eDNA-Based Detection of New Zealand Freshwater Mussel (Kākahi) Populations using Digital PCR

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Abstract

Globally, freshwater mussels are one of the most endangered taxa due to pollution, nutrient loading, habitat fragmentation, and species introduction attributable to anthropogenic activities. New Zealand freshwater mussels (kākahi) are ecological engineers and integral species in freshwater ecosystems. However, kākahi populations are expected to decline 70% by 2024 in part due to their unique reproductive cycle involving an obligatory phoretic relationship with declining native fish. Kākahi distribution and abundance throughout New Zealand is poorly understood due to time-consuming surveying techniques of concealed and morphologically cryptic species. A rapid and reliable method is required for the detection and identification of these 'Threatened' kākahi populations for conservation and management. Therefore, this study aimed to i) validate an environmental DNA (eDNA) based detection method for New Zealand freshwater mussels using digital PCR (dPCR); ii) determine the most appropriate sampling season for kākahi eDNA; and iii) determine whether eDNA could be used to detect effects of fish barriers on kākahi distribution.

In vivo trials determined that filtration of field samples within 24 hours of collection minimised eDNA degradation and that prefiltration effectively removed inhibitory detritus and glochidia. The lowest concentration of eDNA which could accurately be detected to infer abundance (≥3.2 copies/µL) or presence (0.32-3.19 copies/µL) of kākahi eDNA was determined. Duplicate field samples were collected from the Waingaro River and Kahuhuru Stream at sites downstream of fish barriers and upstream of low-flow or all-flow barriers in December, May, and August to investigate spatial and temporal variation in eDNA concentrations. As hypothesised, December was the most effective month to sample, yielding the highest eDNA concentrations due to reduced dilution and heightened water temperatures. Based on eDNA, kākahi species distribution did not appear correlated with fish barriers, but all-flow barriers likely impeded fish host movement to significantly reduce the total kākahi eDNA concentrations found upstream.

eDNA detection can be used by regional councils to effectively infer presence/absence of kākahi populations and direct further conventional surveying efforts to the most impactful locations. This method allows rapid determination of distribution and densities at a national scale to aid conservation management of this taonga (treasured) species.

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Chapter One - Introduction and Literature Review

1.1 Introduction

There are three freshwater mussel species (kākahi, also known as kāeo or torewai) indigenous to New Zealand: *Echyridella menziesii*, *Echyridella aucklandica*, and *Echyridella onekaka* (Marshall et al., 2014). Kākahi are an integral component of freshwater ecosystems and are considered threatened species, but their distribution and abundance remain relatively poorly understood (Walker et al., 2001). Although kākahi are widespread throughout New Zealand in streams, rivers, and lakes (Marshall et al., 2014), surveying is challenging because they partially bury themselves in soft sediments in hard-to-access locations (Strayer & Smith, 2003). Species detection and identification can be challenging and time-consuming because conventional surveys require identification based on shell morphology, which depends partly on water quality, flow, and wave-action, making them morphologically cryptic (Graf & Cummings, 2006). A quick and reliable method is required for the detection and identification of kākahi populations for conservation and management.

Ferreira-Rodríguez et al. (2019) listed the top 20 research priorities for assessing the freshwater mussel conservation status on a global scale. The top two intrinsic priorities (indicators of population status) included performing accurate species identification, alongside determining species population size and current distribution. This thesis aims to validate a detection method to meet these two priorities and will result in a standardised presence/absence detection method for surveying key habitats over time to determine the decline or spread of kākahi populations. To my knowledge, no New Zealand-based studies have attempted to validate a molecular method that is non-invasive, rapid, and cost-effective with high specificity. Field workers remain reliant on conventional

surveying methods which are time and cost-intensive and difficult to scale up effectively. A validated method for use in future systematic regional and national scale surveys of all kākahi populations will allow conservation and management strategies to be developed to ensure these taonga species do not continue to decline or become extinct.

Advancements in environmental DNA (eDNA) technologies present a promising avenue to achieving these goals. eDNA is a combination of genomic DNA from many organisms present in an environment as cellular DNA from intact cells or extracellular DNA from natural release or cell degradation (Levy-Booth, 2007). It can be extracted directly from a water sample (Rees et al., 2014), making it a non-invasive, cost-effective, and highly specific component for a molecular detection method (Ficetola et al., 2008). eDNA studies for aquatic species is an emerging field aiming to inform management and conservation schemes for aquatic systems (Rees et al., 2014), requiring no specialist taxonomist or field knowledge. It can be detected by amplifying the target eDNA in a sample via polymerase chain reactions to allow species identification.

This project will focus on the *in vivo* and *in situ* validation and application of eDNA methodologies in detecting and monitoring freshwater mussels in the greater Waikato region. The *in vivo* section will be conducted as a tank study to ensure kākahi eDNA can be detected in a closed system effectively and will include optimising the method for field sampling. The *in situ* section will validate that kākahi eDNA can be detected in a natural system and investigate the distribution of kākahi eDNA between seasons and locations.

The objectives of this thesis are to:

1. Validate that this methodology could effectively detect New Zealand freshwater mussels in tank and field settings.

2. Determine the seasonality effect on kākahi eDNA concentration to ascertain the best timing for field sampling.
3. Determine whether eDNA can used to detect the effect of fish barriers on the distribution of *E. menziesii* and *E. aucklandica* populations.

The hypotheses underpinning the research are as follows:

1. Summer (December) is the best period to collect field samples due to low flow (i.e., less dilution of eDNA).
2. Kākahi species distribution is negatively correlated with the presence of fish barriers.

This thesis comprises of five chapters. The first chapter is an introduction into the intricacies of kākahi and their detection, including their cultural, ecological, and biological importance in freshwater ecosystems. The second chapter explores the use of eDNA and digital PCR (dPCR) in highly specific species detection and reviews the methodologies used in other freshwater mussel eDNA detection studies to determine the most appropriate method for this thesis. Chapter 3 then outlines the finalised method and tests the efficacy of detecting kākahi eDNA via dPCR in a closed system. To optimise the *in vivo* methodology, small scale experiments were used to investigate the rate of kākahi eDNA degradation, prefiltration efficacy, and lower limit of dPCR detection. The optimised method is utilised in the fourth chapter to investigate the *in situ* seasonal differences in kākahi eDNA, whether fish barriers influence the distribution of kākahi species, and whether the concentration of kākahi eDNA can be used to infer relative abundance of live populations. The conclusions and future directions of this project are outlined in the final chapter, summarising the main outputs of this thesis.

1.2 Freshwater Mussels

1.3 Introduction

Freshwater mussels (Order: Unionida) are found globally and are considered to be one of the most internationally diverse freshwater invertebrates, characterised by a phoretic larval stage on host fish (Bogan, 2007). There are 840 estimated species of freshwater mussels worldwide, including 33 species in Australasia which belong mainly to the Hyriidae family (Graf & Cummings, 2007). Freshwater mussels inhabit a diverse range of environments, existing in soft sediment lake and stream beds to cobble substrate in fast-flowing rivers. However, the majority of freshwater mussels prefer to inhabit clear, highly oxygenated streams with sand and gravel substrate (Nowak & Kozlowski, 2013).

Freshwater mussels are keystone species in freshwater habitats, acting as a food source, habitat for macroinvertebrate growth, sediment stabiliser, habitat modifier via bioturbation and biodeposition, and filter feeder to clarify the water column (Nowak & Kozlowski, 2013). Population distribution of many species is not well understood because they embed themselves in sediments in hard-to-access locations, which makes conventional surveying challenging (Strayer & Smith, 2003). For many freshwater mussels species, identification is an additional challenge because their shell morphology is influenced by water quality, flow, and substrate composition, making kākahi morphologically cryptic (Graf & Cummings, 2006).

Although they are keystone species, freshwater mussels had not been studied in detail until they were listed in the U. S. Endangered Species Act, which resulted in attention from research scientists, policy makers, and environmental monitoring groups. As a result, freshwater mussel research expanded rapidly in the 1980s as groups began to investigate their conservation, ecology, physiology, and toxicology in order to inform management of the declining species (Lopes-Lima et al., 2014). Freshwater mussels are

one of the most endangered taxa globally (Lopes-Lima et al., 2014) and are continuing to decline due to a variety of anthropogenic factors (Modesto et al., 2018). Because freshwater mussels gain nutrients via filtration of the water column, they are exposed to dissolved and suspended contaminants that arise due to water quality degradation (Watters, 1999). As a result, 41% of freshwater mussel species worldwide are classified as 'Critically Endangered', 'Endangered', 'Vulnerable', or 'Threatened' species (Modesto et al., 2018). This is likely due to a global increase in anthropogenic impact on freshwater systems, such as impoundment, toxic pollution, increased nutrient loading, and non-native species' introduction (Strayer, 2014). Due to kākahi being keystone species, their conservation status is of concern; their conservation management is a vital step in protecting New Zealand's freshwater ecosystems.

1.3.1 Ecological Importance

Freshwater mussels serve many important functional roles within freshwater stream and lake habitats. They perform several active (filter feeding, excretion, biodeposition, and bioturbation) and passive roles (soft tissue and shell material use) which are depicted in Figure 1-1.

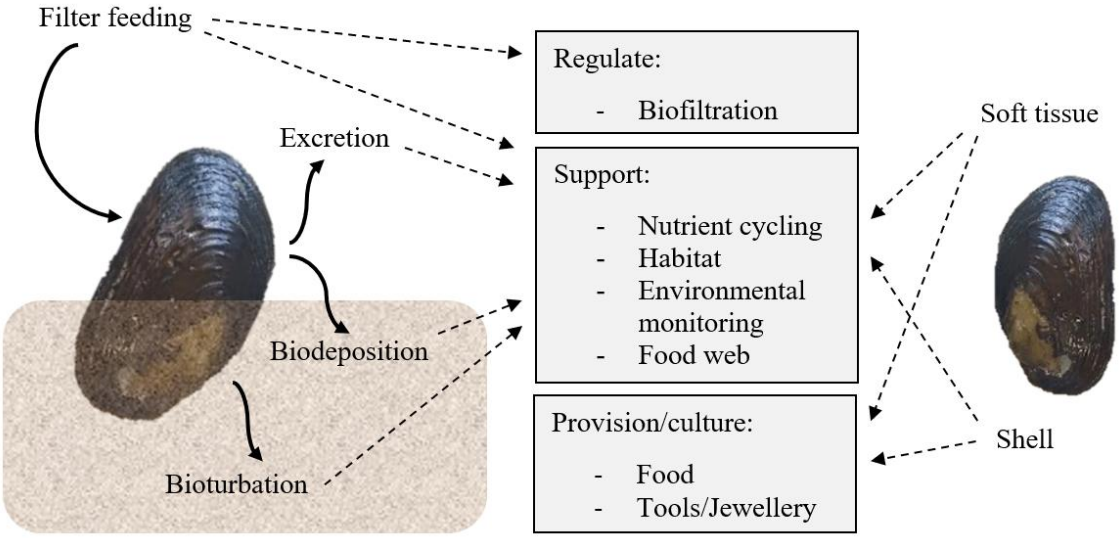

Figure 1-1. Functional roles of freshwater mussels in freshwater habitats. Modified figure from Vaughn (2017).

The act of filtration by freshwater mussels can significantly decrease the concentration of detritus, bacteria, phytoplankton, and particulate organic matter in the water column (Strayer et al., 1999). Filtrates are converted into shell and soft tissue biomass, biodeposits (faeces or pseudo-faeces), and dissolved nutrients (from excretion) (Strayer, 2014). The filtration rate of freshwater mussels differs between species, although they are relatively similar. Roper and Hickey (1995) noted that filtration rates range from 0.97 to 1.66 L mussel^{-1} hr^{-1} for *E. menziesii*, while Cyr et al. (2017) found they filter water at a rate of 0.4-1.3 L mussel^{-1} hr^{-1}, which is consistent with North American and European species (Cyr et al., 2017; Roper & Hickey, 1995). When the water temperature is lower during winter months, the activity level of freshwater mussels is reduced to conserve energy, resulting in minimal filtration and a lower rate of eDNA shedding compared to summer (Wacker et al., 2019). Ogilvie (1994) demonstrated that an increase in filtration rate by *E. menziesii* (then known as *Hyridella menziesii*) occurs as water temperatures increase. The average filtration rate increased from 0.41 L/hr/g in 18 °C water to 1.91 and 2.68 L/hr/g in 21 and 25 °C water, respectively (Ogilvie, 1994).

The volume of water filtered per day can exceed the daily stream discharge (Welker & Walz, 1998), resulting in extensive purification by a single mussel population. This biofiltration can result in biological oligotrophication due to decreasing total phosphorus and phytoplankton while increasing water clarity at a scale large enough to cause a change in trophic state (Welker & Walz, 1998). Large populations can significantly impact cycling, storage, and bioavailability of nutrients, as well as abiotic and biotic conditions of freshwater environments (Nowak & Kozlowski, 2013; Vaughn, 2017).

Suitable habitats for freshwater mussels must have stable sediments, low shear stress, and be relatively sheltered against high-water-flow events. As a result of sparse suitable habitats along stream systems, their distribution is intermittent, although they can form

dense beds once located in suitable environments. These dense mussel patches can greatly increase biodiversity due to the bottom-up release of nutrients, availability of their shells as suitable habitats for other organisms, and the increased stability of substrates, which collectively facilitate the colonisation of these mussel-rich habitats by other organisms (Aldridge et al., 2007; Vaughn, 2017). The attached algae support other grazing animals, and crevices within the mussel shell provide flow and predation protection for macroinvertebrates (Allen et al., 2012). As a result, the macroinvertebrate communities in areas with dense mussel populations are much more diverse and abundant than comparable habitats that do not contain any freshwater mussels (Aldridge et al., 2007; Vaughn, 2017).

Because freshwater mussels are widespread, live for decades, are sessile, and feed on particles suspended in water columns from upstream discharge, bioaccumulated contaminants within the mussel shell can infer the environmental history of the area for monitoring and management purposes (Vaughn, 2017). They can act as bio-indicators of past environmental changes to infer future possibilities over vast spatial and temporal scales due to the change in physical and chemical conditions that are encompassed in the geochemical make-up of their shells (Brown et al., 2005). In addition, they capture information which tracks changes in trophic complexity and the effect of natural and anthropogenic environmental changes on food webs (Delong & Thorp, 2009).

New Zealand freshwater mussels, also known as kākahi, kāeo, or torewai, hold significant cultural importance to Māori (McDowall, 2002). They are considered a taonga (treasured) species due to the plethora of functions they served to the pre-European Māori, including food, tool, and medicinal resources (Hiroa, 1921; McDowall, 2002). Year-round harvesting of kākahi was thought to have depleted the extensive mussel beds present throughout New Zealand but provided a stable protein source, tools to cut hair and

umbilical cords, and methods to prepare harakeke (flax) for fishing nets and woven baskets (Hiroa, 1921).

1.4 New Zealand Kākahi

New Zealand is home to three endemic freshwater mussel species from the unionid family Hyriidae (Graf & Cummings, 2006): *Echyridella menziesii, Echyridella aucklandica*, and *Echyridella onekaka* (Marshall et al., 2014). The conservation status of each of the three kākahi species was assessed in 2018, identifying *E. menziesii* as 'Declining', *E. aucklandica* as 'Nationally Vulnerable', and *E. onekaka* as "At Risk – Naturally Uncommon' (Grainger et al., 2018). In 2014, it was predicted that within a decade there would be a 10-70% decline in *E. menziesii* (Grainger et al., 2014), which follows the trends of overseas freshwater mussels that are declining worldwide (Lopes-Lima et al., 2017; Strayer & Smith, 2003).

Environmental conditions create differing shell morphologies amongst kākahi species, which taxonomists perceived as different species and resulted in historical overestimates of diversity (Fenwick & Marshall, 2006; Marshall et al., 2014). Globally, freshwater mussel shell morphology has been described as plastic because it can be highly variable in response to the local environment (Roper & Hickey, 1995), which has posed challenges for taxonomists when morphologically identifying New Zealand freshwater mussels (Dell, 1953). Although Dell concluded there were only two New Zealand freshwater mussel species (*Hyridella aucklandica* and *Hyridella menziesii*), the phylogenetic relationships and number of species remained unclear until Fenwick and Marshall (2006) reported they were actually part of a new genus, *Echyridella*, separate from the Australian *Hyridella* genus to which they were previously ascribed (Dell, 1953). In 2014, it was confirmed via DNA sequencing that there were three extant native freshwater mussels in New Zealand (Marshall et al., 2014), depicted in Figure 1-2.

Figure 1-2. New Zealand freshwater mussel species: *Echyridella aucklandica* (left), *E. menziesii* (middle), and *E. onekaka* (right, sourced from Fenwick and Marshall (2006)).

Kākahi can be found throughout New Zealand, although the distribution differs between species (Figure 1-3). *E. menziesii* is the most common species and has been reported throughout both the North and South Island, as well as the Great Barrier Island of New Zealand (Gray, 1843) in a vast range of habitats from lakes to small fast-flowing streams (Walker et al., 2001). Comparatively, *E. aucklandica* is less widespread, clustering mainly in the Far North District of Kaeo with outlier populations in Lake Manapouri to Lake Hauroko in Fiordland, Whanganui, and Lake Wairarapa (Walker et al., 2001). The least common species, *E. onekaka*, has very few populations and is restricted to north-western South Island (Fenwick & Marshall, 2006).

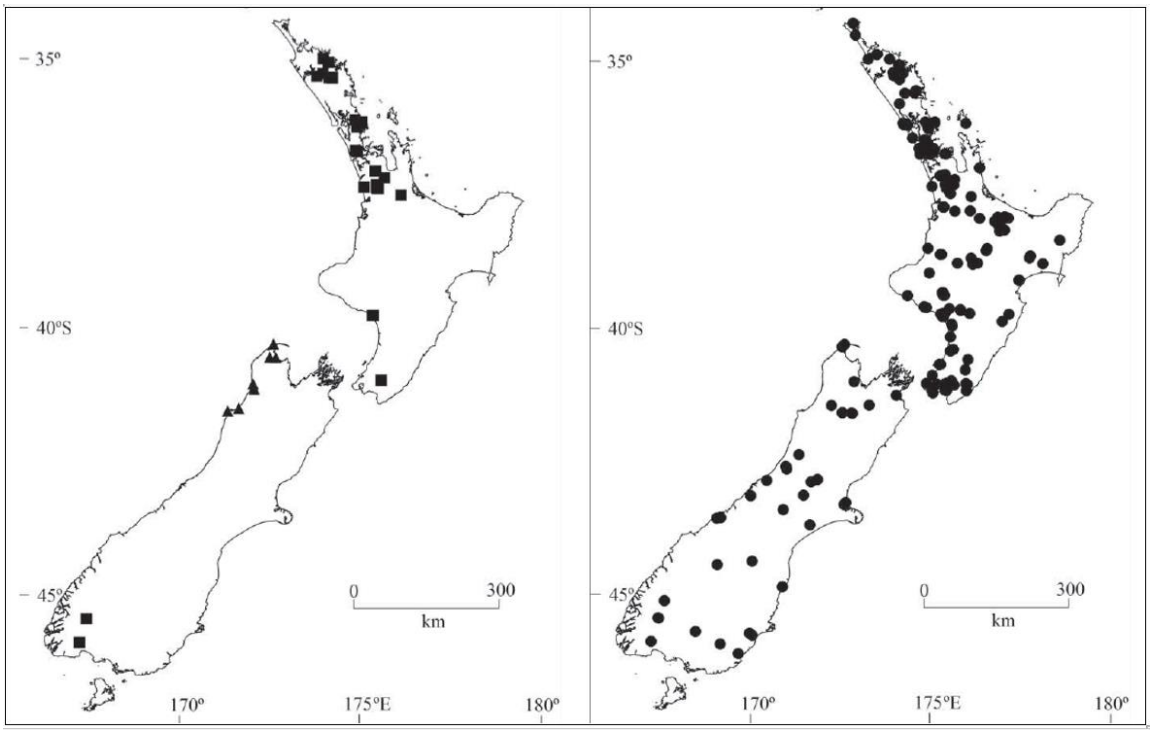

Figure 1-3. Figures from Marshall et al. (2014) depicting recorded kākahi distribution in New Zealand. Left: Distribution of *Echyridella aucklandica* (Gray, 1843) (■) and *E. onekaka* (Fenwick & Marshall, 2006) (▲). Right: Distribution of *E. menziesii* (Gray, 1843) (●).

It is suspected that all three New Zealand freshwater mussels have long lifespans, but there is limited evidence of this. It has been confirmed that *E. menziesii* has lived to as old as 33 years in Lake Waipapa and the Waikato River (Roper & Hickey, 1994), while in Lake Waipori the most frequent range was 20-25 years with the majority ranging from 15-35 years of age (Grimmond, 1968). Specimens of an overseas freshwater mussel species (*Margaritifera margaritifera*) that are similar in size to *E. aucklandica* and *E. menziesii* are frequently found to be aged over 100 years (Geist, 2010). This suggests that the lifespan of New Zealand freshwater mussels may be much longer than reported.

1.4.1 Lifecycle of the New Zealand Kākahi

The lifecycle of a freshwater mussel is complex, including the fertilisation, release of glochidia which is a phoretic phase in which the mussel larvae must attach to a fish host, and years of juvenile growth (Figure 1-4).

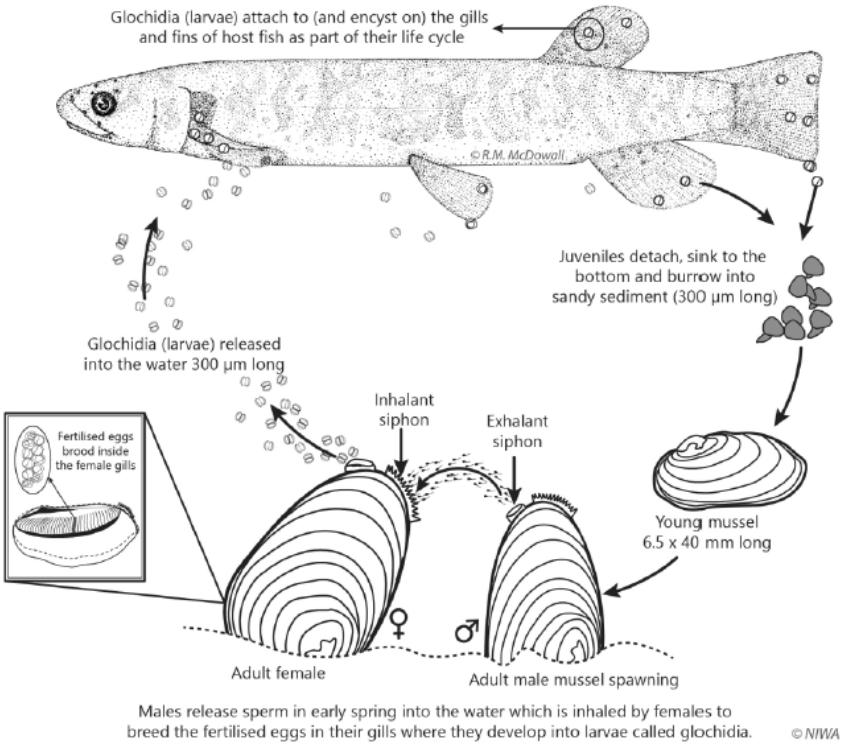

Figure 1-4. New Zealand freshwater mussel, *Echyridella menziesii*, lifecycle – Figure sourced from NIWA (2018).

