PESTSMART

The utility of eDNA as a tilapia surveillance tool

Invasive Animals CRC Project: 1.W.1

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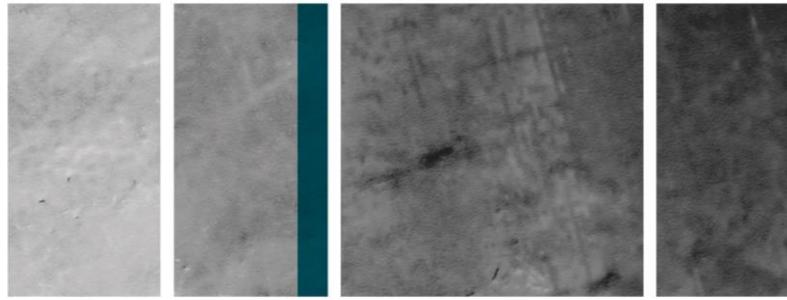Richard J. Saunders
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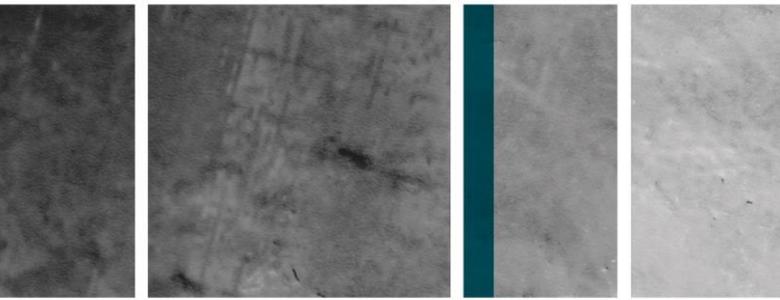
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Executive Summary

Background

Invasive fishes pose a major threat to aquatic ecosystems worldwide. Their impact can be severe in tropical regions, such as northern Australia, where over 20 invasive fish species are recorded from freshwater rivers and streams. The prevention of new incursions of invasive species is the goal, however when prevention fails, early detection of incursions is critical for successful control measures. To this end, environmental DNA (eDNA), the DNA that an organism leaves behind in the ecosystem, is proving to be a promising early detection tool for invasive aquatic species and has been used successfully to detect incursions of temperate invasive species. This research project aimed to modify conventional eDNA protocols developed by the University of Notre Dame (USA) for application in tropical environments to detect the invasive pest fish tilapia and to develop an understanding of the detection sensitivity of the method.

Methods development

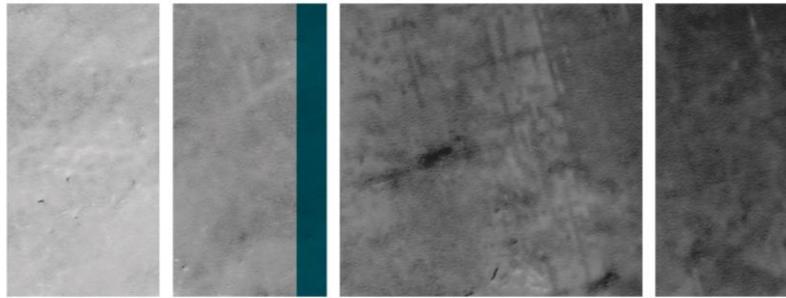Broadly, the method involved collecting and filtering two litre water samples and testing the filtrate for the presence of tilapia eDNA with a species specific probe. Essential to implementation was the successful development of a species specific probe and selection of the appropriate filter types for turbid tropical environments. A tilapia specific probe was developed. Fine filters (from 3 to 10 μm) were inefficient in tropical environments, due to clogging from suspended particles and algae, but 20 μm filters were tested and proved successful for extraction of eDNA from water samples.

The interpretation and application of eDNA surveillance methods required a knowledge of the rate of eDNA degradation and the sensitivity of the method. Thus, a set of controlled aquarium experiments were done that demonstrated eDNA persisted in the environment for between 15 and 29 days dependent on the temperature tested (23, 29 and 35°C). Further, to test sensitivity, low numbers of caged tilapia were introduced to large ponds (0.4 ML) to determine the probability of detection. The method proved effective and in experimental testing was demonstrated to successfully detect one fish/0.4 ML (after four days).

Eureka Creek

Eureka Creek, in the Mitchell River catchment of the Gulf of Carpentaria, is an important site because it was the location of an incursion of tilapia identified in 2008. An eradication program was implemented to remove tilapia from the creek and prevent the spread into other Gulf of Carpentaria river catchments. Follow-up sampling using traditional methods suggested that the eradication program was successful but some concerns remained. Thus, as part of this project a new eDNA survey of Eureka Creek was conducted to follow-up the eradication program. Three locations surrounding the original infestation were sampled. At each location three 2 L water samples from three sites were collected and tested for the presence of tilapia eDNA. No tilapia eDNA was detected in any of these samples adding further weight to the evidence that tilapia were successfully eradicated from Eureka Creek.

Comparison

To contrast the effectiveness of eDNA as a surveillance tool with the primary traditional sampling tool for tilapia, that of electrofishing, a survey was conducted using both methods at 14 sites, in the lower Fitzroy River catchment, where a recent invasion of tilapia was reported. Tilapia were detected by eDNA methods in eight of the 14 sites surveyed. Two
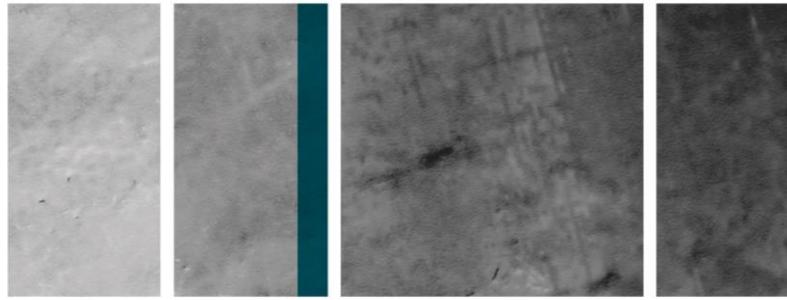
sites had no detectable tilapia eDNA and the remaining four sites failed to meet quality control standards and results from these sites were not confirmed. Positive detections were obtained for the eDNA survey from all three sites where tilapia were detected using electrofishing and at a further five sites where electrofishing failed to detect tilapia. Environmental DNA surveillance has proven to be an effective early detection tool for tilapia incursions and likely to be more sensitive than traditional survey methods but requires considerable care and precision in its implementation due to the potential for sample contamination.

Outputs

Environmental DNA technology was successfully adapted for the specific purpose of tilapia surveillance and this has resulted in a high quality service that will be beneficial to many organisations and associations to help early detection of tilapia incursions. Already, the uptake of eDNA as a method of tilapia surveillance and monitoring has occurred; the Fitzroy Basin Association and Catchment Solutions have employed the eDNA services developed from this project to survey the recent invasion of tilapia in central Queensland.
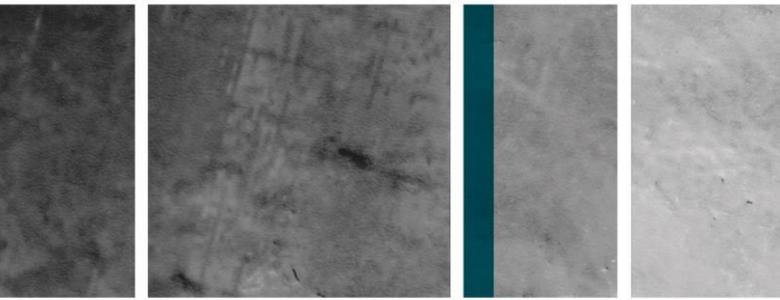
Recommendations

Future research recommendations highlighted from this project include:

1. Further development and testing of eDNA markers is necessary, particularly in light of the recent paper by Ovenden et al. (2014) that suggests the wide spread *Oreochromis mossambicus* species in Australia may in fact be three separate species that co-occur and possibly even interbreed. The eDNA markers developed in the project were based on the two tilapia species thought to be the only invasive species in Australia. How the markers perform on sequences and genomic DNA of these other *Oreochromis* species is unknown.

2. It will be important to move toward probe based qPCR (e.g. TaqMan® Assay) as this technology uses two primers and a probe that has a fluorescent marker attached. The probe increases PCR specificity and potentially eliminates any non-specific binding to bacteria as was sometimes seen using the current primer sets.

3. Further investigation and research will be required to develop greater understanding of environmental factors that may influence eDNA detectability in tropical environments. Particularly, the impact of UV, turbidity, pH and microbial load need to be considered in future studies.

4. Developing the methodology to be more user friendly when in remote areas and so that sampling can be done by outside sources and sent to the laboratory for analysis. This might include further research looking at persistence of eDNA in water samples once collected and stored in cool dark conditions, or frozen and stored until laboratory analysis can take place.

5. Further development of the technology to include other invasive species, current (i.e. aquarium species), or that pose a further threat to tropical aquatic ecosystems (i.e. climbing perch, *Anabas testudineus* and striped snakehead, *Channa striata*).

6. While early detection technology such as that developed in this study is important and can help target education programs, its application in the field will be limited unless new and effective eradication tools can be developed; an improved ability to locate and eradicate the pest species at the lower levels of detection will be essential if eDNA methods are to lead to more effective tilapia control.
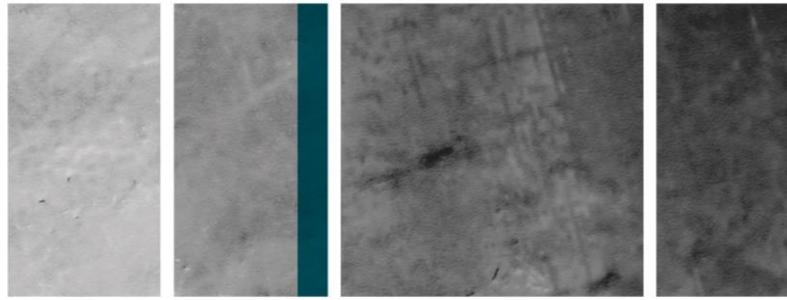
Abbreviations

ANOVA   analysis of variance

bp   base pairs

CPUE   catch per unit effort

CT   cycle threshold

df   degrees of freedom

DNA   deoxyribonucleic acid

DO   dissolved oxygen

EC   equipment control

eDNA   environmental deoxyribonucleic acid

FBA   Fitzroy Basin Authority

gDNA   genomic nucleic deoxyribonucleic acid

GEE   generalised estimation equation

GLM   generalise linear model

JCU   James Cook University

mtDNA   mitochondrial deoxyribonucleic acid

NTC   no template control

P   probability

PCR   polymerase chain reaction

qPCR   quantitative polymerase chain reaction

RNA   ribonucleic acid

RO   reverse osmosis

UND   University of Notre Dame
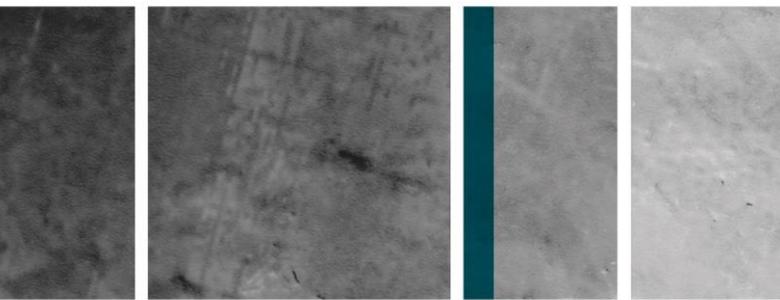
1. Introduction

1.1. Project background

Invasive species pose a major threat to aquatic ecosystems worldwide. Tilapia, an African group of fish belonging to the Cichlidae family, are highly invasive fish that have established feral populations in almost every country to which they have been introduced (Russell *et al*. 2010). Tilapia (*Oreochromis* spp.) are also listed in the top 100 most invasive species worldwide (http://www.issg.org/database/species). The Mozambique tilapia (*Oreochromis mossambicus*) was first recorded in Australia in the late 1970s in the Townsville area; later in the 1990s another species of tilapia, *Tilapia mariae*, was reported in several rivers of north Queensland (Russell *et al*. 2010). Both species of tilapia are now well established in Australia, although *O. mossambicus* is most widespread, found along many parts of the east coast of Queensland and in the Pilbara region of Western Australia (Russell *et al*. 2010; Russell *et al*. 2012). A recent paper by Ovenden *et al*. (2014) indicates that the wide spread *Oreochromis mossambicus* in Australia may in fact be three separate species that co-occur and possibly even interbreed. The persistent spread of tilapia is a matter of national importance and, if left unchecked, could have an adverse effect on coastal and inland fisheries and the environment. Several studies have demonstrated the detrimental impacts of introduced tilapia that are now occurring here in Australia, similar to what has been seen for carp in southern Australia, such as competitive displacement, habitat alteration and potentially introduction of disease and parasites (reviewed by Russell *et al*. 2012).

The best measures for managing the spread of invasive species is through prevention rather than post establishment control programs (Thuesen *et al*. 2011). Unfortunately, the spread of invasive animals, like tilapia, is difficult to avoid as tilapia spread via both natural occurrences (e.g. during flood events) and particularly through human mediated pathways (e.g. releases from ornamental aquaria or ponds, used in bait fishing and deliberately stocked in dams and ponds). In these circumstances it is crucial to detect invasions early to maximise the chances of successful eradication and avoid widespread establishment. Therefore, there is a critical need to develop a reliable and efficient early detection method for aquatic invasive species. Current surveillance methods for tilapia involve physically sampling fish in water bodies using methods such as electrofishing and netting in locations of suspected infestation. These methods, however, are inefficient at detecting low density infestations and positive detections usually mean the population of tilapia is already well established. Alternatively, recent developments in molecular detection methods are proving to be a reliable tool for detecting low density aquatic species (Ficetola *et al*. 2008, Goldberg *et al*. 2011; Wilcox *et al*. 2013), including detection of invasive aquatic species (Jerde *et al*. 2011, Jerde *et al*. 2013, Tréguier *et al*. 2014).

Molecular detection methods of aquatic organisms are based on the principle that they shed cells and consequently DNA into the water body, termed environmental DNA or eDNA. Water samples are collected and detection of eDNA is based on extracting the DNA from the water and using polymerase chain reaction (PCR) assays to determine the presence of the target species DNA. This technology offers the potential to be more sensitive and cost-efficient than traditional surveillance measures such as electrofishing and netting, as eDNA can be used to regularly monitor water-bodies with limited field infrastructure and labour requirements and can be carried out on a wide scale. If used routinely, eDNA as a surveillance tool for tilapia (and other pest fish species) in Australia may enable new outbreaks to be detected much
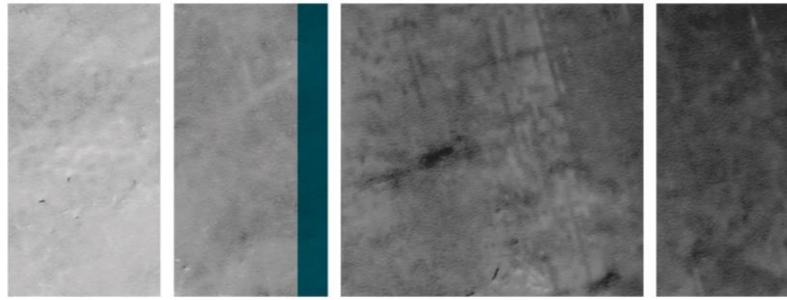
earlier than by using traditional methods and (potentially) at a stage where the populations are geographically contained and not well established. Under such circumstances, eradication programs have a higher probability of success.

Environmental DNA is still an emerging technology. It has recently been used extensively to track the invasion of Asian carp in the Great Lakes of North America (e.g. United States of America (USA) Fish and Wildlife Service eDNA Monitoring Program, 2014), as well as other species in the USA, Japan and Europe (Goldberg *et al*. 2011, Dejean *et al*. 2012, Takahara *et al*. 2012, Pilliod *et al*. 2013a, Pilliod *et al*. 2013b, Tréguier *et al*. 2014). In 2012, the University of Notre Dame (UND) conducted a preliminary assessment of the efficacy of eDNA surveillance as a cost effective method for detecting feral tilapia in north-eastern Australia (IA CRC project 4.F.19). However, this project was unsuccessful as tilapiine-specific primers were not used, resulting in co-amplification of Australian native fishes. Consequently, there are still many unknowns on the effectiveness of eDNA as a routine tilapia monitoring/surveillance tool. To expand on the previous work of UND, the current project aimed to further develop and refine eDNA methods for tilapia detection, with developments continually being made throughout the project, and to understand the sensitivity of the detection method with the ultimate goal of utilising eDNA to detect and monitor tilapia in waterways throughout north-eastern Australia.

1.2. Project outcomes

1. The transfer to, and successful use of, eDNA technology for tilapia detection in an Australian genetics laboratory.

2. A knowledge of the sensitivity, versatility and limitations of the use of eDNA technology for tilapia under Australian conditions.

1.2.1. Project objectives

1. Replicate/improve upon the eDNA technology developed by UND in an Australian genetics laboratory. (Chapter 2,3,5)
2. Use eDNA to confirm the success (or otherwise) of a tilapia eradication program. (Chapter 4)
3. Determine the environmental persistence of tilapia eDNA. (Chapter 6)
4. Make a quantitative comparison with electrofishing as a surveillance tool. (Chapter 7)
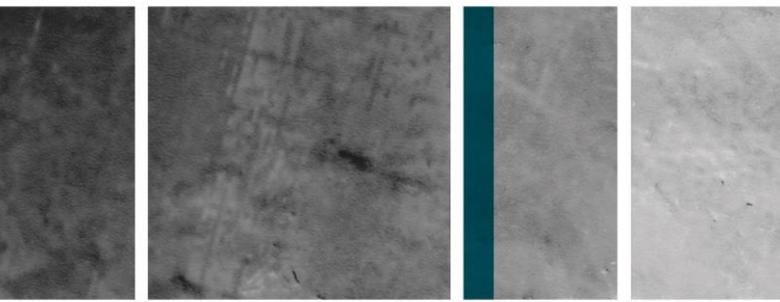
2. Development of tilapia-specific genetic markers

2.1. Introduction

Developing genetic markers that are robust and highly specific is critical in the utility of eDNA as a surveillance and monitoring tool. This is because when a water sample is taken, not only is the target species DNA collected, but so is DNA from all other organisms in the water. It is important that the genetic markers only bind to the target species DNA during the polymerase chain reaction (PCR) so false positive detections do not occur. Markers designed for eDNA samples should consist of primers that amplify small fragments of mitochondrial DNA (mtDNA). Mitochondrial DNA markers are chosen because there are hundreds to thousands of copies of mtDNA per cell as opposed to a single copy of nuclear DNA. Thus, mtDNA is much more abundant in the environment and provides a greater chance of capturing the target species DNA (Tringe & Rubin 2005). Genetic markers designed in this way can provide information on presence/absence of target species in a water sample and therefore the water body itself. The aim in Section 2 was to develop a set of PCR markers that can be used to detect tilapia in water bodies across northern Australia. These markers needed to be specific to tilapia with no non-target PCR amplification of Australian aquatic species and to have potential for use in real-time PCR amplification.

