.3	155.3	198.7	184.0	32.5	5.4
Brown trout	0.0	185.8	253.7	324.3	283.0	85.7	14.3
Pouched lamprey	2.2	37.3	26.0	50.0	5.2	6.8	1.1
Bluegill bully	0.0	0.0	0.0	0.0	28.2	12.5	2.1
Giant or shortjaw kokopu	21.8	9.3	0.0	0.0	0.0	0.0	0.0
Giant bully	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Shortjaw kokopu	5.8	26.5	0.0	13.2	0.0	0.0	0.0
Torrentfish	0.0	18.0	0.0	0.0	0.0	0.0	0.0

Depending on where the samples are taken longitudinally some species might be missed even with six replicates and as little as 165 m between sampling points (upstream to downstream, 2022 data) and this will be dependent on biomass both locally and upstream. The distance signals for inanga and torrentfish (5 km) and snapper (11.8 km) should be viewed as distances that an eDNA signal can travel based on substantial "artificial" biomasses at one single location. These single source bulk biomasses are unlikely to occur for fish in New Zealand streams naturally. As such the results in this study confirm that lotic eDNA metabarcoding can measure biodiversity at spatiotemporal scales (Perry et al. 2024) or reach scales and will be influenced by discharge and biomass (Van Driessche et al. 2023).

This spatiotemporal scale will assist in the development and use of environmental reporting metrics like a potential eDNA Fish-IBI (Index of Biological Integrity) and an eDNA MCI (Macroinvertebrate Community Index) to together with those that have already been developed (Taxon-Independent Community Index or TICI, Wilkinson et al. 2024). Additionally, the use of eDNA at spatial and temporal scales may be refined by the use of environmental RNA (eRNA; Miyata et al. 2021, Veilleux et al, 2021). As RNA decay is faster than DNA it provides more accurate information in space and time. The application of eRNA will provide new research opportunities (Takahashi et al. 2023).

Based on the tributary sites in this study, sampling within the mainstem below tributaries will likely miss the detection of species found in these tributary sites, this will likely depend on the size of the tributaries relative to the size of the mainstem and also, be dependent on the biomass found within the tributaries (Jane et al. 2015, Wood et al. 2020). There was however some evidence that sampling well below the confluence of a tributary (1 km in this study) may result in some detections and this could be the result of plume dynamics from species found in the tributaries.

The selection of sites for eDNA studies can be ad-hoc, the results from this study may help with selecting sites to get the best coverage and avoid false negatives. Results will however likely vary from one site to another based on environmental factors (river morphology, catchment land use, water chemistry) fish diversity and relative abundance (Bylemans et al. 2018). Hydrology-based models have been developed to assist with spatial design for eDNA sampling strategies (Carraro et al. 2021, Carraro and Altermatt, 2024), which may also assist, but will be dependent on the questions that need to be answered.

3.6 Conclusion

This study contrasts how far eDNA signals can travel in a wadeable lotic system via planted fish (both planned and unplanned) with what natural spatiotemporal scales look like obtained via koaro signals and overall fish community compositions from closely staged longitudinal sampling. The results indicate this is dependent on both discharge and biomass and can also be influenced by primers used between samplings (different sampling years) and fish species (as not equal). The snapper signal was detected 11.8 km downstream and the caged inanga and torrentfish 5 km. The natural signal for koaro in the Paparahia stream was detected 2.2 km downstream, 1.1 km downstream in the Wainui stream and 250 m downstream in the Manganui tributaries which corelates with the discharge for these streams. We see constant eDNA signals from species found throughout the streams sampled that dominate the fish biomass to those that are occasional to rare as is also reflected in their biomass. The results from these natural signals confirms that eDNA metabarcoding can measure biodiversity at spatiotemporal scales. The 5 km post sampling site (2021) showed that sequence reads and detection can be influenced by a dominant source, in this case cattle, that effectively “swamped” the reads at this site reducing the detection of other species. Though this type of situation would be rare, this example shows that it can happen and exploring the data as a whole rather than focusing just on one group of interest will highlight theses anomalies.

The 2021 caged fish trials together with the snapper signal provides an example of plume dynamics which correlates with overseas studies that were done at the same time as the field trials in this study. The early publication of these studies in relation to this study helps us to understand the processes of plume dynamics in relation to distance and decay. After the removal of the caged fish in this study we quickly see this eDNA signal reduce. The eDNA signal of habitat specific koaro in the Manganui tributaries (together with planted common bully) did show up in samples 1 km downstream from the tributary confluence with the mainstem Manganui. Indicating that sampling at a distance below a confluence may be beneficial when trying to incorporate the signals from tributaries into sampling strategies, however more work would be needed here.

The passive verses active sampling (syringe) comparison showed that in this type of habitat (limited in anthropogenic effects) the fine filters of the syringes appeared to provide a more effective collection method for eDNA sampling. This result may be different in other systems and would need to be tested. Additionally, a finer passive filter material could be developed for use in cleaner systems which may show different results. However, for ease of use the single site visit using the syringe samplers is a more efficient use of time compared with the set and return process for the passive samplers. Additionally, contamination becomes a factor if using more than one passive sampler in the same system and steps would be required to avoid contamination. It is clear from the sampling in this study that eDNA is a very sensitive tool and care must be taken between sampling not to contaminate samples particularly if moving between catchments or sampling upstream of another. The negative control samples show that despite all practicable steps being taken, contamination can still occur and if true negative controls (taken at the same spot as field samples i.e., beside the stream) this is just a reality of eDNA sampling. Should any future work be conducted using caged fish in New Zealand streams I would recommend the use of hard framed “cages” that allow the free flow of eDNA material while protecting fish from hungry eels.

Chapter 4. Research summary and conclusions

The objective of Chapter 2 was to optimise eDNA sample replication for the consistent characterisation of freshwater fish and macroinvertebrate communities in lotic systems. The results of this study indicated that six replicates were needed to consistently detect 89.5% fish species using field-based syringe eDNA sampling and preservation. For macroinvertebrates, six replicates were required to identify 86% of taxa identified to the NEMS (National Environmental Monitoring Standards) level used for the Macroinvertebrate Community Index (MCI: usually genera) while eight replicates were required to detect 89% of NEMS taxa. Additional analysis (Appendix C) looking at the replication required to assess terrestrial species (birds and mammals) from eDNA samples required 10 replicates to reach 91.4% for mammals and 13 replicates for birds to reach 91.6% of species richness. The comparison of field replication and extraction methods 'standard' and the 'boosted' (the composite pooling of preservation buffer) showed no significant difference between methods for species richness for both fish and macroinvertebrates. Pre-study it was hypothesised that the 'boosted' method might produce better results than the 'standard' sampling method based on a greater level of filtration, but this wasn't the case indicating that sufficient water volumes are being filtered using the 'standard' method. An altitudinal-biodiversity gradient effect was observed for fish, meaning less replicates could be required at elevations greater than 100 m to reach 90% species richness, however further work would be required to confirm these results. In summary,
for fish and macroinvertebrate biodiversity this study suggests that six replicates are a reasonable trade-off between effective community characterisation and cost in New Zealand lotic systems.

As hypothesised for the second objective (Chapter 3), the results of this study confirmed that the distance an eDNA signal will travel downstream before decay occurs will be dependent on discharge and biomass. The 2021 caged fish experiment together with the unplanned snapper detections showed how far an eDNA signal can travel (up to 11.8 km), from a fixed source and biomass in a wadeable lotic system. Natural spatiotemporal scales observed from koaro and general longitudinal sampling indicate that eDNA metabarcoding can measure biodiversity at spatiotemporal scales. Species detection for rare or patchy species at low biomass will be dependent on their locality to sampling point and influenced by discharge/stream order. In larger systems eDNA detection for rare species will be both assisted (reduced retention) and constrained (higher dilution effect) with the opposite effect in smaller systems (increased retention and reduced dilution). Based on the results of this study and reviewed literature, retention maybe more of a limiting factor than dilution or even decay. The way in which eDNA moves downstream, is likely dependant on the substrate to water surface ratio, with retention of eDNA being higher in these smaller streams or smaller discharges, that have this higher ratio. For koaro in the Manganui upper tributary this eDNA signal reduced by half every 50m moving downstream.

The detection and signal strength between fish species will be dependent on primers leading to primer bias. When looking at sequence reads and hit detections, some fish species use all five of the fish primers available in this study while for others only one primer is used. In the 2021 sampling a “swamping” effect was seen at the 5 km site caused by cattle, this chance occurrence highlights its negative effect on the detection of other species and highlights the need for sound replication.

The role of plume dynamics was not originally considered (or a term at conception) in this study, but results show a pattern that is consistent with overseas studies and is a welcome addition to this study. The role of plume dynamics in relation to particle fragmentation, transportation and decay will enable better interpretation of results and aid in the development of sampling strategies. The eDNA signal from tributary sources was largely diluted by the mainstem, but there was some evidence that sampling 1 km downstream of the tributary confluence may help in the detection of species found in these habitats.

The passive verses active sampling (syringe) comparison favoured the syringe method in this low turbidity environment and this result was likely due to some eDNA material passing through the passive filters especially in the clearer water found in the tributary sites. The use of passive samplers increased the risk of contamination by being above sampling points, resulting in possible contamination at some sites despite efforts to eliminate/reduce this possibility. This result together with the results from the negative controls highlights the sensitive nature of eDNA metabarcoding.

Six replicate sampling was used at all sites for the second objective (Chapter 3) in this study based on findings from Chapter 2. The results from this second objective confirm that the use of six replicates provides a good level of confidence in the results. The sequence reads and replicate hits per species from these replicate samples at each site helps to establish a general measure of abundance that, in general matches fish biomass from electric fishing and the general knowledge of these sites.

This study will help in determining the spatial scales of eDNA in lotic systems in a New Zealand context, there will however be differences from one site to the next based on hydrology and environmental conditions. eDNA samples taken (especially at six replicates) will incorporate species well beyond the 150 m sampled at a reach level for traditional State of the Environment (SoE) sampling (electric fishing, netting, spotlighting) particularly at larger discharge sites at lower elevations. But based on this study, biodiversity can be measured at spatiotemporal scales via eDNA metabarcoding. An obvious next step in enhancing the interpretative power of eDNA is to establish an eDNA Fish-IBI (Index of Biological Integrity) and an eDNA MCI (Macroinvertebrate Community Index) based on NEMS taxa for environmental reporting throughout NZ.

Having the ability to standardise eDNA protocols (via replication and/or sampling strategies) enables comparisons across studies and across time, standardised methods also provide all users a guide to follow and not just professionals. Early adoption of methods specifically around replication as put forward in this study will help achieve these results however, this will also depend on the questions being asked. A “standardisation” for eDNA can only be based on what methods are currently in play. The innovative nature of eDNA means new, more effective methods for the detection of species richness and beyond, may arrive, meaning relatively new standards may need to be replaced to keep up with these likely changes. Any new methods will likely be able to process older, stored samples and highlights the importance of biobanking.

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Appendices

Appendix A

Table A-1. Additional details for national high replicate eDNA sites (n=54), sampled December 2020 to May 2021 (*sites excluded from the data analysis). Substrate HB = hard bottom, SB = Soft bottom.