During fertilisation, the male gametes are released into the water column and drawn into the females' inhalant siphons during filtration (Barnhart et al., 2008). Depending on the species, after a female's eggs are fertilised, it can take 2-6 weeks or up to 8 months to form 2,000 to 10,000,000 glochidia per mussel (Haag, 2013). However, the survival rates of most freshwater mussel species' glochidia can be very low, with up to 99.99% of glochidia failing to locate and attach to a suitable host (Bauer, 1994; Haag, 2012; Modesto et al., 2018). The release of the kākahi glochidia is triggered by environmental cues such as changes in water temperature (Walker et al., 2001) and can differ by days to months in stream systems within the same region (Hanrahan, 2019). This glochidia dispersal can be

passive (standard release into the water column) or active, with modifications to attract host fish such as rhythmic contractions of mantle lures, conglutinate packages of glochidia that resemble fish food, lures to trap fish hosts between valves during release, or mucus strands to entangle fish hosts (Modesto et al., 2018).

Male kākahi begin to spawn in late winter (August), releasing their sperm into the water column to fertilise nearby female kākahi (Clearwater et al., 2011). After fertilisation, glochidia grow inside the female's brood pouch for several months, and release can occur between October and late March (Clearwater et al., 2011), although this peaks between February and March depending on the local conditions (Clearwater et al., 2014; Hanrahan, 2019). The release of *E. menziesii* glochidia is heavily dependent on water temperature or sudden environmental changes (Hanrahan, 2019), and *E. aucklandica* likely demonstrates a similar response (Clearwater et al., 2011). In three Waikato streams, the peak glochidia release varied, but the average release was in February when water temperatures were >18.8 °C (Hanrahan, 2019).

There has been no field methodology developed to detect when kākahi have released their glochidia, other than time-consuming and invasive collection and opening of shells to observe their brood pouch. Because glochidia release can be extremely varied (Clearwater et al., 2011; Hanrahan, 2019), at certain times of the season streams may have greatly varied kākahi eDNA concentrations regardless of population size. By sampling streams for eDNA in early summer (December), dilution effects may be reduced and peak glochidia release may be avoided so comparisons can be made between locations.

Depending on the species and water conditions, glochidia are viable for a few hours to 14 days once released into the water column (Bauer, 1994; Haag, 2012). Once *E. menziesii* glochidia are released from a female mussel, they must find a suitable host within 2 to 4 days (Clearwater et al., 2014) and become encysted in host epithelial cells on the fish gills

or fins where they remain for 9 to 22 days (Bauer, 1994; Clearwater et al., 2014). However, many glochidia are prematurely removed from their fish host due to physiological and immunological incompatibility or due to mechanical disruption (Bauer, 1994). This makes the magnitude of glochidia released critical to the turnover of new juvenile mussels (Walker et al., 2001), and high glochidia numbers can be considered a survival strategy that compensates for failed attachment. The glochidia undergo metamorphosis into the juvenile form while encysted on the fish host, before detaching and settling on the stream bed where they feed through their ciliated foot, unlike adult mussels which obtain nutrients via water filtration (Walker et al., 2001).

The interaction between the glochidia and fish host is critical for freshwater mussel dispersal (Haag, 2012). This stage is a distinctive difference between freshwater mussels and all other bivalves, which do not require a host for dispersal (Haag, 2012). Modesto et al. (2018) describes the interaction as phoretic commensalism, as the host allows transport of the symbiont rather than nutrition or protection (Modesto et al., 2018). Some freshwater mussel species only have one or a few suitable host fish, while others can attach to any fish species, which is likely due to adaptations of glochidia to survive physiological responses of the host fish (Barnhart et al., 2008).

1.4.2 Kākahi Fish Hosts

A study by Hanrahan (2019), which sampled fish in three Waikato streams, found all *E. aucklandica* glochidia were attached to common smelt (*Retropinna retropinna*), whereas *E. menziesii* glochidia were detected on redfin and common bully (*Gobiomorphus Cotidianus)*, longfin and shortfin eels *(Anguilla australis* and *A. dieffenbachii*), īnanga (*Galaxias maculatus*), and torrentfish (*Cheimarrichthys fosteri*). These findings were supported by Brown et al. (2017), who alongside the previously mentioned species also detected *E. menziesii* glochidia on Canterbury galaxias (*Galaxias vulgaris*), banded

kōkopu (*Galaxias fasciatus*), and kōaro (*Galaxias brevipinnis*). Moore and Clearwater (2019) also confirmed these findings, defining *E. menziesii* as host generalists which cast their glochidia passively so that a range of fish species could become hosts to viable juvenile mussels. In comparison, *E. aucklandica* are most likely host specialists, with field observations and lab studies demonstrating *E. aucklandica* glochidia only grew and excysted on smelt (Melchior, The University of Waikato, unpublished data, 2020). Although a single *E. aucklandica* glochidia was found attached to an inanga and redfin bully in the field, they were not encysted nor able to successfully metamorphose on inanga or redfin bully in lab experiments (Melchior, The University of Waikato, unpublished data, 2020).

E. menziesii produce large glochidia (277 µm) which are released individually bound in mucus threads that float through the water column to entangle passing fish hosts, whereas *E. aucklandica* glochidia are smaller (99 µm) and released as functional conglutinates that mimic host diet as an infection strategy (Figure 1-5) (Melchior et al., 2019). These differing glochidia release strategies demonstrate how freshwater mussels can deploy tactics that enhance their chance of encystment on the correct host fish for survival.

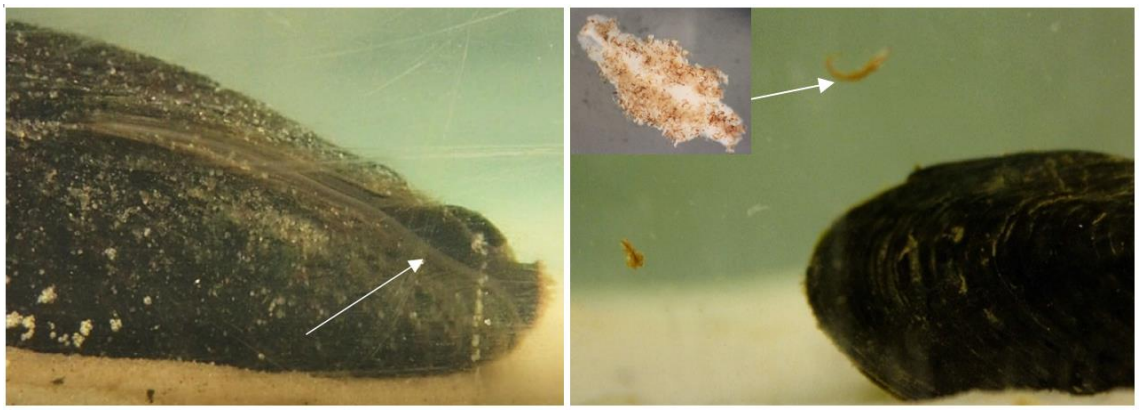

Figure 1-5. Kākahi glochidia release. Left: Adult *Echyridella menziesii* releasing glochidia bound to mucus strands attached to exhalent siphon (white arrow). Right: Conglutinates released by adult *Echyridella aucklandica* (white arrow). Pictures from Melchior et al. (2019).

E. menziesii glochidia are also able to attach to non-native invasive species such as brown bullhead catfish (*Ameiurus nebulosus*), rudd (*Scardinius erythrophthalmus*), and goldfish (*Carassius auratus*) (Moore & Clearwater, 2019). Moore and Clearwater (2019) found that, although the attachment rate of *E. menziesii* to the three invasive species compared to native hosts were the same, they did not produce ecologically significant kākahi juveniles due to poor transformation rates. These invasive species are unsuitable hosts and are capable of disrupting the obligate glochidia stages of the kākahi lifecycle by acting as a glochidia sink, and indirectly competing against or predating the native host species (Poos et al., 2010). As a result, where non-native fish species dominate fish communities, kākahi populations may decline severely or face extinction due to the disruption of the required interaction between fish hosts and phoretic glochidia.

1.4.3 Causes of Mussel Decline

Freshwater mussels are globally one of the most endangered taxa (Lopes-Lima et al., 2014) and are continuing to decline worldwide due to a range of anthropogenic factors (Modesto et al., 2018). In 2018, 41% of freshwater mussel species worldwide were classified as 'Critically Endangered', 'Endangered', 'Vulnerable', or 'Threatened' species (Modesto et al., 2018). This has been recognised by researchers and conservationists and has become a widespread conservation concern (Dudgeon et al., 2006) due to their status as keystone species, which means their decline may result in cascading declines in biotic diversity and abundance of entire freshwater ecosystems (Chowdhury et al., 2016; Nowak & Kozlowski, 2013; Vaughn & Hakenkamp, 2001).

An extensive literature review by Modesto et al. (2018) identified the major anthropogenic threats to freshwater mussels, which included habitat loss, fragmentation and degradation, overexploitation, pollution, climate change, and introduction of non-native species. Although several of these factors have direct impacts on the freshwater

mussels (Haag, 2012; Strayer, 2008), indirect impacts mediated by declining fish host populations also play a major role (Modesto et al., 2018). Similarly, another extensive review of mussel decline pooled all factors into six categories: exploitation by humans, mussel habitat alteration or destruction, energy and food availability, exotic or invasive species, population phenomena (recruitment, host availability, genetic change), and global climate change (Downing et al., 2010). Both reviews support each other in naming the phoretic relationship between glochidia and fish host critical in the decline of freshwater mussels (Downing et al., 2010; Modesto et al., 2018).

There are several features of this unique lifecycle that make freshwater mussels susceptible to decline. Their larval stage is reliant on the presence of suitable host fish at the time of release; the juvenile stage is vulnerable to predation, drift, siltation, and sediment toxicity; the adult stage is susceptible to contaminated water and sediments during filter and deposit feeding; and adult populations are reliant on stable substrates that are likely to be disturbed during flood events (Nowak & Kozlowski, 2013). Each of these factors, or a combination of them, may explain the declining conservation status of the freshwater mussel, as unique life histories are sensitive to fluctuations in environmental conditions (Koh et al., 2004).

Because there is a clear obligate dependency on specific fish host species for kākahi dispersal, kākahi are not only vulnerable due to their own weaknesses, but also the weaknesses of their hosts. Although there are 54 native fish species that inhabit New Zealand freshwater streams and rivers, 74% have been classified as either threatened or at risk of extinction, an increase from 65% in 2009 (Goodman et al., 2014). The reasons for this decline in freshwater fish is due almost entirely to human-induced pressures such as eutrophication, river alterations, exotic species invasion, overharvesting, and

destruction of habitat (Joy et al., 2019). As a result, New Zealand aquatic environments are considered ‘one of the most endangered habitats in the world’ (IUCN, 2012).

1.4.4 Fish Barriers

The fate of kākahi is entangled with the ability of suitable freshwater fish hosts to frequent the same habitat and disperse them. 46% of New Zealand’s freshwater fish species are diadromous, migrating between freshwater and the ocean for spawning and/or larval growth (McDowall, 2000), which indicates how critical fish passage is for the reproductive cycle and dispersal of New Zealand fish species. McDowall (1993) studied New Zealand fish distribution in relation to altitude and demonstrated that some diadromous species were present in stream networks as high as 1,000 m (longfin eel) and 1200 m elevation (kōaro), while smaller migratory fish were only found up to 250 m elevation or 150 km inland (īnanga). All three of these migratory species are confirmed host fish for kākahi (Brown et al., 2017; Hanrahan, 2019), highlighting the importance of bidirectional fish passage for freshwater fish species that are viable hosts for kākahi glochidia. Therefore, impediments to fish dispersal throughout river networks has the potential to impact kākahi populations reliant on migratory host species for recruitment (potentially leading to localised extinctions upstream of barriers to fish passage).

Anthropogenic barriers to fish migration can include culverts, weirs, fords, dams, and tide/flood gates (Franklin et al., 2018), and can exclude migratory species from previously accessible upstream habitat/s. These structures need to consider many design features to ensure fish passage, such as length, diameter, substrate, and gradient. Similarly, natural fish barriers such as waterfalls or rapids may selectively or entirely impede fish passage. These natural and artificial barriers create discontinuous stream systems and may stop fish from dispersing mussel larvae to upstream habitats.

Fish passage is managed by both the Department of Conservation and regional councils under the Fisheries Regulations 1983 and Resource Management Act 1991. NZ Fish Passage Guidelines (Franklin et al., 2018) set objectives for best-practice fish passage design, including minimum design standards and guidance for new and existing structures, and provide guidance for efficient and safe fish passage for all aquatic organisms at each life stage. It is also important to consider that, by ensuring fish passage for all species, invading predatory fish may also gain access to waterways and reduce the native fish populations. Protection of native fish and biodiversity is paramount, so some structures have been retrofitted or designed to limit successful passage by selected non-native species, by utilising the climbing abilities of juveniles of several native species (David & Hamer, 2012).

As kākahi lifecycles are dependent on native fish, if fish passage is impeded in stream networks, freshwater mussel recruitment may not occur upstream of barriers. Kākahi populations may eventually become locally extinct above anthropogenic barriers or be absent above naturally occurring ones. For this reason, kākahi presence could be used to infer if host fish are or have previously been able to migrate upstream to that location, and differences in kākahi species distribution could be explained by the extent of fish passage disruption. Host-generalist *E. menziesii* will likely be present upstream of small fish barriers as their fish hosts are larger or adapted to climbing so are likely to overcome barriers, whereas the host-specialist *E. aucklandica* will not likely be present upstream of extensive fish barriers as they rely on smelt for dispersal. Common smelt migrate as small juveniles and are generally unable to move past even moderate barriers (McDowall, 1995). However, the ability of *E. aucklandica* glochidia to attach to both juvenile and adult smelt (Melchior, The University of Waikato, unpublished data, 2020) may allow a small proportion of *E. aucklandica* to be transported upstream of barriers.

1.4.5 Difficulties in Field Surveying of Kākahi

Alongside the difficulties of identifying New Zealand freshwater mussel species due to cryptic morphologies, detecting mussel presence is also challenging with conventional surveying methods. Traditional visual searches for freshwater mussels require the field workers to wade, swim, or dive at locations where mussels are expected and physically or visually locate the mussels. If there is low mussel abundance, poor visibility in turbid or deep water, high flow due to storm or seasonal events, or mussels are partially or fully buried in the sediment, then this detection method is unreliable (Strayer & Smith, 2003).

The Waikato Regional Council has developed three standardised protocols for kākahi monitoring in wadeable streams that each infer differing degrees of information: Protocol 1 – presence/absence, Protocol 2 – species density/size structure, Protocol 3 – presence, density, size structure, and habitat (Catlin et al., 2017). Protocol 1 is least intensive yet still includes a 30-minute visual survey at each site and a Habitat Assessment Field Data Sheet that takes a further 10 minutes. Due to this lengthy protocol, the traditional visual search is difficult to scale up because each suitable habitat must be searched and each mussel found must be collected, identified, measured, and returned to the environment. Not only does this surveying method require considerable time, but workers must also be trained in morphological identification and adhere to strict health and safety protocols.

An alternative to conventional freshwater mussel sampling and identification that reduces cost and time and has a better detection rate would allow for a wide-scale population survey. One promising option is a molecular technique that targets environmental DNA (eDNA) shed by the freshwater mussels. This removes the need for direct observation of the species and can be achieved by taking a single water sample at each location that does not negatively affect the kākahi. A rapid and specific method is required for species-specific identification of populations for conservation management.

Chapter Two - eDNA Methodology Development

2.1 Introduction

The use of environmental DNA (eDNA) for species-specific detection is a relatively new technique, first utilised by Willerslev et al. (2003), meaning divergent methodologies are being used to complete similar studies because there is no universally adopted protocol (Sansom & Sassoubre, 2017). These methodological differences make it a challenge to consolidate or compare results and strategies (Wacker et al., 2019). Due to the diversity of methodological approaches used in freshwater mussel eDNA studies (Currier et al., 2018; De Ventura et al., 2017; Egan et al., 2013; Gasparini et al., 2020; Schill, 2019; Shogren et al., 2019; Stoeckle et al., 2016; Wacker et al., 2019), a reliable method to collect, filter, and extract eDNA from stream water is required. This chapter explores the benefits and challenges associated with eDNA detection and the methodology utilised to detect freshwater mussel eDNA from stream water. The most appropriate techniques from other studies were identified to develop and validate a standardised New Zealand approach to kākahi eDNA detection. Once proven to be the most effective or appropriate, these techniques were integrated into the final methodology for *in vivo* and *in situ* samples.

2.2 Environmental DNA (eDNA)

eDNA is the combination of DNA originating from organisms present in an environmental sample, such as sediment, soil, water, or snow (Rees et al., 2014). Metazoan species constantly expel DNA into their environment via urine (Valiere & Taberlet, 2001), faeces (Poinar et al., 1998), hair and skin cells (Lydolph et al., 2005), as well as secretions, blood, degrading carcasses, and gametes (Bohmann et al., 2014). eDNA can exist as cellular DNA in intact cells or extracellular degraded DNA (Levy-

Booth, 2007), although it has been proposed that the majority of eDNA from metazoans exists inside mitochondria as mitochondrial DNA (mtDNA) (Turner, Barnes, et al., 2014) because mtDNA is protected from endonuclease degradation that rapidly degrades nuclear DNA in all other cells during apoptosis (Murgia et al., 1992). Because eDNA is shed from organisms, detection of a species using eDNA is non-invasive and cost-effective compared to conventional surveying and does not negatively affect the organism. Because organisms can shed eDNA in any environment, it can be used to address several research questions which range from molecular biology, ecology and species management, palaeontology, and environmental sciences (Thomsen & Willerslev, 2015).

2.2.1 History of eDNA in Species Detection

The use of eDNA to detect organisms was first conceptualised in 1986 as a way to obtain the nucleic acids of microorganisms from environmental samples (Olsen et al., 1986). This allowed scientists to overcome the misrepresentation of microbial population composition caused by many microbes being unculturable (Brock, 1987). eDNA allowed the first glimpse at the true microbial composition in various environments. It wasn't until 2003 that eDNA studies transitioned from microorganism to metazoan communities, when environmental DNA of extant mammals, birds, and plants was detected in New Zealand sediments (Willerslev et al., 2003). The first study which detected metazoan DNA in freshwater systems detected human, cow, pig, and sheep DNA to infer faecal contamination of surface water (Martellini et al., 2005). A study in 2008, which detected eDNA of invasive American bullfrogs in France (Ficetola et al., 2008), was the first study to use eDNA detection for invasive species management and resulted in a surge in molecular genetic studies of aquatic ecosystems with species conservation and management in mind.

2.3 eDNA Detection in Aquaculture and Freshwater Mussel Studies

The use of eDNA to infer a species' presence in an aquatic system has commonly been used in aquaculture studies to detect rare, invasive, pathogenic, genetically modified, and elusive species, or species that are difficult to monitor using conventional methods (Bohmann et al., 2014). There are many advantages to eDNA detection of species in comparison to conventional surveying methods, such as high sensitivity, cost effectiveness, non-invasive nature, and no taxonomic expertise required for morphologically cryptic species. Conventional surveying requires the organisms to be detected in the environment in the exact moment that the surveyors are present, which may be difficult for elusive, rare, or physically hard-to-find species that may be buried, fast-moving, or camouflaged. eDNA detection mitigates false negatives of conventional surveying due to the inability to locate the organism, because if the species is present and leaves detectable concentrations of eDNA, it will be identified as present, regardless of whether it is not in that location at the time of sampling. This ensures a much higher sensitivity of detection than conventional methods.

Most conventional surveying methods require the capture and release of organisms so that population density, health, and size characteristics can be obtained for the species of interest. Depending on the type of downstream analysis that is required for the study, this may also require the culling of these organisms. eDNA detection avoids this disruption, as the animal does not need to be handled in any way to detect it, reducing the chance that the species will be negatively impacted. Conventional surveying of kākahi following the least intensive standardised Waikato regional guidelines takes 30 minutes per site, excluding measurement and identification, and may require additional searching if no kākahi are found (Catlin et al., 2017). The reduction in time required to collect a water sample compared to conventional surveying is drastic and allows many stream samples

to be collected in a single day so that a broader population distribution understanding can be obtained. Water samples can be collected by field workers untrained in morphological identification or laboratory processing, reducing the expertise and time required. The water samples can be sent from different regions to a centralised location for processing, or a standardised protocol can allow the analysis of samples at any laboratory with the required instruments. These attributes meet the criteria for a quick and reliable method for the detection and identification of kākahi populations for conservation and management.

2.3.1 Mussel eDNA

The first known study which detected eDNA of freshwater mussels was published in 2013 and utilised the technique of light transmission spectroscopy (LTS) to detect eDNA from the invasive zebra mussel, *Dreissena polymorpha*, in filtered lake water (Egan et al., 2013). More sensitive detection methods (such as quantitative PCR) have since been used to detect other species of freshwater mussels throughout the world (Currier et al., 2018; De Ventura et al., 2017; Egan et al., 2013; Gasparini et al., 2020; Schill, 2019; Shogren et al., 2019; Stoeckle et al., 2016; Wacker et al., 2019). Freshwater mussels expel eDNA into their environment as sloughed tissue (due to filtration), shell material, faeces, pseudo-faeces, and material such as sperm or glochidia (Clearwater et al., 2011; Currier et al., 2018; De Ventura et al., 2017; Melchior et al., 2019; Sansom & Sassoubre, 2017). The shedding rates of freshwater eDNA can vary greatly for a single species, from 5.4×10^4 to 2.4×10^6 copies·hr^{-1}mussel^{-1} (Sansom & Sassoubre, 2017). Although the factors which influence the eDNA shedding rates are unknown, it has been suggested that rates could differ due to species, size, life stage, mussel density, age, and filtration rates (Sansom & Sassoubre, 2017).