2.2. Methods

Genetic samples (fin clips) from 24 common fish species found across northern Australian were acquired for this project, as well as samples from the two tilapia species (*Oreochromis mossambicus* and *Tilapia mariae*) that are invasive in Australia (Table 2.1). This collection of fish samples acts as a physical data base to test the specificity of any given primer set. DNA was extracted from all fish samples using a chloroform/isoamyl alcohol DNA extraction protocol (modified from Adamkewicz & Harasewych 1996) and stored for use in primer testing. The collection is an ongoing venture maintained at the Molecular Ecology and Evolution Laboratory at James Cook University (JCU) and may be beneficial to further developments of eDNA technology in the region.
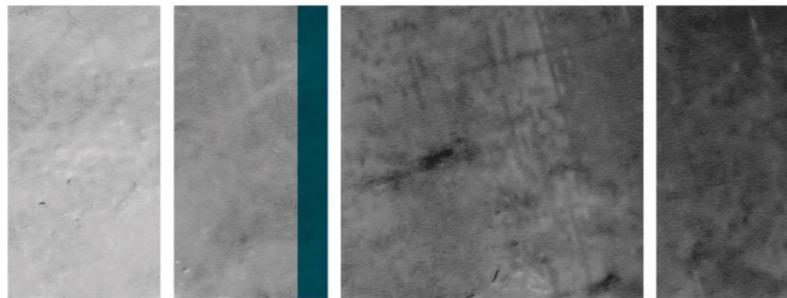
Table 2.1. Fish fin clips collected from north Queensland waterways; included is the sample number ID, common name, scientific name and family to which the species belongs.

Sample ID	Common Name	Scientific Name	Family
1	Striped gudgeon	*Gobiomorphus australis*	Eleotridae
2	Dwarf Flathead gudgeon	*Philypnodon macrostomus*	Eleotridae
3	Flathead gudgeon	*Philypnodon grandiceps*	Eleotridae
4	Empire gudgeon	*Hypseleotris compressa*	Eleotridae
5	Mozambique tilapia	*Oreochromis mossambicus*	Cichlidae
6	Spotted tilapia	*Tilapia mariae*	Cichlidae
7	Jungle perch	*Kuhlia rupestris*	Kuhliidae
8	Freshwater catfish	*Tandanus tandanus*	Plotosidae
9	Fingermark bream	*Lutjanus johnii*	Lutjanidae
10	Estuary cod	*Epinephelus taurina*	Serranidae
12	Barramundi	*Lates calcarifer*	Latidae
13	Seven spot archer	*Toxotes chatareus*	Toxotidae
14	Bloomfield cod	*Guyu wujalwujalensis*	Percichthyidae
15	Golden perch (yellow belly)	*Macquaria ambigua*	Percichthyidae
16	Barred grunter	*Amniataba percoides*	Terapontidae
18	Snake head gudgeon	*Giurus margaritacea*	Eleotridae
19	Black catfish	*Neosilurus ater*	Plotosidae
20	Mary River cod	*Maccullochella mariensis*	Percichthyidae
21	Firetail gudgeon	*Hypseleotris gallii*	Eleotridae
22	Sooty grunter	*Hephaestus fuliginosus*	Terapontidae
23	Obbes' catfish	*Porochilus obbsesi*	Plotosidae
24	Agassiz's Glassfish	*Ambassis agassizii*	Chandidae
25	Fly-specked hardyhead	*Crateocephalus stercusmuscarum*	Atherinidae
26	Bony bream	*Nematolasa erebi*	Clupeidae

The first step in developing genetic markers for tilapia surveillance was to test previously used primers designed by UND. The UND primers were tested for their ability to discriminate tilapia DNA from other fish species that are common in northern Australian waterways. The UND primers (Tilapia-CO1-F1 5'-TGG GGC ACC AGA CAT GGC CT-3' and Tilapia-CO1-R1 5'-GCG AGA TTG CCT GCG AGG GG-3') were tested using standard PCR methods against 23 native fish samples and the two target tilapia species, *Tilapia mariae* and *Oreochromis mossambicus*. PCR was performed in a final volume of 10 µl, using 1 µl of DNA template, 0.1 µl of BIOTAQ DNA polymerase (Bioline 5u/µl), 1 µl NH$_4$ reaction buffer (Bioline 10x conc.), 0.3 µl of MgCl$_2$ (Bioline 50 mM), 0.2 µl dNTPs (Bioline 10 mM), 0.2 µl of each primer (10 µM) and 7 µl of ddH$_2$O. PCR thermal cycling conditions were 95 °C for 2 min followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 seconds and 72 °C for 30 seconds, and a final extension of 72 °C for five minutes. The results of the PCR were checked for amplification success on a 1.5% agarose gel via gel electrophoresis. The unsuccessful results of UND primers (see results) lead us to source and assess published primer sequences that may be useful for tilapia eDNA surveillance. Two primer sets, "Ptr-like" (Forward 5'-CGGGTAGTGAATGTGAGTGCG-3' and Reverse 5'-ACCCAAGACACCCAGCTCCA-3') and "Pax9" (Forward 5'-TCCCACGGCTGTGTCAGYAA-3' and Reverse 5'-ACAGAGTGCGAGGAAGGCCA-3'), published by Meyer & Salzburger (2012) were also
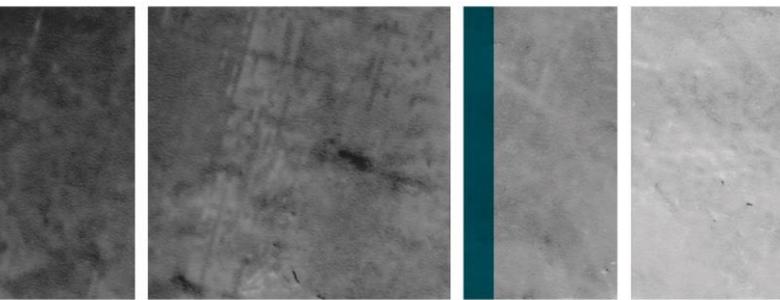
tested against the native fish collection and two tilapia species using the same PCR conditions above. The resulting PCR products were checked on an agarose gel and were found to also be non-specific, leading to the design of novel tilapia specific primers.

New primers were designed by aligning published sequences from various genes including, control region, 16S ribosomal RNA (16S) and 12S ribosomal RNA (12S) within the mitochondrial genome from both tilapia species, *T. mariae* and *O. mossambicus*, along with available sequences from common freshwater native fish species found in north Queensland. The aligned sequences were then manually scanned for regions that displayed differentiation between the tilapia species and the native fish species. Regions that were identified were then assessed in Primer 3 (http://frodo.wi.mit.edu/primer3/) as possible primer pairs. In total, five primer pairs were designed and ordered for further laboratory testing (Table 2.2). The CO1 primer pair was designed to amplify both species of tilapia, whereas the 16s and 12s primer pairs were designed to amplify individual tilapia species. Specifically, 16s oreo was designed to amplify DNA from *O. mossambicus* only and 16s mariae to amplify *T. mariae* only. Similarly for the 12s primers, 12s oreo was designed to be specific to *O. mossambicus* and 12s mariae specific for *T. mariae*.

The newly designed primers were tested in the laboratory for their ability to amplify tilapia genomic DNA and not the native fish samples using PCR (under the same conditions above) and gel electrophoresis. The results of these tests determined which primer set was most suitable for tilapia eDNA surveillance and to proceed for further optimisation.

Table 2.2. Tilapia specific primer sets designed to amplify various regions of mitochondrial DNA (mtDNA).

Primer set name	Forward (5'-3')	Reverse (5'-3')
Tilapia control	TTCCAACAACATTTACAAACCA	TAACATTTAGAAATCAACTGATGGTG
16s oreo	CTTCAGACGCCAGAACAG	GCTTGGAGTTGTAACTCTGG
16s mariae	CTTTAGACACCAGAACAG	GCTTGGAGTTGTAACTCTGG
12s oreo	AGCTTACCCTGTGAAGGAAC	CAGGGAATGTAGCCCATTTC
12s mariae	AGCTTACCCTGTGAGGGAAC	CAGGGAATGTAGCCCATTTC

2.3. Results & Discussion

All previously developed DNA markers trialled for tilapia eDNA analyses were found to be unsuitable for the species. This was due to non-specific binding of the primers to multiple species of fish other than tilapia. More specifically, the UND primers did amplify tilapia DNA (numbers 5 and 6, Fig. 2.1.); however, the primers also amplified DNA from at least 13 of the 23 native fish samples tested against (Fig. 2.1). The UND primers used in previous eDNA assessments are therefore unreliable due to the likelihood of false positive detections from non-specific binding of the primers. Therefore, investigation of alternative primers was required. Similarly, the two sets of published primers "Ptrlike" and "Pax9" that were trialled were also inapt for tilapia eDNA detection, as they did amplify DNA from tilapia, but also multiple native fish species (Fig. 2.2). As all available primers were inadequate for tilapia discrimination in mixed template samples the project needed to develop new novel primers suitable for eDNA analyses.

Five new primer pairs were designed specifically for this project and all showed potential as useful genetic markers for tilapia eDNA surveillance. None of the five primer pairs displayed
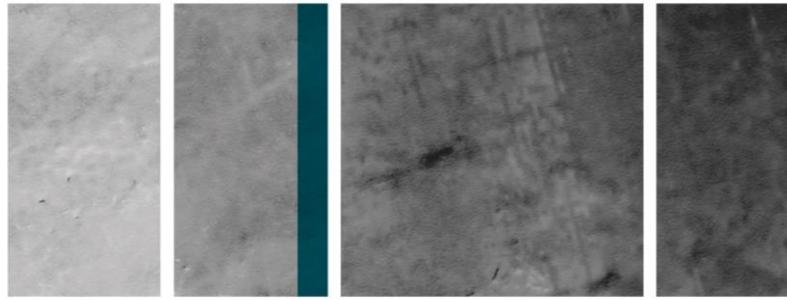
significant non-target species amplification (Fig. 2.3). However, some pairs amplified the target tilapia DNA (*O. mossambicus* #5 and *T. mariae* #6) better than others without any PCR optimisation. CO1 and 16s mariae both resulted in weak amplification of *T. mariae* only and failed to amplify *O. mossambicus*. 16s oreo amplified both tilapia species with strong amplification bands observed. 12s mariae also displayed strong amplification of *T. mariae*, but did not amplify *O. mossambicus* and 12s oreo resulted in weak amplification of both tilapia species and some minor non-target amplification of fingermark bream (Table 2.1, Fig. 2.3 - #9). Following further optimisation of the primers by altering PCR conditions, 16s oreo was chosen to be used in eDNA tilapia analyses, however, both 16s mariae and 12s mariae were also suitable and may be used as additional markers.
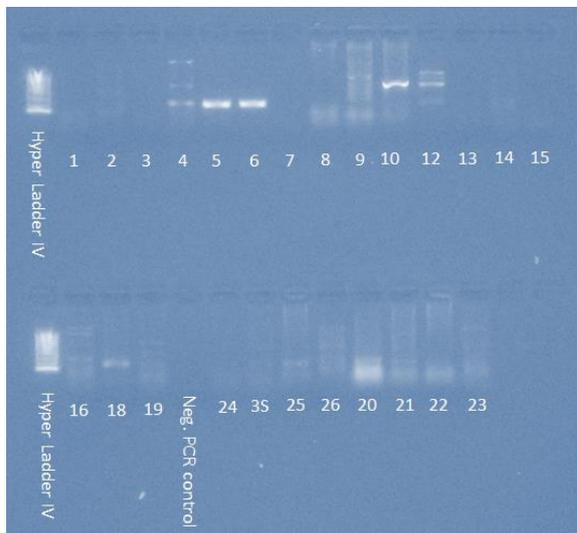
Figure 2.1. Gel image showing the result of the PCR test using the University of Notre Dame primers against the fish sample collection (Table 2.1). Positive amplification is indicated by white bands. Numbers correspond with the fish sample ID's in Table 2.1.
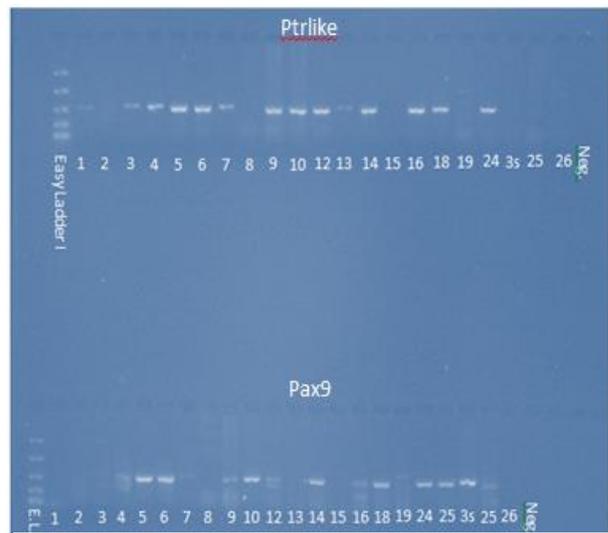
Figure 2.2. Gel image showing the result of the PCR test using published primers "Prtlike" and "Pax9" against the fish sample collection (Table 2.1). Positive amplification is indicated by white bands. Numbers correspond with the fish sample ID's in Table 2.1.
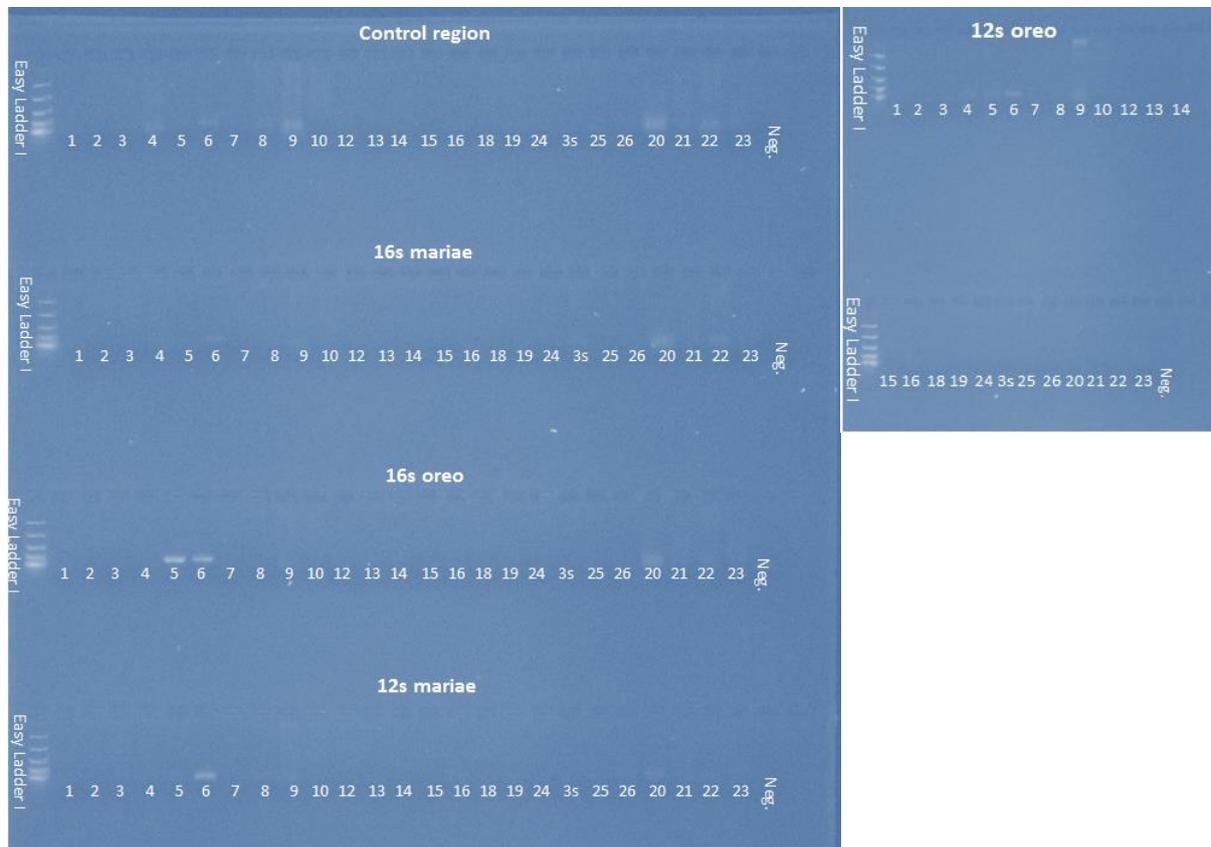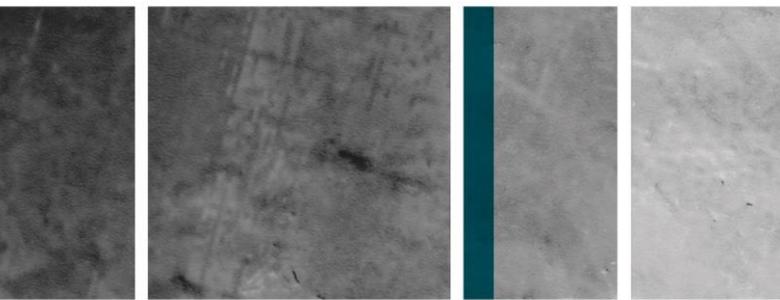
Figure 2.3. Gel image showing the results of the five newly designed primer pairs when tested using PCR against the fish sample collection (Table 2.1). Positive amplification is indicated by the white bands and the numbers correspond with fish sample ID's in Table 1.