Map ID	Site ID	Job ID	Territorial Authority	Site name	Lat	Lon	Collection date	Standard (ml)	Boosted (ml)	Sub-strate	Elevation (m)	Stream order	NZSEGMENT	NZREACH	FENZ LEVEL FOUR ALPHA	Dominant land use (5 km)
1	RC031	601114	Northland Regional Council	Tapapa Stream	-35.19295451	173.4806506	2021-01-28	1000	2000	HB	80.4	3	1006293	1005772	C5.1a	Native Vegetation
2	RC029	601109	Northland Regional Council	Waipoua River	-35.65249605	173.570394	2021-01-29	850-1000	1800-2000	HB	99.6	4	1018149	1016579	C5.1b	Native Vegetation
3	RC027	601111	Northland Regional Council	Waiponamu stream	-35.74427694	174.2931542	2021-02-02	1000	2000	HB	58.5	3	1020553	1018851	C5.1a	High Pastoral
4	RC028	601112	Northland Regional Council	Tangowahine Stream	-35.84428975	173.9359479	2021-02-02	1000	2000	HB	18.8	5	1022656	1020873	C4.2b	High Pastoral
5	RC030	601113	Northland Regional Council	Ruakaka River	-35.86944231	174.4026121	2021-01-27	650-800	1320-1800	SB	17.6	4	1023322	1021503	A3.1b	High Pastoral
6	RC035	601289	Auckland Council	Avondale Stream	-36.923647	174.69076	2021-03-17	300-400	310-800	SB	20.0	3	2039302	2006297	A1.1a	Urban
7	RC036	601287	Auckland Council	Piha Stream	-36.95342	174.475644	2021-03-10	500-1000	1400-2000	HB	19.6	3	2039826	2006509	C5.1a	Native Vegetation
8	RC037	601288	Auckland Council	Ngakoroa Stream	-37.19548	174.973634	2021-03-09	550-1000	1450-2000	SB	159.1	3	2045595	2009507	A4.2a	High Pastoral
9	RC006	601050	Waikato Regional Council	Komakorau Stream	-37.699054	175.329883	2021-01-20	160-200	260-475	SB	30.1	4	3059477	3015932	B1.2a	High Pastoral
10	RC051	601611	Bay of Plenty Regional Council	Whanarua Stream	-37.68458853	177.7887414	2021-05-11	1000	2000	HB	24.0	2	4062208	4001746	C5.1a	Native Vegetation
11	RC005	601049	Waikato Regional Council	Waikato River @ Hamilton	-37.793849	175.290637	2021-01-25	1000	2000	HB/SB	20.2	7	3063520	3017901	A1.2a	Urban

12	RC039	601551	Bay of Plenty Regional Council	Mimiha Stream	-37.8672189	176.6946157	2021-04-23	1000	2000	SB	16.5	3	4070381	4004561	C6.1a	High Pastoral
13*	RC038	601552	Bay of Plenty Regional Council	Karaponga Stream*	-38.00962068	176.7079927	2021-04-30	1000	2000	SB	39.0	3	4079858	4008127	C4.2c	High Pastoral
14	RC050	601609	Bay of Plenty Regional Council	Ohutu Stream	-38.39083617	176.8317664	2021-05-10	1000	2000	HB	241.9	3	4112447	4021130	C12.1b	Native Vegetation
15	RC052	601239	Waikato Regional Council	Manganui Trib @ Small 3	-38.51715248	174.7105166	2020-12-14	1000	2000	HB	180.0	3	3118742	3037993	C7.1c	Native Vegetation
16	RC053	601240	Waikato Regional Council	Manganui River @ Big 3	-38.51715248	174.7105166	2020-12-14	1000	2000	HB	180.0	3	3118742	3037993	C7.1c	Native Vegetation
17	RC054	601241	Waikato Regional Council	Manganui River @ 410_8	-38.59365209	174.6835227	2020-12-15	1000	2000	HB	60.4	4	3125700	3040629	C6.2a	Native Vegetation
18	RC010	601330	Hawke's Bay Regional Council	Nuhaka River	-39.005279	177.764983	2021-03-31	1000	2000	HB	20.4	4	8167155	8012981	C4.2d	High Pastoral
19	RC007	601096	Taranaki Regional Council	Maketawa Stream	-39.23622104	174.1843902	2021-02-01	1000	2000	HB	375.3	2	6178774	6005874	C2.1a	Native Vegetation
20	RC011	601286	Hawkes Bay Regional Council	Waingongoro Stream	-39.382179	176.3325	2021-03-19	1000	2000	HB	497.9	3	8192205	8021421	C6.4a	High Pastoral
21	RC008	601403	Taranaki Regional Council	Kaupkonui Stream	-39.54029426	174.0826127	2021-04-15	1000	2000	HB	59.8	5	6198171	6011674	C2.1a	High Pastoral
22	RC009	601404	Taranaki Regional Council	Tangahoe River	-39.62909427	174.3490832	2021-04-15	510-700	1070-1210	HB	21.2	5	6206136	6014041	A4.2a	High Pastoral
23	RC014	601158	Hawkes Bay Regional Council	Ngaruroro River @ Fernhill	-39.58803	176.769977	2021-03-21	1000	2000	HB	20.0	6	8207185	8024814	C4.2d	High Pastoral
24	RC012	601277	Hawkes Bay Regional Council	Kuripapango Stream	-39.807778	176.98567	2021-03-21	1000	2000	HB	16.5	1	8220246	8028428	A1.1b	Native Vegetation
25	RC013	601323	Hawkes Bay Regional Council	Papanui Stream	-39.855926	176.715002	2021-03-26	1000	2000	SB	80.0	5	8221860	8029023	A4.1c	High Pastoral
26	RC025	601182	Horizons Regional Council	Tamaki River @ Reserve	-40.1200834	176.031067	2021-02-24	1000	2000	HB	401.0	3	7231345	7034557	C7.1d	Native Vegetation
27	RC026	601245	Horizons Regional Council	Turitea Stream @ Massey Farm Bridge	-40.385336	175.607949	2021-03-17	1000	2000	HB	39.0	3	7239293	7040071	C6.4a	High Pastoral
28	RC024	601216	Horizons Regional Council	Waikawa Stream @	-40.706966	175.23451	2021-03-10	1000	2000	HB	61.2	4	7248627	7048450	C7.1c	High Pastoral

				North Manakau Road												
29*	RC048	601400	Greater Wellington Regional Council	Whareroa Stream*	-40.955839	174.970188	2021-04-16	500	1000	SB	13.6	4	9255944	9005999	A4.2a	High Pastoral
30	RC046	601337	Greater Wellington Regional Council	Waipoua River	-40.953588	175.6729873	2021-04-07	1000	2000	HB	104.0	5	9256259	9006301	C6.4a	High Pastoral
31	RC049	601378	Greater Wellington Regional Council	Wainui Stream	-40.9739501	174.9616078	2021-04-13	1000	2000	HB	12.1	3	9256465	9006480	A4.2a	Native Vegetation
32	RC023	601162	Nelson City Council	Whangamoa River @ Kokorua	-41.129	173.541	2021-02-23	1000	2000	HB	19.9	4	10009423	10007714	C6.4b	Forestry
33	RC021	601252	Nelson City Council	Wakapuaka River @ Hira	-41.214	173.399	2021-03-18	1000	2000	HB	49.6	4	10012362	10009633	C6.4b	Forestry
34	RC047	601520	Greater Wellington Regional Council	Kaiwharawhara Stream	-41.259917	174.788556	2021-05-07	1000	2000	HB	19.7	4	9263955	9013529	C5.2a	Native Vegetation
35	RC020	601102	Nelson City Council	Maitai River @ Groom	-41.287	173.328	2021-02-04	1000	2000	HB	47.1	4	10014643	10011156	C6.4b	Forestry
36	RC018	601082	Nelson City Council	Poorman Valley Stream @ Seaview	-41.303	173.226	2021-02-03	1000	2000	HB	4.8	2	10016187	10011985	C5.2b	High Pastoral
37	RC019	601098	Nelson City Council	Poorman Valley Stream @ Barnicoat	-41.317	173.25	2021-02-04	1000	2000	HB	74.6	2	10016393	10012419	C5.2a	Native Vegetation
38	RC022	601354	Nelson City Council	Roding River @ Caretaker	-41.357	173.26	2021-04-09	1000	2000	HB	202.7	4	10018126	10013741	C7.1c	Native Vegetation
39	RC032	601164	Marlborough District Council	Are Are Creek	-41.46738	173.8264	2021-02-24	1000	2000	HB	33.8	4	11022708	11017507	C6.4b	Forestry
40	RC042	601479	Environment Canterbury	Blue Duck Creek	-42.27923926	173.7669203	2021-03-30	1000	2000	HB	14.8	2	13064994	13006067	C1.1b	Native Vegetation
41	RC015	601203	West Coast Regional Council	Redjacks Creek	-42.437996	171.478171	2021-03-01	1000	2000	HB	81.2	3	12073197	12027614	C2.1b	Native Vegetation
42	RC040	601478	Environment Canterbury	Landslip Creek	-42.69208608	172.0452815	2021-03-31	1000	2000	HB	592.0	3	13084962	13017470	G3.1a	Native Vegetation
43	RC016	601204	West Coast Regional Council	Okutua Stream	-43.200577	170.266371	2021-02-21	1000	2000	HB	20.2	2	12111395	12041149	C2.2a	Native Vegetation
44*	RC041	601477	Environment Canterbury	Fork Stream, Talus Spring*	-43.85172455	170.3522441	2021-04-22	1000	2000	HB	1052.1	3	13146902	13504544	H6.4a	High Pastoral
45	RC033	601131	Environment Canterbury	Lower Rakaia	-43.89980816	172.2071035	2021-02-02	1000	2000	HB	2.4	7	13300010	13058524	A4.4a	High Pastoral

46	RC017	601207	West Coast Regional Council	Haast River	-43.937771	169.295797	2021-02-19	1000	2000	HB	73.4	6	12152655	12052210	J8.3a	Native Vegetation
47	RC034	601132	Environment Canterbury	Lower Ashburton @ Hakatere	-44.05245243	171.8045024	2021-02-05	1000	2000	HB	7.7	6	13155682	13061477	A4.2a	High Pastoral
48	RC001	601130	Environment Canterbury	Lower Rangitata River	-44.18753881	171.5084259	2021-02-09	1000	2000	HB	2.2	6	13163160	13063572	A4.4a	High Pastoral
49	RC044	601504	Otago Regional Council	Dunstan Creek	-44.93954022	169.7651639	2021-05-03	1000	2000	HB	430.2	5	14214374	14022310	D4.1a	High Pastoral
50	RC043	601503	Otago Regional Council	Kauru River	-45.10909124	170.7417559	2021-04-22	1000	2000	HB	100.0	5	14224934	14029391	C8.4a	High Pastoral
51	RC004	601316	Environment Southland	Oreti River at McKellars Flat	-45.32618168	168.208478	2021-03-24	1000	2000	HB	599.4	5	15245827	15016746	G1.3a	Native Vegetation
52	RC045	601505	Otago Regional Council	Clutha River @ Clydevale	-46.10141607	169.5326871	2021-05-04	1000	2000	HB/SB	20.1	8	14299081	14067409	A5.2a	High Pastoral
53	RC003	601315	Environment Southland	Oreti River at Branxholme	-46.29545184	168.2841205	2021-03-23	1000	2000	HB	10.6	7	15312337	15057362	G1.4c	High Pastoral
54	RC002	601317	Environment Southland	Waituna Creek at White Pine Road	-46.52024252	168.5427042	2021-03-23	200-250	377-530	HB	10.0	1	15318961	15061719	B2.1a	High Pastoral

Table A-2. Group reads per method.

Group	Standard reads	Boosted reads	Combined reads	% of total reads
Other	6763806	6467960	13231766	33.5658
Plants	3084086	2996699	6080785	15.4255
Fish	2358506	2148401	4506907	11.4329
Worms	1877277	1862996	3740273	9.4882
Ciliates	1284185	1168214	2452399	6.2211
Insects	861423	883224	1744647	4.4258
Molluscs	737269	742786	1480055	3.7545
Birds	662483	602777	1265260	3.2097
Mammals	384444	418729	803173	2.0375
Heterokont algae	393767	392013	785780	1.9933
Cnidarians	390312	388388	778700	1.9754
Diatoms	313791	303202	616993	1.5652
Green algae	224978	183806	408784	1.0370
Crustaceans	176491	182243	358734	0.9100
Red algae	112961	105783	218744	0.5549
Fungi	115854	98939	214793	0.5449
Rotifers	93648	107914	201562	0.5113
Bacteria	77645	54406	132051	0.3350
Cryptomonads	45417	50312	95729	0.2428
Bryozoans	47308	48246	95554	0.2424
Flatworms	27573	22610	50183	0.1273
Springtails	24891	24703	49594	0.1258
Mosses	21666	19669	41335	0.1049
Amoebae	14681	12962	27643	0.0701
Mites and ticks	10027	11066	21093	0.0535
Liverworts	5612	4720	10332	0.0262
Centipedes	1555	1852	3407	0.0086
Spiders	1081	888	1969	0.0050
Amphibians	726	809	1535	0.0039
Hornworts	90	350	440	0.0011
Lizards and snakes	67	77	144	0.0004

Table A-3. Freshwater fish species found in the eDNA samples ranked in order of highest combined reads with conservation status (Dunn et al. 2018). *Introduced, **non-resident native.