The shedding rate of freshwater mussel eDNA also varies extensively between seasons because it is dependent on environmental factors such as water temperature and life cycles (Shogren et al., 2019; Wacker et al., 2019). When the water temperature is lower during winter months, the activity level of freshwater mussels is reduced to conserve energy, resulting in minimal filtration and a lower rate of eDNA shedding than in summer (Wacker et al., 2019). It was suggested by Wacker et al. (2019) that the 20-fold increase in eDNA concentration of *Margaritifera margaritifera* between late spring to late summer was due to higher shedding rates as a result in water temperature. However, this may have been overestimated because freshwater mussels release glochidia during the summer periods when water temperature increases (Clearwater et al., 2011; Hanrahan, 2019), and the river flow is much lower, which further amplifies any increases in eDNA shedding rate.

Freshwater mussels have a hard calcite shell that protects the soft tissue from constant exposure to the environment and they remain partially or fully buried in sediments for much of their life history. Due to this, the rate of eDNA expelled is expected to be less than most aquatic organisms because the soft tissue (which sloughs eDNA) is only exposed during filtration or burrowing (Sansom & Sassoubre, 2017). eDNA shedding is less correlated with the size of the mussel and more with the filtration or activity rate. After Sansom and Sassoubre (2017) removed mussels from their tanks, the filtered tank water contained a high concentration of eDNA. However, the mussels had produced very little faeces or pseudo faeces (Sansom & Sassoubre, 2017). This suggests that filter feeding is the main source of eDNA, rather than pseudo faeces, contrary to what Currier et al. (2018) and De Ventura et al. (2017) suggested.

2.4 eDNA Degradation and Transport in Aquatic Environments

Although eDNA may be shed at a very high concentration, the challenges of eDNA degradation, transport, and dilution must be considered to ensure it is detectable in the environment. Several factors interact to alter the original amount of eDNA produced that is detectable at the time of sampling. This is summarised in Figure 2-1, which depicts how eDNA is influenced by degradation and transport over time in an aquatic environment.

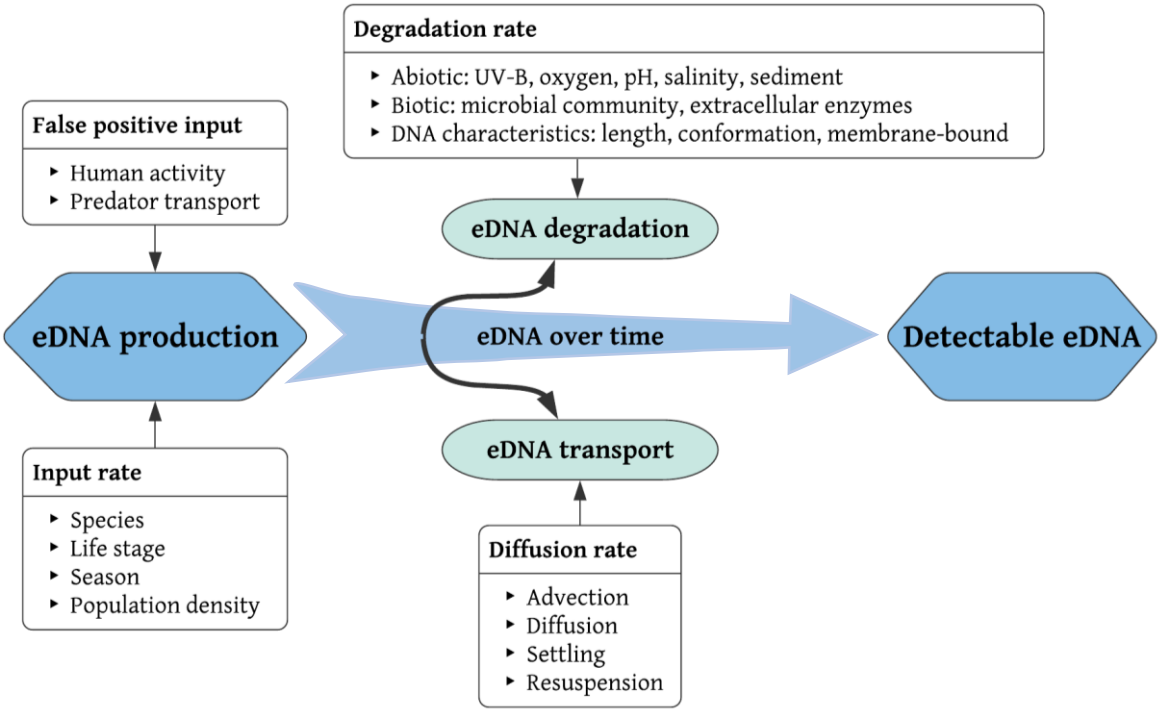

Figure 2-1. Conceptual model of factors which affect eDNA detection from a stream environment.

Sansom and Sassoubre (2017) found that the decay rate of *Lampsilis siliquoidea* freshwater mussel eDNA ranges from 9.7×10^{-3} to 5.3×10^{-2} per hour for mussel densities of 16 to 110 mussels per m^2. They implied that eDNA degradation is dependent on the time of exposure to the freshwater environment (Sansom & Sassoubre, 2017). Aquatic habitats that are colder, more protected from solar radiation, and more alkaline are likely

to hold detectable amounts of eDNA longer than those that are warmer, sunnier, and neutral or acidic (Strickler et al., 2015). In anoxic conditions, such as deeper sediments, nuclease degradation of nucleic acid is reduced, allowing longer preservation of eDNA (Corinaldesi et al., 2011). Anderson-Carpenter et al. (2011) demonstrated that plant DNA from 4600-year-old freshwater lake sediment could be detected because it had been preserved under anoxic conditions. The availability of oxygen has an influence on the preservation of eDNA preservation and degradation due to microbial activity (Corinaldesi et al., 2011).

Although it may seem like the quick degradation of eDNA in aquatic environments is a hindrance to detection, it has a very significant advantage over the persistence of eDNA in soils and sediments. Rapid degradation of eDNA in water enables eDNA detection to inform conservation tactics because positive detection is likely to be associated with the contemporary presence of the species, while misleading signals from past populations may not be detected (Ruppert et al., 2019; Thomsen & Willerslev, 2015).

Once eDNA is released into the freshwater environment, it becomes part of a very complex hydrological system which undergoes dilution, diffusion, settling, and mixing events. These processes all act to alter the initial concentration of eDNA and may allow the detection of eDNA at a significant distance away from the point source. There must be a sufficient distance between the mussel producing the eDNA and the sampling site so that the eDNA can mix in the water column and be detected at any depth, yet short enough distance so that there is minimal loss of eDNA as a result of decay and sedimentation. Wacker et al. (2019) found that eDNA from a large mussel aggregation (1,000 individuals) could be detected up to 1.7 km downstream with no substantial loss in concentration, although they did hypothesise that this may have been due to smaller

populations along the river contributing fresh eDNA. However, other studies showed very mixed results, with both variable distances at which eDNA can be detected (Deiner et al., 2016; Nukazawa et al., 2018; Pilliod et al., 2014; Pont et al., 2018; Stoeckle et al., 2016), and significant changes in eDNA concentration as stream water is transported downstream (Jane et al., 2015a; Sansom & Sassoubre, 2017; Tillotson et al., 2018; Wilcox et al., 2016). While there have been reports of freshwater mussels being detected no more than 50 metres downstream (Pilliod et al., 2014), there has also been a study which detected freshwater mussel eDNA up to 100 km downstream (Pont et al., 2018). There is a debate over whether freshwater mussel eDNA is stable in concentration downstream: studies have reported decreasing concentration (Nukazawa et al., 2018; Tillotson et al., 2018) downstream, whereas other studies have reported stable concentrations over 3 km (Sansom & Sassoubre, 2017) and 9 km (Deiner et al., 2016), which supports Wacker et al. (2019). These differences could be attributed to differing methodology, study species, discharge, gradient, or stream sediment composition, and emphasise the need for a standardised method.

2.5 eDNA Detection as a Molecular Technique

2.5.1 Comparison Between Digital PCR and Quantitative PCR

Polymerase Chain Reaction (PCR) is a widely used technique utilised in molecular biology to selectively amplify a specific segment of DNA to create an exponentially greater number of DNA copies using oligonucleotide primers, polymerase enzymes, and deoxyribonucleotides (Garibyan, 2013). Quantitative PCR (qPCR) is the most common PCR technique used to detect a species of interest and estimates the concentration of DNA amplicons in real time by detection of a fluorescent signal (Hindson et al., 2011), allowing for rapid quantification of a single species (Rees et al., 2014; Wood et al., 2013).

However, the quantification is relative to an internal calibration standard and not absolute (Quan et al., 2018). Alongside qPCR, digital PCR (dPCR) is another common method utilised to detect species of interest and enables absolute quantification of target DNA in a sample by partitioning a PCR reaction mix into thousands of sub-reactions (Quan et al., 2018). The two more common dPCR platforms are droplet-digital (ddPCR) and QuantStudio dPCR, although there are currently six commercialised dPCR platforms available (Dong et al., 2015). There are several differences between the techniques which are summarised in Table 2-1.

The dPCR reaction mix, which often includes the genus-specific primers and species-specific probes to detect and amplify DNA of individual species, is spread evenly on a single dPCR chip or within water-oil immersion droplets so that the 20,000 wells or droplets can undergo discrete and individual sub-reactions (Hindson et al., 2011). The partitioning of DNA targets is done so that some sub-reactions contain target DNA for amplification while others do not, and of the sub-reactions that do contain target DNA, there is likely only one copy (Hindson et al., 2011). After the PCR cycles are completed, each sub-reaction is analysed independently to generate a ratio of positive to negative signal detectable by fluorescence that enables statistically defined accuracy (Dube et al., 2008; Quan et al., 2018; Whale et al., 2013). The randomly distributed positive and negative signals follow a Poisson distribution, enabling the initial target template concentration to be calculated (Hindson et al., 2011). To quantify the positive signals, fluorescently labelled Taqman probes made up of a specific sequence of the desired PCR product and a fluorescent reporter molecule are utilised (Quan et al., 2018). When a successful amplification occurs, the probe attaches to the amplicon which causes the fluorescent reporter molecule to become cleaved so that it can fluoresce and be detected as a positive signal (Hindson et al., 2011).

Table 2-1. Comparison of polymerase chain reaction methods used for detection of aquatic species' eDNA - updated from Wood et al. (2013).

	Quantitative PCR	Digital PCR
Quantification	Relative – internal reference/standard	Absolute
Measurement	Real-time fluorescence detection	End-point positive/negative fluorescent detection
Reproducibility	Reliant on standard	Reproducible
Inhibition	Sensitive to inhibitors	Tolerant to inhibitors
Specificity	Primer + probe Highly specific	Primer + probe Highly specific
Detection level	Low	Very low
Time	Rapid diagnostic	Rapid diagnostic

qPCR assumes that amplification efficiencies of the sample and standard are equivalent, although this is not always the case leading to inaccurate results (Svec et al., 2015). In contrast, dPCR enables absolute quantification of the DNA template because it is based on binomial statistics that statistically define its accuracy (Quan et al., 2018) and alleviate shortcomings of qPCR (Kalinina et al., 1997; Sykes et al., 1992; Vogelstein & Kinzler, 1999). dPCR is more resilient to a diverse range of inhibitors that bind to DNA or polymerase to prevent amplification, because partitioning of the sample dilutes inhibitors so they are less likely to affect individual PCR (Dingle et al., 2013; Nixon et al., 2014; Rački et al., 2014; Taylor et al., 2017; Zhao et al., 2016). dPCR quantifies the targets at the end, while qPCR is quantified in real-time. The efficiency of qPCR amplification can vary between samples due to minor delays at the start of amplification if targets are limited or hard to access (Hindson et al., 2011). Meanwhile, because dPCR partitions the targets into sub-reactions, the delay in amplification due to accessibility of the target does not affect the end-point positive signal produced, making quantification via dPCR more reliable than qPCR (Hindson et al., 2011).

Another advantage of dPCR for environmental samples is that dPCR can accept more eDNA template per reaction (Hindson et al., 2011). eDNA is often used to detect an invasive, rare, or elusive species which means the target DNA is a relatively low percentage of the total DNA in the sample. To account for this, more DNA should be tested to reduce the chance of a false negative result, but too much DNA inhibits the reaction. dPCR reactions divide the total reaction volume into thousands of sub-reactions so more eDNA template can be added: 0.6-1.6 template copies per QuantStudio well is recommended so more eDNA can be analysed than qPCR without inhibition (Hindson et al., 2011). A greater amount of eDNA (containing target and non-target eDNA that acts as an inhibitor) can be analysed in a single reaction due to dilution into sub-reactions. Since most sub-reactions have no target DNA, the ratio of target DNA to inhibitor is higher in the positive reactions, thus reducing the effect of inhibition. Overall, dPCR is advantageous over qPCR for environmental samples so will be used in this study.

2.5.2 Marker Gene Detection

A sequence of a marker gene that is unique to the individual species of interest is required for species-specific identification of target eDNA (Wood et al., 2013). By targeting conserved regions of DNA within a species genome, the primer only anneals and amplifies species-specific DNA, not the variable DNA of other species (Pentinsaari et al., 2016). Because environmental samples could have many hundreds of different species' DNA present, while only one or a few species may be of interest, specific primers reduce the noise of other species' DNA and allow greater detection. Mitochondrial DNA (mtDNA) is favoured for species detection due to its rapid mutation rate which creates sequence divergence that is detectable between even closely related species (Hebert et al., 2003). More importantly, mtDNA is appropriate for eDNA detection because there are many more copies of mtDNA than nuclear DNA per cell, resulting in a greater amount of

mtDNA in the environment available for detection (Rees et al., 2014). There may be up to 1000 mitochondria present in each cell, and within each mitochondrion, there may be 2-10 copies (Robin & Wong, 1988). This extensive variation in mitochondria abundance reflects the energy demand of each cell, which can exist within different tissue types with differing energetic responsibilities (Montier et al., 2009). There is no reported copy number of mtDNA in freshwater mussel species, although it can be assumed that the number varies greatly due to energy demand of different tissue types. The most common marker gene for animals is the mitochondrial cytochrome c oxidase I (COI) (Hebert et al., 2003), which was chosen as the marker gene for kākahi eDNA detection.

However, this also means that the abundance of detectable kākahi eDNA cannot be used to accurately infer the abundance of live kākahi due to mtDNA (detectable as eDNA) and live kākahi ratios being nonsynonymous. Previous studies have reported successful presence/absence detection of species using eDNA (Ikeda et al., 2016; Jerde et al., 2011; Pilliod et al., 2013; Takahara et al., 2012), which infers species richness. However, biomass and abundance estimates provide greater information by quantifying the species present in a population (Sansom & Sassoubre, 2017). There have been several studies which have used eDNA concentrations to infer abundance using mesocosm experiments and water sampling in conjunction with conventional surveying (Doi et al., 2015; Kelly et al., 2014; Pilliod et al., 2013; Port et al., 2016; Sassoubre et al., 2016; Takahara et al., 2012; Thomsen et al., 2012; Wilcox, McKelvey, et al., 2015). However, no study has stated that eDNA detection alone can infer species abundance; a combination of mesocosm lab experiments (investigating eDNA shedding, decay, and deposition rates) and field sampling may be required to accurately quantify abundance (Sansom & Sassoubre, 2017).

2.5.3 Previous Development

The general methodology required to detect kākahi eDNA via dPCR was previously developed by Hu (2017). This included the development of genus-specific primers and species-specific TaqMan probes to selectively amplify and detect *E. aucklandica*, *E. menziesii,* and *E. onekaka*. However, the methodology was only validated using gDNA (genomic DNA extracted from kākahi tissue) and PCR amplicons. Further validation was required to confirm the reliability and accuracy of results and to test if the technique could detect kākahi eDNA from filtered water. After outlining the findings by Hu (2017) below, the questions remaining to be investigated are expanded on.

Genus-specific primers (57F-322R) were designed to selectively amplify a 265-bp sequence of kākahi mtDNA COI genes. This was done by aligning all available *Echyridella* COI gene sequences from GenBank and selecting two separate base sequences (forward and reverse primer sequences) that were common between all three kākahi species. The sequence of the 57F primer is GAGTTGGGGCAGCCTG (5'-3'), while the sequence of the 322R primer is ACAGTYCACCCAGTCCCAA (5'-3'). A Primer-BLAST test for the 57-322R primer pair found that these primers have three matches for possible non-target species amplification of other freshwater mussel species (*Triplodon corrugatus, Hyridella australis,* and *Diplodon deceptus*). However, these species are found only in South America or Australia, so would not affect detection of kākahi in New Zealand streams. There were further unintended target species, but they were from snails, clams, spiders and worms which were not known to be present in New Zealand. The primers were tested by conducting PCR using kākahi gDNA followed by gel electrophoresis and sequencing, which validated that amplification of the three kākahi species was successful using the designed 57F-322R primers.

Species-specific TaqMan probes were then designed, which allow detection of the three kākahi species independently. Separate probes for each species were developed using Geneious by utilising divergent sequences within the region amplified by the 57F-322R primers. Table 2-2 shows the primer-probe combination used to selectively amplify each species' DNA, and the reporter dye used to fluorescently detect it.

Table 2-2. Primer probe pair and reporter dye in species-specific kākahi digital PCR assays (Hu, 2017).

Target species	Primer pair	Probe	Probe sequence (5'-3')	Reporter dye
E. aucklandica	57F-322R	A251P	CCAGCGTTATTCTTGTTGTTAA (antisense)	VIC
E. menziesii	57F-322R	M165P	CAATCATTATAGGCATCACC (sense)	FAM
E. onekaka	57F-322R	O211P	TCCGGAGCACCCAACATCAAAGGA (antisense)	FAM

When the general method set out by Hu (2017) was reproduced for this study, it worked as expected. However, further experimentation was required to ensure that the method could be utilised effectively in a field study. A robust analysis was required to determine the lowest concentration of kākahi DNA that could reliably be detected. It was evident that kākahi gDNA (genomic DNA) could be amplified effectively, but no testing had been done on eDNA which likely contain target sequences at much lower concentration; confirmation that sufficient eDNA for dPCR detection was produced by kākahi in a closed system (aquaculture tank) was required, which would also allow testing of the filtration methodology. The decay rate of kākahi eDNA needed to be determined to ensure that after collection and transport of field samples, the water would still accurately represent the field concentration. And finally, after ensuring the entire methodology worked effectively to detect accurate and reliable kākahi eDNA concentrations in a tank study, testing was required to ensure that field samples contained enough detectable eDNA

regardless of flow, mixing, sedimentation, and other hydrological factors that disperse eDNA. After investigating these points (Chapter 3), the methodology could be implemented in the field (Chapter 4) with assurance of reliable results.

2.6 DNA Extraction Methodologies for Freshwater Mussel eDNA

Many iterations of molecular techniques have evolved to meet specific method requirements that differ in efficiency, time, cost, throughput, and sample specificity. As a result, the method used to answer a research question may diverge while the output is the same. An extensive search was done in June 2020 for studies that utilised eDNA to detect freshwater mussels via PCR, which uncovered twelve papers that were published between 2013 and 2020. Half of these papers were published in the past two years, suggesting that the use of this technique for freshwater mussel species is a current topic of global interest.

It must be noted that most studies had differing research focuses, such as shedding and decay rate modelling (Sansom & Sassoubre, 2017), the distance of signal transport and seasonality of eDNA detection (Wacker et al., 2019), conservation management (Stoeckle et al., 2016), and invasive species tracking (De Ventura et al., 2017). Alongside differences in research aims, the studies focused on a range of freshwater mussel species, river types, countries, and scales. As a result, the methodologies reflect preferences driven by these factors. The most important differences in the methodological approach are summarised in Table 2-3. By selecting the appropriate and desirable attributes of each of these protocols, a standardised approach for eDNA detection of kākahi can be created. This methodology could then be applied to eDNA detection of many aquatic species within New Zealand freshwater systems.

Table 2-3. A summary of freshwater mussel eDNA detection experimental designs implemented in previous studies.