3. Field testing of tilapia eDNA technology

3.1. Introduction

There are two common approaches for collecting and concentrating eDNA from water samples. These approaches are; small volume (generally 15 ml) water samples added to ethanol (absolute) and sodium acetate (3 M) and concentrated via centrifugation, or alternatively a larger volume (generally 2 L) water sample that is filtered through a filter membrane to capture eDNA. The field trials were conducted to prove and further develop the eDNA technology used by UND in the JCU laboratory by project staff, as well as to validate the genetic markers in the field. The UND eDNA protocol utilises the filtration method, where a 2 L water sample is collected from the water surface and filtered through a 1.5 µm glass microfiber filter. However, when implementing this protocol a number of issues were brought to light. The major issues faced during the field testing were: high amounts of suspended
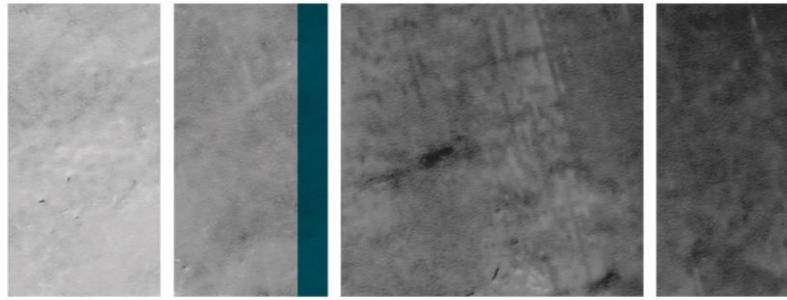
solids in water samples resulting in rapid clogging of filter papers, PCR inhibitors affecting amplification reactions and contamination within negative control samples.

PCR inhibitors are compounds that are co-purified with DNA molecules that inhibit the amplification reaction during PCR (Lodge *et al*. 2012). This may lead to inefficient amplification and potentially lower concentrations detected during quantification PCR or complete lack of amplification and a false negative result. PCR inhibitors, as well as contamination within control samples, whilst being common problems with environmental samples, are often overlooked or not addressed in the aquatic eDNA literature (e.g. Ficetola *et al*. 2008; Dejean *et al*. 2011; Goldberg *et al*. 2011; Jerde *et al*. 2011; Takahara *et al*. 2012; Thomsen *et al*. 2012). These three major issues faced during this project have been overcome and the final protocol developed reflects this (Appendix A).

3.2. Methods

Water samples were collected on several sampling trips between January 2012 and June 2014 from various sites in the Townsville area, including Ross River, Ross River Dam, Alice River, Willows Golf Course ponds, The Lakes, Palmetum and Anderson Park (Fig. 3.1). These sites were chosen due to their close proximity to the JCU campus, as well as having known infestations of tilapia. Water samples were collected following the UND field guide for eDNA sample analysis (also Jerde *et al*. 2011). Samples were filtered using filter papers of varying pore sizes to determine which size was large enough to efficiently filter 2 L of environmental (river) water whilst still able to trap enough eDNA to detect the target species. Filter pore sizes tested included 0.45 µm, 0.7 µm, 1.5 µm, 3 µm, 10 µm and 20 µm. Positive control samples were included in initial sampling efforts to ensure the methods were effective and to test for false negative results due to sample processing. The positive control samples consisted of 2 L of RO laboratory water and 2 L of environmental (river) water that were spiked with DNA extracted from tilapia fin tissue prior to being filtered.
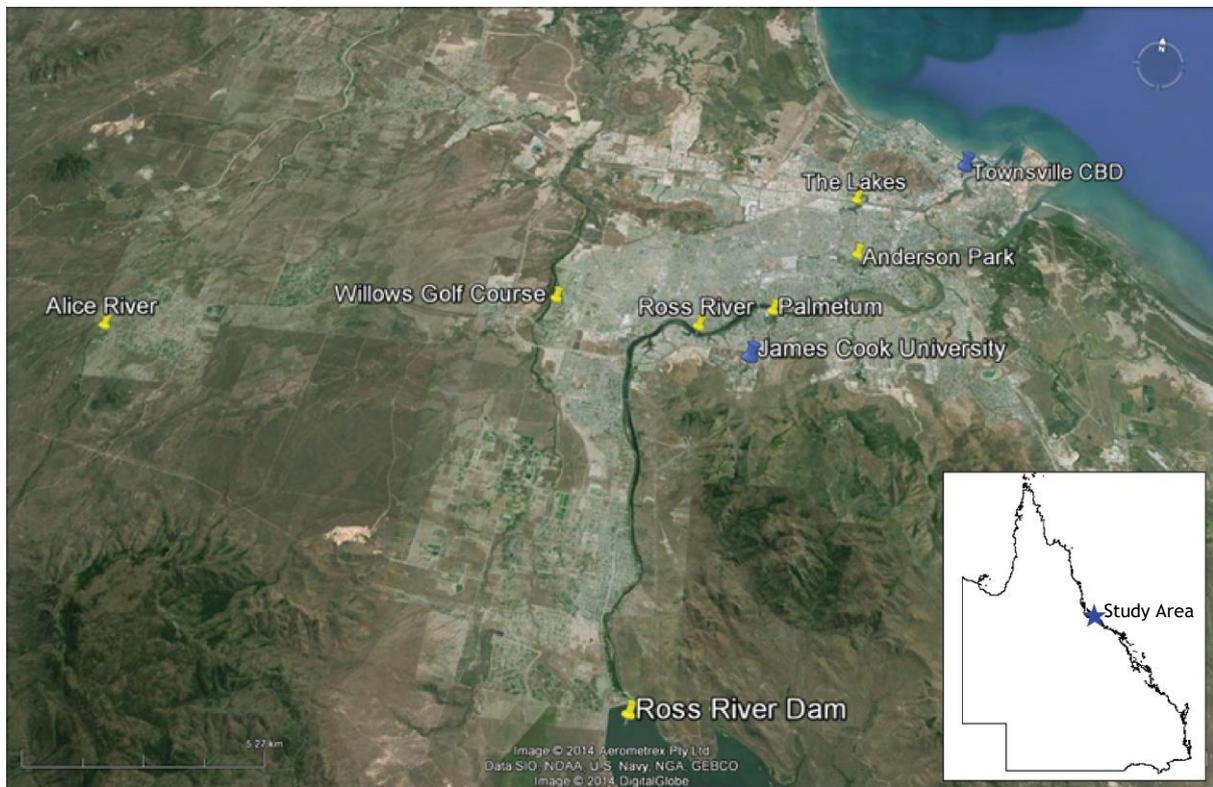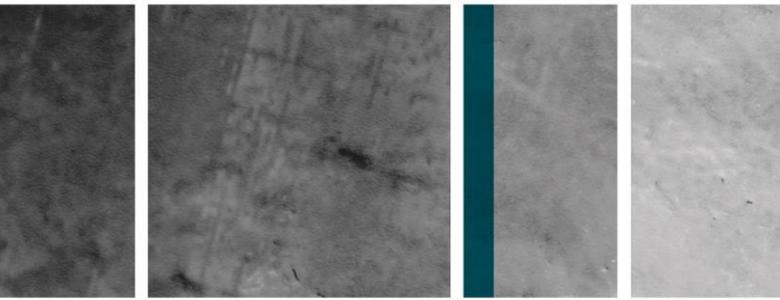
Figure 3.1. Map of Townsville (blue star on insert Queensland map) showing the initial eDNA sampling locations (yellow markers) during field testing and their close proximity to the James Cook University campus (blue marker).

Once water samples were filtered, DNA was extracted from the filters using the QIAGEN DNeasy blood and tissue kit (item no. 69506). This is a common DNA extraction kit used in many studies for extracting environmental samples (e.g. Guy *et al*. 2003; Goldberg *et al*. 2011; Takahara *et al*. 2012; Thomsen *et al*. 2012; Goldberg *et al*. 2013; Pilloid *et al*. 2013). Later, a cheaper alternative DNA extraction kit, Bioline ISOLATE II genomic DNA isolation kit (item no. BIO-52067) was sourced and a comparison test with the QIAGEN Kit on its performance was undertaken. The cheaper Bioline kit performed just as well as the QIAGEN kit permitting all later eDNA samples to be extracted at a lower price (see Appendix A for detailed DNA isolation method).

Analysis of eDNA samples to detect the target species DNA was by PCR and gel electrophoresis. Initial PCR protocols used Bioline BIOTAQ DNA polymerase and accompanying NH_4 reaction and $MgCl_2$ (see Appendix B for PCR recipes and thermal cycling conditions); however, results from these reagents failed to detect tilapia DNA in known tilapia infested water samples, as well as in positive control samples. The lack of positive results from these samples was thought to be a result of PCR inhibitors. A series of steps were undertaken to overcome the inhibitors by trialling commonly used additives (QIAGEN Q-solution and Bovine Serum Albumin) in the PCR, as well as trying different DNA polymerases (Thermo Fisher Phire Hot Start II DNA Polymerase, item no. F-124S and QIAGEN Type-It Microsatellite PCR kit, item no. 206246) (see appendix B for trialled PCR recipes and thermal cycling conditions). Results from the PCR were checked using gel electrophoresis on a 1.5% agarose gel and photo
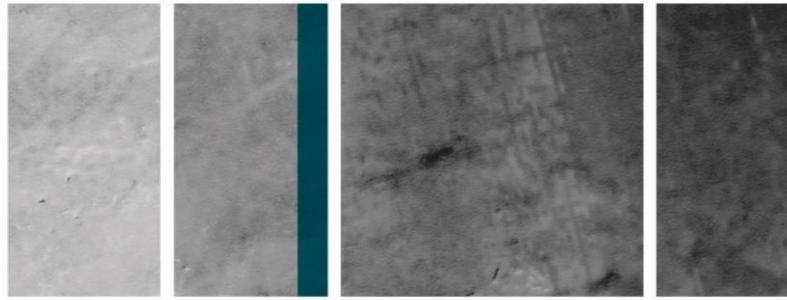
documented. Finally, to ensure the PCR product being detected was from tilapia sources, a series of eDNA samples were sequence analysed (Australian Genome Research Facility, Brisbane) and the resulting sequences aligned and compared against published tilapia sequences in Genbank.

Once the filtration, DNA extraction and PCR conditions were optimised for detection of tilapia DNA from water samples, several collections from a variety of water bodies were undertaken to confirm the methods used were consistent under a range of conditions. Sample sites included those listed previously and in Fig. 3.1. These sites all have well established populations of tilapia. At each site multiple 2 L water samples were collected and transferred to the laboratory for processing. Filtration was done the same day as samples were collected to avoid eDNA degradation. DNA extraction was carried out using either the QIAGEN DNeasy kit or the Bioline ISOLATE II kit. PCR was performed using QIAGEN's Type-it microsatellite PCR kit and results checked on a 1.5% agarose gel.

3.3. Results & Discussion

3.3.1. Trialling different filter sizes

Initial water samples collected from Ross River in Townsville were filtered using 0.45 µm filter papers. The filter papers became rapidly clogged resulting in less than 50 ml of water able to be filtered through a single filter paper. As a result, 0.45 µm filter paper was considered inadequate to filter environmental water samples such as those from Ross River. A second attempt at filtering water samples collected from Ross River was carried out using filters with 0.7 µm, 1.5 µm and 3.0 µm pore sizes. The filtration efficiency of these filter sizes were slightly better (a single filter paper able to filter approximately 200 ml of water) and the difference in filtration efficiency between the three sizes varied only slightly. The 3.0 µm filter, being the largest pore size, filtered most rapidly, however, it still required a minimum four filter papers to filter the least turbid samples (e.g. Ross River and Alice River) and up to nine filter papers for more turbid samples such as from Anderson Park. Due to the cost and time required to filter 2 L of river water using 3.0 µm filters, a larger 10 µm sized filter paper was trialled. The 10 µm filter paper allowed efficient filtration of most water samples, whilst still producing clear positive results compared with using the 3.0 µm filters (Fig. 3.2). For highly turbid samples, such as those from farm ponds, an even large filter of 20.0 µm was necessary to filter 2 L of water. For example, during the pilot study to determine eDNA detection limits (see section 7.2.), farm ponds were used and the water in these ponds was highly turbid with high amounts of algae. This results in the 10.0 µm filter papers becoming clogged after less than 300 ml. Water samples from these ponds consequently required filtration using 20.0 µm filter papers. The 20.0 µm pore size did not influence the detectability of tilapia eDNA as positive detections were consistently made (Fig. 3.3).
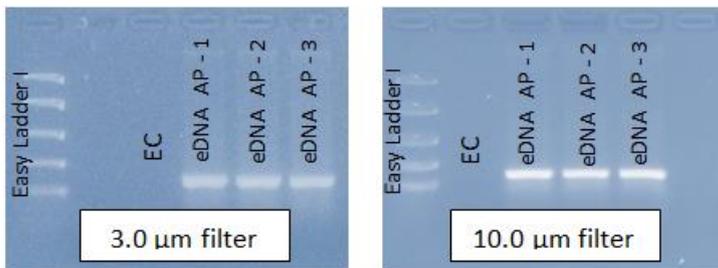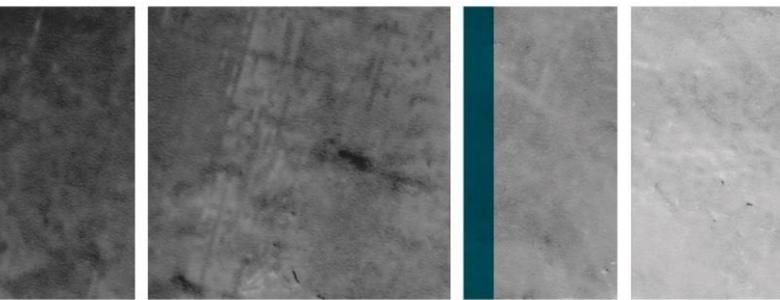
Figure 3.2. Gel image showing the results of PCR from Anderson Park eDNA samples that were filtered using a filter with 3.0 µm pore size and 10.0 µm pore size. Positive PCRs are indicated by white bands. Both sizes of filter paper resulted in clear positive detections for tilapia and therefore the 10.0 µm filter could be used to filter turbid water samples.
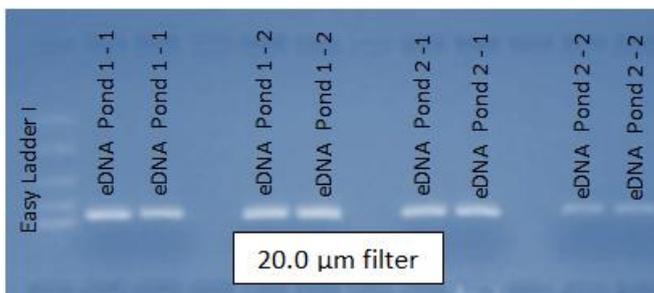
Figure 3.3. Gel image showing the results of PCR from eDNA samples collected from farm ponds stocked with tilapia that were filtered using 20.0 µm pore size filters. Positive PCRs are indicated by white bands. All eDNA samples collected from the two ponds produced distinct positive bands.