Species name	Common name	Reads (standard)	Reads (boosted)	Reads (combined)	Site occurrence	Average reads per site	Status
Anguilla dieffenbachii	Longfin eel	398980	450437	849417	51	16655	At Risk
Gobiomorphus breviceps	Upland bully	152626	172872	325498	17	19147	Not Threatened
Gobiomorphus huttoni	Redfin bully	128336	140120	268456	33	8135	Not Threatened
Anguilla australis	Shortfin eel	124437	120925	245362	43	5706	Not Threatened
Salmo trutta*	Brown trout*	86070	94462	180532	32	5642	Sports fish
Retropinna retropinna	Common smelt	80397	94290	174687	21	8318	Not Threatened
Galaxias maculatus	Inanga	81296	81318	162614	28	5808	At Risk
Cheimarrichthys fosteri	Torrentfish	78137	69605	147742	29	5095	At Risk
Galaxias brevipinnis	Koaro	42155	61306	103461	20	5173	At Risk
Oncorhynchus mykiss*	Rainbow trout*	45395	47279	92674	13	7129	Sports fish
Gobiomorphus hubbsi	Bluegill bully	48055	44609	92664	17	5451	At Risk
Galaxias divergens	Dwarf galaxias	29149	30639	59788	4	14947	At Risk
Galaxias fasciatus	Banded kokopu	20680	26487	47167	13	3628	Not Threatened
Gobiomorphus basalis	Crans bully	9002	20153	29155	3	9718	Not Threatened
Gambusia affinis*	Mosquitofish*	16809	12102	28911	6	4819	Pest
Cyprinus rubrofuscus*	Koi carp*	10181	17762	27943	2	13972	Pest
Carassius auratus*	Goldfish*	13282	11817	25099	7	3586	Pest
Gobiomorphus cotidianus	Common bully	11139	6934	18073	4	4518	Not Threatened
Mugil cephalus	Grey mullet	4368	8883	13251	5	2650	Not Threatened
Arripis trutta	Kahawai	7189	5149	12338	4	3085	Not Threatened
Galaxias argenteus	Giant kokopu	5310	4052	9362	6	1560	At Risk
Galaxias vulgaris	Common river galaxias/Canterbury galaxias	2691	3725	6416	2	3208	At Risk
Geotria australis	Pouched lamprey	2852	3317	6169	19	325	Threatened

Perca fluviatilis*	European perch*	367	5552	5919	5	1184	Pest/Sports fish
Gobiomorphus gobioides	Giant bully	1627	1879	3506	10	351	At Risk
Galaxias southern	Southern galaxias	926	1277	2203	1	2203	Threatened
Rhombosolea retiaria	Black Flounder	1249	759	2008	5	402	Not Threatened
Scardinius erythrophthalmus*	Rudd*	417	1445	1862	3	621	Pest/Sports fish
Galaxias postvectis	Shortjaw kokopu	856	767	1623	6	271	Threatened
Galaxias cobitinis	Lowland longjaw galaxias	1052	542	1594	1	1594	Threatened
Stokellia anisodon	Stokell's smelt	668	423	1091	2	546	At Risk
Oncorhynchus tshawytscha*	Chinook salmon/King salmon*	593	200	793	6	132	Sports fish
Galaxias gollumoides	Gollum galaxid	298	400	698	2	349	Threatened
Aldrichetta forsteri	Yelloweye mullet	249	286	535	4	134	Not Threatened
Anguilla reinhardtii**	Speckled longfin eel**	47	407	454	5	91	Non-Resident native
Galaxias paucispondylus	Alpine galaxias	228	187	415	2	208	Threatened
Galaxias anomalus	Roundhead galaxias	189	104	293	1	293	Threatened
Forsterygion nigripenne	Estuarine triplefin	154	0	154	1	154	Not Threatened
Ameiurus nebulosus*	Brown bullhead catfish*	74	66	140	1	140	Pest
Galaxias sp. D/Pomahaka Galaxias	Clutha flathead/Pomahaka galaxias	0	24	24	1	24	Threatened

Table A-4. Freshwater Invertebrate species/taxa (Stark taxa) found in the eDNA samples ranked in order of highest combined reads.

Common name	Reads (standard)	Reads (boosted)	Reads (combined)	Site occurrence	Average reads per site
Oligochaeta	1032099	1075289	2107388	51	41321
Hydra	381556	374549	756105	51	14826
Potamopyrgus	339072	359423	698495	50	13970
Cladocera	63726	45787	109513	42	2607
Deleatidium	37529	34065	71594	33	2170
Copepoda	31686	31637	63323	47	1347
Nematoda	29062	27561	56623	51	1110
Hydropsyche	31090	22315	53405	27	1978
Nemertea	28311	23632	51943	41	1267
Collembola	24676	24323	48999	45	1089
Pycnocentrodes	27813	19334	47147	41	1150
Coloburiscus	14822	17565	32387	33	981
Olinga	8577	13814	22391	26	861
Austrosimulium	9926	7477	17403	30	580
Physella	8224	8633	16857	26	648
Nesameletus	7081	6964	14045	18	780
Acarina	5456	7158	12614	34	371
Hydrobiosis	5990	6216	12206	35	349
Chironomus	4319	6816	11135	38	293
Atalophlebioides	5011	5238	10249	11	932
Austroperla	4274	5321	9595	18	533
Hudsonema	4055	3423	7478	29	258
Bryozoa	3907	2738	6645	16	415
Platyhelminthes	3606	2887	6493	41	158
Chironomidae	1393	3355	4748	43	110
Paratya curvirostris	1589	1798	3387	14	242

Austroclima sepia	1479	1266	2745	9	305
Nesameletidae	967	1416	2383	6	397
Dixidae	1607	656	2263	16	141
Aphrophila sp. EJD-2015	1213	980	2193	7	313
Triplectides	969	1044	2013	13	155
Psilochorema	997	927	1924	24	80
Polyplectropus	991	911	1902	29	66
Zephlebia	1044	790	1834	7	262
Ameletopsis	1050	761	1811	11	165
Ichthybotus	589	1200	1789	11	163
Sphaeriidae	656	943	1599	15	107
Syrphidae	711	749	1460	20	73
Sororipyrgus kutukutu	626	779	1405	2	703
Leptophlebiidae	632	668	1300	12	108
Gyraulus	434	823	1257	11	114
Culicidae	585	646	1231	7	176
Latia	686	541	1227	8	153
Paracalliope	77	820	897	3	299
Rallidens	576	309	885	8	111
Tardigrada	444	430	874	12	73
Spaniocercoides	487	349	836	1	836
Hirudinea	739	50	789	5	158
Zelandobius	375	399	774	11	70
Paranephrops	343	339	682	8	85
Elmidae	476	109	585	9	65
Hygraula nitens	279	255	534	6	89
Oxyethira	441	83	524	12	44
Hydraenidae sp. EJD-2015	145	372	517	12	43
Paroxyethira	275	220	495	4	124

Neurochorema	288	203	491	10	49
Melanopsis	272	161	433	4	108
Costachorema	171	225	396	14	28
Amphipoda	170	182	352	6	59
Ostracoda	169	170	339	12	28
Echyridella	104	210	314	3	105
Confluens	111	160	271	4	68
Antipodochlora braueri	71	190	261	6	44
Hydrobiosidae	117	124	241	6	40
Cryptobiosella	74	139	213	2	107
Diplectrona	59	151	210	2	105
Rhabdocoela	104	101	205	5	41
Beraeoptera	79	88	167	5	33
Zelandoperla	101	63	164	9	18
Obtusopyrgus alpinus	0	150	150	1	150
Archichauliodes	133	15	148	6	25
Hydroptilidae	107	34	141	3	47
Oeconesidae	20	121	141	4	35
Hydrobiosella	74	66	140	4	35
Stenoperla	54	83	137	7	20
Pycnocentria	96	40	136	8	17
Orthopsyche fimbriata	93	38	131	4	33
Isopoda	55	74	129	7	18
Oeconesus	87	37	124	6	21
Helicopsyche	46	70	116	4	29
Triplectidina	0	114	114	2	57
Mauiulus	78	30	108	4	27
Neocurupira sp. EJD-2015	52	54	106	1	106
Spaniocerca	79	4	83	4	21

Orthocladiinae	32	50	82	5	16
Collembola	34	44	78	9	9
Rakiura vernale	33	40	73	1	73
Glyptophysa sp. NZla2	39	31	70	3	23
Lymnaeidae	0	68	68	3	23
Hydrophilidae	57	8	65	7	9
Staphylinidae	43	17	60	5	12
Xanthocnemis	42	13	55	5	11
Stictocladius sp. EJD-2015	25	28	53	2	27
Oniscigaster	49	3	52	3	17
Tabanidae	29	23	52	4	13
Philorheithrus	42	3	45	4	11
Oecetis	23	16	39	3	13
Zelandoptila moselyi	22	16	38	2	19
Notonemouridae	5	32	37	4	9
Plectrocnemia	13	23	36	3	12
Sigara	15	20	35	4	9
Tanytarsus	10	25	35	3	12
Hydrochorema crassicaudatum	16	18	34	2	17
Limonia	34	0	34	1	34
Austrolestes	22	8	30	3	10
Procordulia	9	18	27	1	27
Hemicordulia	0	23	23	3	8
Acroperla	0	22	22	1	22
Nesoperla fulvescens	19	0	19	1	19
Polypedilum	18	0	18	1	18
Zelolessica	16	0	16	1	16
Ephydridae	0	15	15	2	8
Ceratopogonidae	6	6	12	3	4

Rhantus suturalis	8	3	11	1	11
Psychodidae	4	5	9	2	5
Corynoneura	0	6	6	1	6
Ischnura aurora	0	6	6	1	6
Alloecentrella magnicornis	5	0	5	1	5
Ferrissia	5	0	5	1	5
Gundlachia	5	0	5	1	5
Kempynus latiusculus	0	5	5	1	5
Tiphobiosis	0	5	5	1	5
Megaleptoperla	4	0	4	1	4
Nematomorpha	0	4	4	1	4
Polypedilum	0	4	4	1	4
Taraperla ancilis	0	4	4	1	4
Aoteapsyche	0	3	3	1	3
Austronella	0	3	3	1	3
Stratiomyidae	0	3	3	1	3

Appendix B

Electric fishing verses eDNA

Methods

Following eDNA collection, electric fishing was conducted at three sites in the Waikato Region, two reference sites, the Manganui River Big 3 and Manganui small 3 and one random site, the Komakorau Stream (Table B1). All reference sites have been fished annually since 2010 providing 12 years of electric fishing data, and random sites have been fished every three years on a rotating basis providing three years' worth of electric fishing data (over nine years). The Komakorau stream is turbid in nature and at this site only 160 to 200mls of water was able to be filtered per by syringe. One litre per syringe was achieved at all other electric fishing sites. Each site is fished consistently using a standard single pass 150m electric fishing protocol (Joy et al. 2013). Sampling at each electric fishing reach commenced directly above the eDNA sampling point and continued in an upstream direction. All species captured were identified to species level. All species were measured (mm) up to the first 50 for each species, when 50 individuals were reached, the first 10 of the same species were measured from there on, in each of the 15m sub reaches as per protocols (Joy et al. 2013). All details were entered into a standardised electronic data "fish template" via field laptops (Toughbook, CF-20 Panasonic). Following data quality checks these data were then loaded into corporate databases (KiEco, KISTERS, Inc.) and the NZFFD (NZ Freshwater Fish Data Base). Species presence/absence results were compared with the eDNA results (standard method at six reps) for freshwater fish for the same sites. An additional three sites were also added to the analysis giving a total of six electric fishing sites (Table B1) to compare the eDNA data with reach level SoE (State of the Environment) sampling. Two of these sites were not sampled on the day of eDNA sample collection. The Waingongoro Stream (Hawke's Bay, SoE program) was electric fished 23 days prior to eDNA sampling and comes with an additional two previous samplings (in 2019 and 2020). The Manganui River at 410_8 (Waikato Region, random site) was not fished the same season as the high replicate eDNA samples were taken (December 2020) and was last fished in December 2019 and comes with an additional two electric fishing samplings (2016 and 2013 both in December) but species captured at this site have remained relatively consistent throughout. The Paparahia Stream (Waikato Region, reference site) was not part of this high replicate study but has been included as six standard eDNA replicates were taken directly prior to annual SoE electric fishing in March 2021 and this site comes with an additional 11 years' worth of electric fishing data making it a valuable addition to the analysis.