Sample collection	Negative controls	Water preservation	Filtration	eDNA extraction	PCR type	Reference
Volume: 0.5 L	None	Transported on ice, filtered <48 hrs.	Polycarbonate 0.5 µm	CTAB method	Conventional PCR	Egan et al. (2013)
Volume: 2 L Replication: x 3 Depth: Mid depth Position: Mid river	Lab: 3 x 2 L distilled waters filtered	Transported on ice, stored <4°C. Filtered <6 hrs.	Glass fibre 0.22 µm	DNeasy Blood and Tissue Kit (Qiagen)	qPCR	Stoeckle et al. (2016)
Volume: 1 L Replication: x 3 Depth: 1 m	Field: 1 x 1 L distilled water transported with samples. Lab: Filtration, extraction, and qPCR	n/a – filtered on site	Glass fibre 0.7 µm	DNeasy Blood and Tissue Kit (Qiagen)	qPCR	De Ventura et al. (2017)
Volume: 2 L Replication: x 2-8	Field: 1 x 2 L distilled water bottle opened in field, closed and submerged in stream. Lab: Extraction and qPCR	Transported on ice, stored <4°C. Filtered <24 hrs.	Glass fibre 1.5 µm	DNeasy Blood and Tissue Kit (Qiagen)	qPCR	Gingera et al. (2017)
Volume: 0.25 L Replication: x 3	Field: 2 x 0.25 L distilled waters transported with samples Laboratory: qPCR	Transported on ice, stored <4°C. Filtered <12 hrs.	Cellulose acetate 0.45 µm	PowerWater DNA isolation kit (Qiagen)	qPCR	Xia et al. (2018)
Volume: 1 L Replication: x 2 Depth: Pair of surface/subsurface	Field: 1 x 1 L distilled water transported with samples Lab: qPCR	Transported on ice (<10 hrs), stored <4°C. Filtered <24 hrs.	Glass fibre 1.2 µm	PowerWater DNA Isolation kit (Qiagen)	qPCR	Currier et al. (2018)
Volume: 500 mL Replication: x 4	None	Treated with flocculant,	n/a Flocculant dissolution	ZR Soil Microbe DNA	qPCR	Schill (2019)

		transport on ice, frozen at -20°C.		MicroPrep procedure		
Volume: 1 L Replication: x 3 Position: 1 x left, middle, right sample	Field: 1 x 1 L distilled waters transported with samples Lab: qPCR	n/a - filtered on site	Glass fibre 0.22, 0.45, 0.8, 1.2 and 2.0 µm - 0.45 µm gave best results.	DNeasy Blood and Tissue kit. (Qiagen)	ddPCR (droplet digital PCR)	Wacker et al. (2019)
Volume: 500 mL Replication: x1 Other notes: No sampling during glochidia release	Field: 1 x 0.5 L distilled waters transported with samples Lab: Extraction and qPCR	Transported on ice, stored <4°C.	Cellulose nitrate 1.2 µm	DNeasy PowerWater Kit (Qiagen)	qPCR	Shogren et al. (2019)
Volume: 50 mL Replication: x10 at different depth	Lab: qPCR	Transported on ice, stored <4°C.	n/a Centrifuged and pellet collected	gMAX Mini Genomic DNA Sample Kit (IBI Scientific).	qPCR	Amberg et al. (2019)
Volume: 3.79 L Replication: x3 Other notes: Surface	Field: 1 x 3.79 L distilled waters transported with samples Lab: Extraction and qPCR	Transported on ice, filtered immediately.	Glass fibre 1.2 µm	Investigator Lyse & Spin Basket Kit (Qiagen), gMAX Mini Genomic DNA Sample Kit (IBI Scientific).	qPCR	Sepulveda et al. (2019)
Volume: 1 L Replication: x4 Other notes: Surface	Field: 1 x 1 L distilled waters transported with samples Lab: Extraction and qPCR	Transported on ice, filtered immediately.	Cellulose nitrate 5.0 µm	DNeasy Blood and Tissue kit (Qiagen)	qPCR	Gasparini et al. (2020)

There was extensive variation in volume, replication, depth, and river condition between methodologies. The most common sample volume was 1 L, although it was demonstrated that volumes as low as 50 mL contained enough eDNA for detection (Amberg et al., 2019). In general, there was very little clarification on the sampling location within the water column or the flow dynamics of the sampled rivers. From the papers which did note sampling location within the water column, there was no common theme. It was confirmed that mussel eDNA could be detected in water samples that were collected from surface, mid-column, and deep-water zones, as well as midstream and stream edge. Because no consensus could be made from reviewed data, preliminary testing was conducted (data not shown), where 1-L water samples from low-flow and high-flow zones within the Ohautira Stream, Waingaro, Waikato (37.762392, 174.98124) were collected and analysed in August 2019. From these samples, it was confirmed that 1 L of stream water contained enough kākahi eDNA for detection and that low-flow areas (e.g., edges of pools and undercut banks) retained more kākahi eDNA than high-flow areas.

Water preservation methods between studies were similar. Every study which filtered the stream waters off-site transported the samples on ice to keep them cool and refrigerated the samples at <4°C until filtration to reduce eDNA degradation. The time between sample collection and filtration ranged from within 6 to 48 hours. Because kākahi eDNA is subject to several previously mentioned decay factors (see Figure 2-1), it is imperative that the sampled water is filtered promptly to minimise alteration in eDNA concentration. To ensure that the decay of kākahi eDNA between sampling and filtration would not affect the accuracy of results, a preliminary degradation study was conducted (see Chapter 3) and confirmed that if a sample was filtered within 24 hours of collection, there was no significant degradation in detectable kākahi eDNA. As the samples would all be collected and filtered on the same day, they were expected to be filtered within 6 hours of collection.

Only two of the studies filtered water on site and used syringe filters that have a much lower capacity than in-lab filter methods. This resulted in many filters being used per sample and an increased chance of cross-contamination in a non-sterile environment. For this reason, in-lab filtration was chosen.

The size of the filter pores can influence the recovery of eDNA in water samples (Wacker et al., 2019). Of the ten methods which used filtration to isolate the eDNA, the filter size ranged from 0.22 µm to 5.0 µm. Wacker et al. (2019) compared filter size when sampling for freshwater pearl mussel and found that 2.0 µm and 0.22 µm pore size recovered significantly lower concentrations of eDNA than 0.45 µm filters, but there was no significant difference in recovery between 0.45, 0.8 or 1.2 µm filters. This suggests that even though different filter sizes were used in each study, this would not significantly contribute to differing eDNA recovery or quantification if the filter pore size was between 0.45 and 1.2 µm. It was decided that 0.5 µm glass fibre filter papers would be utilised in this study because glass fibre filters were the most common material in all twelve studies, and the filter size was very close to the 0.45 µm pore size that Wacker et al. (2019) determined to be the most efficient size, so the effectiveness was expected to be high.

None of the methods mentioned pre-filtration to remove detritus materials that would clog the filter membrane. Shogren et al. (2019) was the only paper which planned the sampling time period around gamete release because at certain times of the season more mussel eDNA would be released into the stream system as sperm or fertilised glochidia. Sampling outside this time avoids an increase in population signal due to seasonal gamete release, which for kākahi can occur days to weeks apart in different streams in the same region (Hanrahan, 2019). It is important to avoid the peak period when glochidia release is expected, which is February for *E. menziesii* (Hanrahan, 2019), to ensure a surge in glochidia does not cause inaccurate comparisons between sites. However, this release can

occur between late October to late March depending on environmental cues (Clearwater et al., 2011; Hanrahan, 2019) so a pre-filtration step (200 µm, determined in Chapter 3) is critical to remove glochidia that may skew results. The prefilter will also remove sediments and detritus which may inhibit downstream lab processes.

All but one study used an extraction kit rather than a more complex extraction method. Using a commercially available kit removes variation within the process and enables a universal strategy that is easy to implement. The DNeasy Blood and Tissue Kit (Qiagen) and DNeasy PowerWater DNA Isolation Kit (Qiagen) were the most commonly used. To ensure consistency the DNeasy PowerWater DNA Isolation Kit (Qiagen) was chosen because this detection method may be used by many scientists in differing labs if it becomes the routine way to detect kākahi throughout New Zealand.

All studies used qPCR except for Wacker et al. (2019), who utilised ddPCR. dPCR has much greater sensitivity and lower detection limit that qPCR so would produce more accurate results (Hindson et al., 2011). Because dPCR offers distinct advantages over qPCR (discussed previously) this is the PCR method that will be used throughout the experiments.

There was no consistency in the type of controls each study had, and two did not mention any controls (Egan et al., 2013; Schill, 2019), reducing the reliability of their results. The most common field-negative/procedural control was a bottle of distilled water taken from the lab, opened on site and transported in the same container as the field samples to determine if there was any species-specific eDNA contamination during the sampling process in the field. This also acts as a procedural control to detect if there is any contamination from PCR amplicons during DNA extraction. A more appropriate field-negative may have been sampling from a location where there is no known presence of

the species of interest, although conventional surveying does not always infer the absence of a species, so this may not be reliable. Although not all studies mentioned a no-template PCR negative control, they are imperative to ensure that there is no cross-contamination between samples during the PCR. Similarly, a positive PCR control is imperative as it reduces the chance of a false negative and confirms that the laboratory protocol does work. A positive PCR control is a sample known to contain DNA from the species of interest to ensure that any negatives are due to DNA concentrations below detection level rather than a failed PCR (Darling & Mahon, 2011).

Field-positive controls (samples from locations of known kākahi populations) will be included in the methodology so that some inference can be made on whether the ratio of conventionally surveyed *E. aucklandica* and *E. menziesii* presence is reflected by the eDNA results. If these field-positive samples contain kākahi eDNA, they will also indicate that any negative or low concentration samples are not a result of methodological or environmental factors within each stream which inhibited the dPCR. A field-negative/procedural control and dPCR negative (no-template) and dPCR positive (gDNA) control will be included for every sampling session and dPCR run to ensure the reliability of results in the present study.

The review was used to determine the final methodology steps (Figure 2-2).

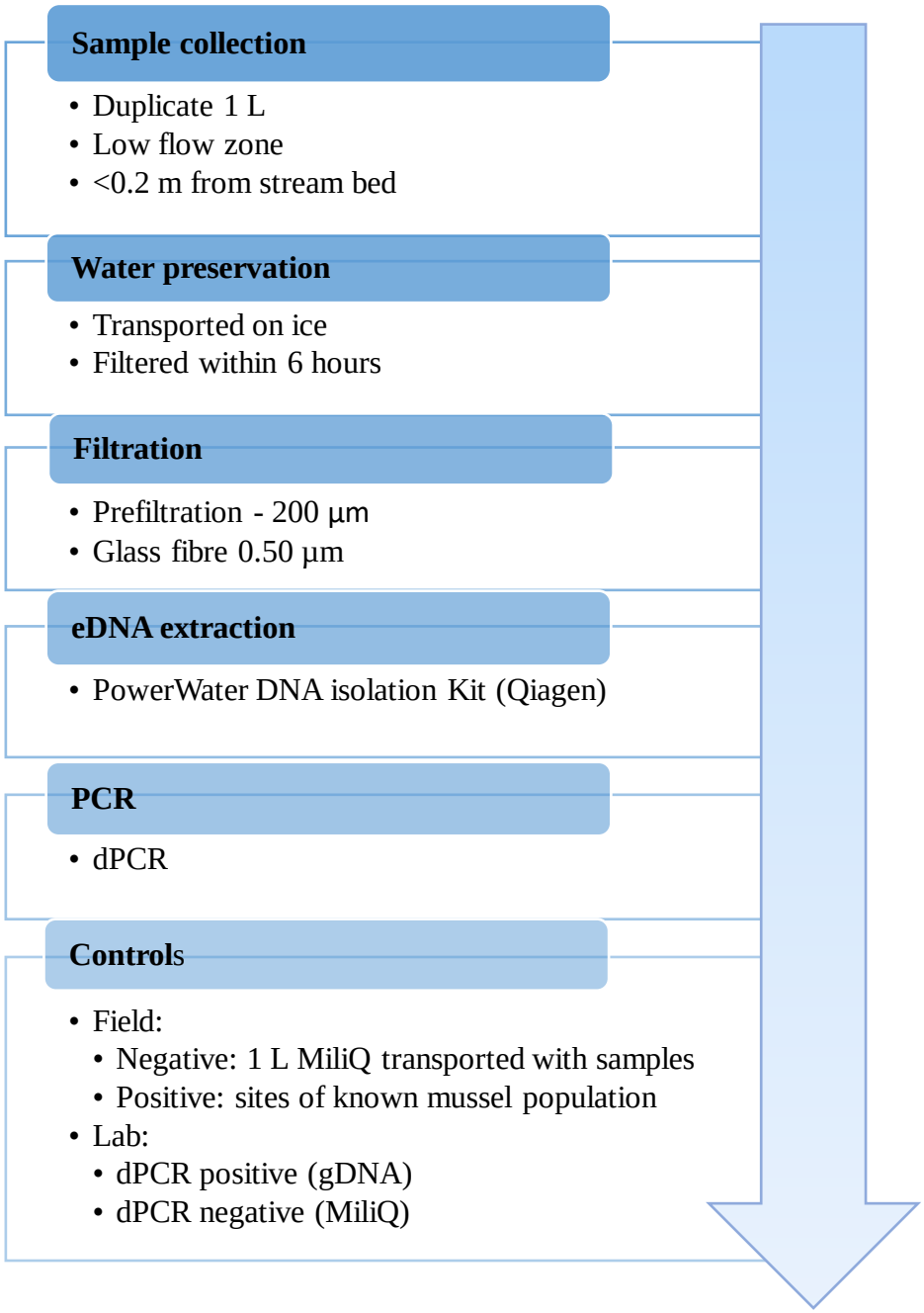

Figure 2-2. The methodological pipeline developed to analyse the eDNA concentration of kākahi from freshwater field samples.

Chapter Three - eDNA Methods Optimisation and Validation

3.1 Introduction

Preliminary testing was conducted before field samples were analysed to ensure that the eDNA collection and lab methodologies were optimised. This guaranteed that assays using the following methods produce reliable results. Although the review of methodologies (Chapter 2) and previous development of the kākahi dPCR assay (Hu, 2017) did determine the majority of the eDNA methodology, some aspects needed to be optimised or validated. This included:

- Lower limit of detection – to infer the lowest concentration of kākahi DNA that could accurately be detected and reported.
- Confirmation of method for eDNA samples – it had only been used to detect genomic DNA (gDNA) or PCR amplicons, not eDNA from filtered water.
- Optimisation of prefiltration – to reduce detritus and glochidia which may inhibit dPCR or skew results.
- eDNA degradation rate – to determine the maximum time before a field sample must be processed and indicate if results reflect historic or current kākahi populations.

3.2 dPCR Assay Performance

3.2.1 Introduction

To ensure reproducible results between previous development (Hu, 2017) and current analysis, preliminary testing using the kākahi dPCR method developed by Hu (2017) was conducted. In addition, the lower limit of detection needed to be determined to infer the lowest concentration of kākahi DNA that could accurately be detected and reported.

3.2.2 Methods

3.2.2.1 Digital PCR Methodology

All dPCR reactions were run using the QuantStudio™ 3D Digital PCR platform (Thermo Fisher Scientific). The dPCR reaction mix differed depending on if it was a single-species dPCR (Table 3-1) or two-species (i.e., duplex) dPCR (Table 3-2), and the volume of DNA differed depending on if it was eDNA or gDNA (Table 3-3). The total dPCR reaction mix was made up to 14.5 µL with MiliQ water. The reaction mix was loaded onto a QuantStudio™ 3D Digital PCR Chip v2 using the QuantStudio™ 3D Digital PCR Chip Loader and PCR was conducted using the ProFlex™ 2x Flat PCR System.

Table 3-1. Reagent volumes and concentrations for the reaction mix for a single species digital PCR analysis.

Reagent	Volume (µL)	Concentration
QS Master Mix	7.25	--
TaqMan probe (single species)	0.363	10 µM
Forward primer	0.625	10 µM
Reverse primer	0.625	10 µM
MiliQ water	to make total volume 14.5 µL	--
DNA	See Table 3-3	--

Table 3-2. Reagent volumes and concentrations for the reaction mix for a duplex digital PCR analysis.

Reagent	Volume (µL)	Concentration
QS Master Mix	7.25	--
TaqMan probe (*E. menziesii*)	0.363	10 µM
TaqMan probe (*E. aucklandica*)	0.363	10 µM
Forward primer	0.625	10 µM
Reverse primer	0.625	10 µM
MiliQ water	to make total volume 14.5 µL	--
DNA	See Table 3-3	--

Table 3-3. DNA volumes added to digital PCR reaction mix.

DNA added	Volume (µL)	Concentration
gDNA (single positive)	1	1 ng/µL
gDNA (duplex positive)	2	1 ng/µL
eDNA (field)	5	unknown

The reactions were all run using the thermal cycling programme specified in Table 3-4. After the thermocycler had finished, the dPCR chips were read using the QuantStudio™ 3D Digital PCR Chip Reader. The reaction mix was shielded from light during creation, chip loading, and chip reading to ensure the photosensitive dye was not prematurely degraded.

Table 3-4. Thermocycler settings for digital PCR analysis.

Stage	Step	Temperature (°C)	Time (mm:ss)
Hold	DNA polymerase activation	96.0	10:00
Cycling (39 cycles)	Anneal/extend	60.0	2:00
	Denature	98.0	0:30
Hold	Final extension	60.0	2:00
Hold	Storage	10.0	∞

Results were processed using the QuantStudio™ 3D Analysis Suite™ and further processed and analysed using Microsoft Excel. The dilution was set as µL of DNA added divided by the total volume (14.5 µL), so differed between single, duplex, and DNA types. The threshold for quantification was manually altered if the auto calculation did not correctly assign positive fluorescent signals generated by probes their correct dye.

3.2.2.2 DNA extraction

Kākahi gDNA was extracted from frozen mussel foot tissue using a Qiagen DNeasy® Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). eDNA was extracted from 0.5 µm filter papers using a Qiagen DNeasy® PowerWater Kit (Mo Bio Laboratories,

Carlsbad, CA, USA). There were no deviations from the method protocol for either extraction.

All extracted gDNA and eDNA samples were quantified using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA) following the recommended protocols for the double-stranded DNA high-sensitivity (dsDNA HS) assay. The DNA quality, purity, and quantity were also analysed using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). After extraction, all DNA samples were stored in a -20 °C freezer until use.

3.2.2.3 Lower Limit of Detection methodology

Extracted gDNA which contained only *E. menziesii* or *E. aucklandica* DNA was amplified via conventional PCR on a DNA Engine® Thermal Cycler (Bio-Rad Laboratories Ltd, CA, USA). The reaction volumes for the reagents in each PCR mix were: 2.5 µL 10 X buffer, 2.5 µL dNTP mix (2 mM), 1.25 µL $MgCl_2$ (50 mM), 0.2 µL Taq Platinum DNA Polymerase (5 U/ µL), 1 µL of each 57F - 322R primer (10 µM), 2 µL gDNA, 14.55 µL MiliQ water. The samples were amplified using the following settings: Incubation at 95ºC for 3 min, 34 cycles of: denaturation at 95ºC for 30 seconds, annealing at 60ºC for 30 seconds, elongation at 72ºC for 30 seconds, followed by a final extension at 72ºC for 5 min. Following amplification, 10 µL PCR products were run in 1% TAE Gels consisting of 0.3 g agarose, 30 mL 1x TAE buffer, and 0.4 µL SyberSafe (10,000X concentrate) under 70 V for 25 minutes.

The bands of the gels were viewed using the Safe Imager™ Blue-Light Transilluminator (Invitrogen), excised using a sterilised blade, and placed into a 1.5 µL tube. The gel band was purified using the UltraClean® 15 DNA Purification Kit (MoBio Laboratories Inc., Carlsbad, USA) and quantified using a Qubit Fluorometer (Life Technologies, Carlsbad,

CA, USA) following the recommended protocols for the double stranded DNA high sensitivity (dsDNA HS) assay. The DNA quality, purity, and quantity were also analysed using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

The user guide of the QuantStudio™ 3D Digital PCR System recommends 0.6-1.6 DNA copies per well, so the target concentration for dPCR analysis should not exceed 32,000 copies per chip. The quantified value from the Qubit Fluorometer and Nanodrop ND-1000 Spectrophotometer was used to calculate the copy number of PCR amplicons in each sample so that an initial dilution of 32,000 copies/µL could be made. The formula below was used to determine the number of copies per microlitre of known PCR product:

$$Number\ of\ \text{copies}/\mu\text{L} = \frac{\text{ng}/\mu\text{L x } 6.022\text{x}10^{23}}{Length\ of\ PCR\ amplicon\ x\ 1x10^{9}\ x\ 650}$$

Further dilutions (3,200, 320, 32, 3.2, 0.32 copies/µL) were created so that there were six dilutions of purified *E. aucklandica* and *E. menziesii* amplicons.

These dilutions were analysed by dPCR (with 1 µL of template DNA) to determine if the actual detected DNA concentration correlated with what was expected. These results allowed the lower Limit of Detection (LoD) to be set for eDNA samples.

3.2.2.4 Control samples

To determine if the serial dilution results were reliable, control samples were analysed alongside the LoD samples. The results are reported in the appendix (Supplementary Table 6-1). The dPCR positive control amplified eDNA for both kākahi species in comparable concentrations. It can be assumed that any samples which amplified low or no eDNA simply had limited eDNA present and the assay worked as expected. The dPCR negative control contained no detectable kākahi eDNA, which suggests there was no

contamination during the dPCR process. These results imply that the dPCR methods are robust and the LoD results are reliable.

3.2.3 Results and Discussion

The PCR method worked as expected on kākahi gDNA samples compared to previous dPCR results (Hu, 2017), assessed by analysis of positive and negative dPCR samples (not reported) to confirm that the method worked effectively and analysis could continue. The number of copies per microliter of known amplicon concentration was determined using the QuantStudio™ 3D AnalysisSuite™ Software, and the expected copies per microlitre were compared to the actual copies per microlitre (Table 3-5).

Table 3-5. The expected and actual kākahi DNA concentration detected by dPCR and the 95% confidence interval (CI) for lower Limit of Detection determination.

DNA Concentration (copies/µL)	1	2	3	4	5	6
Estimated template	32,000.00	3,200.00	320.00	32.00	3.20	0.32
E. aucklandica eDNA	27,813.90	2,594.77	277.00	29.09	2.45	0.79
E. menziesii eDNA	29,156.46	3,163.30	292.55	25.71	3.04	0.83
E. aucklandica eDNA 95% CI	393.00	78.40	12.99	2.06	0.16	0.13
E. menziesii eDNA 95% CI	245.00	54.80	18.08	2.08	0.19	0.19

To better analyse the data, the results were $\log_{10}$ transformed and plotted in Figure 3-1.

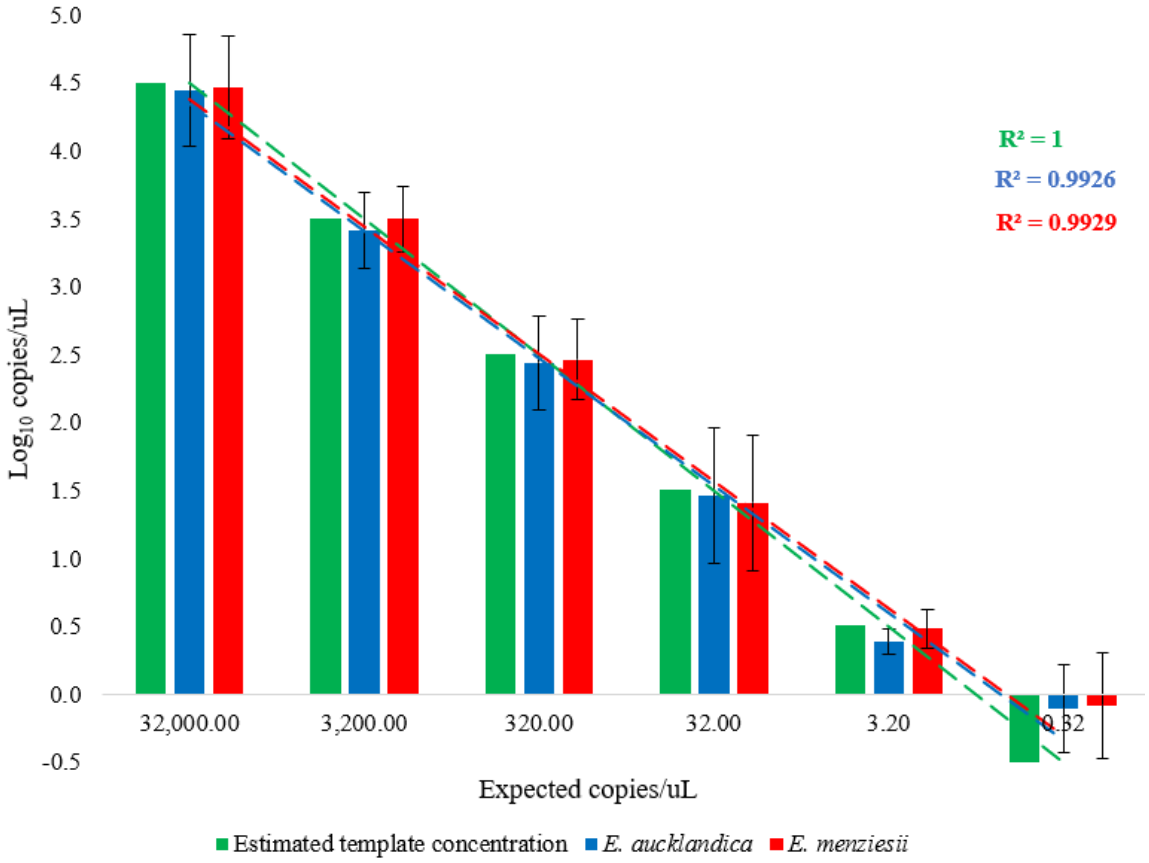

Figure 3-1. Lower limit of detection dPCR concentrations (Log10 copies/L) in a serial dilution. Green depicts the expected DNA concentration, blue and red shows the actual concentration of *E. aucklandica* and *E. menziesii* detected. Error bars depict the 95% confidence interval determined by the QuantStudio™ 3D Analysis Suite™.