3.3.2. PCR optimisation

Initial PCR attempts using Bioline BIOTAQ DNA polymerase and the associated buffers failed to amplify any eDNA samples from Ross River as well as the river water spiked with DNA extracted from tilapia fin tissue (Fig. 3.4). The reaction did successfully amplify tilapia DNA in the RO water positive control sample as well as the positive PCR control. These results suggested the environmental river water samples included compounds that inhibited the PCR when using the Bioline BIOTAQ enzyme and buffer. When a common PCR additive, Q-solution, was added to the BIOTAQ reaction recipe, the results of the PCR did not improve as the eDNA samples still failed to amplify. Next in the series of PCR trials was Phire Hot Start II polymerase which was chosen due to this product being described as having a "high inhibitor tolerance" (Phire Hot Start II product description, Thermo Scientific). Phire enzyme was trialled stand-alone and with Q-solution and BSA added into the reactions. Using Phire enzyme did not overcome PCR inhibitors as the eDNA samples all resulted in negative detections (Fig. 3.5). Finally, Type-IT Microsatellite PCR kit was trialled which is commonly used for genotyping applications. The Type-IT kit samples returned positive results from water samples taken from known tilapia infestation sites and those spiked with eDNA
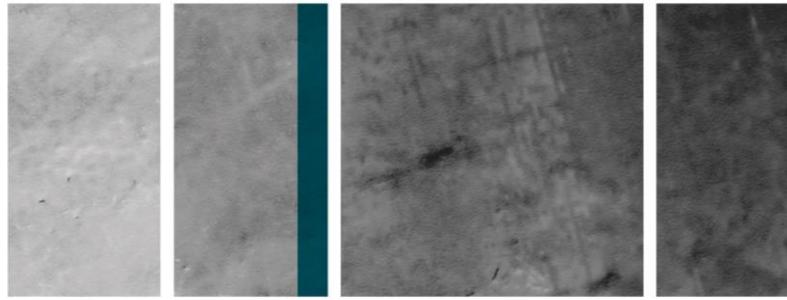
(Fig. 3.6). After further optimisation of the Type-IT PCR protocol a number of replicate eDNA samples from known tilapia infested waters (Ross River and Alice River) confirmed the suitability of the Type-IT kit for eDNA samples (Fig. 3.7). Similarly, Goldberg *et al*. (2011) found QIAGEN's multiplex PCR kit was suitable for testing eDNA samples.
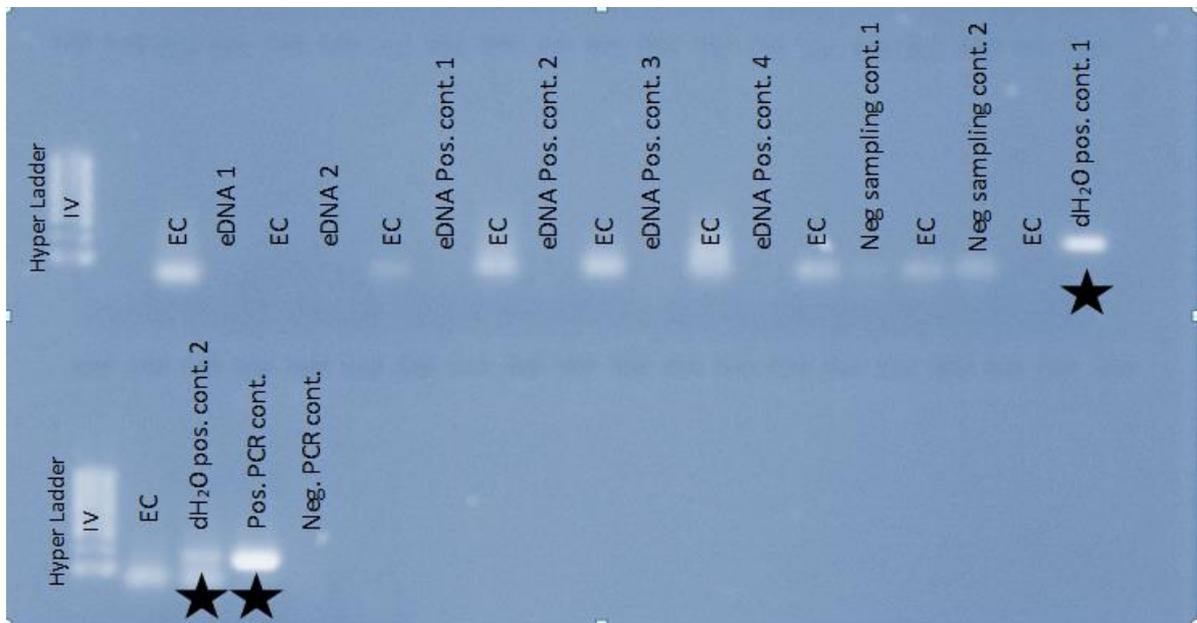
Figure 3.4. Gel image showing the results of PCR on eDNA samples collected from Ross River using Bioline BIOTAQ DNA polymerase. Positive PCRs are indicated by the white band. Using the BIOTAQ polymerase, the RO water positive control samples resulted in positive detection for tilapia as well as the positive PCR control (black stars), however, all eDNA samples (eDNA 1 &2) failed to amplify even when spiked with DNA extracted from fin tissue (eDNA Pos. cont. 1 - 4).
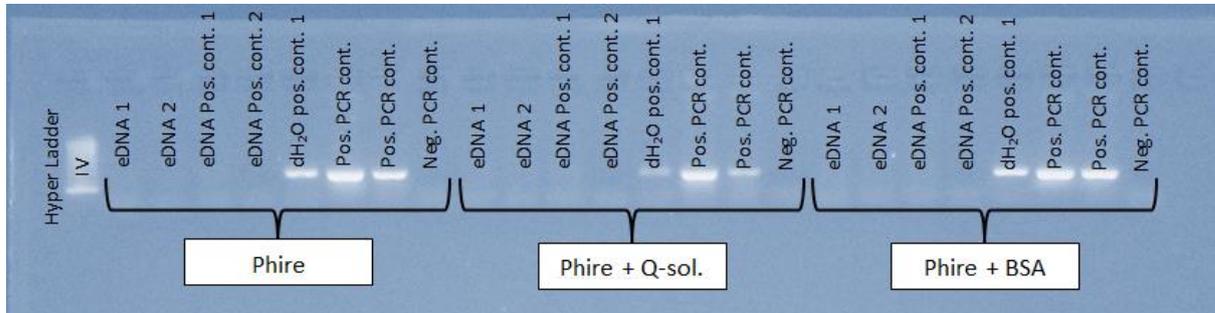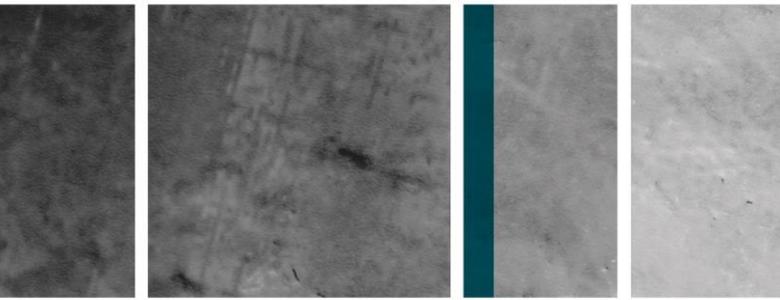
Figure 3.5. Gel image showing the results of the PCR on eDNA samples collected from Ross River using Thermo Fisher Phire DNA polymerase alone and with either Q-solution or BSA added into the reactions. Positive PCRs are indicated by the white band. All of the Phire recipes trialled failed to amplify eDNA samples (eDNA 1 & 2) as well as the eDNA positive control samples (eDNA Pos. cont. 1 & 2). RO water positive control sample and positive PCR controls samples all amplified as expected.
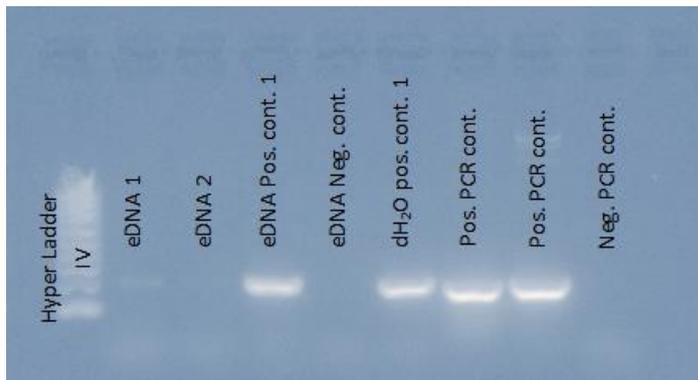
Figure 3.6. Gel image showing the results of the PCR on eDNA samples collected from Ross River using QIAGEN Type-IT microsatellite PCR kit. Positive PCRs are indicated by the white band. Using the Type-IT PCR kit the eDNA positive control sample resulted in a clear bright band, indicating the successful detection of tilapia DNA. Additionally, a non-spike eDNA sample from Ross River (eDNA 1) also showed a faint band. The RO water positive control and positive PCR controls were also all positive. All negative controls were clear of contamination.
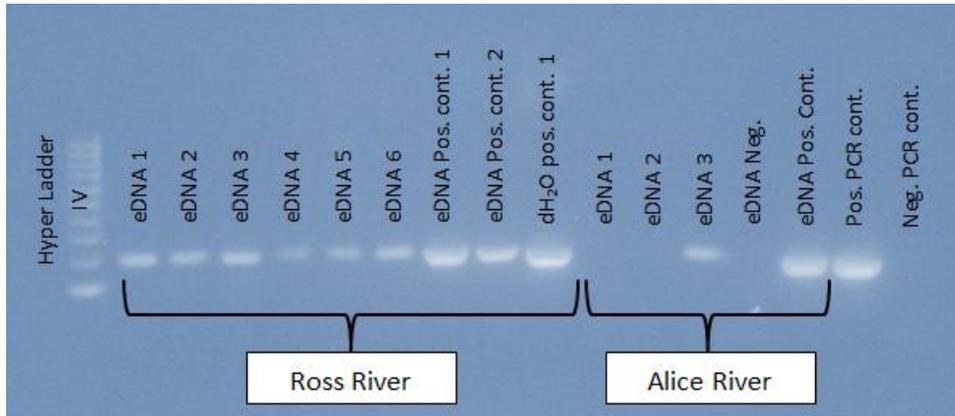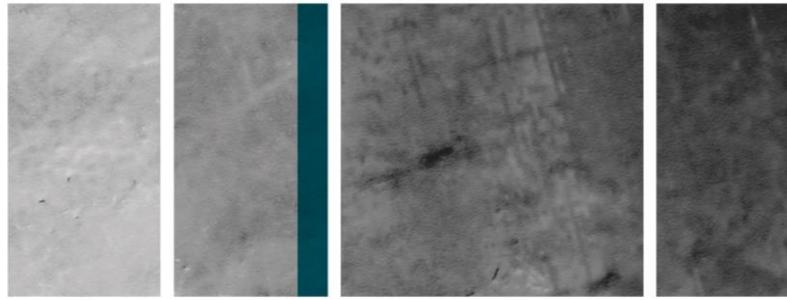
Figure 3.7. Gel image showing the PCR results of Ross River and Alice River eDNA samples using QIAGEN Type-IT Microsatellite PCR kit. Positive PCRs are indicated by the white band. All six eDNA samples collected from Ross River resulted in positive detections for tilapia. Alice River eDNA samples resulted in one out of three samples returning positive detections for tilapia.

3.3.3. Overcoming contamination

Contamination was observed in negative equipment controls on several occasions during the proofing stage of this project (Fig. 3.8). PCR product from contaminated samples that were sequence analysed to confirm the source of contamination was from tilapia DNA. The results indicated a 100% match to tilapia DNA sequences. This necessitated the introduction of further precautionary measures over and above those suggested in the UND protocols. These measures included using freshly prepared bleach solution for each batch of samples, running bleach through the filtering apparatus in between each sample to be filtered (step 23 in "Filtration of water samples for eDNA analysis" in the eDNA laboratory manual, Appendix A), using filtered pipette tips throughout the entire eDNA analysis, preparing PCR within a UV hood (step 1 in "PCR amplification of eDNA samples" in eDNA laboratory manual, using PCR tubes with individual attached lids (but no longer applicable to qPCR analysis), ensuring electrophoresis equipment is cleaned before use and leaving empty wells in between samples and controls when running agarose gels (not applicable when using qPCR analysis). The introduction of these additional measures resulted in the elimination of contamination of negative equipment controls (Fig. 3.9).
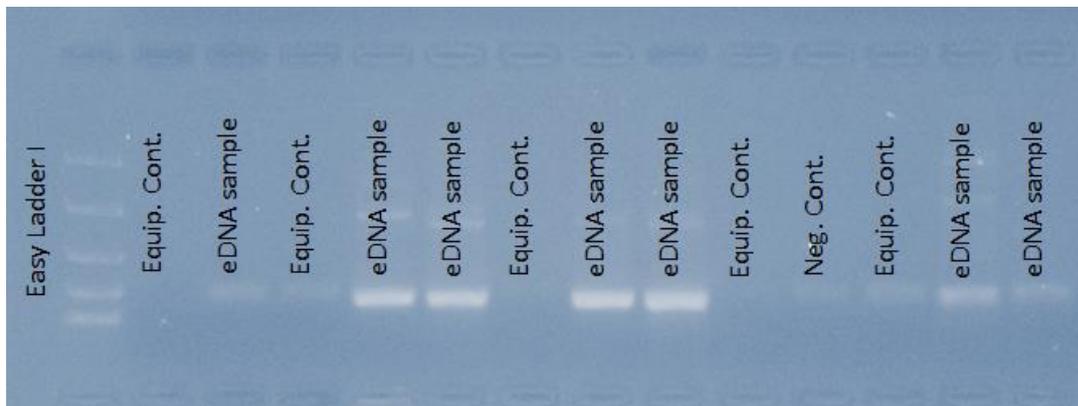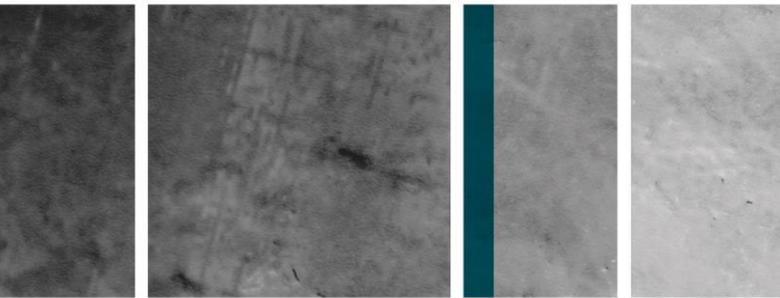
Figure 3.8. Gel image showing the contamination of negative equipment control samples, "Equip. Cont." as well as the negative sampling control, "Neg. Cont.". Amplified PCR product is indicated by the presence of a white band.

Figure 3.9. Gel image showing no contamination of negative equipment control samples after additional quality assurance measures were put in place. Amplified PCR product is indicated by the presence of a white band.

3.3.4. Field testing at different sites

Field testing the eDNA methods confirmed the utility and efficacy of eDNA to detect tilapia in various water bodies where their presence was known and well established. Environmental DNA analyses from these sites were consistently positive (Fig. 3.10) and matched published tilapia sequences on GenBank when PCR products were sequenced and analysed. Throughout the field testing, positive detections were apparent as consistent strong bands and with generally no non-specific amplification. Where non-specific amplification did occur the amplicon was very different in size (observed between 1000-2000 bp) making it easily distinguished from the tilapia-specific amplicon of 189 bp. These non-specific PCR products were sequence analysed and returned results that was not tilapia DNA and was most similar to various types of bacteria.
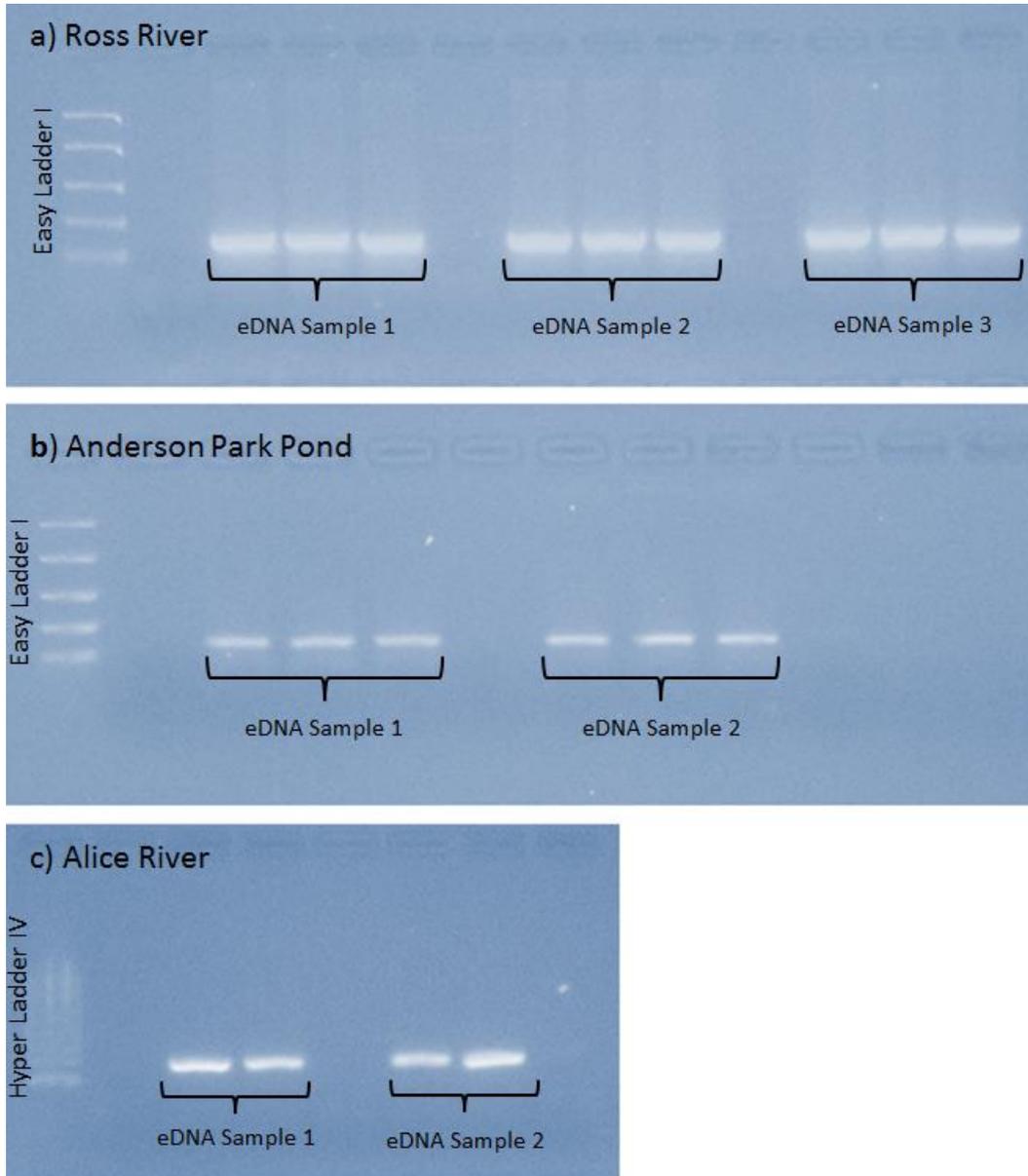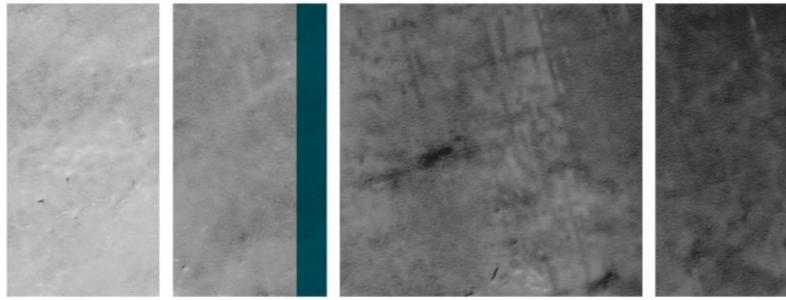
Figure 3.10. Gel image showing results of field testing the eDNA technology on water samples collected from Ross River (a), Anderson park (b) and Alice River (c). Consistent positive results (presence of white band of 189 bp in size) were detected from these sites with known tilapia infestations.
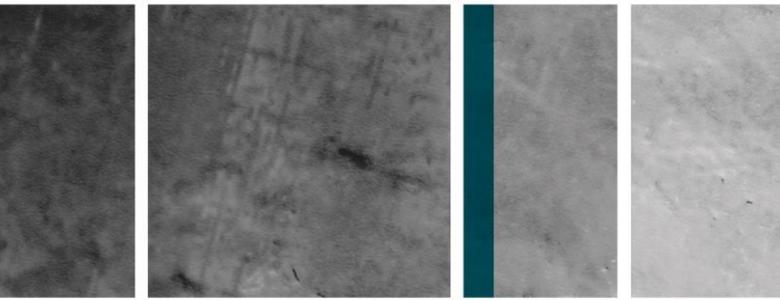
4. Tilapia eradication program – Eureka Creek case study

4.1. Introduction

Eureka Creek, in the Mitchell catchment of the Gulf of Carpentaria, is an important site because it was the location of a small incursion of tilapia (*O. mossambicus* and *T. mariae*) found in 2008 (Burrows 2009; Pearce *et al*. 2009). Establishment of a population in the Gulf of Carpentaria drainage would facilitate spread of tilapia across much of northern Australia. An eradication program was carried out to destroy and remove tilapia from the creek and prevent the spread into Gulf catchments. Follow-up sampling using electrofishing suggested that the eradication program had been successful (Burrows 2009). However, subsequent eDNA surveillance by UND in 2011 found a high proportion (77%) of presumptive positive detections in Eureka Creek samples, although this result was unreliable as it was based on inappropriate primers that co-amplified DNA of numerous native fishes (see Chapter 2). An additional eDNA survey of Eureka Creek was carried out in the current project to follow-up the unreliable UND assessment.