Results

An average of 2.83 more fish species are detected with eDNA compared to electric fishing per site from the six sites assessed or 32.6% in total (59 species with eDNA and 42 electric fishing) and this reduces to 1.16 species per site for all year's (considering all species caught over the years sampled) or in total 15.4% (52 species electric fishing). The 'extra' species found with eDNA at these sites make sense ecologically and all extra species found with eDNA can be considered either rare/patchy or pelagic (highly mobile) or both. Two species were not detected with eDNA but caught via electric fishing at two sites (one species per site). One single juvenile brown trout (*Salmo trutta*) was caught at Manganui big 3 and two shortfin elvers were caught at Manganui small 3, both species at these sites can be categorised as rare. Shortfin eel signal at Manganui small 3 was however detected in the eighth replicate (71 reads, Table B1).

Discussion

In general, it is difficult to compare the results from eDNA sampling with electric fishing results in NZ as the distances sampled are not comparable and this is expanded upon in David et al. (2021). The six sites fished in this study are all part of regional SoE programmes with standardised sample lengths of 150m while the eDNA signal for most species present will extend well beyond this. How far beyond the signal extends will differ from species to species based on biomass, proximity to sampling location, shedding rates and environmental variables. From the six sites assessed 32.6% more fish species were found using eDNA. This result is similar to more recently obtained data (J. Smith unpub. data) from 44 stream sites (Waikato Region, stream order range 1 to 4) that were electric fished (single pass, 150m) directly after eDNA sampling (six replicates) which showed that 35.2% more species were detected with eDNA. Some of these 'extra' species found at these sites are rare/patchy or occupy different habitat niches to the area sampled by electric fishing. For example, galaxiid (koaro, banded kokopu and shortjaw kokopu) adult habitat is often found in steeper first and second order streams that flow into larger streams. When electric fishing these larger streams these species often are not found but the eDNA signal flowing into them can be strong if they are close by. This is especially true in late summer when any juvenile migrations upstream are over. These galaxiid species can often also be rare and patchy in certain streams. Other species tend to be found as sporadic individuals making them difficult to catch via electric fishing at some locations when only 150m of stream is fished, for example bluegill bullies, giant bullies, and in some cases common bullies. Trout found at these sites are also sporadic, highly mobile, and mainly juvenile. Single pass electric fishing in NZ catches on average 51% of a given fish population (Jowett and Richardson, 1996) and less again according to Graynoth et al. (2012) when night sampling is not included. Thus, it is possible some of these rare and patchy species may be missed by electric fishing altogether especially small bodied benthic bullies. For these rare and patchy species eDNA sampling has a real advantage in detection and in the narrowing down of their locations compared to electric fishing especially when considering the distance/area advantage eDNA has. However, when the biomass of a rare species is very low eDNA metabarcoding may not detect its presence as seen at two sites where electric fishing detected two rare species (at each these locations) but eDNA did not, at six replicates. This was also the case when sampling the Paparahia stream (Waikato Region, March 2020) when two bluegill bullies were caught electric fishing and not detected with eDNA, however this was only with three replicates taken and an average of 183 ml of water filtered per replicate due to a discoloured stream. (David et al, 2021). More recent data (J.Smith unpub. data) found that 5 species or 2% of the total species were not detected via eDNA metabarcoding from a total of 44 electric fished sites. Again, as with other sampling events these species detected via electric fishing and not by eDNA metabarcoding were rare with a low biomass.

When trying to detect or locate very rare or newly invasive species more than six replicates may be required as several rare/patchy species only occurred in the 7th or 8th replicate of this study. All species present in all six replicates at these six sites are consistently found in the 150m electric fishing reaches and most would be considered abundant to common with some of the more mobile pelagic species (trout, inanga, and common smelt) occasional. These similarities between fish species abundance via electric fishing and the species hits and read counts with eDNA is discussed in a NZ context in more detail in David et al. (2021), but in short more work in needed in this area to validate fish abundance determination via eDNA.

Table B-1. SoE freshwater fish electric fishing sites and eDNA comparison, six standard reps. Note: The first three sites with eDNA taken directly before electric fishing (Same day). Waingongoro (*) with electric fishing 23 days prior to eDNA sampling, Paparahia Stream (**) not part of high rep study but with six reps prior to electric fishing, Manganui 410_8 (***) last fished in 2019. 'Number of samplings' indicates the number of times each site has been electric fished and 'average all years' is the average number of species caught over these samplings. Each rep at these sites is 1000mls, apart from Komakorau (*) at 160 to 200mls per rep due to the turbid nature of this stream.

		eDNA (six replicates)			Electric fishing (150 m single pass)				
Site	Species	Hits	Average reads	Reads St Dev	Same day	Average all years	Stdev all years	Number of samplings	Notes
Manganui Big 3 Reference site Waikato Regional Council 601240	Redfin bully	6	1396.17	675.91	34.00	15.60	15.60	12 (2010-2021)	
	Longfin eel	6	1239.00	519.48	16.00	24.16	7.21		
	Shortfin eel	4	134.50	126.64	4.00	8.41	7.91		
	Koaro	1	90.67	150.00	0.00	0.00	0.00		
	Rainbow trout	2	32.33	61.71	0.00	0.50	1.17		
	Shortjaw kokopu	2	31.33	48.61	0.00	0.16	0.58		
	Pouched lamprey	2	10.83	22.84	16.00	5.00	7.20		
	Bluegill bully	0	0.00	0.00	0.00	0.08	0.29		Present in one boosted rep (reads = 193)
	Brown trout	0	0.00	0.00	1.25	1.25	2.05		
Manganui small 3	Longfin eel	6	1773.67	728.43	7.00	12.16	4.62	12 (2010-2021)	
	Redfin bully	6	1089.83	870.21	6.00	15.16	8.89		
Reference site	Koaro	6	604.17	259.58	4.00	5.58	2.42		

Waikato Regional Council	Brown trout	1	41.67	102.06	0.00	2.30	3.65		
601239	Shortjaw kokopu	1	11.83	28.99	0.00	0.66	0.77		
	Shortfin eel	0	0.00	0.00	2.00	0.66	0.88		Present in 8th rep (read = 71, standard)
Komakorau Stream	Koi carp	6	906.50	1736.00	2.00	1.25	0.95	4 (2012, 2015, 2018, 2021)	
	Mosquitofish	6	614.67	1199.57	300.00	202.00	143.43		
Random site	Shortfin eel	6	522.00	1018.54	61.00	76.00	11.86		
Waikato Regional Council	Longfin eel	6	207.00	398.84	5.00	2.75	20.60		
601050	Common smelt	6	186.50	440.31	0.00	10.25	10.65		
	Giant kokopu	3	62.17	145.75	0.00	0.50	0.58		
	Inanga	2	16.83	45.81	2.00	4.50	4.35		
	Rudd	2	5.17	13.89	0.00	1.50	1.73		
	Crans bully	0	0.00	0.00	0.00	0.75	1.50		
	Speckled longfin eel	0	0.00	0.00	0.00	0.00	0.00		Present in one boosted rep (reads = 126)
Waingongoro Stream*	Redfin bully	6	1393.67	565.48	149.00	120.33	25.01	3 (2019 to 2021)	
	Longfin eel	6	708.83	305.79	14.00	16.00	11.14		
Hawke's Bay Regional Council	Koaro	6	230.33	141.86	1.00	2.00	0.58		
601286	Shortfin eel	4	83.00	75.55	72.00	26.30	39.70		

	Torrentfish	4	64.67	78.81	2.00	6.00	0.00		
	Inanga	6	60.33	37.75	6.00	14.00	4.16		
	Bluegill bully	3	43.00	87.22	0.00	0.00	0.00		
	Giant bully	2	17.00	26.94	0.00	0.00	0.00		
	Banded kokopu	0	0.00	0.00	0.00	0.00	0.00		Present in 7th rep (reads = 101)
	Common bully	0	0.00	0.00	0.00	0.66	1.15		
Paparahia Stream**	Longfin eel	6	2656.83	520.38	22.00	33.00	11.74	12 (2011 to 2022)	
	Redfin bully	6	2099.83	503.86	232	289.66	109.84		
Reference site	Shortfin eel	5	139.66	116.54	12.00	14.83	5.64		
Waikato Regional Council	Inanga	3	94.66	144.25	6.00	4.83	6.07		
	Torrentfish	4	75.33	129.67	0.00	2.33	2.57		
	Banded kokopu	6	46.50	192.17	6.00	4.08	3.55		
	Pouched lamprey	2	37.33	65.02	21.00	3.25	5.59		
	Koaro	3	21.16	31.30	0.00	0.00	1.00		
	Giant kokopu	1	10.66	26.12	3.00	2.00	1.20		
	Common bully	1	2.33	5.71	30.00	6.25	13.01		
	Bluegill bully	0	0.00	0.00	0.00	1.83	1.27		
Manganui 410_8***	Longfin eel	6	2681.00	230.90	420.00	231.67	170.45	3 (2013, 2016, 2019)	
	Redfin bully	6	1190.33	153.44	239.00	315.00	252.72		
	Shortfin eel	6	446.50	88.82	305.00	214.67	83.26		
Random site	Inanga	6	387.33	187.16	22.00	105.67	78.23		

Waikato Regional Council	Torrentfish	6	331.00	220.24	33.00	23.00	11.79		
601241	Rainbow trout	6	247.50	120.63	3.00	8.00	10.44		
	Pouched lamprey	4	67.50	69.09	7.00	9.00	4.36		
	Brown trout	4	33.50	35.04	0.00	1.00	1.00		
	Giant bully	1	24.50	60.01	0.00	0.00	0.00		
	Koaro	1	6.50	15.92	0.00	0.00	0.00		
	Bluegill bully	1	5.83	14.29	0.00	0.00	0.00		
	Common or Crans bully	0	0.00	0.00	0.00	0.33	0.58		Present in one boosted rep (reds = 29)
	Common smelt	0	0.00	0.00	0.00	1.00	1.73		

Appendix C

eDNA detection of mammals and birds

Results

Mammals and birds represent 2.0% and 3.2% of the total reads analysed in this study respectively. Mammals including 'human' reads totalled 7.3% this 5.3% of 'human' derived reads were excluded from the analysis. In total six assays were used to detect mammals, and birds. Primers used for mammals and birds are presented in Table C1.

Table C-1. eDNA primers used for mammals and birds.

Mammals	Birds
RV	RV
LV	LV
WV	WV
ZV	DG
CI	ZV
DG	CI

Accumulation plots were prepared for mammals and birds comparing the two methods (standard and boosted, Figure C1 (A and B). For terrestrial mammals and birds, 10 and 13 replicates were required to attain 91.4 and 91.6 % richness, respectively. There was no significant difference between the methods for mammals and birds at 6 or 8 replicates other than for mammals at 8 eight replicates (ANOVA, df = 1, F = 5.24, n = 816, *p* = 0.02) where the standard method achieved enhanced species detections. As with fish and NEMS (MCI) taxa the species proportional richness range for mammals and birds generally improved with each replicate sample. However, both mammals and birds have a much lower mean species richness than fish and NEMS taxa.

For mammals, when compared to fish and NEMS taxa a more protracted accumulation plot was evident starting at a lower base (richness for the 1st replicate), which slowly continued increasing without flattening. Birds, starting lower still (richness for 1st replicate) remains on a steeper more linear trajectory to that of mammals. Overall, no significant difference was found (ANOVA, df = 1, F = 0.08, n = 3264, *p* = 0.769) between the two sampling methods (standard and boosted) therefore each replicate taken can effectively be treated as a standard replicate giving a total of 16 replicates per site. Figure C1(C) highlights the difference between groups and number of replicates needed to reach a certain group proportional richness with all 16 replicates combined. To reach 90% richness 10 replicates are needed for mammals and 13 for birds. Variation at the 95th percentile for mammals and birds (Figure C1, A and B) reduces with increased replication. Variation for both mammals and birds start high at one replicate (77.1 and 90.0%) and reduces modestly by 27.1 and 23.4% respectively over six replicates but remains high compared to fish and MCI taxa (main text). Table C2 summarises mean proportional richness between mammals and birds as a whole and by stream order and elevation. Detection of birds improves in larger lowland streams. The replication difference for each group (species occurrence) over all 16 replicates is summarised at the end of Table C2.

Table C-2. Mean species/taxa proportional richness (%) by stream order, elevation, and replication difference for fish, NEMS taxa, mammals and birds at one and six replicates (standard method) and by stream order and elevation. The last two columns are number of species per replicate (16 replicates) with range (minimum to maximum) and average.