The R^2 (coefficient of determination) value is a statistical representation of how close the data is to the fitted regression line and the proportion of variation explained by the independent variable. The 'expected' dataset was determined by mathematical calculation (10-fold dilution series from 32,000 copies/µL) rather than dPCR assay, so the R^2 value is 1. The R^2 value of the *E. aucklandica* (0.9926) and *E. menziesii* (0.9929) regression line is very close to 1, which indicates dPCR results are highly log-linear and accurate.

The 95% confidence intervals for each species were relatively constricted and captured the expected concentration of DNA, except for 0.32 copies/µL. Although the Log_{10} transformed concentrations for each species for the 0.32 copies/µL dilution appear to differ significantly from the calculated estimate, the dPCR results indicate they are around double the estimated concentration (0.83 and 0.79 copies/µL for *E. menziesii* and *E. aucklandica*, respectively). These results suggest that if a sample underwent dPCR, and the detected eDNA concentration was 3.2 copies/µL or greater, the result is an accurate representation of the true concentration. However, if the detected eDNA concentration was between 3.2 and 0.32 copies/µL it would not be accurate but may be used to infer the presence or absence of kākahi.

Because this thesis is aiming to validate a method that can detect the presence or absence of kākahi, the reduced reliability to accurately quantify the copy number at such low levels is not a hindrance. The abundance of detectable kākahi eDNA cannot be used to infer the abundance of live kākahi due to mtDNA (detectable as eDNA) and live kākahi ratios being nonsynonymous. This concept will be further investigated in Chapter 4, by comparing field eDNA concentrations and live mussel abundance.

3.3 Optimising Filtration Methodology for Kākahi eDNA

3.3.1 Introduction

A field water sample may contain many particulates that are suspended in the water column or are resuspended from the sediment due to sampling practice. A reliable method to exclude detritus and reduce unwanted signals from kākahi glochidia eDNA must be devised so that comparisons can be made between samples at differing locations. The field water samples will be filtered through a 0.5 µm glass fibre filter, which was identified as the most reliable pore size for freshwater mussel eDNA capture (Wacker et

al., 2019). The filter must be capable of collecting kākahi eDNA while not clogging easily or collecting excessive detritus or contaminants. (Wilcox, Carim, et al., 2015) identified 1.2 µm pore size filters as being the smallest pore filter that could capture eDNA while simultaneously allowing particulates bound in the water to pass through. However, other studies have noted that clogging can still occur with pore sizes much larger than 1.2 µm, depending on the environmental features and quality of the water (Bruno et al., 2017; Dunker et al., 2016; Turner, Miller, et al., 2014). These particulates can include humic acids bound to soil particles introduced onto the land from fertiliser application which enter streams via runoff (Little et al., 2014). Humic acids can inhibit PCR amplification via many pathways, such as reducing DNA polymerase activity, binding to nucleic acids, and interacting with the fluorescent signal of DNA binding dyes (Jane et al., 2015b; Opel et al., 2010; Sidstedt et al., 2015). Thus, a prefiltration step must be included to remove soil particulates before they become bound to the 0.5 µm filter and have the potential to inhibit the detection of low concentration kākahi eDNA.

Collection of some kākahi eDNA (glochidia) must also be avoided as it creates misleading population signals. Kākahi eDNA may be released into the environment as sloughed tissue (due to filtration), shell material, faeces, and pseudo-faeces (Sansom & Sassoubre, 2017), or as sperm or glochidia during sexual reproduction (Melchior et al., 2019). Unless they are undergoing significant decay, the only large particulate source of kākahi eDNA in the water column would be the glochidia (Wacker et al., 2019). It has been noted that the timing of glochidia release can be extremely varied (Clearwater et al., 2011; Hanrahan, 2019) such that at certain times of the season different streams may have greatly varied kākahi eDNA concentrations regardless of population size. Although it can be roughly predicted that the peak kākahi glochidia release is in February when water temperatures are >18.8 °C (Hanrahan, 2019), release can occur between October to late

March (Clearwater et al., 2011). A method to exclude kākahi glochidia and reduce sudden spikes in kākahi eDNA must be introduced so that comparisons can be made between samples which may have different glochidia release.

3.3.2 Methods

3.3.2.1 Kākahi collection from the field

E. menziesii and *E. aucklandica* were collected by hand on 23 September 2019 from populations in the Ohautira Stream (37°45'44.6"S 174°58'52.5"E), part of the Waingaro catchment near Raglan, Waikato. The kākahi were assessed for species, sex, and reproductive status by gently opening the mussel shell to inspect the brood pouch (described by Melchior et al. (2019)). Two male and two female kākahi of each species (8 total) were collected. Kākahi were kept immersed in stream water during collection, inspection, and transport to the Aquaculture laboratory, University of Waikato, Hamilton.

The kākahi were housed in 2.5 L tanks with 3 cm depth of silica sand as seen in Figure 3-2. Water was gradually transitioned over 4 days from stream water to dechlorinated tap water and was constantly aerated. Every second day, ten percent of the water was removed and replaced by fresh dechlorinated tap water to reduce the build-up of ammonia and other waste products. The tanks were at a constant room temperature of 15 °C and a 16:8 hr light:dark cycle to mimic ambient stream conditions. The kākahi were fed a mixture of Reed Mariculture Nanno 3600 and Mariculture Shellfish diet at around 4700 cells·ml·mussel·day (Ganser et al., 2015).

Figure 3-2. Kākahi housed in 2.5 L tanks releasing eDNA in a closed system before water collection to validate dPCR detection of kākahi eDNA.

After eDNA samples were collected from the mussels for the following experiments, they were removed from their tanks, and their shell lengths were measured using callipers. The kākahi were removed from their shells, and wet weight of their soft tissue was measured. The tissue was placed in a drying oven at 60 °C for 24 hours and then dry weight measured (Supplementary Table 6-2).

3.3.2.2 eDNA prefiltration optimisation

Bulk water from the kākahi tanks was collected after they acclimatised (4 days) and used to determine the optimal pre-filtration method for eDNA recovery. The bulk water was homogenised by inversion, and 1 L was consecutively filtered through a 400, 300, 200, 125, 75, and 47 µm mesh to determine if any of these sizes would collect kākahi eDNA, followed by a final filtration through a 0.5 µm filter which would collect the remaining eDNA. These samples were then extracted following the method outlined in 3.2.2.2 and amplified using the conventional PCR method outlined in 3.2.2.3. Following amplification, PCR products were run in a 1% TAE gel as described in 3.2.2.3

3.3.3 Results and Discussion

The prefiltration of tank water did not result in the collection of kākahi eDNA. None of the filter pore sizes from 400 µm to 47 µm showed amplification of detectable kākahi eDNA during filtration, while the 0.5 µm filter did (Figure 3-3). The positive control, which was run on the gel alongside the filter samples, also produced a band in the expected region of the amplified PCR product (265 bp). This suggests that the prefilters (400 µm to 47 µm) did not collect detectable eDNA that could be amplified and visualised on gels.

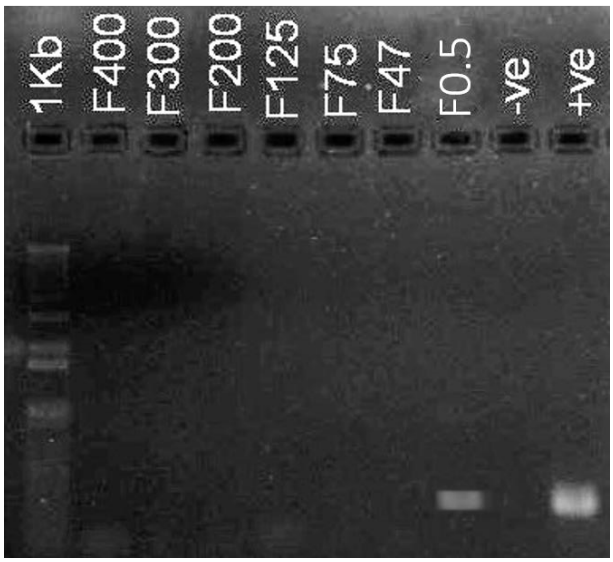

Figure 3-3. The gel electrophoresis image of prefiltration samples of kākahi eDNA tank water. Lane one contains the 1Kb ladder indicating amplicon size, F400 to F0.5 indicate the filter size (400 µm to 0.5 µm), white bands indicate presence of amplified kākahi eDNA.

Analysis was conducted at a time point when no glochidia would be present so that it could be confirmed that kākahi eDNA from filtration and other activities was collected on the 0.5 µm filter and not removed from the sample by prefiltration. The results in Figure 3-3 demonstrates that no large (400 µm to 47 µm) particulate sources of kākahi eDNA were present in the kākahi tank water that would be excluded from the final filter.

E. menziesii produce large glochidia (277 µm) that are released individually bound in mucus threads that float through the water column to entangle passing fish hosts, whereas *E. aucklandica* glochidia are smaller (99 µm) but are released as large functional conglutinates that mimic host diet as an infection strategy (Melchior et al., 2019). By introducing a 200 µm prefilter before the filtration with a 0.5 µm pore size occurs, we expect that glochidia will be excluded from the sample. This 200 µm prefilter will also exclude a large proportion of detritus material and particulates that may inhibit downstream lab methods.

It was determined that eDNA filtration would follow the method outlined below:

Water samples will be filtered using a 0.5 µm pore size glass-fibre filter (Advantec, Japan). Before water is poured into the filtration apparatus (Sartorius D-3400, Germany), they will be pre-filtered through a 200 µm sieve to remove detritus, which may inhibit downstream processes, or glochidia, which may skew results. During filtration, a single 0.5 µm filter will be used for each sample, which will then be carefully folded (filtered side in) and placed in a 1.5 mL tube and labelled for storage in a -20 °C freezer until DNA extraction. Filtration apparatus will be washed with bleach and rinsed with MilliQ water between samples to remove DNA contamination.

3.4 DNA Degradation Over Time

3.4.1 Introduction

Although eDNA may be shed at a very high concentrations, one major challenge is that it degrades over time in freshwater systems (Eichmiller et al., 2016; Pilliod et al., 2014; Sansom & Sassoubre, 2017; Strickler et al., 2015) so the amount detected in a water sample may not represent the actual concentration at the time of sampling. Factors such as temperature, solar radiation, pH, microbial activity, and DNA characteristics can

influence the rate at which eDNA decays (Corinaldesi et al., 2011; Strickler et al., 2015), but the most important factor is the time that eDNA is exposed to the freshwater (Sansom & Sassoubre, 2017). To ensure that the eDNA concentration detected in field samples is an accurate representation of the eDNA concentration of the stream water at the time of sampling, the rate of kākahi eDNA degradation had to be determined. This information would be used to limit the maximum time between collection and filtration/freezing of field samples and ensure that kākahi eDNA could be detected effectively using dPCR in a closed system without extensive hydrodynamic interactions. It could also indicate if results reflect historic or current kākahi populations.

3.4.2 Methods

After the bulk water for the filtration optimisation was collected, the kākahi were kept in their tanks for 96 hours to produce eDNA via filtration and faeces. A bulk container of water was collected from all tanks (1.5 L from each tank) and homogenised by inversion. The container was kept in the dark at 20 °C until filtration to mimic transportation conditions. At 0, 24, 48, 96, and 144 hours, the bulk sample was mixed, and a 1 L sub-sample taken. This sample was prefiltered through a 200 µm sieve then filtered using a 0.5 µm glass fibre filter and stored at -20 °C until eDNA extraction. eDNA was extracted and analysed on the QuantStudio™ 3D Digital PCR platform following the method outlined in 3.2.2.2.

R Studio (v1.3.959) was used for all statistical analyses and all figures were created using either Microsoft Excel or R Studio. Any datasets to be compared were tested for normality and variance to ensure assumptions were met for statistical tests. Shapiro-Wilk tests were conducted to check if the data was normally distributed, and if not, it was log-transformed and retested to ensure it was normal. This was followed by an F-test to determine if the

data had equal variance. The significance level for all statistical tests was 5% (p-value < 0.05). To compare two subsets, a two-sample T-test was conducted on normally distributed data with equal variance, and if the p-value was <0.05, the difference between the subsets was deemed statistically significant.

To determine if the results were reliable, control samples were analysed alongside the eDNA degradation samples. The results are reported in the appendix (Supplementary Table 6-1). The dPCR positive control amplified eDNA for both kākahi species in comparable concentrations. It can be assumed that any samples which amplified low or no eDNA simply had limited eDNA present and the assay worked as expected. The dPCR negative control contained no detectable kākahi eDNA, which suggests there was no contamination during the dPCR process. These results imply that the dPCR methods are robust and the eDNA degradation results are reliable.

3.4.3 Results and Discussion

The change in kākahi eDNA copies per litre over six days demonstrates how kākahi eDNA degrades over time in a closed freshwater system (Figure 3-4).

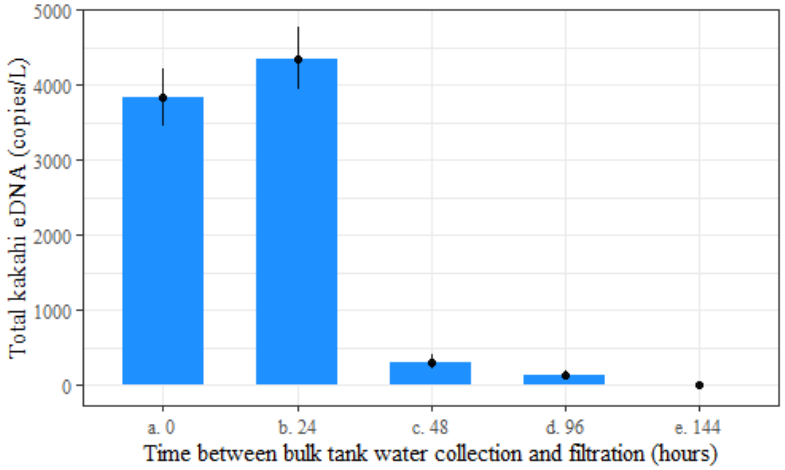

Figure 3-4. Degradation of kākahi eDNA in a closed system over time. Error bars depict the 95% confidence interval determined by the QuantStudio™ 3D Analysis Suite™.

A Shapiro-Wilk normality test was conducted on the total kākahi eDNA copies per litre data which indicated the data was not normally distributed ($p = 0.03652$). The data was subsequently Log_{10} transformed, which a Shapiro-Wilk normality test indicated was now normally distributed ($p = 0.4951$). Figure 3-5 shows the Log_{10} transformed degradation in eDNA copies per litre over six days.

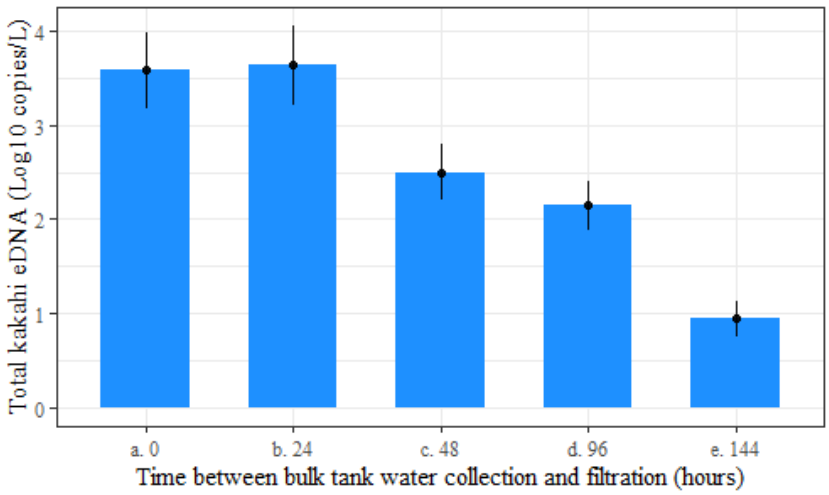

Figure 3-5. Log_{10} transformed degradation of kākahi eDNA in a closed system over time. Error bars depict the 95% confidence interval determined by the QuantStudio™ 3D Analysis Suite™.

Kākahi eDNA was found to degrade in a log-linear trend with a significant reduction between 24 and 48 hours (Figure 3-5). This is consistent with the decay of *Lampsilis siliquoidea* freshwater mussel eDNA, which also decayed in a log-linear trend and fell below detection limits after 168 hours (Sansom & Sassoubre, 2017). The decay rate constant of kākahi eDNA was calculated to be 4.5×10^{-2} per hour, comparable to the decay rate constant of *Lampsilis siliquoidea* freshwater mussel eDNA which ranged from 9.7×10^{-3} to 5.3×10^{-2} per hour. Although this is the only other reported decay rate constant for freshwater mussel eDNA, the kākahi eDNA decay rate constant is also consistent with other studies in freshwater systems such as 1.5×10^{-2} to 0.1 per hour for common carp (*Cyprinus carpio*) (Eichmiller et al., 2016), 6.8×10^{-2} to 7.9×10^{-2} for Idaho giant

salamander (*Dicamptodon aterrimus*) (Pilliod et al., 2014), and 0.2 $x10^{-2}$ to 1.4 $x10^{-2}$ hour for American bullfrog (*Lithobates catesbeianus*) (Strickler et al., 2015).

Between 24 and 48 hours, the concentration of detectable kākahi eDNA underwent more than a 1-log reduction, equivalent to a 92.7% reduction in total eDNA (Figure 3-5). However, between 0 and 24 hours, there was no significant change in detectable kākahi eDNA. This suggests that if a field sample was filtered (and stored or extracted) within 24 hours of collection, the results would be a reliable reflection of the kākahi eDNA that was present in the water column at the time of sampling. Although there was no reason given as to why filtration occurred within a certain timeframe after collection, all papers reviewed in Chapter 2 that filtered the sample water did so within 48 hours. To ensure that the detectable kākahi eDNA results are as accurate as possible to the initial stream concentration, all field samples should be filtered within 24 hours of collection. However, because there is not an extensive number of samples collected in the field study section and transport distance are reasonable, all samples are to be filtered within 6 hours of collection.

Kākahi eDNA was proven to decay rapidly in freshwater, confirming that detected kākahi eDNA is the result of a current population at the time of sampling, not a historic relic of an extinct population that once existed in that location. The detection method can be utilised to investigate the change in population sizes and distribution over time for conservation and management purposes, meeting two of the top 20 global freshwater mussel research priorities (Ferreira-Rodríguez et al., 2019): performing accurate species identification, alongside determining species current distribution.

Chapter Four - Seasonality of Kākahi eDNA and Relationship Between Fish Barriers and Kākahi Distribution

4.1 Introduction

This chapter aims to answer the two main objectives of this study: i) determine the seasonality effect on kākahi eDNA and ii) establish whether eDNA can be used to detect an effect of fish barriers on the distribution of *E. menziesii* and *E. aucklandica* populations. In doing so, the best time period for field sampling can be established to ensure future sampling is done at the most appropriate time for reliable results. Alongside this, we can examine whether the presence/absence of kākahi species may be used to infer the presence/absence of native fish. It is hypothesised that early summer (December) is the best period to collect field samples due to low flow and less dilution of kākahi eDNA, and that the distribution of kākahi species is correlated with the presence of fish barriers due to host fish climbing ability.

4.2 Field Sample Sites

To assess suitable sampling locations, a map of the Waikato region was generated using QGIS (QGIS Development Team, 2020), which depicts kākahi conventional survey field counts and fish barrier data (MFE / NIWA REC1 derived layer) provided by the Waikato Regional Council (WRC) (Figure 4-1). From this, several locations within the Waikato region were identified as being potentially suitable for sampling based on fish barrier and kākahi populations presence in the stream system. These locations were visually surveyed in November 2019 to ensure the fish barrier data (from 2003-2005) was still valid and to determine the most suitable sites for field sampling.