4.2. Methods

Sample collection of Eureka Creek included a total of 30 replicate 2 L water samples collected from three sites, upper, mid and lower Eureka creek. Each site consisted of nine 2 L creek water samples and one 2 L negative sampling control of RO water. Positive sampling controls were also included to identify potential PCR inhibitors in the Eureka Creek water. Positive control samples consisted of a 2 L water sample from each site, as well as a 2 L RO water sample each spiked with 50 μL of extracted genomic DNA from *O. mossambicus* fin tissue (416 ng/μl concentration). Samples were kept cool and were not exposed to sunlight until processing.

Filtration methods of the water samples collected from Eureka Creek followed those given in the laboratory manual in Appendix A of this report. These methods are a modified version of UND eDNA field guide and Jerde *et al*. (2011). Water samples were vacuum filtered through a 10 μm membrane filter (Merck Isopore membrane filter 47mm, item no. TCTP04700) within 24 hours of collection. 500 ml of deionised water was filtered through the equipment prior to each water sample being filtered. This is the equipment control (EC) that ensures any contamination of the filtration equipment can be identified to exclude false positives from this type of contamination. Once filtered, the sample filter papers were kept frozen (-20 °C) until DNA extraction. DNA extraction was carried out using the Bioline ISOLATE II Genomic DNA kit and following the methods given in the laboratory manual (Appendix A). Samples were amplified by PCR using 16s Oreo primers designed and tested previously. The PCR was carried out using the QIAGEN Type-IT kit and the following thermal cycling conditions; 95 °C for 5 min followed by 35 cycles of 95 °C for 30 sec, 60 °C for 90 sec and 72 °C for 30 sec, and a final extension of 72 °C for 10 min. All eDNA samples and controls were analysed for tilapia DNA by PCR. Each sample was analysed in triplicate reactions and a positive and negative PCR control were included for each site. The results of the PCRs were checked for presence/absence of tilapia specific products on an agarose gel. Tilapia-specific products are
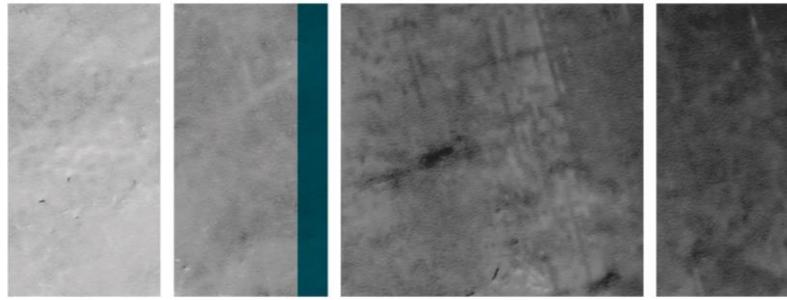
identified as a fluorescent band of 189 bp in size using the size standard ladder (left hand side on each gel). Absence of this band or a band of different size indicates a negative result for tilapia DNA.

4.3. Results & Discussion

All samples from the three Eureka Creek sites investigated (mid, upper and lower Eureka Creek) tested negative for tilapia (Fig. 4.1, 4.2 & 4.3), i.e. none of the samples indicated presence of tilapia (*Oreochromis mossambicus* or *Tilapia mariae* DNA when analysed by PCR and gel electrophoresis. All quality control samples including negative sampling controls for each site, equipment controls and negative PCR controls were all clear of contamination. Positive control samples spiked with *O. mossambicus* DNA all tested positive for tilapia as expected and indicated PCR reactions were unlikely to have been inhibited by any compounds in the creek water (Fig. 4.4). Non-specific amplification was observed in several samples, particularly negative control samples, however, these bands were of a much greater size than the expected size of a tilapia specific PCR product. This product was sequenced to determine the origin and the results of this test indicated the product originated from bacteria in the water and not tilapia DNA. Since 2009, electrofishing (Burrows, 2009) and eDNA surveys (current project) have identified no evidence of tilapia in Eureka Creek suggesting the eradication program has been successful.
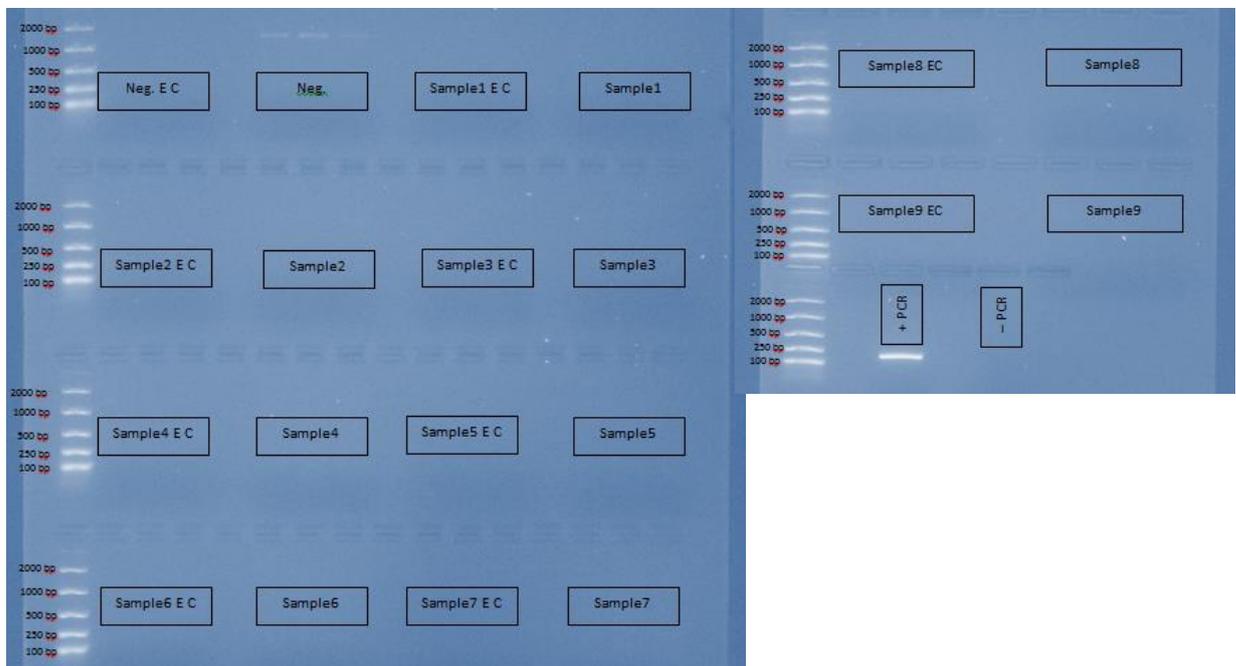
Figure 4.1. Gel image following electrophoresis of PCR product from mid Eureka creek eDNA samples. Results from the analysis indicate all eDNA samples tested negative for tilapia DNA and all quality control samples were clear.
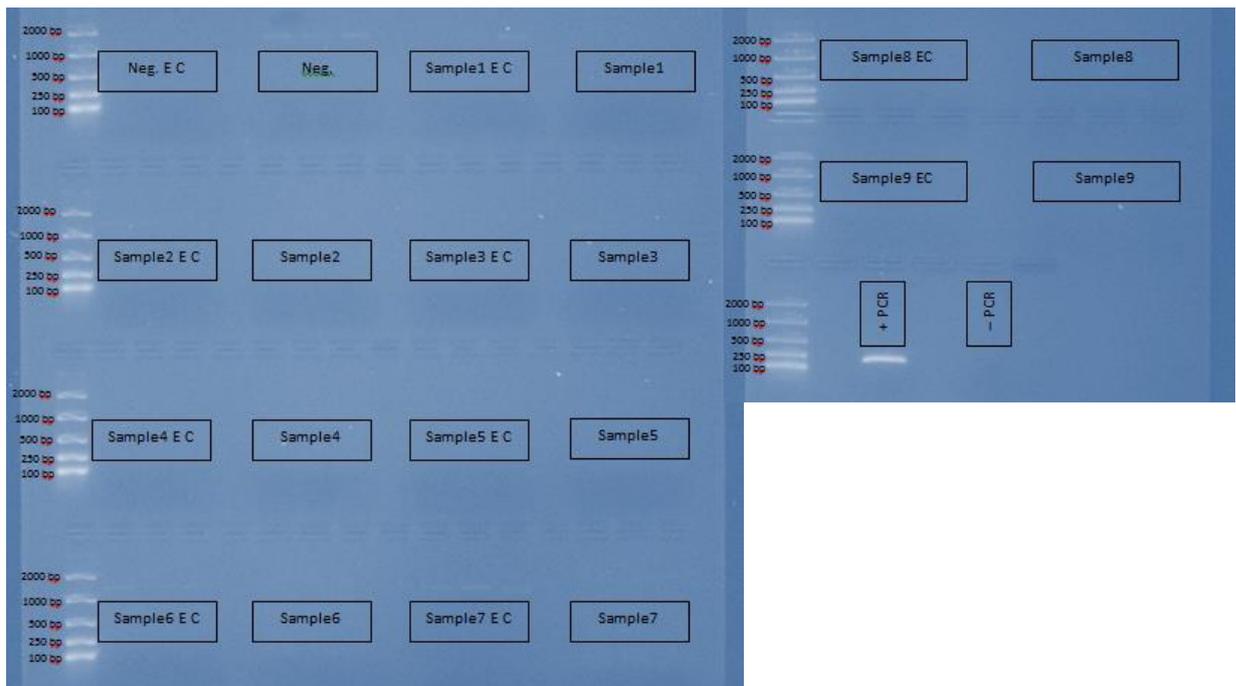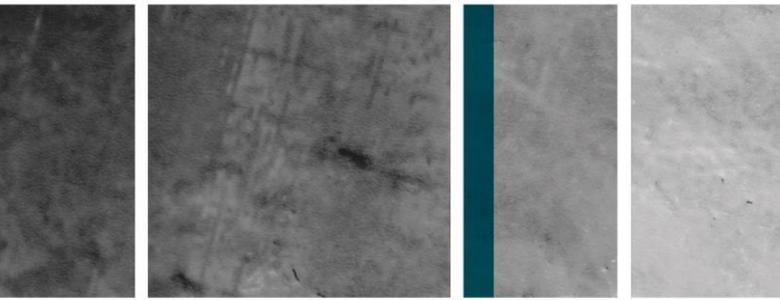
Figure 4.2. Gel image following electrophoresis of PCR product from upper Eureka creek eDNA samples. Results from the analysis indicate all eDNA samples tested negative for tilapia DNA and all quality control samples were clear.
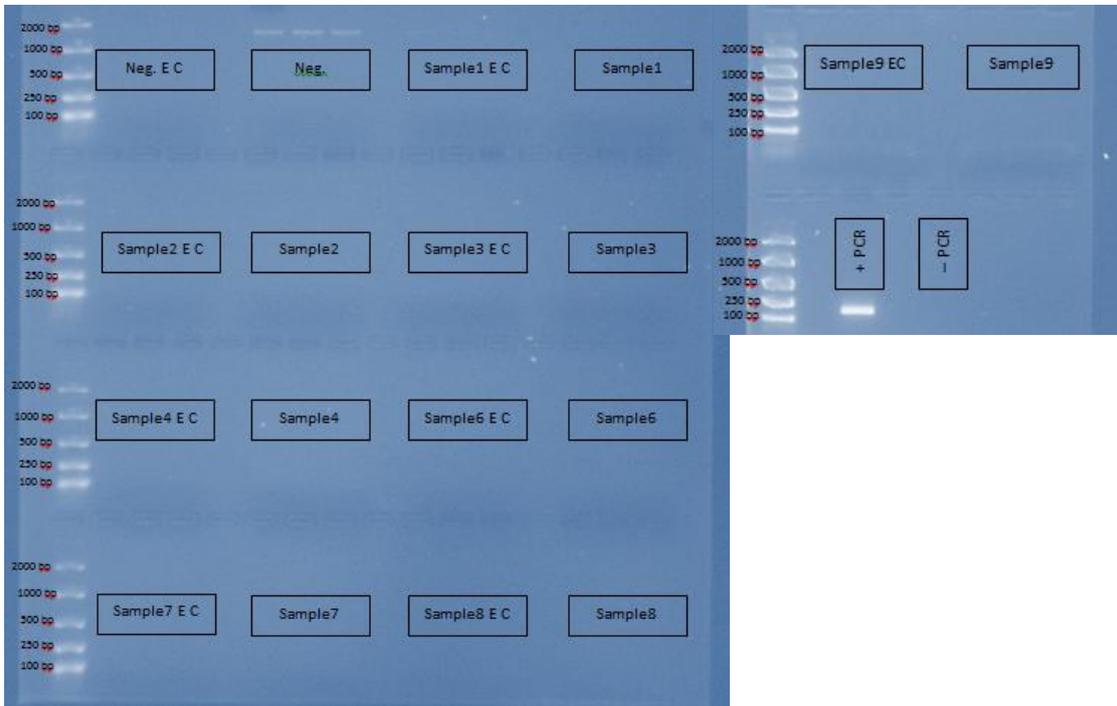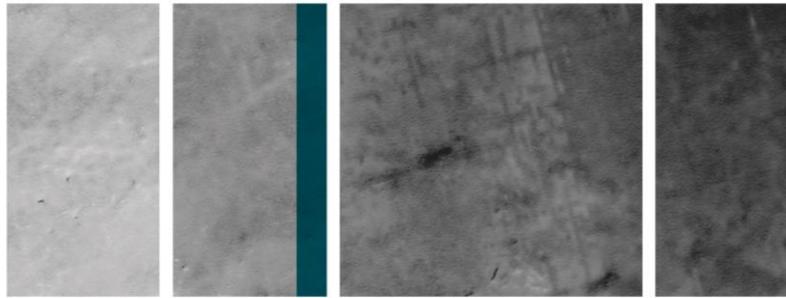
Figure 4.3. Gel image following electrophoresis of PCR product from lower Eureka creek eDNA samples. Results from the analysis indicate all eDNA samples tested negative for tilapia DNA and all quality control samples were clear.
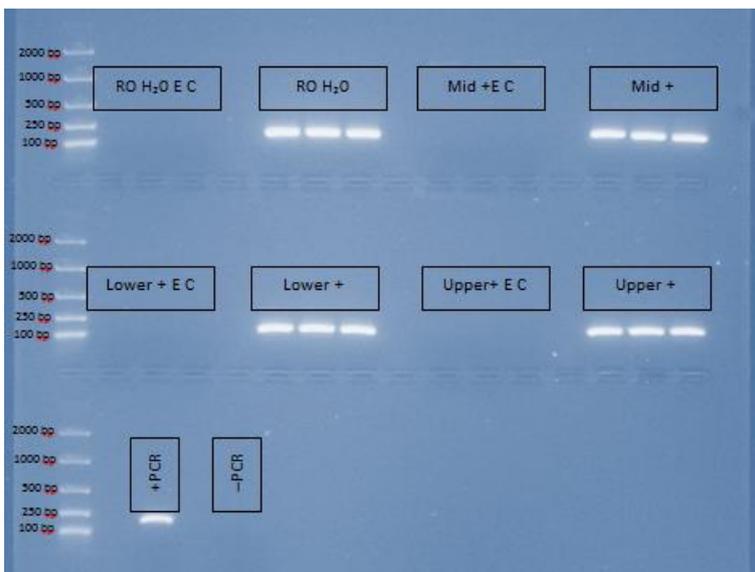
Figure 4.4. Gel image following electrophoresis of PCR product from positive control samples spiked with tilapia gDNA. Results from the analysis indicate all positive control samples tested positive for tilapia DNA, indicating no PCR inhibition occurred, and all quality control samples were clear.
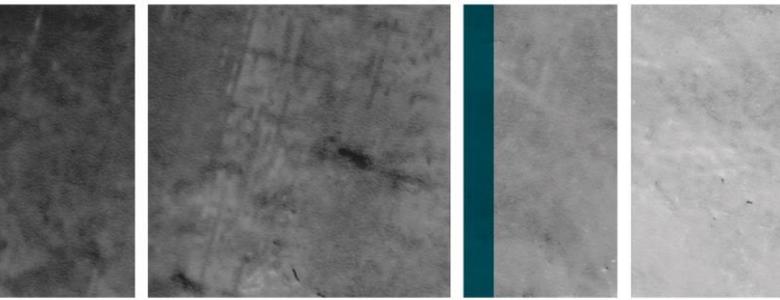
5. From conventional endpoint PCR to real-time quantitative PCR

5.1. Introduction

Endpoint PCR was used in many of the early eDNA studies (Ficetola *et al*. 2008; Goldberg *et al*. 2011; Jerde *et al*. 2011), probably because of its familiarity and ease of take up; however, real-time quantitative PCR (qPCR) is now the primary method of eDNA analysis (Takahara *et al*. 2012; Thomsen *et al*. 2012; Goldberg *et al*. 2013; Pilliod *et al*. 2013; Tréguier *et al*. 2014). Endpoint, or traditional PCR, relies on agarose gels for detection of PCR amplification at the final or endpoint of the reaction. This method has several limitations, a) it can only provide a presence or absence answer by visual inspection on an agarose gel, b) it relies on amplicon size discrimination which may not be very precise and c) means post PCR product is routinely handled. Quantitative real-time PCR (qPCR) in contrast detects PCR amplification whilst the reaction is occurring and in doing so allows the PCR amplification to be quantified. The advantages of qPCR are, a) it is generally more efficient in time and cost compared with end-point PCR as thermal cycling times are reduced, b) results are provided in real-time and agarose gels are eliminated, c) it allows for quantification of samples using standard curves and there is no need for post-PCR handling limiting contamination risks (Bott *et al*. 2010). A melt curve analysis can be done at the end of the quantitation stage of the qPCR analysis but only when using intercalating dyes. This replaces the need for gel electrophoresis to assess amplicon specificity and consistency. Quantitative PCR is also said to be more sensitive than endpoint PCR (Bott *et al*. 2010; Thomsen *et al*. 2012), although this may not always be the case (own observations and Bastein *et al*. 2008). Thus, a series of trials were undertaken prior to the adoption of qPCR technology for tilapia eDNA assessment in this study.