Group	Standard		Stream order				Elevation				Number of species / rep	
	n=51		1-3, n-23		4-8, n=28		>100m, n=13		<100m, n=38		n=16	
	1 rep	6 reps	1 rep	6 reps	1 rep	6 reps	1 rep	6 reps	1 rep	6 reps	min-max	Av
Fish	64.0	89.5	65.3	90.6	64.1	89.5	70.4	91.6	61.9	88.8	0-8	3.2
NEMS invertebrates	57.0	86.0	54.8	87.1	59.5	85.6	57.0	88.6	57.5	85.1	3-17	9.9
Mammals	39.5	78.0	37.6	80.3	41.2	76.0	41.5	80.1	38.8	77.3	1-9	4.1
Birds	32.4	64.7	31.5	60.3	33.2	68.4	23.4	60.1	35.2	66.3	0-13	5.3

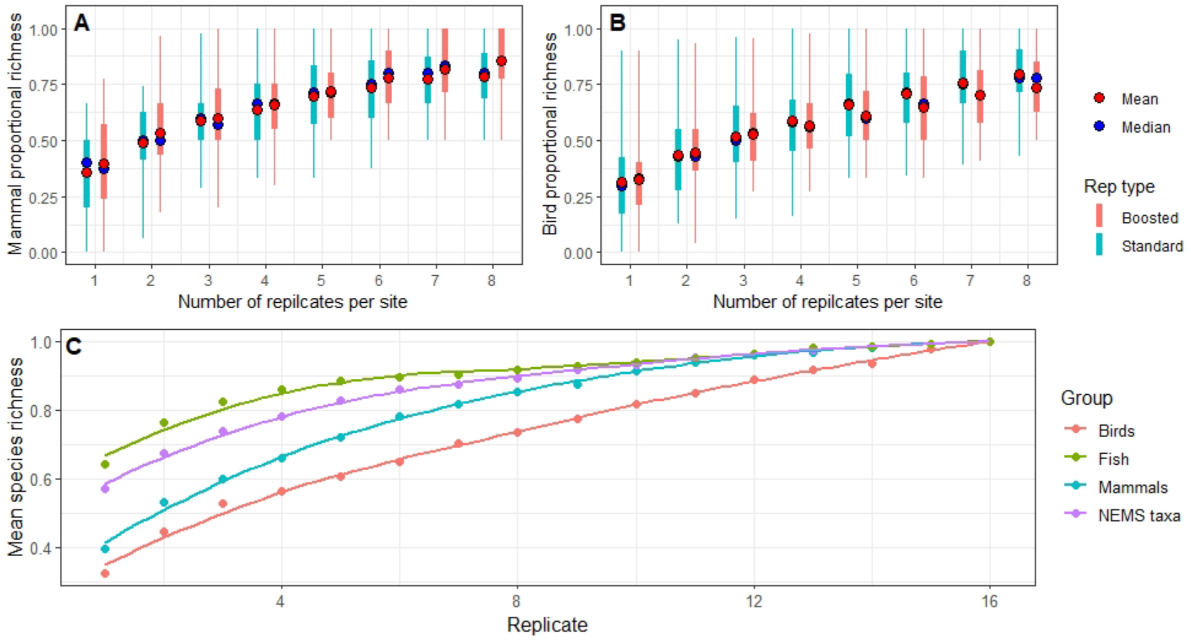


Figure C-1. Proportion of taxa richness detected by eDNA sampling for (A) mammals and (B) birds with standard versus boosted method. Box and whiskers are 50th and 95th percentiles. Red dot = mean, blue dot = median. Proportion of taxa richness for 16 replicates=1. Plot C mean species richness for birds, fish, mammals and NEMS taxa across all 16 replicates.

Mammals

Twenty-three of the 33 terrestrial mammals (excluding humans and zoo species) in NZ were detected in this study. Stocked farm animals were the most detected mammals, reflecting the fact that 47 % of sites were dominated by pastoral land use (Table A1). Human (*Homo sapiens*) eDNA was present at all 51 sites and cattle eDNA (*Bos taurus*) was present at 46 sites and dominated the reads for mammals (Table C3). The highest mammal reads occurred at site 16 (Table A1, Figure 3) which was dominated by farmed cattle that have open access to the river. At this site, cattle had the highest reads of any species not just mammals. Mammal diversity ranged from 3 to 13 species per site (Table C2) and the site with the highest mammal diversity was the Tangahoe River (site 22, Table A1, Figure 3) in Taranaki (High Pastoral dominant land use) with a mix of wild and farmed mammals. Endemic NZ mammals are restricted to two species of bats (O'Donnell et al. 2017), the rarest of these being the threatened – "Nationally Critical (2017)" long-tailed bat (*Chalinolobus tuberculatus*) whose eDNA was detected at one site. Several mammalian predators were detected at various sites in this study: Norway rat (*Rattus norvegicus*) at 27 sites, black rat (*Rattus rattus*) at 34 sites, the common brush-tailed possum (*Trichosurus vulpecula*) at 38 sites and stoats and ferrets at 11 and 1 sites, respectively

(Table C3). On average across all 16 replicates, mammal species variation was 4.1 and ranged from 1 to 9 species at a site (Table C2).

Birds

There are 116 extant land and freshwater birds occurring on mainland (North and South Islands) New Zealand, excluding rare migrants and vagrants. Eighty-one of these are native, and 35 are introduced and naturalised (Robertson et al., 2021). Forty-eight bird species are present in the eDNA results (25 native and 23 introduced) two of which (native) can be classified as marine/coastal species (Southern black-backed gull (*Larus dominicanus*) and the silver gull (*Chroicocephalus novaehollandiae*)) but can often be found inland. The introduced Mallard duck (*Anas platyrhynchos*) generated the most eDNA sequence reads and was detected at 50 sites accounting for 64.5% of the total bird reads. The two sites with the highest number of bird reads occurred in the Nelson City Council area (33, 37, Table A1, Figure 3) with 7 and 12 bird species but 98% and 69.5% of the reads respectively for birds were from the mallard duck. Mallard ducks at these two sites had the highest reads of any species not just birds. At these two sites dominant land cover were "Native Vegetation" and "Forestry" respectively, and the lower sections of these streams above the sample point could be classified as urban and urban lifestyle, so possibly popular spots to feed the local ducks. Bird diversity ranged from 2 to 21 species. The highest bird diversity site was the Tangahoe River (site 22, Table A1, Figure 3) which was dominated by duck species (Mallard, Paradise, Shelduck) sequence reads. Of the 21 bird species occurring at this site, 8 were native. Two "Threatened" and six "At Risk" species (Robertson et al. 2021) occurred in the eDNA results (Table C4). The two threatened species are the whio or blue duck (*Hymenolaimus alacorhynchos*) which was found at three sites and the black fronted tern (*Chlidonias albostriatus*) was present at one site (Table C4). Bird diversity over the 51 sites ranged from 2 to 21 species, replication difference over 16 replicates was an average of 5.3 and ranged from 0 to 13 bird species. Proportional richness at 6 replicates was significantly higher (8.1%) for birds in larger water bodies >4th order (ANOVA, df = 1, F = 6.89, n = 51, p = 0.011, Table C2).

Other species of interest

Other species worth noting included "At Risk" species; Hochstetter's frog (*Leiopelma hochstetteri*) found at one site (site 10, Table A1), the glossy brown skink (*Oligosoma zelandicum*) at one site (site 34) and the copper skink (*Oligosoma aeneum*) at one site (site 31). The introduced Plague skink (*Lampropholis delicata*) was found at three sites, one of three species of native freshwater mussels *(Echyridella menziesii*) occurred at four sites and the introduced freshwater jellyfish (*Craspedacusta sowerbii*) occurred at 35 sites. Several pest plant species have been identified including alligator weed (*Alternanthera philoxeroides*) which occurred at 3 sites (3664-National-Pest-Plant-Accord-manual-Reprinted-in-February-2020-minor-amendments-only (mpi.govt.nz).

Discussion

Aquatic versus terrestrial species detection

Detecting an organism's eDNA depends on the concentration and dispersion of its DNA. Streams are a sink for eDNA signals, which are carried downstream. For fish and aquatic invertebrates, continuous water contact (respiration, feeding, excretion, reproduction) increases their signal strength in eDNA with higher read counts. In contrast, terrestrial species contact stream water only occasionally, and often by chance (drinking, crossing, defecation). As such, their detection in stream water is presumably reliant on many factors that vary from site to site such as access, habitat, gradient, geology, catchment size, rainfall (Staley et al. 2018, Villacorta-Rath et al. 2023), population size and behaviour (Lyet et al. 2021, Yasashimoto et al. 2021). An example of this difference can be seen with two plant species, hornwort (*Ceratophyllum demersum*) a submerged aquatic species, and watercress (*Nasturtium officinale*) a semi-aquatic species which together accounted for just over a quarter of the total plant reads from 178 plant species, the majority being terrestrial. Work by Harper et al. (2019) highlights a similar pattern between terrestrial mammals and semi-aquatic mammals with semi aquatic mammals giving a much greater signal than the terrestrial mammals from samples taken in rivers. This is also seen in the results of this study where semi aquatic birds (mainly ducks) dominate the reads at some sites above all other species including instream aquatic species. In contrast, terrestrial species were seen to dominate the read counts in other sites. This is an example of "species bias", where a certain species can dominate the read counts due to their proximity to the sampling site in terms of distance and their link to the water course i.e., cattle standing in the stream while defecating. These examples highlight the need in having a good understanding of the sites sampled when reviewing results as a community signal inferred from eDNA can be very site specific (Cantera et al. 2019).

Differences in biomass and various factors influencing shedding rates (habitat, physiology, behaviour) can also account for differences between mammals and birds with slightly more bird species per site. But as with fish and invertebrates, there are some major differences in biomass and shedding rates between mammals and birds that will influence their detection and number of sequenced read counts. Ryan et al. (2022) showed larger vertebrates were more likely to be detected with eDNA compared to small vertebrates. The ability to detect terrestrial species will also be site specific (Lyet et al. 2021). For example, it is logical that a tree lined stream is more likely to provide fresh bird eDNA via excretion compared to a stream without riparian cover for tree dependant bird species. Or, as seen in these results, semi aquatic bird species in larger water bodies (stream order >4) can dominate the total eDNA reads of all taxa. This can be explained as a function of a greater water surface area at these bigger sites enabling a greater volume of bird DNA to be detected, especially for wetland and water birds. Just as the eDNA signal varies between streams for aquatic species based on many variables, it also varies for terrestrial species not only in a stream context but also in a terrestrial context (Sales et al. 2020, Lyet et al. 2021).

Bycatch

While recent development of eDNA monitoring in NZ freshwater streams has focused on identifying fish diversity, (David et al. 2021) and in assessing the suitability of eDNA to be used to assess stream health based on Macroinvertebrate Community Index (MCI), (Suren et al in prep), eDNA metabarcoding can also generate valuable "bycatch data" that can be used in a variety of ways. For example, data can be used for conservation (Barnes and Turner 2016, Beng and Corlett 2020), biosecurity objectives (Pochon et al. 2017, Macher et al. 2021) and to assess overall ecosystem health of sites in general by examining all organisms (Cordier et al. 2019, Wilkinson et al. submitted, 2023). Cross agency planning of eDNA monitoring/sampling may lead to mutual benefits in both data outcomes and shared costs. Early identification of an invasive species can prevent ecological damage and the (usually significant) costs involved in their subsequent management and/or removal (Vander Zanden et al. 2010). Caution, however, should be taken particularly for terrestrial species with respect to false negatives and positives as interaction with water can be in certain situations fleeting (Barnes and Turner 2016, Macher et al, 2021). Any bycatch data obtained should initially be used as a screening tool, more sampling would be needed to confirm presence and more sensitive DNA tools could also be used (qPCR, ddPCR).

Introductions of mammalian predators, particularly rats, possums, and mustelids in the late 18th century and first half of the 19th century in NZ either on purpose or by accident has caused significant ecological damage to native flora and fauna, resulting in extensive habitat and species loss (Wodzicki and Wright, 1984). Fifty-seven bird species alone have become extinct in NZ, and many species of land birds are now 'Threatened' or 'At Risk' as a result (Robertson et al. 2021). Conservation of the remaining native species and control of mammalian predators is an ongoing and expensive task. For example, the 2022 NZ Department of Conservation budget provided 91 million dollars (NZD) for mammalian predator control and a further 30 million for the control of deer and goats (Department of Conservation, 2022), while another agency (OSPRI) spent an additional 35 million dollars (NZD) in 2020-2021 for possum control as part of their TB Free campaign. Given the ability of eDNA in detecting mammals within catchments, there is potential to use eDNA as another tool to assess the relative efficiency of mammalian control operations by observing whether such operations end up with a significant reduction in mammalian eDNA signals in target catchments. Similarly, eDNA could be used as a monitoring tool to detect when mammalian populations have reached levels high enough to be deemed unacceptable, triggering another control operation. Thus, the use of eDNA to help detect presence/absence information on pest species could be another valuable tool to assist managers in the fields of conservation and biosecurity. However, it should be noted that in this study no sensitivity testing was done for mammals or birds and 100% of species richness is only based on eDNA detection.