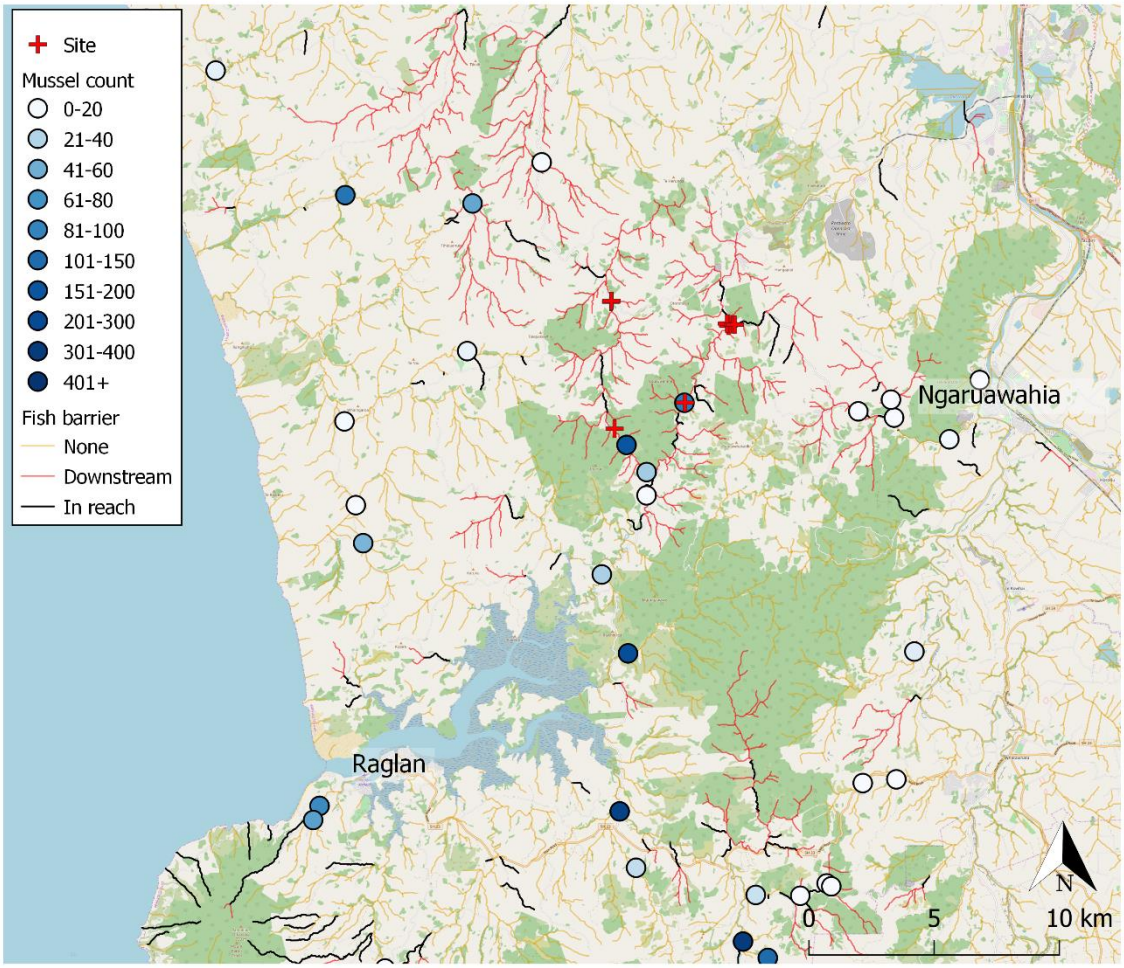

Figure 4-1. Map of north-west Waikato kākahi and fish barrier prevalence. Red crosses indicate sites visually surveyed for sampling suitability; coloured circles indicate the population size of kākahi conventionally surveyed in stream reaches. The fish barrier level of each waterway is indicated by yellow (none), red (downstream) and black (in reach) lines. Native forest is depicted as dark green, plantation forest as light green, and pasture as cream underlay.

Ten sampling sites in the Waingaro River catchment were chosen, including seven sites along Waingaro River (site A-F, J) and three sites (G-I) along Kahuhuru Stream (Figure 4-2). The Waingaro catchment is situated west of Ngaruawahia and flows into the northern arm of the Whaingaroa Harbour (LAWA, 2020). The main two stems include the Waingaro River and Kahuhuru Stream, which are a combined length of 46.6 km and connect with 170 km of tributary streams within an extensive catchment area of 123 km^2. Much of the catchment is steep rolling topography, although Waingaro River and

Kahuhuru Stream pass through pasture, plantation forest (Radiata pine), and indigenous forest (LAWA, 2020). At the time of sampling, livestock farming was the dominant land use for all sites, and most sites were partly shaded with minor riparian planting. However, no sites had riparian fencing, which leaves both Waingaro River and Kahuhuru Stream accessible to wandering livestock that may have contributed to the bank erosion present at most sites.

The study included three sites downstream of fish barriers (A, B, G), two sites upstream of low-flow fish barriers (C, H), and three sites upstream of all-flow fish barriers (D, E, F) (Figure 4-2). Low-flow barriers are a natural or man-made obstruction in the river/stream that inhibits fish passage during months when there are lower water depths, whereas all-flow barriers may obstruct fish passage all year round. Sites I and J were positive control sites with previous surveyed kākahi population data, provided by the WRC. These conventional surveys were conducted by WRC field scientists in January 2018 following the WRC standardised protocol for kākahi monitoring in wadeable streams (Catlin et al., 2017), and located 218 live kākahi at site I (3:1 ratio of *E. menziesii* and *E. aucklandica*) and 97 at site J (7.8:1 ratio of *E. menziesii* and *E. aucklandica*).

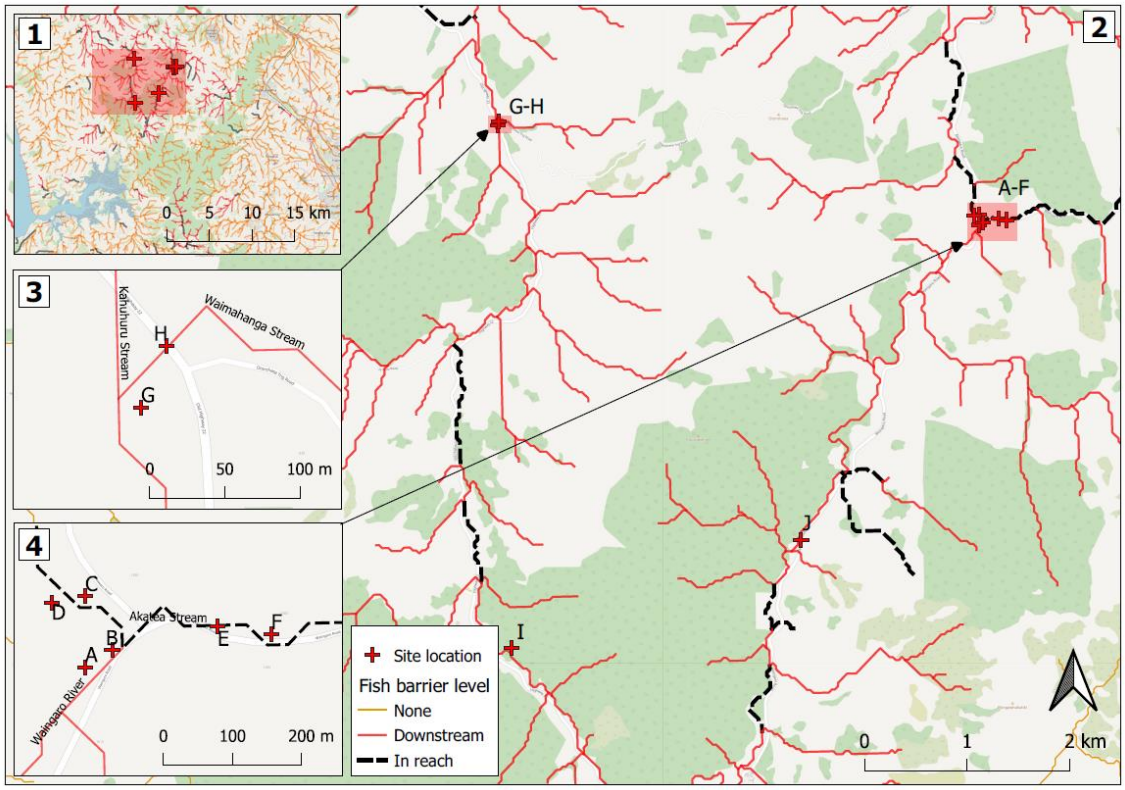

Figure 4-2. Sampling sites (+) for stream water collection in Waingaro, Waikato. Pane 1 shows the location in comparison to Raglan and Ngaruawahia, pane 2 shows all sampling sites relative to each other, pane 3 shows the sampling sites G-H along Kahuhuru Stream, and pane 4 shows sampling sites A-F along Waingaro River. The fish barrier level of each waterway is indicated by yellow (none), red (downstream) and dashed (in reach) lines. Native forest is depicted as dark green, plantation forest as light green, and pasture as cream underlay.

Figure 4-3 depicts each sampling site and conveys the scale of some fish barriers within each reach during high flow. During low flow (summer), the impact of the fish barriers is much greater due to lower water levels, highlighting several natural rock/waterfall barriers which separated the sites along Waingaro River (A-F). Sites A and B were downstream of all fish barriers, while sites C and D were upstream of broken natural (low-flow) fish barriers and sites E and F were upstream of extensive natural (all-flow) fish barriers. A manmade culvert (low-flow) fish barrier separated site G (downstream) and H.

Prior to collecting water samples, an assessment of the qualitative features of each site was undertaken to ensure they were of high enough quality for kākahi to dwell there. The qualitative features of each site are described in Table 4-1, including land use, canopy cover, and channel dimensions. A Rapid Habitat Assessment (Clapcott, 2015) of each site was conducted (Supplementary Table 6-3, Supplementary Figure 6-1), and a resultant Habitat Quality Score (HQS) was calculated by assessing the quality of each site reach for deposited fine sediment, hydraulic heterogeneity, invertebrate habitat diversity and abundance, fish cover diversity and abundance, bank erosion and vegetation, and riparian width and shade. The HQS can range from 1-100, with a higher score indicating greater habitat quality, and is broken into four classes: Poor (1-25), Fair (26-50), Good (50-75), and Excellent (76-100). Indicators of high-quality habitats include aspects such as habitat diversity for fish cover and invertebrates, riparian buffer width, shading, and diversity, and hydraulic heterogeneity. Whereas indicators of low quality habitats include fine deposited sediment and bank erosion (Clapcott, 2015). Six sites were classed as Fair habitat quality (C - E, G - I), four were Good (A, B, F, J), and the average HQS of sites sampled was 46.4, indicating that all sites were adequate habitats for kākahi populations.

Table 4-1. Qualitative features of sampling locations A – J along Waingaro River and Kahuhuru Stream, Waingaro, Waikato.

Site	Stream/river	Fish barrier status	Land use	Canopy cover	Width (m)	Depth (m)	HQ score	Mussels visible
A	Waingaro	Downstream	Pasture	Partly shaded	2.5	0.5-1.5	53	Dead *E. menziesii*
B	Waingaro	Downstream	Pasture	Partly shaded	2.5	0.5	54	E. *menziesii*
C	Waingaro	Upstream – low flow	Pasture	Open	2	1-1.5	48	No
D	Waingaro	Upstream – all flow	Pasture	Partly shaded	2.5	1-1.5	27	No
E	Waingaro	Upstream – all flow	Pasture	Partly shaded	2	0.5	49	No
F	Waingaro	Upstream – all flow	Pasture	Partly shaded	2	0.5	51	No
G	Kahuhuru	Downstream	Pasture	Partly shaded	2	0.5	39	No
H	Kahuhuru	Upstream – low flow	Pasture	Partly shaded	2.5	0.5	47	No
I	Kahuhuru	n/a - control	Native forest	Partly shaded	2	0.5-1	38	>50 mixed species
J	Waingaro	n/a - control	Pine forest	Significant shade	3	0.5-1	58	No

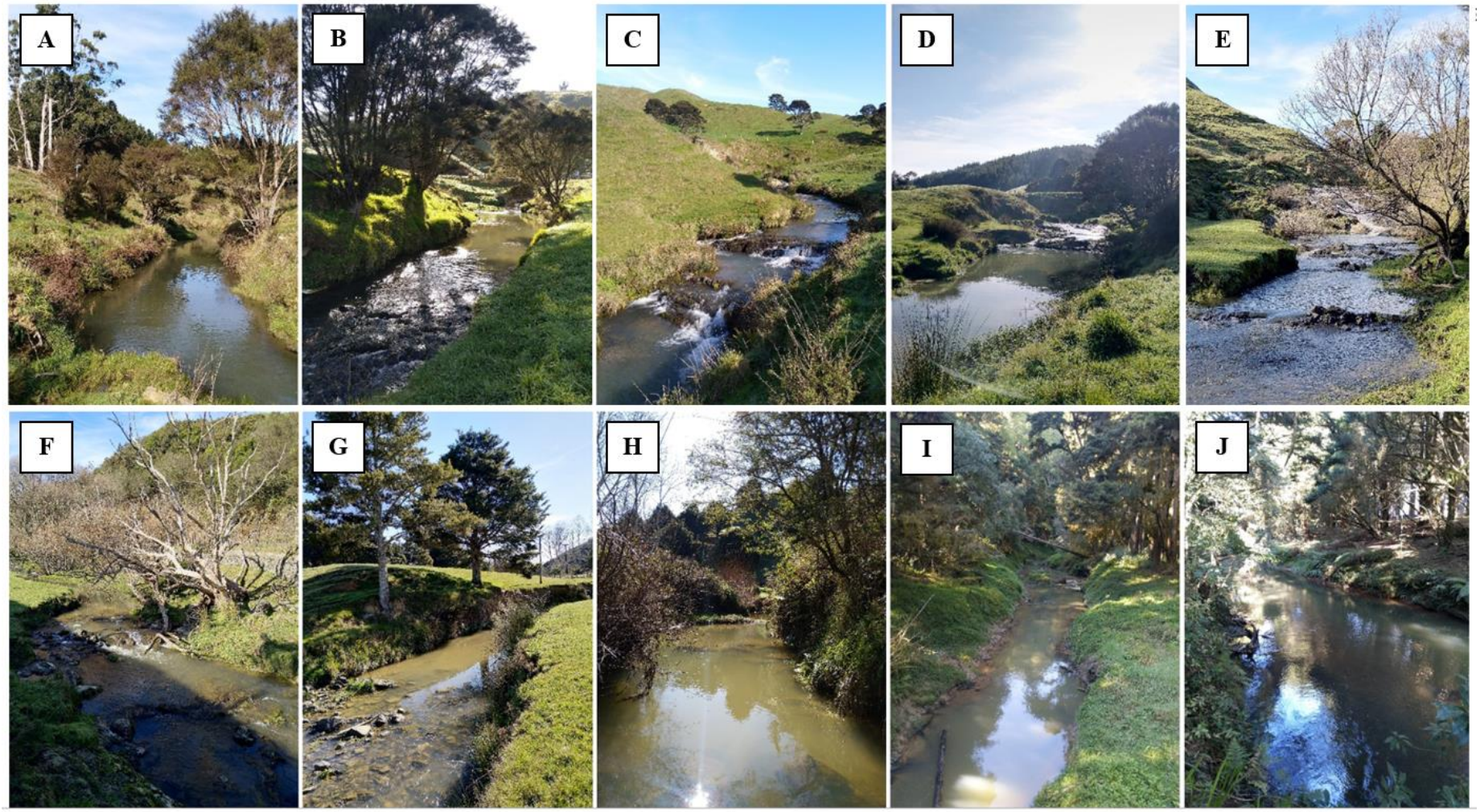

Figure 4-3. Field sample locations along Waingaro River (A-F, J) and Kahuhuru Stream (G-H), Waingaro, Waikato Region, New Zealand.

Pictures were taken in mid-winter, August 2020 (high flow)

4.3 Field Sample eDNA Methodology

Field water samples were collected from each site on three separate occasions in December (summer), May (autumn), and August (winter) to encompass different time points of the kākahi reproductive cycle. The second and third sampling sessions were shifted back by two months from the original plan (March and June) due to COVID-19 restrictions but remained within the reproductive cycle time periods of interest. Unfortunately, sampling could not be done in March as hoped, which may have captured peak glochidia release. Sampling occurred on:

- Friday, December 12th, 2019 (presumed limited glochidia release)
- Friday, May 15th, 2020 (presumed no glochidia/sperm release)
- Wednesday, August 5th, 2020 (presumed sperm release)

Field sampling days were selected to avoid the interference of high rainfall events on eDNA recovery, so sampling was only undertaken if no rain had fallen in the previous 72 hours to ensure that pooled eDNA or kākahi were not washed from the stream before collection could occur. Duplicate 1 L water samples were collected at each site in a zone of low flow or high retention where eDNA may likely concentrate (e.g., pool, curved side of stream) within 0.2 m from the stream bed. Samples were collected from downstream to upstream to reduce the resuspension of sediments that could inhibit dPCR or block the water filter. Sample bottles were pre-bleached and rinsed with MilliQ water to remove any contaminant DNA. A single field-negative/procedural control was collected on each sampling day by filling a 1-L sampling bottle with Milli-Q water in the lab before sampling, which was taken into the field and opened on site and stored with all other sample bottles. Sample bottles were transported together in a chiller box on ice until filtration in the lab, which occurred within 6 hours of sampling to reduce the degradation of the kākahi eDNA.

Filtration, eDNA extraction, and dPCR analysis of each field sample followed the methodology described in Chapter 3. The dPCR mix included 5 μL of eDNA per chip. Two dPCR controls were included: a positive control using 1 μL of *E. aucklandica* and *E. menziesii* gDNA, and a negative control containing no template. If either of these controls failed (no amplification in positive or amplification in negative), the dPCR results for any samples analysed in the same set were discarded.

4.3.1 Statistical Analysis

R Studio (v1.3.959) was used for all statistical analyses, and figures were created using either Microsoft Excel or R Studio. Any datasets to be compared were tested for normality (Shapiro-Wilk test) and variance (F-test) to ensure assumptions were met for statistical tests with a significance level for all statistical tests of 5% (p-value < 0.05). To compare two subsets, a two-sample T-test was conducted on normally distributed data with equal variance. If multiple subsets were compared, a two-way ANOVA was conducted, followed by a post-hoc TukeyHSD test to determine which sets differed.

4.3.2 Controls

To ensure that the results were reliable, a field-negative/procedural control and dPCR positive and negative sample were analysed alongside the field samples. All controls amplified as expected (Supplementary Table 6-4), suggesting the field samples results were a reliable reflection of actual stream kākahi eDNA concentration. Locations with known kākahi populations were included as field-positive controls (site I and J). Regardless of the month, each field-positive sample contained detectable kākahi eDNA for both *E. aucklandica* and *E. menziesii* (see section 4.4) which implies that any samples with no or low detection of kākahi eDNA were not a result of methodological or environmental factors within each stream inhibiting eDNA detection.

4.4 Results

4.4.1 Site Replication and Statistical Analysis

At each sampling site, duplicate 1 L water samples were taken to reduce the chance of downstream error for a single sample affecting the resulting comparisons. Statistical analysis of the December samples was conducted to confirm if using an average of these duplicates would precisely reflect the site values. After meeting the assumptions of the two-sample T-tests (using Shapiro-Wilk normality tests and F-tests for variance), the results showed no statistically significant difference between the duplicate eDNA data sets for *E. aucklandica* ($p = 0.9571$), *E. menziesii* ($p = 0.8933$), or total kākahi ($p = 0.9069$) eDNA concentration. This confirmed that the duplicates were precise. From this point on, an average of site duplicates was used for all statistical analysis and considered a precise reflection of the sampled results for each site. All statistical assumptions were met (normal distribution and equal variation $p = >0.05$) for data subsets, grouped by season, species, fish barrier status, and total kākahi eDNA, so further statistical analysis could be conducted.

4.4.2 Environmental Monitoring Data

Rainfall, depth, flow, and water temperature records for Waingaro River was obtained from a location downstream of where Kahuhuru Stream joins Waingaro River. This site is approximately 8.5 km downstream from the control sites in both Kahuhuru Stream (site I) and Waingaro River (site J), producing environmental data that is reflective of both stream systems within the Waingaro catchment. This environmental data (Table 4-2) was sourced from the WRC Environmental Monitoring dataset, which is accessible online or via request (http://waikatoregion.govt.nz) for site 1167-4.

Table 4-2. Environmental monitoring data for the Waingaro River on the day and month of sampling.

	Rainfall total (mm)		Depth average (m)		Flow average (m^3/sec)		Water temperature average (°C)	
	Day	Month	Day	Month	Day	Month	Day	Month
12/12/19	0.00	94.0	0.65	0.80	0.352	0.854	17.2	17.2
15/05/20	0.00	120.0	0.55	0.62	0.161	0.325	12.8	12.5
05/08/20	0.00	216.5	0.81	1.18	0.585	2.296	8.9	9.2

There was no rainfall in the 72 hours prior to sampling (meeting WRC fieldwork guidelines) to ensure that any retained eDNA was not washed out of the system. As a result, the daily flow average for each sampling date was lower than the monthly average. The average river flow was lowest in May (0.161 m^3/sec), moderate in December (0.352 m^3/sec), and considerably higher in August (0.585 m^3/sec). The river flow in May was around a third of August, and about half the flow of December. The average depth was greatest in August, followed by December and May.

4.4.3 Comparison Between Kākahi Species and Season of Sampling

The difference in eDNA concentrations detected in December, May, and August were graphed, depicting the proportion of total kākahi eDNA that was *E. aucklandica* or *E. menziesii* (Figure 4-4).

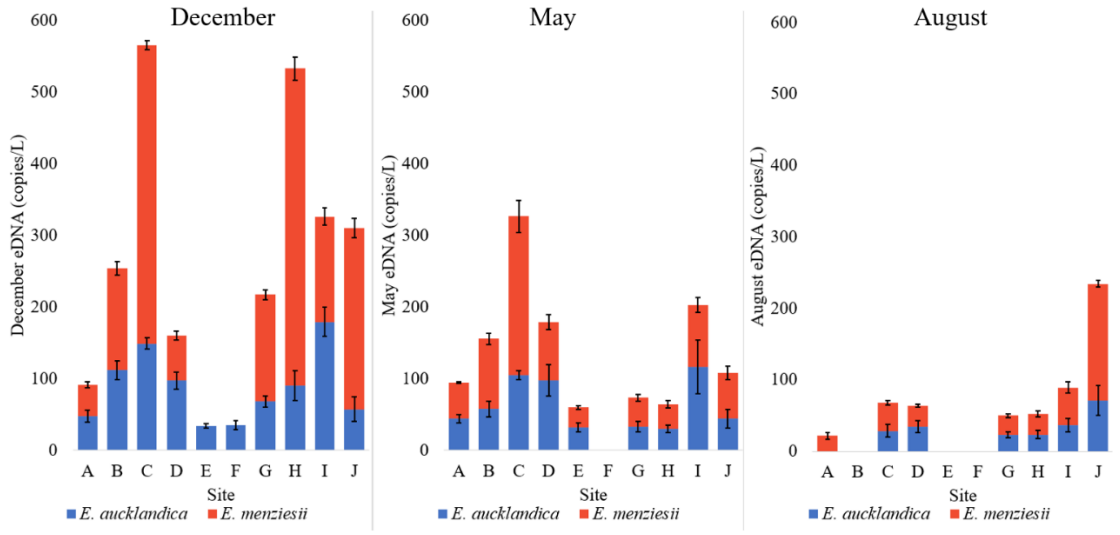

Figure 4-4. The total kākahi eDNA detected at each site in December (left), May (centre), and August (right) depicting the proportion of *E. aucklandica* (blue) and *E. menziesii* (red) eDNA. Error bars depict the 95% confidence interval determined by the QuantStudio™.