5.2. Methods

In total six different qPCR chemistries were tested for use in eDNA detection and quantification (see Appendix C for qPCR reaction recipes and protocols):

- Bio Rad SsoFast EvaGreen Supermix (item no. 172-5203)
- Bioline SensiFAST SYBR no-ROX kit (item no. BIO-98002)
- Life Technologies Power SYBR Green PCR master mix (item no. 4368577)
- QIAGEN Type-IT microsatellite PCR kit (item no. 206246) with PICO Green (item no. P11496)
- QIAGEN QuantiNova SYBR Green PCR Kit (item no. 208052)
- QIAGEN QuantiFast SYBR Green PCR Kit (item no. 204054)

The first trial of qPCR analysis was performed using Bio Rad SsoFast chemistry on eDNA samples collected from tilapia aquaria. Following initial trials on aquarium samples, water was collected from ponds stocked with tilapia and underwent the same qPCR analysis. The qPCR successfully amplified eDNA samples collected from aquaria (see results 5.3), but failed
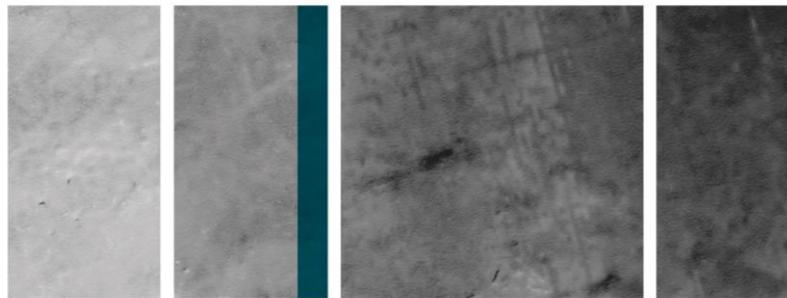
to amplify the eDNA samples collected from ponds when the DNA was analysed undiluted. These results indicated PCR inhibition (as a 1:10 dilution of the DNA successfully amplified) was occurring in the pond samples. Following this, several other qPCR chemistries (listed above) were trialled to determine which chemistry best amplified neat eDNA samples from environmental water samples (ponds, lakes, rivers etc). The various chemistries were trialled using the same eDNA samples collected from 2 L of pond water and used previously for the SsoFast trial. The chemistry was evaluated by its ability to amplify eDNA samples and the limit of detection for the standard curve. Based on the evaluation the most appropriate chemistry was chosen and further optimised for use in qPCR analysis.

5.3. Results & Discussion

Six different qPCR chemistries were trialled and two were suitable for eDNA analysis; Qiagen Type-IT with Pico Green and QuantiFast (Table 5.1). The other four chemistries trialled were not suitable for eDNA detection and quantification because they did not consistently amplify DNA when the water was collected from river and pond environments and the sample was analysed undiluted (neat). However, initial trials using Bio Rad SsoFast EvaGreen Supermix on water samples collected from aquaria produced reliable results. This chemistry was consequently used during the first controlled experiment (see section 6) where eDNA samples were collected from aquaria. When the SsoFast chemistry was later tested on eDNA samples originating from river or pond water, it failed to amplify the DNA when analysed undiluted. All chemistries, apart from Power SYBR, amplified eDNA when diluted 1:10 or 1:100. However, diluting the eDNA samples prior to analysis was undesirable due to the risk of over diluting already low concentrated eDNA samples. The qPCR efficiencies of all the chemistries tested were generally low. This was because limited PCR optimisation was carried out as well as the same DNA samples were being used for all trials and as the trials continued the DNA was likely degrading. Most of the chemistries produced the same results in terms of the standard curve detection limit. This is the point at which the qPCR reaction reached the minimum amount of target DNA to produce the desired PCR product. After all chemistries were trialled, QuantiFast was chosen due to its ability to amplify neat eDNA samples, acceptable PCR efficiency (improved to 1.0 after optimisation and fresh gDNA was used) and low detection limit. It was also chosen over Type-IT with addition of Pico Green as the QuantiFast is targeted for qPCR analysis and comes in an easy to use 2 X PCR master mix format.

Table 5.1. Initial overview of the qPCR chemistries trialed to assess their suitability for use in analysing eDNA samples.

qPCR chemistry	qPCR efficiency	R^2	Neat eDNA amplification	1:10 eDNA amplification	Detection limit of standard curve	Suitable for eDNA analysis
SsoFast	0.85	0.989	Failed	Passed	0.0000312	No
SensiFast	0.69	0.998	Inconsistent	Passed	0.0000312	No
Power SYBR	0.82	0.997	Failed	Failed	0.0000312	No
Type-IT w/ Pico Green	0.63	0.982	Passed	Passed	0.0000312	Yes
QuantiNova	0.29	0.997	Inconsistent	Passed	0.000625	No
QuantiFast	0.69	0.983	Passed	Passed	0.0000312	Yes
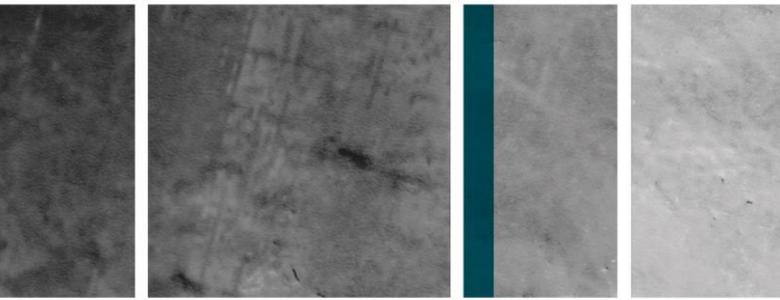
6. Controlled experiments understanding eDNA dynamics

6.1. Introduction

Understanding how different environmental parameters affect the use of eDNA to detect target organisms in aquatic environments is critical in survey design and interpretation of eDNA data. There have been several studies that have investigated various influences on eDNA detectability focussing on factors that affect eDNA shedding rates, environmental parameters that may affect DNA degradation or removal and site occupancy times. Species biomass and abundance have been shown to have significant effect on eDNA detection, where greater biomass results in increased eDNA concentration or increased detection probability (Takahara *et al*. 2012; Thomsen *et al*. 2012; Pilliod *et al*. 2013). Environmental DNA has also been shown to degrade rapidly once the source has been removed, for example, upon removal of the common spadefoot toad (*Pelobates fuscus*) and northern crested newt (*Triturus cristatusin*) from controlled mesocosm experiments, eDNA concentration of each species dropped immediately and the species' eDNA were unable to be detected one to two weeks later (Thomsen *et al*. 2012). Studies that have investigated environmental factors influencing eDNA detectability have focussed solely on temperate species and conditions. Therefore, two controlled experiments were carried out to: 1) gain an understanding of eDNA degradation at typical tropical temperatures and 2) develop an understanding of the sensitivity of the eDNA technology developed in this project.

6.2. Methods

6.2.1. Controlled experiment 1 – eDNA persistence in aquaria

To determine degradation rates of eDNA at tropical temperatures an experiment was conducted in a controlled temperature facility. Mozambique tilapia (*Oreochromis mossambicus*) were stocked in 12 x 60 L tanks at a range of temperatures that are typical of tropical environments. The temperature treatments were 23 °C, 29 °C and 35 °C. Each of the treatments and control tanks were replicated three times and assigned within the experimental room using a randomised block design to reduce the effect of position. Each tank was stocked with three fish that were measured (total length) and weighed prior to being randomly assigned to a treatment and tank. Fish were held in the respective tanks and temperature treatments for five days to allow eDNA to accumulate. After this period the fish were removed to evaluate the degradation rates. Each of the nine tanks were sampled daily with three replicate samples collected per tank as well as a negative control sample. Given the small volumes of the tanks, sample collection followed the eDNA precipitation collection method of Ficetola *et al*. (2008) which consisted of 15 ml of tank water added to a solution of 33 ml of absolute ethanol and 1.5 ml of sodium acetate (3 M). This method of water sampling was chosen so that the volume of the experimental tank was not greatly affected, as well as providing more flexibility in storing and processing the samples. Water samples were taken every 24 hours for the first three days and every 48 hours from day four to 29, or until eDNA levels approached minimum detection limits. The samples were mixed and stored overnight
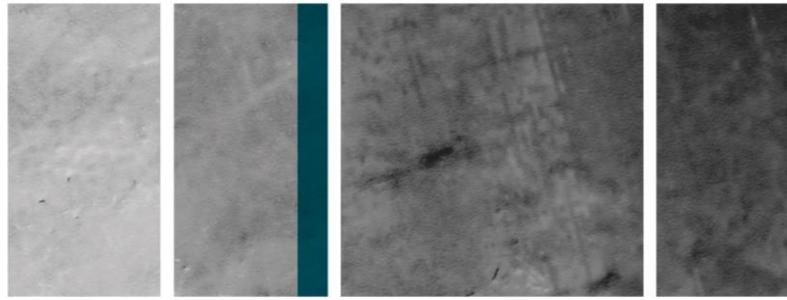
at -20 °C. To recover the DNA from the samples, tubes was centrifuged at 32,000 g at 6 °C for 70 min and the supernatant was discarded. The remaining pellets were extracted using the Bioline Isolate II Genomic DNA kit (cat no. Bio-52067) as per the manufacturer's instructions. Quantitative PCR analysis was performed in triplicate on each of the three samples taken per day giving nine replicates per tank per day using Bio Rad SsoFast EvaGreen Supermix (cat. no. 1725203). See Appendix C for specific qPCR recipe and thermal cycling conditions.

The effect of temperature on initial eDNA concentrations (on day one, after removal of tilapia) was tested using a one-way ANOVA (with log10 transformation of the data). Post-hoc tests were done comparing eDNA concentration at different temperatures using the Tukey-Kramer method of unplanned comparisons. Data on eDNA degradation was analysed with a Generalised Estimation Equation (GEE) using the geeglm function with R, in which eDNA presence was treated as a binomial response variable, with temperature as a main effect and day as a repeated measure with an exchangeable correlation structure (Zuur *et al*. 2010).

6.2.2. Controlled experiment 2 – eDNA detection sensitivity in ponds

Experiment 2 was carried out to gain an understanding of the accumulation rates of eDNA at various stocking densities and the overall sensitivity of eDNA as a detection tool for tilapia. The experiment was carried out in two parts, the first was in a static water system (pond) to determine lower limits of detection and accumulation rates and the second incorporated water flow through the ponds to understand the effect of flow on eDNA detectability. For this experiment, Mozambique tilapia (*Oreochromis mossambicus*) were stocked in outdoor ponds at the Walkamin Research Station on the Atherton Tablelands. The ponds each had a water surface area of 336 m^2, depth of 1.2 m and a total volume of 0.4 ML. A total of eight ponds were stocked with the following densities of tilapia; 1, 2, 4, 8, 16, 32 and 64 fish per pond as well as a control pond with no fish. The experimental treatments (fish density) were randomly assigned to each pond. Fish were measured before being randomly assigned to a treatment and for each treatment the ponds were stocked with equal number of male and female fish to avoid a bias in fish size (male tilapia were of greater size than females). Fish were housed in baskets (up to 8 fish per basket) that were suspended in the ponds to avoid possible escapes and mating.

All ponds were sampled at the start of the experiment to ensure the initial tilapia eDNA levels were zero and thereafter water samples were collected every 48 hours after the initial stocking for a period of 10 days. After 10 days of static flow in the ponds, the second part of the experiment took place with the addition of water flow to four of the experimental treatment ponds (densities 0, 2, 16 and 64). This part of the experiment ran for an additional four days. The simulated water flow was added to each pond by opening the pond inlet and outlet valves. The total volume of water in each pond remained the same, but an additional 267,840 L of water per day was added to each pond. This additional water would allow for a complete turnover of the water in each pond every one and half days. In addition to the "calculated" water that was added during the second part of the experiment, 42 mm of rainfall occurred over the four days (adding an estimated 14,112 additional litres of water).

Sampling of the ponds for eDNA consisted of collecting 2 L of water from two points in the pond which remained the same sampling locations for the duration of the experiment. A negative control sample, 2 L of RO water, was included in each sampling day. The negative control was opened, resealed and submerged in a pond and placed with all other water samples in a transport cooler before processing. Each 2 L water sample was vacuum filtered on site within 4 hours of collection using a 20 μm nylon filter (Merck Millipore, item no. NY2004700), following the filtration method adapted from Jerde *et al*. (2011) (see laboratory
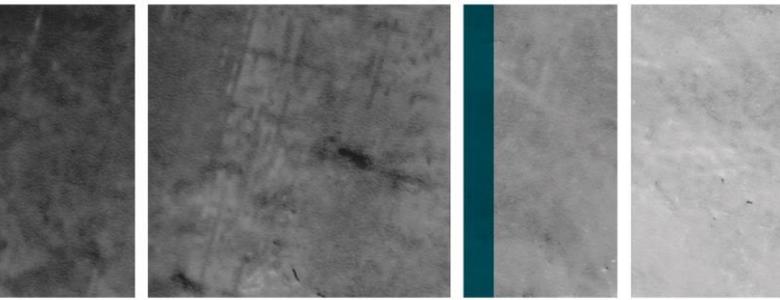
guide in Appendix A). Before each sample was filtered, 500 ml of RO water was filtered through the equipment and filter paper to test for contamination of the filtration equipment. The filter paper was stored for later testing and termed the equipment control, or EC. Filters were stored overnight at -20 °C before DNA was extracted the following morning using a Bioline Isolate II Genomic DNA kit (Bio-52067) as per the manufacturer's instructions. Any samples that exhibited excessively slow filtration and required the use of multiple filters (turbid samples or samples with high levels of algae) were recombined during the DNA extraction process. To analyse the water samples for eDNA detections a three phase qPCR analysis was performed using QuantiFast SYBR Green PCR kit on the Qiagen Rotor Gene Q PCR platform in a dedicated low copy room. Phase 1 tested each sample for PCR inhibition followed by phase 2 which evaluated the presence or absence of tilapia DNA in each sample. Five replicate qPCR reactions were included for each eDNA sample (10 PCR replicates per pond) and included in each run was a set of five DNA standards that ranged from 5 ng/µl down to 3.12×10^{-5} ng/µl. A sample was scored as positive in phase two when the following criteria were met: if there was a distinct amplification curve; if the CT value was equal to or less than the CT value of the lowest standard; and a distinct melt curve peak was present that corresponded to tilapia DNA. Any samples that resulted in positive tilapia detections then had the corresponding EC sample analysed for contamination (Phase 3) following the same qPCR methods as phase 2. See Appendix A for a detailed methodology used for eDNA collection and analysis during this experiment.

Data on the detection of eDNA was analysed with a logistic regression model, in which the probability of a water sample detecting eDNA (= a binary response) was modelled as a function of the number of fish in the pond, the day of the experiment, and the interaction between the two. Tests of the effect of fish number and time were conducted on data collected during the first 10 days of the experiment when there was no water flow to the experiment. Tests of the effect of water flow included water samples collected at the start of the water flow experiment (up to day 10) and the subsequent four days of water flow. Confidence limits on the probabilities of detection were calculated using the Exact Method as this was probability data with binary response of presence or absence and not normally distributed (Jones *et al*. 2014). Possible differences in average fish size per pond were analysed with a one-way ANOVA. All statistical analyses were performed using SPlus 8.2 ©.