Aquatic and terrestrial plants accounted for 15.90% of the total reads with 178 species present in this study and like other "by catch" information highlights any species of interest for conservation and biosecurity. For example, several plant pest species have been identified including alligator weed (*Alternanthera philoxeroides*) which occurred at 3 sites (3664-National-Pest-Plant-Accord-manual-Reprinted-in-February-2020-minor-amendments-only (mpi.govt.nz)). eDNA is also currently being used to detect the invasive algae *Didymosphenia geminata* in North Island streams following its incursion and spread throughout the South Island (Cary et al. 2006).

Replication optimisation

Macher et al. (2021) assessed at by-catch species, mammals, and birds which showed increases of 68.9% and 77.3% respectively over their 18 replicates. This is comparable to our observations of 60.5% and 68.0% for mammals and birds respectively after 16 replicates. Macher et al. (2021) recommended 8 to 10 replicates for fish fauna, with no substantial richness increase above these replicate numbers and additional increases beyond this for mammals and birds. In accordance with our study Macher et al. (2021) also exhibited similar starting points for species richness after one replicate with fish was well above that of birds and mammals, thus making increases for fish with additional replicates above six replicates less dramatic than terrestrial species. Ten replicates are needed to reach 90% richness (91.4%) for mammals and 13 replicates who be needed for birds (91.6%). Both these terrestrial groups have high degrees of variation between replicates, this reduces significantly after 10 replicates for mammals, variation reduces after 4 replicates for birds but remains higher than that of mammals throughout. For terrestrial taxa the number and proportion of species missed is difficult to ascertain and ground truthing would need to be established to have confidence in results.

Table C-3. Mammal species found in the eDNA samples ranked in order of highest combined reads. All mammals apart from the NZ long-tailed bat (O'Donnell, 2001) are introduced.

Species name	Common name	Reads (standard)	Reads (boosted)	Reads (combined)	Site occurrence	Average reads per site	Status
omo sapiens	Human	1291670	1274969	2566639	51	50326	human
Bos taurus	Cattle	205648	231093	436741	46	9494	domestic/wild pest
Cervus elaphus	red deer	55381	72719	128100	31	4132	domestic/wild pest
Rattus norvegicus	Norway rat	26078	31269	57347	27	2124	pest
Trichosurus vulpecula	common brush-tailed possum	25804	25883	51687	38	1360	pest
Sus scrofa	pig	16196	19107	35303	48	735	domestic/wild pest
Canis lupus	dog	19871	15277	35148	7	5021	domestic/wild pest
Rattus rattus	black rat	14146	16172	30318	34	892	pest
Rusa unicolor	sambar deer	13696	16383	30079	1	30079	domestic/wild pest
Ovis aries	sheep	7248	13702	20950	25	838	domestic/wild pest
Oryctolagus cuniculus	rabbit	3632	5626	9258	13	712	domestic/wild pest
Mus musculus	house mouse	3720	3436	7156	18	398	pest
Capra hircus	goat	1413	1855	3268	6	545	domestic/wild pest
Felis catus	cat	769	1189	1958	8	245	domestic/wild pest
Dama dama	fallow deer	990	895	1885	8	236	domestic/wild pest
Lepus europaeus	brown hare	1152	400	1552	6	259	pest
Mustela erminea	stoat	1231	200	1431	11	130	pest
Erinaceus europaeus	common hedgehog	720	368	1088	9	121	pest
Chalinolobus tuberculatus	NZ long-tailed bat	0	392	392	1	392	Native, threatened, Nationally Critical
Hemitragus jemlahicus	Himalayan tahr	0	165	165	2	83	pest
Equus caballus	horse	116	27	143	2	72	domestic/wild pest
Bos indicus	zebu cattle	36	0	36	1	36	domestic
Mustela putorius	European polecat/ferret	23	0	23	1	23	pest
Cervus nippon	sika deer	0	19	19	1	19	domestic/wild pest

Table C-4. Bird species found in the eDNA samples ranked in order of highest combined reads with conservation status (Robertson et al. 2021). *Introduced, **non-resident native.

Species name	Common name	Reads (standard)	Reads (boosted)	Reads (combined)	Site occurrence	Average reads per site	Conservation Status
Anas platyrhynchos*	Mallard duck*	296328	266935	558677	50	11174	
Porphyrio melanotus**	Pukeko/Australasian swamphen**	39810	40130	78146	27	2894	Not Threatened
Turdus merula*	Blackbird*	29059	23244	51249	35	1464	
Zosterops lateralis	Silvereye	17978	15874	33661	29	1161	Not Threatened
Tadorna variegata	Paradise Shelduck	15113	14451	29460	27	1091	Not Threatened
Tadorna ferruginea	Ruddy shelduck*	8619	7254	15708	20	785	
Phalacrocorax carbo	Black Shag	6573	6546	13010	9	1446	At Risk
Hemiphaga novaeseelandiae	Kereru	7924	3451	11339	16	709	Not Threatened
Turdus philomelos	Song thrush*	4852	4201	8987	22	409	
Larus dominicanus	Southern black-backed gull	4095	4890	8626	9	958	Not Threatened
Fringilla coelebs	Common chaffinch*	3781	1957	5738	24	239	
Prosthemadera novaeseelandiae	Tui	3809	1628	5437	14	388	Not Threatened
Sturnus vulgaris	Common starling*	1906	2933	4744	14	339	

Passer domesticus	House sparrow*	2083	2384	4440	14	317	
Anser anser	Greylag goose*	1576	2960	4344	10	434	
Microcarbo melanoleucos	Little shag	1672	2241	3913	7	559	At Risk
Branta canadensis	Canada goose*	2674	1143	3796	7	542	
Streptopelia chinensis	Spotted dove*	1903	1326	3153	3	1051	
Hirundo neoxena	Welcome swallow	939	2162	3071	12	256	Not Threatened
Rhipidura fuliginosa	Fantail	1573	896	2469	11	224	Not Threatened
Anthornis melanura	Bellbird	793	1026	1819	3	606	Not Threatened
Columba livia	Pigeon*	786	925	1711	10	171	
Hirundo tahitica	Pacific swallow*	388	1156	1544	8	193	
Prunella modularis	Dunnock*	195	1084	1279	4	320	
Hymenolaimus malacorhynchos	Whio/blue duck	116	1073	1189	3	396	Threatened
Anas chlorotis	Brown teal	648	528	1176	4	294	At Risk
Gerygone igata	Grey warbler	317	770	1087	4	272	Not Threatened
Ardea novaehollandiae	White-faced heron	553	334	865	8	108	Not Threatened
Chloris sinica	Greenfinch*	288	335	623	9	69	
Emberiza citrinella Linnaeus	Yellowhammer*	99	519	618	8	77	

Cygnus atratus	Black swan	312	263	575	7	82	Not Threatened
Sterna hirundo	Common tern	530	0	530	1	530	Vagrant
Phalacrocorax varius	Pied Shag	64	318	382	1	382	At Risk
Alauda arvensis	Eurasian skylark*	334	0	334	2	167	
Acridotheres tristis	Myna*	75	227	302	7	43	
Chroicocephalus novaehollandiae	Silver gull	215	103	264	2	132	At Risk
Carduelis carduelis	Goldfinch*	72	183	255	3	85	
Gymnorhina tibicen	Magpie*	100	85	185	5	37	
Anas gracilis	Grey teal	102	60	162	4	41	Not Threatened
Fulica atra	Eurasian coot	36	124	160	1	160	At Risk
Anser cygnoides	Swan goose*	0	120	120	1	120	
Phasianus colchicus	Pheasant*	0	83	83	1	83	
Circus approximans	Swamp harrier	66	0	66	1	66	Not Threatened
Petroica macrocephala	Tomtit	55	0	55	2	28	Not Threatened
Cairina moschata	Muscovy duck*	15	27	42	3	14	
Platycercus eximius	Eastern rosella*	0	26	26	1	26	

Chlidonias albostriatus	Black fronted tern	16	0	16	1	16	Threatened
Anas clypeata	Northern shoveler	12	0	12	1	12	Vagrant
Species name	Common name	Reads (standard)	Reads (boosted)	Reads (combined)	Site occurrence	Average reads per site	Conservation Status

Appendix D

Table D-1. Manganui River 2021 sampling. Locations and timing of samplings with water quality measurements (Pre, Post and after fish removal). Species: in = inanga, tor = torrentfish. 2U = upper tributary and 2L = lower tributary. Sampling after fish out *denotes eDNA samples analysed.

Site name	GPS location (WGS-84)		Pre sampling 06/04/21	Time fish in 07/04/21	Post sampling 08/04/21	Hours since fish in	Sampling after fish out	Species	Temperature (°C)		DO (mg/L)		Conductivity (µS/cm)	
	Lat	Long							Pre	Post	Pre	Post	Pre	Post
Cage	174.707731E	-38.519165S		10:10				in, tor						
.150 km	174.707683E	-38.520049S	15:31	10:10	11:40	25hr 30m	10:58*	in, tor	16.2	14.3	9.32	9.9	90.1	89.9
500m	174.708666E	-38.522820S	14:57	10:10	11:06	24hr 56m	10:42	in, tor	16.5	14	9.38	10.05	90.4	90.3
1km	174.709993E	-38.525602S	14:23	10:10	10:22	24hr 12m	10:06	in, tor	16.5	13.8	9.19	9.91	90.4	90.1
2km	174.712071E	-38.530979S	13:40	10:10	09:43	23hr 33m	09:10	in, tor	16.5	13.4	9.36	9.93	98.9	90.5
3km	174.712188E	-38.535933S	12:20	10:10	08:59	22hr 49m	08:45	in, tor	16.5	13.2	9.21	9.88	90.3	91.1
4km	174.707513E	-38.541994S	11:50	10:10	08:29	22hr 19m	08:15	in, tor	17.2	13.3	9.4	10.04	89.9	91.3
5km	174.701650E	-38.546359S	10:20	10:10	07:57	21hr 39m	07:46	in, tor	16.6	13.6	9.18	9.47	89.9	91.6
9.5 km	174.691393E	-38.578723S	09:41	10:10	07:18	21hr 08m	07:16	in, tor	16.8	14.2	9.61	9.16	97	98.6
2U	174.712397E	-38.535056S	12:48						14.7		9.25		98.9	
2L	174.707517E	-38.543120S	11:21						15.7		9.56		101.2	

Table D-2. Paparahia Stream 2021 sampling. Locations, distance, and timing of sampling with water quality measurements.

Site name	GPS location WGS-84		Distance (m)	Sampling 22-23/03/21	Temperature (°C)	DO (mg/L)	Conductivity (µS/cm)
Waterfall	174.657427E	-38.514373S		23rd, 11:51	14.5	9.52	153.2
			470				
SOE	174.654343E	-38.517453S		22nd, 14:45	14.7	9.48	154.1
			1600				
Mainstem	174.645119E	-38.527928S		22nd, 13:38	16.9	10.4	168.9
			1700 (total 3770)				
Coast	174.635048E	-38.531494S		22nd, 12:30	15.9	9.65	1360
Trib. 1	174.653666E	-38.5179409S		22nd, 19:01	14	9.68	186.1
Trib. 2	174.6452416E	-38.5282423S		23rd, 09:06			

Table D-3. Manganui River Tributary sampling. Locations, distance, and timing of samplings with water quality measurements (Pre and Post). Species: bk = banded kokopu, cb = common bully. Ds = downstream, us = upstream and conflu. = confluence.