To investigate the effect that the month of sampling had on the detectable eDNA concentration of *E. aucklandica* and *E. menziesii*, a two-way ANOVA test was conducted on the kākahi eDNA data, grouped by species and season. Sites upstream of all-flow barriers were excluded so that only sites with expected kākahi eDNA could contribute to the statistical analysis. Neither species had an eDNA concentration that was consistently higher than the other ($p = 0.078$), and there was no interaction effect between species and season on the detectable eDNA ($p = 0.323$). However, there was a statistically significant difference in eDNA concentration due to the sampling season ($p = 0.0008$).

To investigate the seasonal effect further, a TukeyHSD test was conducted to determine which seasons were significantly different. The p-values for this analysis are shown in Table 4-3, which indicates a significant difference in eDNA concentration between December and May ($p = 0.033$) and December and August ($p = 0.001$). By combining the graphed results and statistical tests, it can be concluded that the kākahi eDNA concentration in December was significantly higher than in May and August.

Table 4-3. Tukey HSD p-values for the effect of sampling season on kākahi eDNA concentrations. Data for all sites except upstream all-flow barriers were included in the analysis. Green indicates a statistically significant difference, while orange is not significant at P<0.05.

ANOVA p-value = <0.001	December	May	August
December		0.033	0.001
May	0.033		0.345
August	0.001	0.345	

The total kākahi eDNA (sum of all seasons) detected for each site was then graphed, depicting the proportion of eDNA that was *E. aucklandica* or *E. menziesii* (Figure 4-5). Conventional survey data were included to allow comparisons between eDNA detection of each species and physical surveying and indicated a higher number per 50 m reach of live *E. menziesii* were present at sites I and J (164, 86 respectively) in comparison to live *E. aucklandica* (54, 11 respectively). The eDNA concentration of each species at site J correlates with the relative abundance of the conventional survey count. However, at site I there is no correlation. It is important to note that the conventional survey data were collected by WRC field scientists in January 2018, so population densities may have since changed.

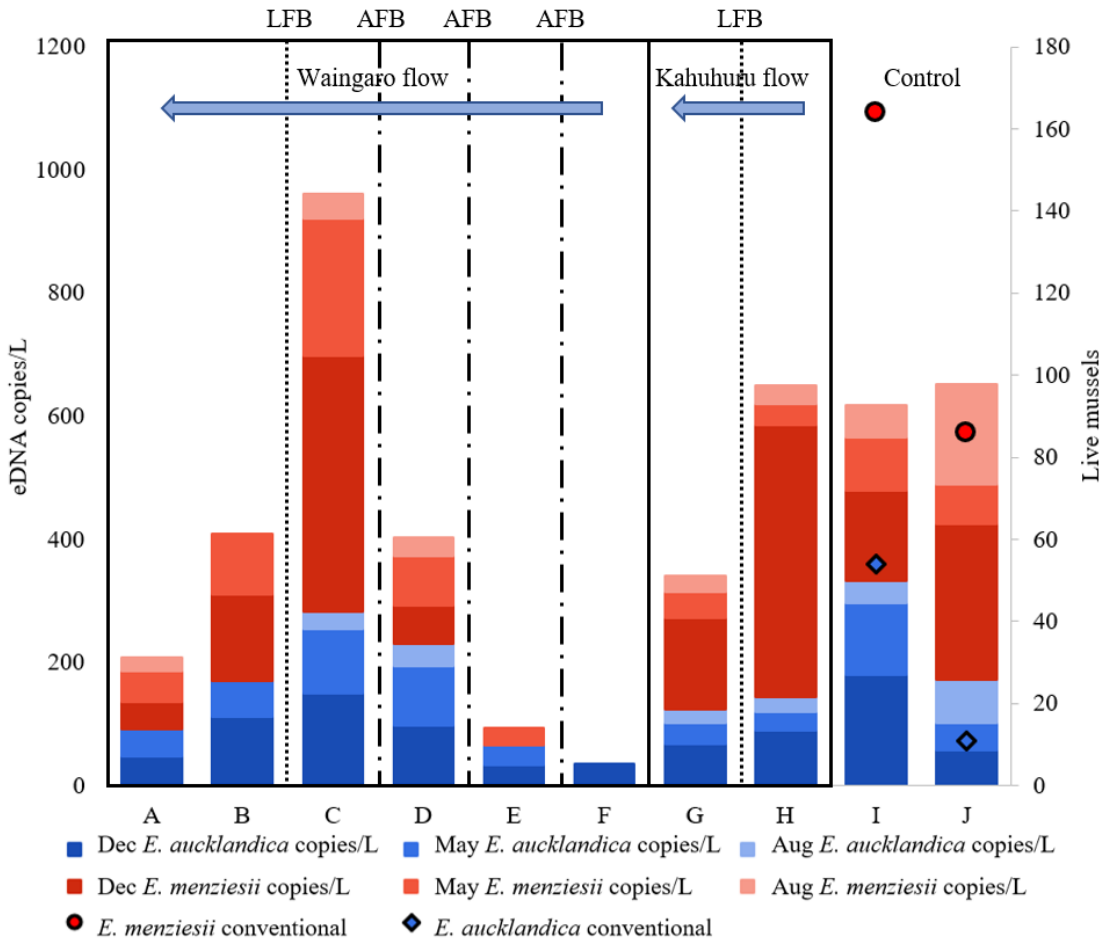

Figure 4-5. Total kākahi eDNA detected at each site depicting the proportion of *E. aucklandica* (blue) and *E. menziesii* (red) eDNA. The gradient change in each colour indicates the season the eDNA was collected, from darkest (December) to lightest (August). The points at site I and J indicate the number of *E. aucklandica* (◆) and *E. menziesii* (●) observed in a conventional survey by the Waikato Regional Council. Solid borders depict the sites within the same catchment and blue arrow shows the direction of water flow, dotted lines indicate low-flow barriers (LFB) while dash-dot lines indicate all-flow barriers (AFB).

4.4.4 Comparison of Sampling Season, Fish Barrier Status, and Kākahi Species

The concentrations of detected kākahi eDNA were graphed, depicting the proportion of eDNA detected in December, May, or August, separated into sub-clusters of each location (Figure 4-6).

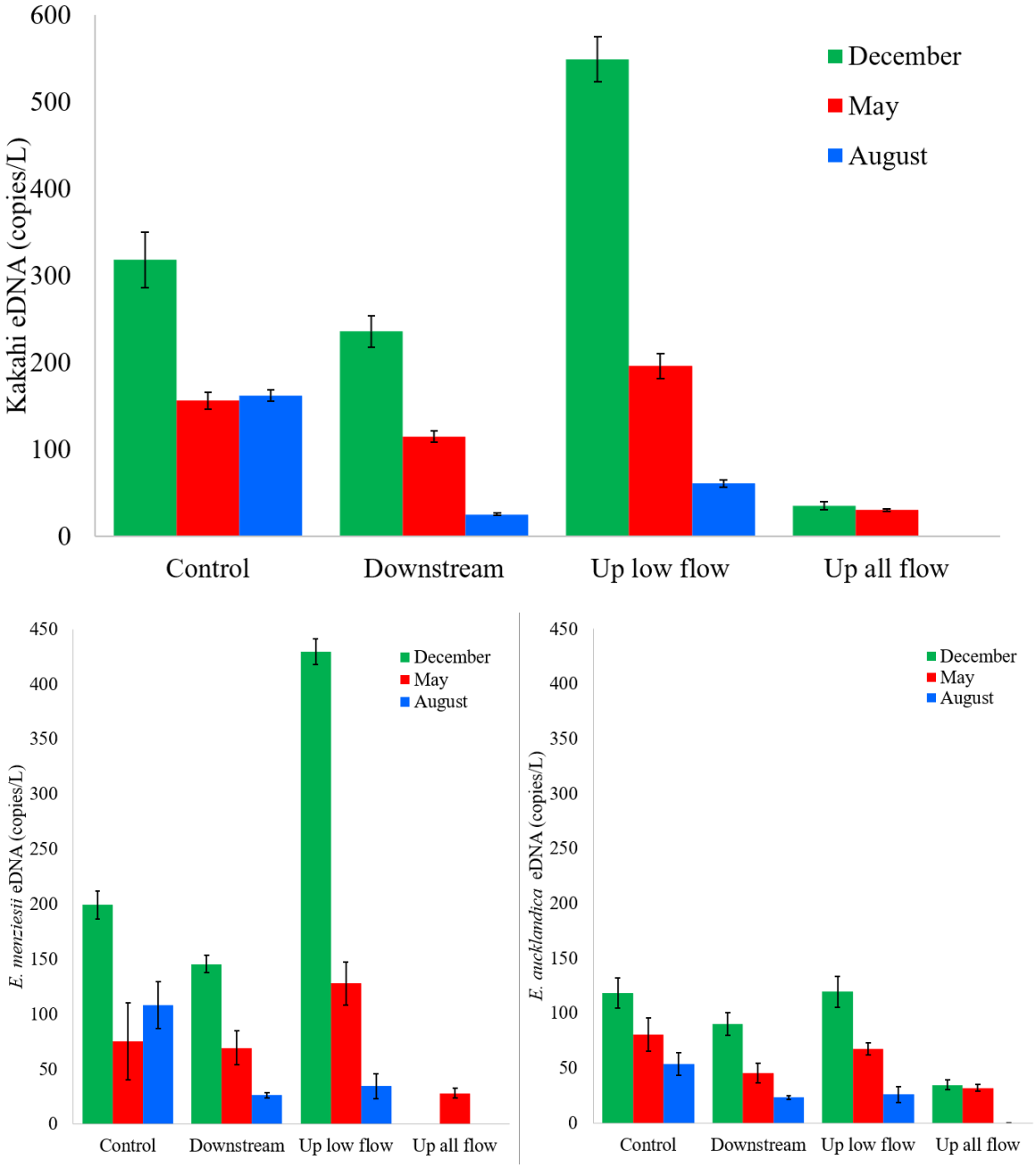

Figure 4-6. Top: Average kākahi eDNA concentrations for each fish barrier subset detected in December (green), May (red), and August (blue). Bottom: Average *E. menziesii* (left) and *E. aucklandica* (right) eDNA concentrations for each fish barrier subset detected in December (green), May (red), and August (blue). Subsets include the positive control sites, sites downstream of fish barriers, and sites upstream of low-flow and all-flow fish barriers. Error bars depict the 95% confidence interval determined by the QuantStudio™ 3D Analysis Suite™.

To determine if there was an interactive effect between season and fish barrier, or fish barrier status and species distribution on the total detectable kākahi eDNA concentration,

a two-way ANOVA was conducted. Control sites were excluded, so only locations with differing fish barrier status (downstream, upstream low-flow, and upstream all-flow) could contribute to the statistical analysis.

There was no significant difference in eDNA concentrations due to the combined season and barrier interaction ($p = 0.058$), nor was there a difference due to the interaction of species and fish barrier ($p = 0.104$), and neither species had an eDNA concentration that was consistently higher than the other ($p = 0.072$). However, the difference in eDNA concentration due to the sampling season ($p = 0.004$) and barrier status ($p = 0.001$) were significantly different. A TukeyHSD was used to determined which seasons and locations were significantly different. The p-values for this analysis are depicted in Table 4-4 and Table 4-5. For seasonal data, there is a significant difference in eDNA concentration between December and May ($p = 0.007$), and December and August ($p < 0.001$). For barrier status, there is a statistically significant difference between each of the three location types ($p < 0.01$ for all comparisons).

Table 4-4. Tukey HSD p-values for the effect of sampling season on kākahi eDNA concentrations. Data for all sites except controls were included in analysis. Green indicates a statistically significant difference, while orange is not significant at P<0.05.

ANOVA p-value = 0.004	December	May	August
December		0.007	<0.001
May	0.007		0.200
August	<0.001	0.200	

Table 4-5. Tukey HSD p-values for the effect of sampling location on kākahi eDNA concentrations. Data for all sites except controls were included in analysis. Green indicates a statistically significant difference at P<0.05.

ANOVA p-value = 0.001	Downstream	Upstream – Low-flow	Upstream – All-flow
Downstream		0.006	0.010
Upstream – Low-flow	0.006		<0.001
Upstream – All-flow	0.010	<0.001	

By combining the graphed results and statistical tests, it can be concluded that the kākahi eDNA concentration in December was significantly higher than in May and August, while the May concentration was not significantly different than in August. This is synonymous with the findings of 4.4.3, so regardless of if the control or all-flow barrier subset is removed, a seasonal difference in kākahi eDNA was detected. It can also be concluded that the kākahi eDNA concentration was reduced significantly due to fish barrier status in the following trend: upstream of low-flow sites > downstream > upstream of all-flow barrier sites.

4.5 Discussion

4.5.1 Seasonal Differences in Kākahi eDNA Concentration

New Zealand's average rainfall and water temperature vary greatly throughout the year (NIWA, 2020), which will have an effect on the kākahi eDNA concentrations present in waterways due to dilution (Wacker et al., 2019), glochidia release (Clearwater et al., 2011; Hanrahan, 2019), and shedding rate (Sansom & Sassoubre, 2017). These factors may interact or have independent impacts on the resultant kākahi eDNA concentrations. It was hypothesised that early summer (December) is the best period to collect field samples due to low flow and less dilution of kākahi eDNA. This has in part been confirmed, with results indicating that irrespective of kākahi species or the location of sampling compared to fish barriers, there is a statistically significant difference in kākahi eDNA between seasons, with December having a greater concentration than May or August (Table 4-4 and Table 4-5).

Increased rainfall and stream flow in August were likely a factor in the reduced kākahi eDNA concentration due to dilution of the eDNA in the system. High flow dilutes eDNA concentration for a resultant lower concentration, whereas low flow should lead to

relatively higher eDNA concentrations. In contradiction to this theory, December had double the river flow of May, yet the eDNA concentration was significantly greater. This suggests that although the flow in August may have diluted the eDNA concentration, there is another factor that is driving a significantly higher concentration in December in comparison to May.

Although the field water samples were pre-filtered as a precaution to reduce detritus and kākahi glochidia, there is a chance that degraded fragments of glochidia that had not attached to fish hosts may still increase the eDNA concentration in the stream. Glochidia release is heavily influenced by the water temperature (Clearwater et al., 2011; Hanrahan, 2019), and has been shown to peak in February when water temperatures are >18.8 °C (Hanrahan, 2019) although it can occur between October and late March (Clearwater et al., 2011) depending on local conditions. The average daily water temperature during field sampling was highest in December (17.2 °C average) and did not exceed 18.8 °C prior to the December sampling session, so it can be assumed that significant glochidia release had not yet occurred at any sites. It is likely that if fragmented glochidia were present in the streams it would be in low number from premature glochidia release and would not skew the resultant eDNA concentrations and comparisons between sites. It is likely that the significant differences in kākahi eDNA concentration between sampling sessions are not attributed to glochidia release.

An increase in activity and resultant sloughing of tissue during filtration by the kākahi is likely a large factor in the significantly differing kākahi eDNA concentrations between December, May, and August (Figure 4-6). The change in eDNA concentrations is related to the change in average water temperature on the day of sampling. The December eDNA concentration was significantly greater than May and August, as was the average water temperature, which decreased from 17.2 °C in December to 12.8 °C and 8.9 °C in May

and August. This inference is supported by Wacker et al. (2019), who attributed the increasing activity rate as the main factor that resulted in a 20-fold increase in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA between seasons, alongside Ogilvie (1994), who demonstrated that water temperatures and filtration rate by *E. menziesii* increase concurrently. However, it must also be noted that increasing water temperatures can increase the decay rate of eDNA (Strickler et al., 2015), buffering the effects of greater eDNA production.

In summary, it can be assumed that the statistically significant differences in kākahi eDNA concentration between seasons can be attributed to two factors: dilution and activity rate. An increase in flow rate of Waingaro River as a result of greater rainfall in August (Table 4-2) would have diluted the concentration of eDNA in the samples, which would have been further decreased from baseline levels due to a reduction in filtration rate in cool water temperature (8.9 °C). In December, the water temperature was much warmer (17.2 °C) which would have increased the activity levels of the kākahi, leading to more tissue being sloughed from the organisms. This confirms the hypothesis that early summer (December) is the most appropriate period to sample for kākahi eDNA. However, this was not only due to dilution effects but also the water temperature and resultant increase in filtration rate.

4.5.2 A Comparison of Fish Barrier Status and Distribution of Kākahi Species

It was hypothesised that the distribution of kākahi species is dependent on the presence of fish barriers that could exclude certain host fish species. However, it was discovered that there was no statistically significant difference in the concentration of *E. aucklandica* and *E. menziesii* eDNA ($p = 0.078$) at differing fish barrier locations. Although fish barriers did not influence the species differently, they did result in a significant difference in the distribution of total kākahi eDNA (Table 4-5). The highest concentration of kākahi

eDNA was found at sites upstream of low-flow barriers, followed by sites downstream of barriers, while the lowest concentration was at site upstream of all-flow barriers.

E. aucklandica are most likely host-specialists, with field observations and lab studies demonstrating how *E. aucklandica* glochidia were only found to encyst, grow, and excyst on common smelt (*Retropinna retropinna*) (Melchior, The University of Waikato, unpublished data, 2020; Hanrahan, 2019). In contrast, *E. menziesii* are host-generalists, casting their glochidia passively so that a range of fish species could become hosts to viable juvenile mussels (Moore & Clearwater, 2019). *E. menziesii* glochidia have been detected on redfin and common bully (*Gobiomorphus cotidianus)*, longfin and shortfin eels *(Anguilla australis* and *A. dieffenbachii*), īnanga (*Galaxias maculatus*), torrentfish (*Cheimarrichthys fosteri*), Canterbury galaxias (*Galaxias vulgaris*), banded kōkopu (*Galaxias fasciatus*), and kōaro (*Galaxias brevipinnis*) (Brown et al., 2017; Hanrahan, 2019; Moore & Clearwater, 2019). These fish species differ in size and climbing ability, but it has been noted that common smelt and inanga are small and especially susceptible to fish barriers obstructing their migration up and downstream (McDowall, 1995). This difference implies that *E. aucklandica* are less likely to exist upstream of all-flow barriers as they are less likely to attach to fish hosts capable of passing barriers.

Low-flow barriers are a natural or man-made obstruction in the river/stream that inhibits fish passage during summer months when there are lower water depths, whereas all-flow barriers obstruct fish passage all year round. When sampling, it was visually evident that during December and May low-flow barriers would have been prominent enough to obstruct fish passage of smaller fish such as inanga or smelt, but during August the water level was higher and fish would be able to pass. This visual observation is consistent with the environmental monitoring depth data (Table 4-2). The migration periods of many New Zealand fish species and greater water depth are correlated (July to October)

(McDowall, 1995). The obligatory fish host of *E. aucklandica*, common smelt, migrates upstream between late September and December when water depth is increased (McDowall, 1995), suggesting that these fish hosts should be able to migrate upstream of low-flow barriers if velocities are not too high and transport *E. aucklandica* glochidia past these points. However, due to common smelt being poor swimmers (Mitchell & Boubée, 1989), they would be incapable of migrating past all-flow barriers. The field data somewhat supports this theory, with greater concentrations of eDNA found above low-flow barriers and reduced eDNA concentrations upstream of all-flow barriers. Although it was hypothesised that no *E. aucklandica* eDNA would be detected upstream of all-flow barriers, there is the chance that at some point in the past very few common smelt migrated upstream to transport glochidia, or glochidia were able to attach to a less suitable host fish with a greater capability of upstream migration.

Due to sampling restrictions, no surveying of fish presence at each location was conducted. Instead, the probability of common smelt occurrence at each location was investigated (Supplementary Table 6-5) utilising data provided by the WRC and mapped in QGIS from the predicted Fish Index of Biotic Integrity (F-IBI). The predicted F-IBI models characteristics of the fish community for wadeable streams relative to what would be expected if there was no human impact (Joy & Death, 2004), enabling a prediction of the probability of occurrence of certain fish species in a stream or river segment in the absence of physical surveying. The probability of common smelt occurrence supported the conclusion from eDNA detection that fewer smelt were able to migrate upstream of all-flow fish barriers, with locations upstream of all-flow barriers having the lowest average probability of occurrence (0.467), while downstream had the highest probability (0.775).

Of the diadromous fish species that have been identified as *E. menziesii* hosts (Brown et al., 2017; Hanrahan, 2019; Moore & Clearwater, 2019), many migrate upstream during months of greater water depth (July to October), enabling them to surpass the low-flow barriers (McDowall, 1995). These include the redfin and common bully, longfin and shortfin eel, īnanga, torrentfish, banded kōkopu, and kōaro (McDowall, 1995). Regardless of this, many of these fish have great swimming ability or adaptations that enable them to migrate upstream past low-flow or all-flow barriers. Kōaro juveniles, longfin eel, and shortfin eel are anguilliform species capable of 'worming up' stones or vegetation, while kōaro, common bully and redfin bullies are climbers, able to adhere and climb up wet margins of barriers using surface tension (Mitchell & Boubée, 1989). These abilities are likely the reason that some *E. menziesii* eDNA was present upstream of all-flow fish barriers, even though it was significantly less than the concentration downstream of fish barriers.

Unexpectedly, the locations upstream of low-flow barriers had a significantly greater eDNA concentration than downstream locations (Table 4-5). It was assumed that even though some fish hosts may be able to pass barriers during low-flow or due to climbing adaptations, the frequency of migration past these would be reduced, and as a result, so would the kākahi populations. This unanticipated result may be explained by the qualitative features of the field locations that were sampled. The average Habitat Quality Score (HQS) was calculated for each site and was similar for sites downstream of fish barriers (48.7) and upstream of low-flow barriers (47.5). However, there may have been habitat features upstream of low-flow barrier sites which were better suited for kākahi populations. Sites downstream of fish barriers had more bank erosion and deposited sediment than sites upstream of low-flow barriers, which can negatively impact kākahi as

it reduces filtration efficiency (Clearwater et al., 2014). This may be the cause of more kākahi eDNA upstream of low-flow fish barriers.

In summary, it was hypothesised that the distribution of kākahi species was dependent on the presence of fish barriers that could exclude certain host fish species, yet there was no significant difference in the concentration of *E. aucklandica* and *E. menziesii* eDNA at differing fish barrier locations. Although the presence of fish barriers cannot reliably be used to infer the distribution of each kākahi species, the location of these barriers can likely indicate a reduction in kākahi abundance upstream of them.