6.3. Results

6.3.1. Controlled experiment 1 – eDNA persistence in aquaria

DNA was quantified in all temperature treatments following the removal of tilapia on Day 1. The concentration of eDNA (measured in ng/15 ml sample) on Day 1 was much higher in the highest temperature treatment (35 °C), but was very similar in the 23 °C and 29 °C tanks (Fig. 6.1). Temperature significantly influenced eDNA concentrations (ANOVA $F_{2,75}$ = 39.5, p < 0.001). The concentration at 35 °C was significantly higher than both the 29 °C and the 23 °C temperature treatments (p<0.001), neither of which were significantly different from each other (p>0.05). As a result of this large difference in starting eDNA concentrations, the probability of detection of eDNA was significantly affected by temperature (X^2 = 21.42, df = 2, p < 0.001). Detection probabilities were also affected by time (X^2 = 30.05, df=1, p < 0.001) (Fig. 6.2). Once the fish were removed, the eDNA began to degrade and the probability of detection decreased as time increased. However, it is important to note that when degradation rate of the three temperatures were compared over time (by comparing
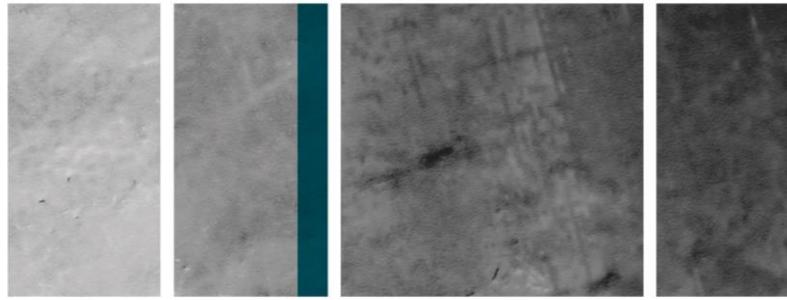
detection probabilities and the interaction of time and temperature), the rate at which eDNA degrades did not significantly differ between the three temperature treatments (X^2 = 1.82, df=2, p = 0.4). Environmental DNA was detected in the 35 °C treatment for at least 29 days post tilapia removal and detection probability remained high for the duration of the experiment at this temperature. Conversely, eDNA detection probabilities dropped in the 29 °C and 23 °C treatments, but was still detectable at low detection rates up until day 15 for 29 °C waters and until day 21 in the cooler 23 °C water temperature. No tilapia eDNA was detected in the control tanks or negative samples for the duration of the experiment.
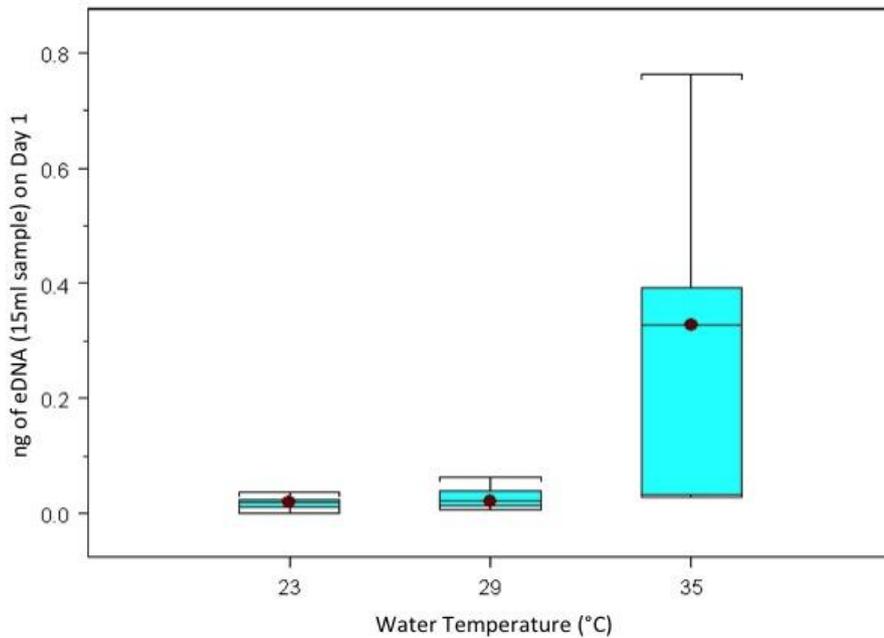
Figure 6.1. Environmental DNA starting concentrations as measured per 15 mL sample on Day 1 of experiment 1. Tilapia were held at three temperature treatments (23, 29, 35 °C) for five days prior to the start of the experiment.
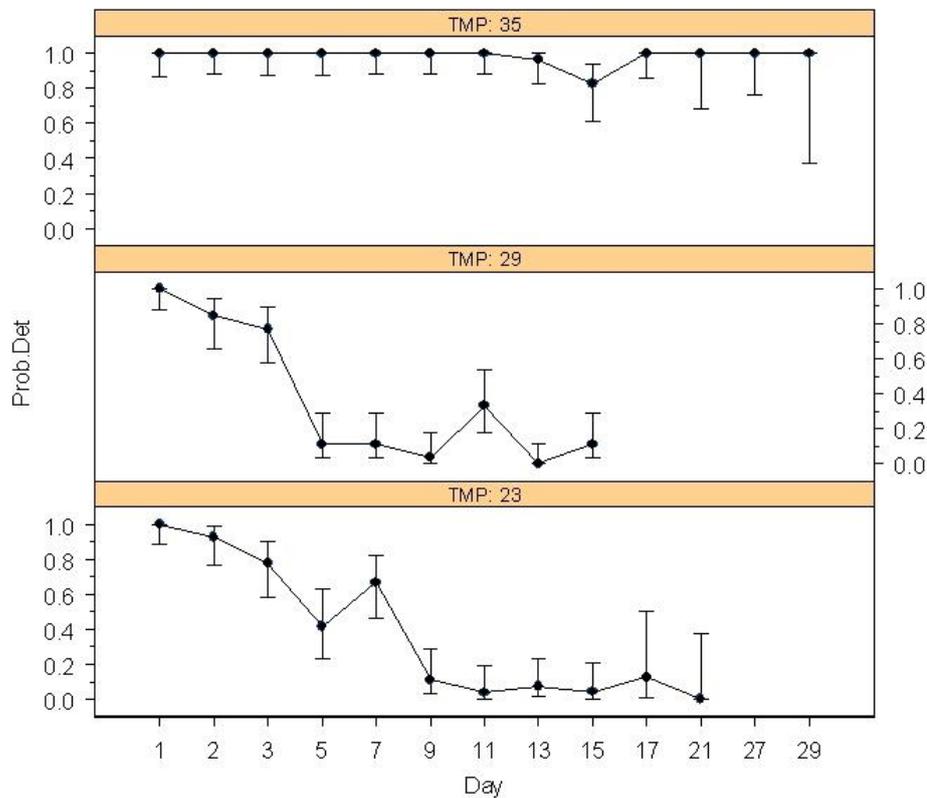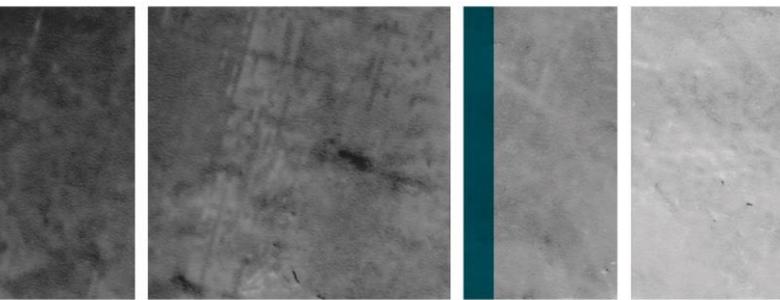
Figure 6.2. Degradation rates of eDNA following the removal of tilapia as a function of time and temperature (23, 29 and 35 °C). The probability of eDNA detection (+/- 95% confidence limits) each day water was sampled from aquaria at the three temperature treatments.

6.3.2. Controlled experiment 2 – eDNA detection sensitivity

Tilapia eDNA was detected in all ponds stocked with the various density treatments, down to one fish in 0.4 ML of pond water. There was no significant difference in average fish size (total body length) between each pond (ANOVA, $F_{6,115}$ = 0.65, p = 0.69). The probability of detecting eDNA in a closed system was significantly influenced by the number of fish in each pond (logistic regression, deviance = 157.50, df = 32, p < 0.001). Detection probabilities in ponds with low densities, 1, 2 and 4 fish, remained low during the 10 day period. Alternatively, detection rates were consistently high in ponds with higher numbers of fish (8, 16, 32 and 64, Fig. 6.3). There was no consistent effect of day on the probability of detecting eDNA (Deviance = 2.76, df = 31, p = 0.09), but there was a significant interaction between fish density and day, indicating that the changes in the probability of detecting eDNA over time was influenced by the number of fish in each pond (Deviance = 54.16, df = 24, p < 0.001). Therefore, higher abundance of tilapia in the environment will increase the probability of detecting them over time.
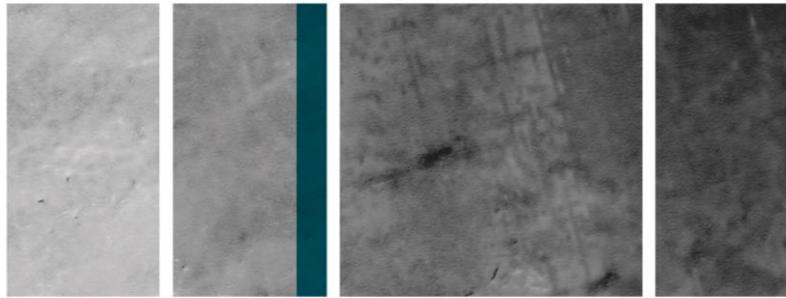
Examination of the effect of water flow on eDNA detection probabilities occurred in four ponds, of which only two (ponds with 16 and 64 fish) had detectable levels of eDNA prior to the introduction of water flow (Fig. 6.3). There was no effect of fish density (Deviance = 0.63, df = 1, p = 0.43) or day (Deviance = 4.01, df = 2, p = 0.14) and no significant interaction between the two (Deviance = 5.73, df = 2, p = 0.06) on the detection probabilities of eDNA. Therefore, the addition of water flow did not affect the ability to detect tilapia eDNA. The control pond that was not stocked with tilapia tested negative for tilapia eDNA throughout the experiment.
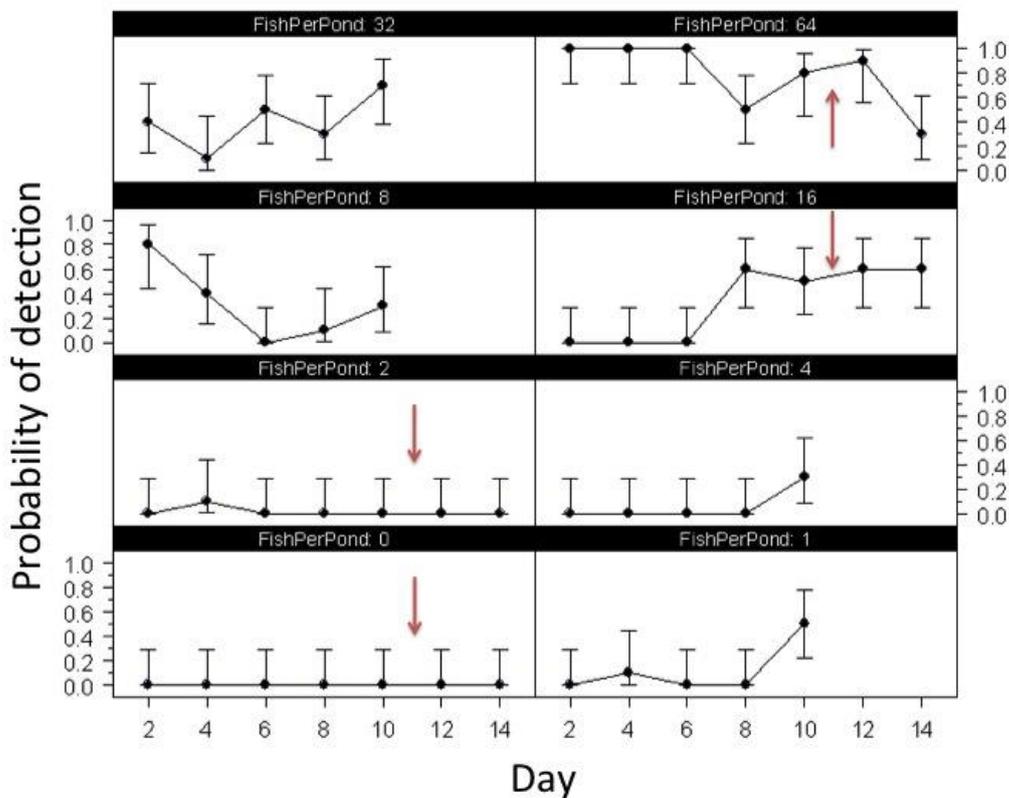
Figure 6.3. The relationship between fish density and time (post fish introduction) on the probability of detecting eDNA in water samples (+/- 95% confidence limits for each treatment pond). The red arrows indicate the time "water flow" commenced in four of the treatment ponds.

6.4. Discussion

Detection of eDNA relies on the balance between organisms shedding DNA into the environment and the loss of DNA from the system either through degradation or water movement. The first experiment investigated the effects of temperature on eDNA degradation. At the beginning of the degradation experiment, the fish were placed into the treatment tanks for five days prior to the start of the experiment. It was anticipated that all nine treatment tanks would have similar levels of eDNA. However, the 35 °C tanks had
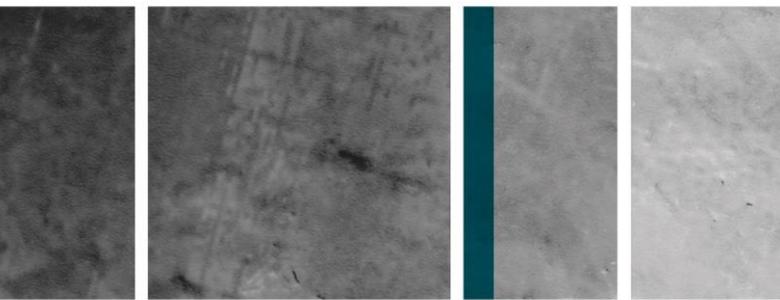
significantly high starting levels of eDNA than the tank at the cooler temperature (23 °C and 29 °C). This indicated that when tilapia are held at very high water temperature, they shed more DNA either from increased metabolism at higher temperature or due to stress. Therefore, at such high temperature the probability of detection increases. Detectability at 35 °C was longer most likely due to the higher starting concentration of eDNA. In controlled environments eDNA detection is likely to last longer than compared with natural conditions due to the many other factors in nature that would have an effect on eDNA being removed from the environment such as UV, microorganisms and water flow (Dejean *et al*. 2011). This was shown by Dejean *et al*. (2011) where eDNA was detectable (greater than 5% detection) in controlled aquaria conditions until day 25 and just 14 days in a semi natural pond environment. In tropical environments where UV radiation is greater and microbial activity high, eDNA degradation is likely to be more rapid.

The effect of temperature on eDNA shedding was contrary to what has been shown in the literature (Takahara *et al*. 2012; Kylmus *et al*. 2013). Tilapia did shed significantly more DNA when held at 35 °C which is near their upper thermal tolerance (Russel *et al*. 2012). Water temperatures of 35 °C can occur in northern Australia in shallow ponds and lakes during warmer months (Burrows and Butler, 2012). Conversely, Takahara *et al*. (2012) found no effect of water temperature on eDNA concentration of common carp held in aquaria, as did Kylmus *et al*. (2013) using Silver and Bighead carp (*Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* respectively). The temperatures used in these two studies were 7 °C, 15 °C and 25 °C and 19 °C, 25 °C and 31 °C respectively. However, when conducting field surveys Takahara *et al*. (2012) found a positive significant effect of temperature and eDNA concentration. Although, this result was more likely to reflect carp distribution within the field site with preference to warmer waters during winter (temperatures recorded average 8.5 °C), rather than increased DNA shedding caused from high temperature (Takahara *et al*. 2012). Therefore, experiment 1 in the current project is the first to show temperature does have a significant effect on eDNA shedding, but only when the organism is near or at its upper thermal tolerance.

The results from experiment 2 highlighted the sensitivity of the specific eDNA techniques and methods used in the current project. The method developed in this research was very sensitive, as one fish in 400,000 L of pond water was able to be detected after just 10 days in the water body and the higher density treatments produced consistently high detection rates. The results from the detection sensitivity experiment match what has been shown with numerous other species in the eDNA literature (Dejean *et al*. 2011; Takahara *et al*. 2012; Thomsen *et al*. 2012; Pilloid *et al*. 2013; Jane *et al*. 2014). Species biomass or abundance has a great influence on DNA concentration in the environment and therefore increased abundance increases the probability of detection. Therefore, when conducting field surveys increasing sampling effort, specifically increasing the number of samples collected and/or the volume of water sampled, may improve sensitivity and increase the likelihood of positive detections when abundance is anticipated to be low, such as invasion fronts.

Regardless of water flow tilapia were detectable during experiment 2, although, this was only shown for the higher density treatments where detection rates were already high. This result might not be the same at low densities when eDNA concentrations are likely to be low. Jane *et al*. (2014) also found that, generally, increased water flow did not affect eDNA concentration from brook trout (*Salvelinus fontinalis*) in flowing streams. The only negative effect of water flow on eDNA copy number was found when water samples were collected close to the source but as distance increased water flow had little impact. Further study is required to determine whether water flow will affect eDNA detection at low densities. This
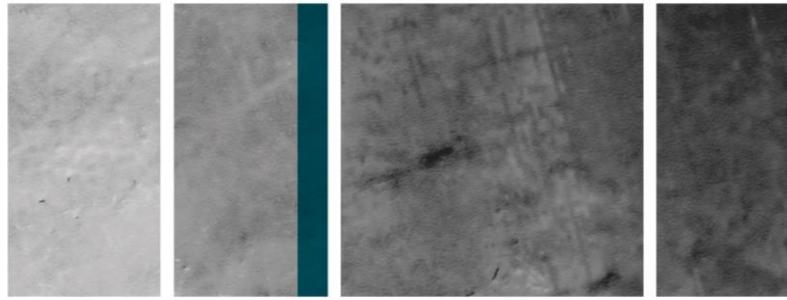
experiment and previous published work support the notion that flowing rivers can be effectively sampled using eDNA survey methods.

The controlled experiments carried out during this project have provided novel and important information for conducting eDNA surveys and interpreting the results. Several environmental parameters such as temperature and species abundance have significant effects on the results obtained and need to be considered when designing surveys and interpreting results. The current techniques used are unable to provide biomass or abundance estimates of tilapia in natural systems, however, in general when detection rates are high it is highly probable the population is well established. When carrying out surveys or monitoring invasion fronts low detection rates are likely to be indicative of low level presence and should not be disregarded as false positives due to contamination or PCR stochasticity.