Site code	Site	GPS location (WGS-84)		Distance (m)	Pre sampling				Post sampling					
		Lat	Long		28/03/22 passives in	29/03/22 passives out	time in (hr.min)	28/03/22 syringe	30/03/22 Fish in	30/03/22 passives in	31/03/22 passives out	time in (hr.min)	31/03/22 syringe	Hours since fish in (hr.min)
Cage	Caged site mainstem	174.707731E	-38.519165S						11:45					
150 m	upper mainstem	174.707683E	-38.520049S	.150 km to 1U = 2850	17:30	14:30	21.00	17:30		14:25	12:00	21.35	11:55	25.10
1U	upper trib. confluence u/s	174.711994E	-38.534990S	1U to 3U = 160	16:55	14:00	21.05	16:55		14:35	12:05	21.30	10:01	23.16
3 U (3 km)	upper trib. confluence d/s	174.712188E	-38.535933S		16:37	13:46	21.09	16:35		14:43	12:10	21.27	09:20	22.35
410_11	Caged site upper trib.	174.710646E	-38.516851S	cage to 2U = 400					12:05					
2U	upper trib. d/s	174.712397E	-38.535056S		19:40	17:00	21.20	19:35		14:45	12:15	21.30	10:28	23.43
410_11	upper trib. stream	174.712985E	-38.533263S	cage to 410_11 = 150	19:05	16:30	21.25	18:50		14:40	12:20	21.40	11:01	24.16
1L (4 km)	lower trib. confluence u/s	174.707251E	-38.543034S	3U to 1L = 1200	14:02	13:02	23.00	16:02		14:56	13:12	22.16	10:22	23.37
3L	lower trib. confluence d/s	174.706755E	-38.533112S	1L to 3L = 160	15:43	12:43	21.00	15:35		15:03	12:55	21.52	11:17	24.32
410_9	Caged site lower trib.	174.714071E	-38.543897S	cage to 2L = 400					10:48					
2L	lower trib. d/s	174.707517E	-38.543120S		19:40	16:40	21.00	19:40		15:40	13:00	21.20	09:45	23.00
410_9	lower trib. stream	174.709708E	-38.543088S	cage to 410_9 = 150	19:00	16:00	21.00	19:00		15:00	13:05	22.05	10:30	23.45
5 km	5 km	174.701650E	-38.546359S	3L to 5km = 760	15:07	12:07	21.00	15:00		16:15	13:55	21.40	08:36	21.51
9.5 km	9.5 km	174.691393E	-38.578723S		14:12	11:12	21.00	14:09		19:45	17:10	21.25	08:20	21.35
410_8	below gorge	174.683874E	-38.593510S	9.5 to 410_8 = 2400	12:40	09:40	21.00	12:40						

Table D-4. SOE site parameters.

Parameter	Site				
	410_8	410_9	410_10	410_11	3009_1
Number of samplings	4	14	14	14	14
Year range	2013-2022	2010-2023	2010-2023	2010-2023	2010-2023
Annual sampling	3 yearly	yes	yes	yes	yes
Reach length (m)	150	150	150	150	150
Average temperature (deg. C)	17.6	14.16	14.72	13.37	13.45
Average dissolved oxygen (mg/L)	9.51	10.02	9.8	9.31	9.41
Average oxygen (%)	100.83	99	98.4	91.08	91.29
Average conductivity (µS/cm)	99.55	92.79	84.85	87.53	140.4
Average gradient (degrees)	0.54	3.7	0.89	2.77	0.96
Average shade (%)	0.41	83.51	65.18	81.24	81.2
Average wetted width (m)	9.07	2.05	4.57	2.45	2.97
Average depth (m)	0.42	0.16	0.34	0.19	0.22
Sediment (qualitative habitat assessment, out of 20)	14.85	16.92	17	18.38	16.31
Substrate (average %)					
Bedrock	0	1.92	1.92	2.92	0.64
boulder	1.67	1.92	0.73	6.46	0.73
cobble	25.14	22.85	21.62	38.23	15.85
large gravel	56	52	61.08	37.62	44.54
small gravel	12.43	18.58	11.69	12	25.23
sand	3	4	3	2.08	9.31
silt	1.6	0.55	0.6	0.5	3.08
clay	2.5	0.18	0	0.2	1.1
Dominant landuse	Pasture	Native forest	Native forest	Native forest	Native forest
Average score per metric	0.55	0.7	0.74	0.7	0.63
Average MCI	115.88	133.08	139.4	133.6	125.8

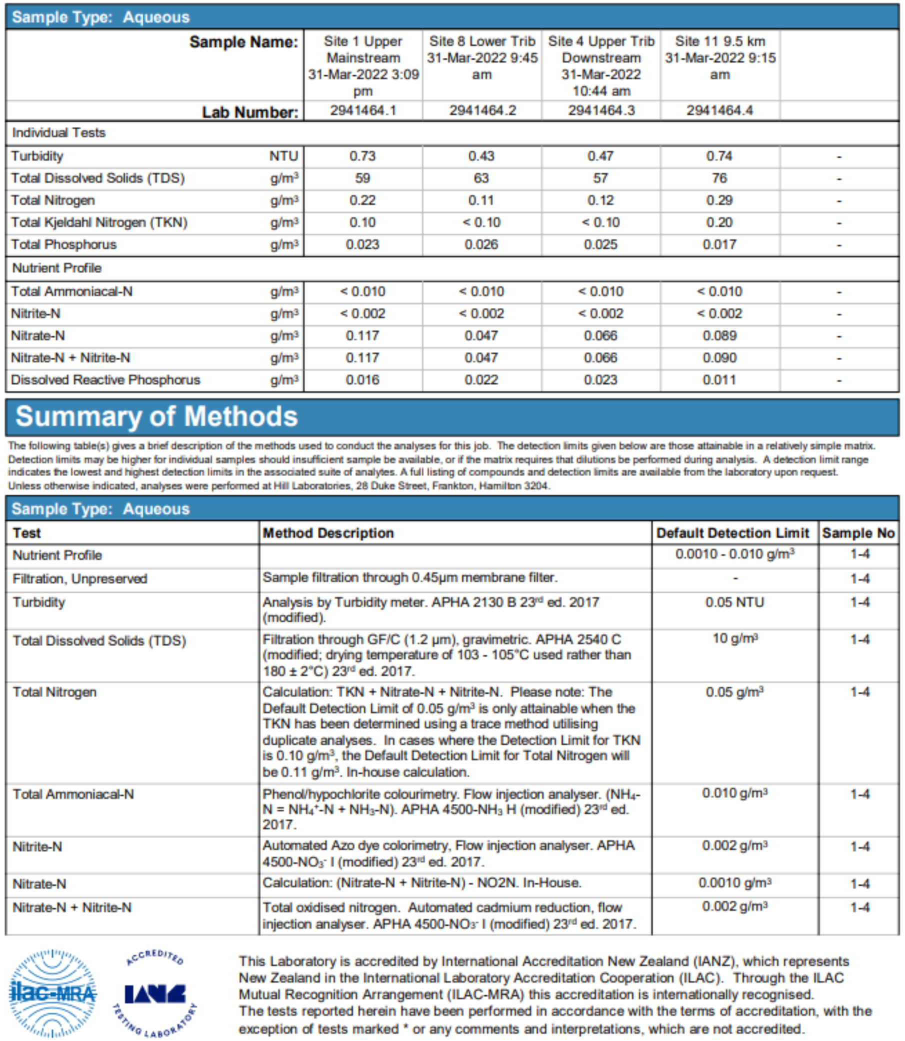
Table D-5. SOE site fish parameters.

		Average number					Average length (mm) + (min and max)					Biomass (g)				
Site	Species	All years	Dec-20	April-March 2021	Dec-21	April-March 2023	All years	Dec-20	April-March 2021	Dec-21	April-March 2023	All years	Dec-20	April-March 2021	Dec-21	April-March 2023
410_8	Brown trout	0.8					54.83 (75-99)					5.8				
	Common bully	0.3					80 (80-80)					1.5				
	Common smelt	1.0					72.83 (52-80)					1.3				
	Inanga	80.8					62.31 (44-102)			65.5 (47-79)		90.1				
	Koura	0.5														
	Lamprey	13.0					74.24 (44-102)			72.68 (61-89)		9.3				
	Longfin eel	230.0					161.43 (75-1180)			153.53 (75-720)		11360.0				
	Rainbow trout	6.0					56.15 (34-93)					10.7				
	Redfin bully	292.5					51.78 (23-116)			51.17 (39-116)		407.9				
	Shortfin eel	217.8					102.11 (75-415)			96.38 (76-134)		400.4				
	Torrentfish	18.3					86.81 (66-123)			90.75 (81-102)		144.1				
410_9	Banded kokopu	0.1	0.0		0.0		96 (96-96)					0.6	0.0		0.0	
	Brown trout	6.1	2.0		1.0		92.73 (59-195)	81.5(80-83)		76 (76-76)		73.1	0.0		3.5	
	Koaro	2.4	2.0		4.0		118.73 (50-171)	110.5(109-112)		135.5 (134-137)		36.1	37.3		118.8	
	Koura	29.2	30.0		28.0											
	Lamprey	0.1	0.0		0.0		95 (95-95)									

	Longfin eel	30.9	38.0		25.0		267.99 (100-960)	378.6 (111-826)		262.85 (100-940)		1533.2	1443.8		814.3	
	Rainbow trout	0.3	1.0		0.0		68.33 (63-80)					1.2	2.7		0.0	
	Redfin bully	25.4	47.0		14.0		78.1 (44-104)	79.3 (60-102)		74.39 (52-97)		134.8	242.1		75.8	
	Shortfin eel	2.4	2.0		5.0		172.77 (96-438)	321 (184-438)		162.5 (161-164)		9.3	7.5		64.8	
	Unidentified galaxid	0.1	0.0		0.0							0.1	0.0		0.0	
410_10	Bluegill bully	0.1	0.0		0.0							0.3	0.0		0.0	
	Brown trout	1.5	0.0		0.0		129.25 (60-253)	57 (57-57)				56.7	0.0		0.0	
	Koaro	0.0	0.0		0.0											
	Koura	45.0	55.0		84.0											
	Lamprey	5.2	16.0		17.0		67.69(33-96)	55.81 (33-90)		74.86 (65-90)		3.3	13.8		9.5	
	Longfin eel	23.0	32.0		32.0		249.91 (105-910)	249.88 (110-820)		263.88 (115-530)		3251.2	5145.3		5525.0	
	Rainbow trout	0.4	1.0		0.0		171 (98-284)					30.8	309.2		0.0	
	Redfin bully	30.5	31.0		25.0		77.70 (34-112)	78.18(34-108)		83.10 (60-112)		161.5	204.8		121.4	
	Shortfin eel	6.6	16.0		3.0		147.20 (96-262)	165.75 (122-225)		126.5 (108-145)		31.5	85.5		8.3	
	Shortjaw kokopu	0.2	0.0		0.0		148.87 (98-233)					28.0	0.0		0.0	
410_11	Brown trout	1.2	0.0		0.0		79.08 (68-86)					20.5	0.0		0.0	
	Koaro	4.9	4.0		5.0		102.01 (85-143)	98.25 (89-116)		99 (87-116)		50.7	35.7		51.2	
	Koura	15.8	13.0		22.0											
	Longfin eel	14.0	18.0		12.0		244.38 (103-498)	205.43 (116-311)		263.88 (115-530)		756.0	1126.1		916.0	
	Rainbow trout	0.2	0.0		0.0		83 (76-95)					1.5	0.0		0.0	

	Redfin bully	15.4	26.0		9.0		75.39 (29-94)	78 (66-87)		76.43 (50-93)		77.1	113.2		59.8	
	Shortfin eel	0.7	2.0		0.0		117.67 (104-249)	146.5 (122-171)		126.5 (108-145)		4.8	0.0		0.0	
	Shortjaw kokopu	0.6	0.0		0.0		162.5 (125-210)					46.4	0.0		0.0	
3009_1	Banded kokopu	3.7		6.0		2.0	128.67 (64-178)		129.17 (106-149)		159 (140-178)	100.1		141.4		91.0
	Bluegill bully	1.6		0.0		0.0	57.94 (42-64)					2.9		0.0		0.0
	Common bully	5.7		30.0		5.0	57.92 (33-79)		52.9 (41-68)		65.8 (33-79)	10.2		53.1		19.7
	Giant kokopu	1.9		3.0		1.0	234.19 (62-301)		263.33 (242-283)		249 (249-249)	432.3		774.9		211.3
	Inanga	4.4		6.0		0.0	85.39 (67-126)		81.83 (70-94)			15.9		20.6		
	Koura	11.1		11.0		31.0										
	Lamprey	4.9		21.0		27.0	75.54 (46-104)		69.05 (55-87)		90.59 (52-104)	4.5		13.1		28.2
	Longfin eel	30.4		22.0		23.0	175.48 (83-847)		178.27 (94-385)		214 (92-847)	1027.9		429.2		2860.2
	Redfin bully	271.0		232.0		159.0	47.81 (28-108)		51.62 (36-68)		49.32 (31-108)	318.5		328.8		241.8
	Shortfin eel	13.3		12.0		8.0	104.87 (73-188)		104.75 (80-131)		119 (91-188)	17.2		17.4		21.1
	Torrentfish	2.3		0.0		0.0	115.08 (103-140)					43.8		0.0		0.0
	Unidentified galaxid	0.1		0.0		0.0						0.0		0.0		0.0

Table D-6. Water quality testing and methods (Hill laboratories), 31 March 2022 at four sites (Upper mainstem 0.150 km, Lower tributary downstream (410_9), Upper tributary downstream (410_11) and 9.5 km).