4.5.3 A Comparison of Conventional Kākahi Count and eDNA Concentration

It has already been stated that it is not possible to reliably infer relative or absolute abundance of freshwater mussels solely from eDNA concentration (Chapter 2), and consequently, the aim of this thesis is to validate a presence/absence detection method for kākahi. Regardless, the conventional surveying count and eDNA concentration of kākahi were compared (Figure 4-5) to determine if eDNA concentration was weakly correlated with the abundance data for kākahi available at two sites surveyed previously. Although the evidence was inconclusive, the lower limit of detection (LoD) tests (Chapter 3) confirmed that detection of kākahi eDNA above the LoD can reliably indicate if kākahi are present in the stream system, but relative abundance cannot be reliably inferred. Nevertheless, eDNA has the potential to complement conventional mussel surveying techniques to enhance the current abundance estimates. eDNA can be utilised as a screening tool to identify sites where intensive conventional surveys are warranted, but caution must be made when comparing samples that were collected at differing times of the year without thorough understanding of climate and flow conditions.

Local abundance estimates from eDNA concentrations can be hampered by a very complex hydrological system which undergoes dilution, diffusion, settling, and mixing events (Jane et al., 2015a; Sansom & Sassoubre, 2017; Tillotson et al., 2018; Wilcox et al., 2016). Alongside this, detection of kākahi is reliant on the presence of mtDNA, which can significantly vary in copy number in eukaryotic cells (Robin & Wong, 1988). Consequently, detectable kākahi eDNA cannot be used to accurately infer the abundance of live kākahi due to hydrological alterations to the concentration and mtDNA and live kākahi ratios being nonsynonymous.

Previous studies have reported successful presence/absence detection of species using eDNA, in support of the findings of this study (Ikeda et al., 2016; Jerde et al., 2011; Pilliod et al., 2013; Takahara et al., 2012) and many have used eDNA concentrations to infer abundance by investigating a combination of parameters (Doi et al., 2015; Kelly et al., 2014; Pilliod et al., 2013; Port et al., 2016; Sassoubre et al., 2016; Takahara et al., 2012; Thomsen et al., 2012; Wilcox, McKelvey, et al., 2015). However, no study has stated that eDNA detection alone can infer species abundance; a combination of mesocosm lab experiments (investigating eDNA shedding, decay, and deposition rates) and field sampling may be required to accurately quantify abundance (Sansom & Sassoubre, 2017). The ability to infer presence or absence of kākahi from the detectable eDNA in a stream sample is a powerful tool in conservation management and can direct further conventional survey efforts to the most effective and impactful locations to understand population sizes with confidence.

Chapter Five - Conclusions and Future Directions

Kākahi are ecological engineers within many of New Zealand's freshwater ecosystems, performing vital active and passive functional roles (Nowak & Kozlowski, 2013). Alongside this, they are considered taonga (treasured) species with significant cultural importance to Māori (Hiroa, 1921; McDowall, 2002), yet they are a threatened species with the potential to decline by up to 70% by 2024 (Grainger et al., 2014). Kākahi distribution and abundance throughout New Zealand are poorly understood (Walker et al., 2001) due in part to the time-consuming surveying techniques required to locate and quantify concealed and morphologically cryptic species (Strayer & Smith, 2003; Graf & Cummings, 2006). To implement conservation and management initiatives, a rapid and reliable method is required for the detection and identification of these threatened kākahi populations. The aim of this research was to validate an eDNA-based detection method for kākahi using dPCR in tank and field settings, which was accomplished via *in vivo* (Chapter 3) and *in situ* (Chapter 4) optimisation.

5.1 *In Vivo* Optimisation of dPCR Detection Technique for Kākahi eDNA

Although previous studies have developed an eDNA-based detection methods for freshwater mussels (Amberg et al., 2019; Gasparini et al., 2020; Schill, 2019; Sepulveda et al., 2019; Shogren et al., 2019; Wacker et al., 2019), this is the only reported molecular detection method for kākahi and the first method to utilise QuantStudio dPCR (determined to be the most effective eDNA detection method in Chapter 2). The aim of the *in vivo* investigation was to optimise the eDNA-based detection method for kākahi to fill knowledge gaps left in the previous development of the kākahi dPCR assay (Hu, 2017) and ensure that reliable results could be produced and reported. These analyses included the lower limit of detection, proof that the method works effectively on eDNA samples rather than genomic DNA, optimisation of the prefiltration step to remove inhibitory

detritus and glochidia, and determination of the kākahi eDNA degradation rate to ensure field sample results reflected the accurate stream concentration.

To address the first aspect of the study, dPCR analysis of a serial dilution of genomic kākahi DNA was conducted to determine the lowest concentration of DNA which could accurately be detected to infer abundance or presence of kākahi eDNA. The dPCR detection sensitivity for *E. aucklandica* and *E. menziesii* eDNA were the same; the lower limit of detection for kākahi eDNA was 0.32 copies/µL, presence could be inferred if the concentration of eDNA was between 3.19 and 0.32 copies/µL, and abundance of kākahi eDNA could be reliably inferred if the concentration was ≥ 3.2 copies/µL. The inability to infer abundance at the lower scale of detection is not a hindrance because this method was designed to indicate the presence or absence of kākahi.

It is imperative that factors which may reduce the ability to make reliable comparisons between results are controlled. To reduce the presence of inhibitory detritus or glochidia in a field sample, it must first undergo prefiltration. Particulates may inhibit PCR amplification (Jane et al., 2015; Opel et al., 2010; Sidstedt et al., 2015) while glochidia release can vary largely due to local conditions (Clearwater et al., 2011; Hanrahan, 2019) and may hamper comparisons between sites at differing locations. The most appropriate filter pore size to exclude detritus and glochidia was found to be 200 µm.

Once eDNA is introduced to the freshwater system, it degrades due to a combination of environmental, biological, and physical factors (Corinaldesi et al., 2011; Strickler et al., 2015), so the detected amount may not represent the actual concentration at the time of sampling. Between 24 to 48 hours, the concentration of detectable kākahi eDNA underwent a 92.7% reduction that was consistent with studies of other freshwater mussels (Sansom & Sassoubre, 2017). The time between sample collection and filtration must not

exceed 24 hours to ensure the detected eDNA concentration is an accurate representation of the initial stream concentration.

5.2 *In Situ* Seasonality and Fish Barrier Effect on Kākahi eDNA

Although the kākahi dPCR assay had been optimised for *in vivo* samples and confirmed to reliably detect eDNA, no analysis had been conducted on field samples, so a better understanding of the intricacies of field samples was imperative. The aim of the *in situ* study was to investigate the seasonality effect on eDNA concentration to ascertain the most appropriate time for field sampling and investigate if fish barriers affect the distribution of each kākahi species. Alongside this, it could be determined if the eDNA concentration of kākahi could infer relative abundance of the population. To address these aims, duplicate field samples were collected from the Waingaro River and Kahuhuru Stream in December, May, and August at sites downstream of fish barriers and upstream of low-flow or all-flow fish barriers to investigate temporal and spatial variation in eDNA concentrations.

Environmental monitoring data demonstrated how the rainfall, water flow, and water temperature within the Waingaro catchment varied greatly throughout the year, which has been demonstrated to have an effect on freshwater mussel eDNA concentrations due to dilution (Wacker et al., 2019), glochidia release (Clearwater et al., 2011; Hanrahan, 2019) and tissue shedding rate (Sansom & Sassoubre, 2017). It was hypothesised that December would be the most appropriate time to sample for kākahi eDNA due to reduced dilution effects, which was confirmed, although, this appeared not only due to dilution effects but also the water temperature and resultant increase in filtration rate.

There was a statistically significant difference in kākahi eDNA between seasons, with December having a greater concentration than May or August (4.7-fold increase). It is

likely that dilution of eDNA in August was caused by an increase in rainfall and stream flow, reducing the concentration of detectable eDNA. However, this did not explain why May, which had the lowest flow, had significantly less eDNA than December; there is another driving factor in the seasonal eDNA differences. The average hourly water temperatures within the Waingaro catchment did not exceed 18.8 °C prior to December sampling, the temperature at which peak glochidia release of Waikato kākahi populations occurs (Hanrahan, 2019). If any glochidia had been prematurely released and were present in the streams it would have been in low quantity, so would not have attributed to the greater eDNA concentration. It was determined that the heightened December eDNA concentrations correlated with increased water temperatures, which can cause an increased filtration rate (thus eDNA shedding) for *E. menziesii* (Ogilive, 1994).

The findings revealed that, of the months compared, December yields high eDNA concentration, such that smaller populations may produce enough eDNA for detection. By sampling prior to February or before water temperatures reach 18.8 °C, which is when peak glochidia release occurs (Hanrahan, 2019), any degraded glochidia that can pass through the prefilter from locally inconsistent release (Clearwater et al., 2011) would not skew comparisons between sites.

Although it was hypothesised that the distribution of *E. aucklandica* and *E. menziesii* would be dependent on the presence of fish barriers which could exclude certain fish hosts, there was no significant difference in the distribution of each mussel species' eDNA at differing fish barrier locations. However, there was a significant difference in the distribution of total kākahi eDNA based on fish barriers presence, such that there was reduced eDNA detected upstream of all-flow fish barriers. This was likely due to the ability of a reduced number of *E. menziesii* host fish to overcome all-flow barriers using adaptations such as climbing up stones or vegetation. Surprisingly, *E. aucklandica* eDNA

was also found upstream of these barriers regardless of the poor swimming ability of their obligatory fish host, common smelt (McDowall, 1995). This suggests that a small number of smelt were able to overcome the barriers, at least in some years, or that *E. aucklandica* glochidia were able to attach to other host fish with a greater capability of upstream migration. The presence of fish barriers cannot reliably infer the distribution of each kākahi species, but the location of fish barriers can likely indicate a reduction in kākahi abundance upstream of them.

Although this detection method aimed to infer presence or absence of kākahi and it is not possible to reliably infer relative or absolute abundance of freshwater mussels solely from eDNA concentration (Sansom & Sassoubre, 2017), weak correlation between eDNA concentration and live kākahi abundance would enhance the detection tool for conservation management. However, results suggest that kākahi abundance and eDNA concentrations of *E. aucklandica* and *E. menziesii* were not correlated, and that population density cannot be reliably inferred using eDNA concentration alone. Regardless, the ability to infer presence or absence of kākahi from the detectable eDNA in a stream sample is a powerful tool in conservation management and can direct further conventional survey efforts to the most effective and impactful locations to understand population sizes with confidence.

5.3 Conclusion

This research contributes new information to the existing knowledge pool of eDNA-based detection of freshwater species, in particular freshwater mussels, which are globally one of the most diverse yet endangered taxa (Lopes-Lima et al., 2014). The findings of this thesis reinforce that this molecular tool may be effective in conservation management and can be used to map the presence of kākahi throughout New Zealand's extensive

freshwater networks to increase understanding of the distribution of each species. eDNA-based detection allows rapid and accurate assessment of multiple sites, overcoming a constraint of time-consuming conventional survey techniques for this concealed and morphologically cryptic species.

5.4 Future Work

This thesis has increased the understanding of eDNA-based detection of kākahi in both field and laboratory settings. It is the first time such a method for kākahi detection has been utilised on field samples within New Zealand. However, this study only investigated kākahi populations in river and streams. Future research could transition to the reliability of this detection method in lake habitats, where differing hydrological effects (e.g., reduced unilateral flow) may result in greater or more accurate detection patterns. This universal method could be used to map the distribution of kākahi throughout the majority of New Zealand's lakes, streams, and river systems enabling a better understanding of the distribution of the less common kākahi species (*E. aucklandica* and *E. onekaka*) throughout New Zealand. Furthermore, this study was temporally limited, as sampling occurred on only three occasions rather than spanning the entire reproductive cycle of kākahi. In future, it would be beneficial to implement a 12-month sampling survey to encompass the entire reproductive cycle and hydrological changes. This would increase the understanding of how eDNA concentration changes in response to environmental conditions (water temperature and flow) and glochidia release.

A limitation of this study was that updated conventional survey counts of kākahi were not collected at any sites due to travel and workplace restrictions caused by COVID-19. The original kākahi counts for site I and J were done in early 2018 and the population size or ratio of *E. aucklandica* and *E. menziesii* may have since changed. The inclusion of such

data for additional sites would have provided a much greater understanding of how the relationship between eDNA concentration and conventional counts could be used to strengthen the use of kākahi eDNA to somewhat infer relative abundance of each species. Alongside this, assumptions were made that kākahi species would be distributed according to the presence of fish barriers that may exclude certain fish hosts, but only data from a predictive model of fish distribution was available to support this comparison. If each stream reach was surveyed for the presence of native or invasive fish (e.g., using electrofishing or fish eDNA), the presence or absence of fish species may consolidate which species are predominant fish hosts for kākahi, and it could be determined which of these were able to swim upstream of apparent all-flow fish barriers.

There are no other molecular assays which identify individual kākahi species, which is invaluable in widescale detection of the species throughout New Zealand. There is limited and perhaps outdated knowledge of the distribution of kākahi throughout New Zealand, which is critical in conservation of the taonga (treasured) organisms. However, it is hoped that this method can be utilised as a regional council tool which is rapid, low effort, non-invasive, highly specific, and requires no specialist taxonomic knowledge to map the presence of kākahi throughout New Zealand's extensive stream, river, and lake networks. This work may contribute to an understanding of the unique distribution of each species and provide a scaffold for conservation and management efforts. Kākahi are long-lived, sessile organisms with complex life histories, so once populations decline it can take decades for kākahi to recolonise habitats. Active conservation and management tactics are vital now to ensure the protection of these national treasures and ecosystem engineers in future freshwater ecosystems.

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Appendix

Supplementary Table 6-1. dPCR control results for the eDNA degradation and Limit of Detection experiments.

	E. aucklandica eDNA copies/µL		*E. menziesii* eDNA copies/µL	
	eDNA degradation	LoD	eDNA degradation	LoD
dPCR positive	1485.1	1362.7	3430.9	3313.7
dPCR negative	0	0	0	0

Supplementary Table 6-2. Characteristics of kākahi housed in tanks for in vivo analysis.

Tank	Kākahi	Species	Sex	Length (mm)	Width (mm)	Depth (mm)	Dry weight (g)
	1a	*E. aucklandica*	Female	80	37	17	1.83
1	1b	*E. aucklandica*	Male	93	42	23	3.016
	2a	*E. menziesii*	Female	59	31	17	0.628
2	2b	*E. menziesii*	Male	57	32	14	0.495
	3a	*E. aucklandica*	Female	82	37	17	1.949
3	3b	*E. menziesii*	Female	58	30	14	0.63
	4a	*E. aucklandica*	Male	77	35	18	1.175
4	4b	*E. menziesii*	Male	52	28	14	0.39

Supplementary Table 6-3. Rapid Habitat Assessment (Clapcott et al. 2015) results for each site used to calculate the Habitat Quality Score (HQS). Green indicates Good HQS, orange indicates Fair HQS.

RHA features	A	B	C	D	E	F	G	H	I	J
Deposited sediment	7	7	5	1	8	7	6	4	1	9
Invertebrate habitat diversity	6	7	5	2	5	5	5	5	3	7
Invertebrate habitat abundance	8	7	7	2	8	8	7	7	3	8
Fish cover diversity	6	6	4	3	3	4	4	5	1	5
Fish cover abundance	5	5	4	4	4	5	4	5	3	5
Hydraulic heterogeneity	4	7	7	1	7	7	3	3	1	3
Bank erosion	6	6	7	6	7	8	2	5	5	5
Bank vegetation	4	2	4	3	2	2	2	4	4	4
Riparian width	2	1	1	1	1	1	1	3	10	5
Riparian shade	5	6	4	4	4	4	5	6	7	7
Habitat Quality Score	53	54	48	27	49	51	39	47	38	58

Habitat parameter	Condition category										SCORE
1. Deposited sediment	*The percentage of the stream bed covered by fine sediment.*										
	0	*5*	*10*	*15*	*20*	*30*	*40*	*50*	*60*	*≥ 75*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
2. Invertebrate habitat diversity	*The number of different substrate types such as boulders, cobbles, gravel, sand, wood, leaves, root mats, macrophytes, periphyton. Presence of interstitial space score higher.*										
	≥ 5	*5*	*5*	*4*	*4*	*3*	*3*	*2*	*2*	*1*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
3. Invertebrate habitat abundance	*The percentage of substrate favourable for EPT colonisation, for example flowing water over gravel-cobbles clear of filamentous algae/macrophytes.*										
	95	*75*	*70*	*60*	*50*	*40*	*30*	*25*	*15*	*5*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
4. Fish cover diversity	*The number of different substrate types such as woody debris, root mats, undercut banks, overhanging/encroaching vegetation, macrophytes, boulders, cobbles. Presence of substrates providing spatial complexity score higher.*										
	≥ 5	*5*	*5*	*4*	*4*	*3*	*3*	*2*	*2*	*1*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
5. Fish cover abundance	*The percentage of fish cover available.*										
	95	*75*	*60*	*50*	*40*	*30*	*20*	*10*	*5*	*0*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
6. Hydraulic heterogeneity	*The number of of hydraulic components such as pool, riffle, fast run, slow run, rapid, cascade/waterfall, turbulance, backwater. Presence of deep pools score higher.*										
	≥ 5	*5*	*4*	*4*	*3*	*3*	*2*	*2*	*2*	*1*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
7. Bank erosion	*The percentage of the stream bank recently/actively eroding due to scouring at the water line, slumping of the bank or stock pugging.*										
Left bank	*0*	*≤ 5*	*5*	*15*	*25*	*35*	*50*	*65*	*75*	*> 75*	
Right bank	*0*	*≤ 5*	*5*	*15*	*25*	*35*	*50*	*65*	*75*	*> 75*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
8. Bank vegetation	*The maturity, diversity and naturalness of bank vegetation.*										
Left bank AND Right bank	*Mature native trees with diverse and intact understorey*		*Regenerating native or flaxes/sedges/tussock > dense exotic*			*Mature shrubs, sparse tree cover > young exotic, long grass*			*Heavily grazed or mown grass > bare/impervious ground.*		
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
9. Riparian width	*The width (m) of the riparian buffer constrained by vegetation, fence or other structure(s).*										
Left bank	*≥ 30*	*15*	*10*	*7*	*5*	*4*	*3*	*2*	*1*	*0*	
Right bank	*≥ 30*	*15*	*10*	*7*	*5*	*4*	*3*	*2*	*1*	*0*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
10. Riparian shade	*The percentage of shading of the stream bed throughout the day due to vegetation, banks or other structure(s).*										
	≥ 90	*80*	*70*	*60*	*50*	*40*	*25*	*15*	*10*	*≤ 5*	
SCORE	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
TOTAL	(Sum of parameters 1-10)										

Supplementary Figure 6-1. Rapid Habitat Assessment field sheet (Clapcott et al. 2015) used to calculate the Habitat Quality Score.

Supplementary Table 6-4. Results of controls for in situ lab and dPCR analysis for each subset of field samples.

	E. aucklandica copies/µL			*E. menziesii* copies/µL		
	December	May	August	December	May	August
dPCR positive	1318.60	1374.3	1386.7	3511.70	3599.6	3544.2
dPCR negative	0	0	0	0	0	0
Field/procedural	0	0	0	0	0	0

Supplementary Table 6-5. Probability of Common Smelt presence at each sampling site determined by the predicted Fish Index of Biotic Integrity (Joy & Death, 2004) modelled by WRC and QGIS.

Site	Reach ID	Fish barrier	Probability of Smelt
A	3014730	Downstream	0.750
B	3014738	Downstream	0.703
C	3014666	Up - Low flow	0.698
D	3014612	Up - All flow	0.741
E	3014703	Up - All flow	0.329
F	3014703	Up - All flow	0.329
G	3014524	Downstream	0.870
H	3014525	Up - Low flow	0.477
I	3015438	Control	0.660
J	3015138	Control	0.603

Supplementary Table 6-6. Detected eDNA concentration (copies/L) and confidence intervals of kākahi at each site in December, May, and August.

			E. menziesii		***E. aucklandica***		**Total kākahi**	
	Site	Fish barrier status	eDNA (copies/L)	confidence interval (+/-)	eDNA (copies/L)	confidence interval (+/-)	eDNA (copies/L)	confidence interval (+/-)
December	A	Downstream	43.9	4.575	47.4	8.525	82.9825	13.1
	B	Downstream	141.65	9	112.025	13.075	253.675	22.075
	C	Upstream - low flow	416.25	6.7	149.05	7.75	565.3	14.45
	D	Upstream - all flow	63.05	6.025	97.025	11.7	160.075	17.725
	E	Upstream - all flow	Below LoD	Below LoD	34.1	2.75	17.05	2.75
	F	Upstream - all flow	Below LoD	Below LoD	35.225	6.4	35.225	6.4
	G	Downstream	149.25	6.85	67.95	7.675	217.2	14.525
	H	Upstream - low flow	442.35	16.25	90.125	20.75	532.475	37
	I	Control	146.45	12.005	179.25	20.35	325.7	32.355
	J	Control	252.375	13.5	57.275	17.225	309.65	30.725
May	A	Downstream	50.5	1.1	44.575	5.825	69.825	6.375
	B	Downstream	97.775	8.05	57.875	10.925	155.65	18.975
	C	Upstream - low flow	221.6	22.575	105.075	5.875	326.675	28.45
	D	Upstream - all flow	80.9	10.325	97.9	21.675	178.8	32

	E	Upstream - all flow	28.15	2.575	31.925	6.3	60.075	8.875
	F	Upstream - all flow	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD
	G	Downstream	40.725	4.8	33	7.075	73.725	11.875
	H	Upstream - low flow	34.075	5.35	30.25	5.15	64.325	10.5
	I	Control	86.7625	10.5	116.4125	37.15	203.175	47.65
	J	Control	63.8	9.2	44.4	13.25	108.2	22.45
August	A	Downstream	21.85	4.8	Below LoD	Below LoD	10.925	4.8
	B	Downstream	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD
	C	Upstream - low flow	39.675	3.2225	28.475	8.925	68.15	12.1475
	D	Upstream - all flow	29.3	2.25	34.55	7.95	46.575	6.225
	E	Upstream - all flow	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD
	F	Upstream - all flow	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD	Below LoD
	G	Downstream	26.425	2.8	23.25	3.95	38.05	4.775
	H	Upstream - low flow	29.225	4.425	23.35	5.575	52.575	10
	I	Control	52.6875	7.575	36.65	9.4	89.3375	16.975
	J	Control	163.35	4.925	70.95	20.825	234.3	25.75