Sample Type: Aqueous

Sample Name:		Site 1 Upper Mainstream 31-Mar-2022 3:09 pm	Site 8 Lower Trib 31-Mar-2022 9:45 am	Site 4 Upper Trib Downstream 31-Mar-2022 10:44 am	Site 11 9.5 km 31-Mar-2022 9:15 am	
Lab Number:		2941464.1	2941464.2	2941464.3	2941464.4	
Individual Tests						
Turbidity	NTU	0.73	0.43	0.47	0.74	-
Total Dissolved Solids (TDS)	g/m^3	59	63	57	76	-
Total Nitrogen	g/m^3	0.22	0.11	0.12	0.29	-
Total Kjeldahl Nitrogen (TKN)	g/m^3	0.10	< 0.10	< 0.10	0.20	-
Total Phosphorus	g/m^3	0.023	0.026	0.025	0.017	-
Nutrient Profile						
Total Ammoniacal-N	g/m^3	< 0.010	< 0.010	< 0.010	< 0.010	-
Nitrite-N	g/m^3	< 0.002	< 0.002	< 0.002	< 0.002	-
Nitrate-N	g/m^3	0.117	0.047	0.066	0.089	-
Nitrate-N + Nitrite-N	g/m^3	0.117	0.047	0.066	0.090	-
Dissolved Reactive Phosphorus	g/m^3	0.016	0.022	0.023	0.011	-

Summary of Methods

The following table(s) gives a brief description of the methods used to conduct the analyses for this job. The detection limits given below are those attainable in a relatively simple matrix. Detection limits may be higher for individual samples should insufficient sample be available, or if the matrix requires that dilutions be performed during analysis. A detection limit range indicates the lowest and highest detection limits in the associated suite of analytes. A full listing of compounds and detection limits are available from the laboratory upon request.
Unless otherwise indicated, analyses were performed at Hill Laboratories, 28 Duke Street, Frankton, Hamilton 3204.

Sample Type: Aqueous

Test	Method Description	Default Detection Limit	Sample No
Nutrient Profile		0.0010 - 0.010 g/m^3	1-4
Filtration, Unpreserved	Sample filtration through 0.45µm membrane filter.	-	1-4
Turbidity	Analysis by Turbidity meter. APHA 2130 B 23rd ed. 2017 (modified).	0.05 NTU	1-4
Total Dissolved Solids (TDS)	Filtration through GF/C (1.2 µm), gravimetric. APHA 2540 C (modified; drying temperature of 103 - 105°C used rather than 180 ± 2°C) 23rd ed. 2017.	10 g/m^3	1-4
Total Nitrogen	Calculation: TKN + Nitrate-N + Nitrite-N. Please note: The Default Detection Limit of 0.05 g/m^3 is only attainable when the TKN has been determined using a trace method utilising duplicate analyses. In cases where the Detection Limit for TKN is 0.10 g/m^3, the Default Detection Limit for Total Nitrogen will be 0.11 g/m^3. In-house calculation.	0.05 g/m^3	1-4
Total Ammoniacal-N	Phenol/hypochlorite colourimetry. Flow injection analyser. (NH_4-N = NH_4^+-N + NH_3-N). APHA 4500-NH_3 H (modified) 23rd ed. 2017.	0.010 g/m^3	1-4
Nitrite-N	Automated Azo dye colorimetry, Flow injection analyser. APHA 4500-NO_3^- I (modified) 23rd ed. 2017.	0.002 g/m^3	1-4
Nitrate-N	Calculation: (Nitrate-N + Nitrite-N) - NO2N. In-House.	0.0010 g/m^3	1-4
Nitrate-N + Nitrite-N	Total oxidised nitrogen. Automated cadmium reduction, flow injection analyser. APHA 4500-NO_3^- I (modified) 23rd ed. 2017.	0.002 g/m^3	1-4

This Laboratory is accredited by International Accreditation New Zealand (IANZ), which represents New Zealand in the International Laboratory Accreditation Cooperation (ILAC). Through the ILAC Mutual Recognition Arrangement (ILAC-MRA) this accreditation is internationally recognised.
The tests reported herein have been performed in accordance with the terms of accreditation, with the exception of tests marked * or any comments and interpretations, which are not accredited.

Table D-7. Negative control results from six random sites, post sampling - Upper tributary stream (410_11), 5 km, 9.5 km) and pre sampling – Upper mainstem (0.150 km), Upper tributary stream (410_11), Lower SOE site (410_8). *Denotes taxa only found in the negative control samples.

					Upper trib stream (410_11, Post	5km Post	9.5km Post	Upper mainstem (0.150 km) Pre	Upper trib stream (410_11) Pre	410_8 Pre
ScientificName	**Rank**	**TaxID**	**CommonName**	**Group**	**522179**	**522185**	**522184**	**522186**	**522176**	**522181**
Euteleosteomorpha	cohort	1489388		Fish	0	0	17	0	0	0
Oncorhynchus	genus	8016	Salmon/Trout	Fish	0	0	106	0	0	0
Anguilla dieffenbachii	species	61127	Longfin eel	Fish	1804	0	0	0	393	0
Oncorhynchus mykiss	species	8022	Rainbow trout	Fish	0	0	238	0	0	0
Cheimarrichthys fosteri	species	206139	Torrentfish	Fish	0	730	6	0	0	0
Cecidomyiidae	family	33406	Gall midges	Insects	557	0	0	0	0	0
Chironomidae	family	7149	Nonbiting midges	Insects	0	0	11	0	0	0
Drosophilidae	family	7214	Pomace flies	Insects	0	0	0	44*	0	0
Bedellia	genus	753281	Narrow-winged moth	Insects	0	38	0	0	0	0
Hemiptera	order	7524		Insects	0	0	225	0	10	0
Lepidoptera	order	7088	Butterflies and moths	Insects	0	0	130	0	0	0
Trichoptera	order	30263	Caddisflies	Insects	11	0	0	0	23	0
Hyadaphis passerinii	species	1425398	Aphid	Insects	0	0	72	0	0	0
Teleogryllus commodus	species	672150	Black field cricket	Insects	0	0	0	0	1366	0
Pterygota	subclass	7496	Winged insects	Insects	0	0	0	0	0	280*
Orthocladiinae	subfamily	43808		Insects	13	0	0	0	0	0
Aphidinae	subfamily	133076		Insects	0	10	0	0	0	0
Calliphorinae	subfamily	43912		Insects	0	0	7*	0	0	0
Hydropsychinae	subfamily	147297	Caddisflies	Insects	562	0	0	0	0	0
Artiodactyla	order	91561	Hoofed Animals	Mammals	0	0	107	0	0	0
Bos taurus	species	9913	Cattle; kau	Mammals	0	0	7237	0	161	0
Sus scrofa	species	9823	Pig	Mammals	0	50	2998	0	0	0
Canis lupus familiaris	subspecies	9615	Dog; pero	Mammals	0	0	30	0	0	0
Gastropoda	class	6448	Gastropods	Molluscs	18	0	0	0	0	0
Galba	genus	401861		Molluscs	0	0	0	0	30	0
Caenogastropoda	subclass	69555		Molluscs	11	0	0	0	0	0
Opisthokonta	clade	33154		Other	370	8	20	854	57	180
Embryophyta	clade	3193	Higher plants	Other	82	0	0	0	0	0
Actinopteri	class	186623		Other	11	0	0	0	0	0

Chromadorea	class	119089		Other	0	0	358	0	0	140
Arachnida	class	6854	Arachnids	Other	0	10	136	0	0	0
Insecta	class	50557	Insects	Other	560	180	0	0	67	102
Kinetoplastea	class	5653	Kinetoplastids	Other	0	0	0	0	13	0
Rhynchomonadidae	family	2728145		Other	0	0	0	0	0	56
Leptophryidae	family	1920164		Other	0	0	0	0	235	0
Euglyphidae	family	39712		Other	6	0	0	0	55	0
Neobodo	genus	312470		Other	0	0	7*	0	0	42
Thaumatomonas	genus	45099		Other	13	0	0	0	0	0
Cercomonas	genus	45109		Other	0	15	0	0	32	0
Spirogyra	genus	3179		Other	37	0	0	0	0	0
Austropallene	genus	136199		Other	0	0	49*	0	0	0
Paravahlkampfia	genus	166957		Other	0	0	7	0	0	0
Fungi	kingdom	4751		Other	439	1238	413	673	2263	283
Viridiplantae	kingdom	33090	Green plants	Other	213	50	421	0	0	0
Metazoa	kingdom	33208	Metazoans	Other	2744	6350	9335	1926	2646	523
cellular organisms	no rank	131567		Other	0	14	87	6	17	199
root	no rank	1	Unidentified	Other	12165	5634	9819	5080	19551	1209
Rhabditida	order	6236		Other	0	0	363	0	0	0
Cercozoa	phylum	136419		Other	23	258	74	0	8	21
Streptophyta	phylum	35493		Other	360	0	0	75	252	0
Annelida	phylum	6340	Annelid worms	Other	8	0	0	0	0	0
Arthropoda	phylum	6656	Arthropods	Other	556	1187	98	191	4604	0
Chordata	phylum	7711	Chordates	Other	58	0	227	0	28	0
Ciliophora	phylum	5878	Ciliates	Other	9	62	83	0	125	108
Chlorophyta	phylum	3041	Green algae	Other	8	0	96	0	0	0
Eukaryota	superkingdom	2759	Eucaryotes	Other	3933	2174	2687	1588	702	3461

Table D-8. Fish molecular targets for all eDNA study sites (2021-22) with totals.

Site	Species (Genus*)	CI	DG	LV	RV	WV	ZV	Totals
Manganui 2022	Banded kokopu		1270	1459	1382	1774		5885
	Bluegilled bully			217	359	535		1111
	Brown trout	7557	15303	5399	15267	12381		55907
	Common bully		1240					1240
	Common smelt				7	5		12
	Common/Cran /Dinahs bully*			523				523
	Giant bully					787		787
	Giant kokopu		24	80	45			149
	Inanga		1816	579	1434	2625		6454
	Koaro		3884	1923		7183		12990
	Longfin eel	41329	196001	92392	200656	135103		665481
	Pouched lamprey	630	12	312	523	186		1663
	Rainbow trout		8810	3089		13029		24928
	Redfin bully		67672	41638	78627	8963		196900
	Shortfin eel	898	14574	44727	17926			78125
	Shortjaw kokopu		230	213				443
	Speckled longfin eel		192		72			264
	Torrentfish	1126	3114	706	1350	1967		8263
Manganui 2021	Banded kokopu			38		122		160
	Bluegill bully			92	1820	122		2034
	Brown trout	472		215		328		1015
	Giant bully					78		78
	Giant or shortjaw kokopu					106		106
	Inanga			8193	45550	10253	189	64185
	Kahawai	198		7	101	686		992
	Koaro			118		564		682
	Longfin eel	9463		19257	154641	27845		211206
	Pouched lamprey	124		345	3209	138	476	4292
	Rainbow trout	1126		1945	15288	7154		25513
	Redfin bully			9371	55552		42	64965
	Shortfin eel	445		2349	16449	2620		21863
	Shortjaw kokopu			64				64
	Snapper			20102	124940	18361		163403
	Torrentfish	1816		10183	50199	8550	48	70796
Paparahia 2021	Banded kokopu			117	10615	779		11511
	Brown trout				81			81
	Common or Crans bully			1490				1490
	common smelt				132	29		161
	Giant kokopu			26	235			261
	Giant or shortjaw kokopu					62		62
	Inanga			82	3378	628		4088
	Kahawai							
	koaro			30		403		433
	Longfin eel	1935		1305	64233	2881		70354
	Pouched lamprey	25			374		5	404
	Rainbow trout							
	Redfin bully			2115	47816			49931
	Shortfin eel	46		7	4720	247		5020
	Shortjaw kokopu							
	Snapper			903	20213	4898		26014
	Torrentfish	54		28	1140	38		1260