While these experiments provide some insight into the environmental influences on the capacity of eDNA methods to detect tilapia, it should be recognised that natural systems are much more variable and numerous other factors are likely to influence detection probability. As the technology is now being applied, continued research will be useful in developing our understanding and should be considered a high priority. In particular, the impact of other abiotic and biotic factors such as UV, turbidity (algal and sediment based), oxidative capacity (e.g. biological oxygen demand, chemical oxygen demand) and pH need to be considered in future studies.
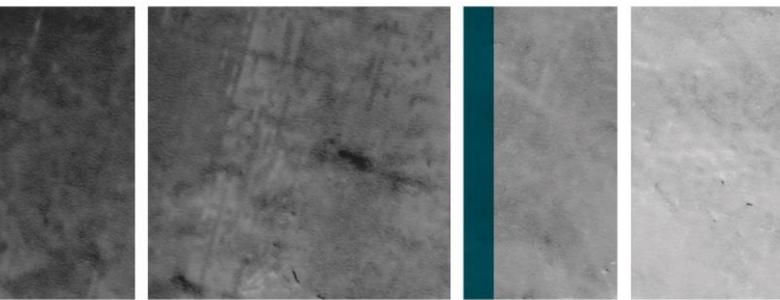
7. Comparison of eDNA with electrofishing

7.1. Introduction

Traditional fish surveillance relies on the community providing reports of new incursions that are followed up using nets and/or electrofishing to capture or detect the target species. These methods are generally quite laborious, time consuming and require a high level of expertise in fish identification (Thuesen *et al*. 2011; Minamoto *et al*. 2012; Thomsen *et al*. 2012) which can be costly. Traditional fish sampling methods are most effective when the target species is in moderate to high abundances, as often the target species evades capture at low densities resulting in inaccurate or false inferences on species absence (Jerde *et al*. 2011). Furthermore, tilapia are suggested to be particularly difficult to capture and detect using methods like electrofishing (pers. comm. Trent Power, Damien Burrows; TropWater). In contrast, eDNA surveillance methods have been shown to be just as efficient, if not more sensitive in detecting a number of target species including amphibians, fish and invertebrates, when compared to traditional methods (Ficetola *et al*. 2008; Jerde *et al*. 2011; Dejean *et al*. 2012; Minamoto *et al*. 2012; Thomsen *et al*. 2012; Tréguier *et al*. 2014). Consequently eDNA is likely to be a useful surveillance tool in circumstances where traditional sampling methods, like electrofishing for tilapia, may not be adequate, or requires huge sampling effort in order to detect the species at low densities and cover extensive survey areas. The aim was to contrast the effectiveness of eDNA as a surveillance tool with the primary traditional sampling tool for tilapia, electrofishing. The objectives were to provide a quantitative evaluation of the sensitivity and a consideration of the practical benefits in conducting each survey method.

7.2. Methods

Electrofishing and eDNA surveys were conducted at 14 sites in the lower Fitzroy River catchment (Figure 7.1) where a recent invasion of tilapia was reported (first reported in 2011). Site description and water quality parameters are provided in Table 7.1. The sites sampled were considered likely to provide a gradient in the level of infestation from the initial location of tilapia sightings and into areas where there have been no reports of tilapia. Both survey methods were carried out concurrently from 11 – 15 August 2014.

7.2.1. eDNA Survey

A total of 70 x 2 L water from the 14 sites within the survey area (see Table 7.2 for list of sites and site descriptions). At each site, five independent 2 L water samples were collected at approximately 20 m to 50 m apart. Sites varied in their habitat type ranging from small lagoons to creeks and large weir pools. Samples were either filtered straight after collection in the field, or kept in the dark on ice until processing could take place; all samples were filtered within nine hours of collection. The filtration protocol used follows the methods given in Appendix A. Water samples were vacuum filtered through a 20 μm nylon net filter (Merck NY2004700), filters were then stored in the freezer until further processing in the laboratory.
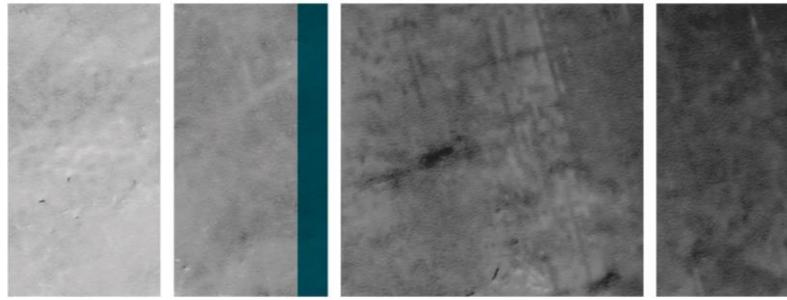
All filtration equipment was sterilised with 10% bleach solution before processing each sample. A negative sampling control was included for each site; this was a 2 L sample of deionised water taken into the field, stored with the samples and processed in the same way as above to test for field contamination (i.e. dunking the filled and capped bottle into the water). Following that, 500 ml of deionised water was filtered through the filtration apparatus, the filter paper was then stored and kept for further analysis. We referred to this as the equipment control (EC) and used it to test for equipment contamination for corresponding positive samples. DNA captured on the filters was extracted and isolated using the Bioline ISOLATE II Genomic DNA kit (item no. BIO-52067) following the manufacturers standard protocol (also given in Appendix A). A three phase qPCR analysis was then carried out for each sample to determine presence or absence of tilapia eDNA using specific 16s tilapiine primers (see Appendix A for detailed methodology). Phase 1 of the qPCR analysis identified whether the sample was being inhibited and consequently required further treatment. Following this was the presence/absence test for tilapia DNA using five replicate PCR reactions per sample. Finally, for every sample that resulted in positive tilapia detection, the corresponding EC was analysed to test for equipment contamination in phase 3 of the analysis.

7.2.2. Electrofishing Survey

The electrofishing survey was conducted from a 4.5 m vessel with a Smith-Root 7.5 FPP electrofisher unit, equipped with two boom arms, 16 dropper anode arrays and a hull cathode. The electrofishing boat was equipped with a generator powered pulsator to generate a pulsed DC waveform of 240 V. The electrofishing method used was a combination of power on, power off for the duration of the sampling effort. A master and a single dip-netter conducted all sampling activity. The sampling was conducted in shallow water to 5 m depth and in such a way that the anode array was nearly always in close proximity to suitable fish habitat such as fallen timber, grasses, macrophytes, bank overhangs and rocky structures. All electrofished tilapia were collected with dip nets with 3 m long handles, and immediately placed into a 100 L recirculating live tank on-board the vessel. At the completion of sampling at each site all fish were euthanized with an overdose of the anaesthetic Aqui-S (Aqui-S New Zealand Ltd.) and immediately chilled with ice. Numbers of tilapia caught were recorded along with fishing effort (time and distance covered by efisher) for catch per unit effort (CPUE) estimates. CPUE was calculated as fish caught per distance of creek sampled per hour of sampling at each site.

Water temperature, pH, dissolved oxygen and conductivity were measured using an Aqua Read AP-200 at three points in the main channel at each site at a depth of 0.1 m. Secchi depths were obtained by lowering a standard 200mm secchi disk into the water column until the disk could no longer be seen and then raised until the contrasting black and white portions were discernible.
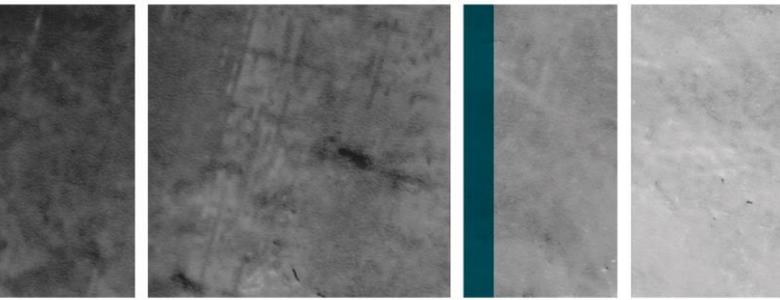
Figure 7.1. Location of sites sampled within the Fitzroy River Basin during the eDNA survey of tilapia. Results of the eDNA survey which returned the positive presence of tilapia DNA are represented by images of tilapia, those that did not are represented by images of tilapia with crosses. Contaminated sites are marked.
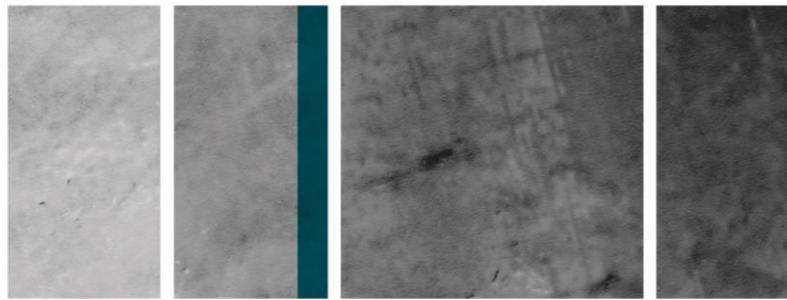
Table 7.1. Site description and water quality parameters during eDNA and electrofishing survey in the Fitzroy River lower catchment area. Water quality parameters are averages of three sites within each system.

	Water quality parameters					Site description			
Site	Temp. (°C)	pH	Conductivity (μs/cm)	Dissolved oxygen (% sat)	Secchi depth (m)	Water body type	Average site width (m)	Site length (m)	Flow (m/s)
Nankin Creek Lagoon	22.4	7.0	470	84.7	4.7	Instream pool	30	1200	0
Moores Creek	21.7	7.3	1560	111.5	>1.5	Instream pool	10	165	<0.1
River Road Lagoon 1	19.9	7.0	507	75.2	0.8	Offstream pool	14	150	0
Raglan Creek	20.8	7.6	1512	97.0	1.2	Instream pool	32	1060	<0.1
Bajool weir pool	20.6	7.7	1220	93.6		Weir pool	25	1230	0
Gracemere Lagoon	18.4	7.6	463	26.5	0.4	Instream pool	10	310	0
Yeppen Lagoon	20.7	8.0	342	98.4	0.6	Offstream pool	75	1500	0
Lion Creek	17.8	7.4	230	80.1	1.6	Weir pool	23	620	0
Splitters Creek	22.1	8.6	539	122.4	1.0	Weir pool	15	675	0
Belmont Creek	17.8	7.4	230	80.1	1.6	Weir pool	12	410	0
Alligator Creek	19.8	7.5	287	91.5	1.8	Weir pool	25	1230	0
Rossmoya Rd Creek	20.4	7.5	203	93.7	0.5	Instream pool	12	275	0
Hedlow Creek	20.5	7.5	1272	94.4	1.4	Instream pool	18	940	0
Eden Bann weir	19.0	7.6	276	91.9	0.8	Weir pool	160	1360	<0.1

7.3. Results

7.3.1. eDNA survey

Tilapia were detected by eDNA methods in eight of the 14 sites surveyed (Table 7.2). Two sites had no detectable DNA and the remaining four sites failed to meet the necessary quality controls and results from these sites could not be confirmed. Of the eight sites that tested positive for tilapia DNA, the sample detection and PCR amplification success was varied. The number of positive samples per sites ranged from 1 – 5 and the proportion of positive PCRs per sites ranged from 0.04 to 0.48, with an average of 0.24 ± 0.06. At two of the sites surveyed (Moores Creek and Raglan Creek) the PCR amplification success rate of the eDNA was very low (0.04, or 1 out of a possible 25 reactions). Conversely, at Yeppen Lagoon the PCR amplification success was much higher at 0.48.
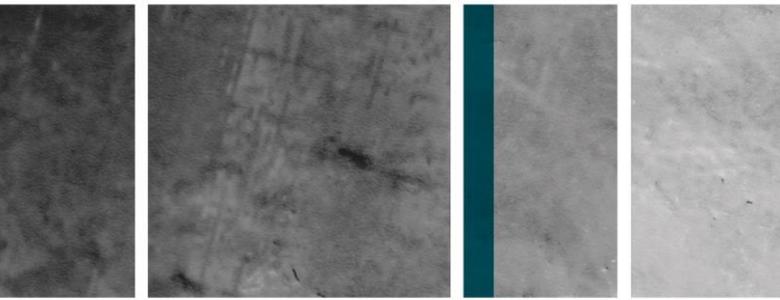
7.3.2. Electrofishing survey

Tilapia were caught at three of the 14 sites surveyed using electrofishing (Table 7.1). The three sites where tilapia were caught were Yeppen Lagoon, Lion Creek and Splitters Creek and the number of tilapia caught at each of these sites were 2, 20 and 40 fish, respectively. The CPUE varied considerably at each site, with Yeppen Lagoon requiring the highest effort in order to catch the two tilapia caught (0.0005 fish/m/hr). Conversely, at Splitters Creek tilapia were much more readily caught with CPUE at this site being 0.2063.

7.3.3. Comparison of eDNA with electrofishing

Positive detections were obtained for the eDNA survey from all three sites where tilapia were detected using electrofishing (Table 7.1). The average number of positive samples for these sites was four out of a possible five samples and amplification success (i.e. proportion of positive PCRs) averaged 0.41 ± 0.05. In addition to the three sites where tilapia presence was confirmed by electrofishing, tilapia DNA was also detected in a further five sites where electrofishing failed to detect tilapia. The sample detection averaged 1.2 out of five for these sites and amplification success 0.14 ± 0.05.
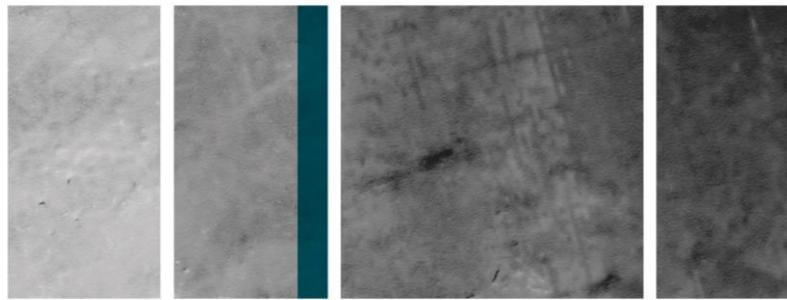
Table 7.2. Detection of tilapia using electrofishing and environmental DNA (eDNA) in the Fitzroy River lower catchment area. Number of tilapia caught and catch per unit effort (CPUE) as the number of tilapia caught per metre of water fished per hour resulting from the electrofishing survey are given. For eDNA the presence or absence of tilapia DNA at each site and eDNA detection rates are provided. Anecdotal sightings of tilapia at each site are also given.

| Site | Electrofishing | | eDNA | | | Anecdotal sightings |
	Number of tilapia caught	CPUE (fish/m/hour)	Tilapia detected	Sample detection positives	PCR detection positives	Tilapia sighted
Nankin Creek Lagoon	0	0	Yes	1/5	5/25	No
Moores Creek	0	0	Yes	1/5	1/25	Yes
River Road Lagoon 1	0	0	No	0/5	0/25	Yes
Raglan Creek	0	0	Yes	1/5	1/25	No
Bajool weir pool	0	0	Yes	2/5	8/25	Yes
Gracemere Lagoon	0	0	No	0/5	0/25	No
Yeppen Lagoon	2	0.0005	Yes	3/5	12/25	Yes
Lion Creek	20	0.0255	Yes	5/5	11/25	Yes
Splitters Creek	40	0.2063	Yes	4/5	8/25	Yes
Belmont Creek	0	0		Contaminated		Yes
Alligator Creek	0	0		Contaminated		Yes
Rossmoya Rd Creek	0	0	Yes	1/5	2/25	No
Hedlow Creek	0	0		Contaminated		No
Eden Bann weir	0	0		Contaminated		No

7.4. Discussion

The ability to survey many sites across large areas efficiently and effectively is important in managing and controlling the spread of invasive animals. Molecular methods for ecological surveys such as using eDNA to detect invasive tilapia have the ability to do this, whilst also being somewhat more sensitive than traditional electrofishing methods. The eDNA technology performed just as well as the electrofishing by detecting tilapia DNA at all three sites where tilapia were also confirmed via electrofishing. The eDNA technique also found tilapia DNA at a further five sites where electrofishing failed to detect their presence, suggesting eDNA may be more sensitive than electrofishing in surveys for tilapia in freshwater. Environmental DNA technology has been proven, in several studies, to be more sensitive than comparative traditional methods for several species. For example, Dejean *et al.* (2012) found presence of bullfrogs in 38 ponds surveyed was five times higher compared to when traditional survey methods were used. Using only traditional methods such as electrofishing for tilapia or visual or auditory surveys for bullfrogs, it is likely that the presence of these species at survey sites can be severely underestimated. Indeed, the extreme sensitivity of eDNA surveillance requires great care be taken to prevent contamination when collecting samples and during sample processing.
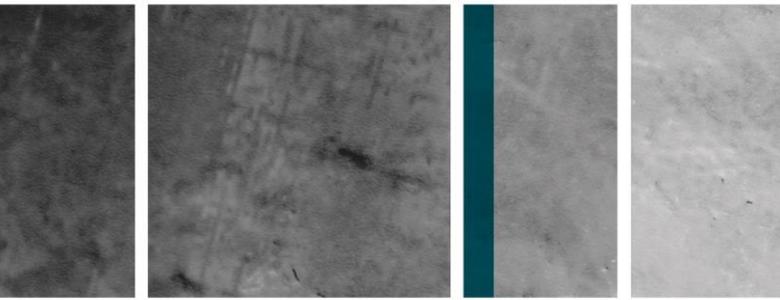
In this example, contamination occurred at four sites (Belmont Creek, Alligator Creek, Hedlow Creek and Eden Bann Weir Pool) resulting in contaminated negative sampling controls. All four of these sites were those that followed the capture of tilapia by the electrofishing vessel. The capturing and handling of tilapia on board the boat likely resulted in traces of tilapia DNA remaining on-board which were not effectively removed before sampling took place at the following sites. Sampling and handling of water samples for the eDNA survey at these sites took place on-board the boat in the front deck area where tilapia were previously caught and handled. Traces of tilapia DNA on-board the boat, or released into the surrounding water (e.g. flushed from the live fish well where the tilapia were also held) was the most likely source of contamination detected in the downstream qPCR tests. Further to this, the one instance following capture of tilapia where no field contamination occurred (that of Rossmoya Road) was that in which eDNA samples were collected from the bank and upstream from the boat, therefore away from any traces of tilapia DNA that may have been on board.

A critical comparison of eDNA against traditional electrofishing sampling methods surveillance needs to consider two key subjects: sensitivity and logistics. In this study, the sensitivity of the methods was assessed in a direct comparison between electrofishing and eDNA in a quantitative comparison. From a logistical perspective, field sampling for eDNA was able to be done in 30 minutes per site by a single person, followed by an additional three hours of sample processing in the laboratory, whereas electrofishing required a minimum of two hours per site by two people. Environmental DNA methods are particularly efficient when scaled up as the reduced site time offers the potential to survey large areas rapidly with a greater likelihood of detection in comparison to electrofishing. Furthermore, when scaled up, the cost of reagents and laboratory time is reduced in large surveys as samples can be bulk processed.

The use of eDNA was found to be an efficient and effective broad-scale tilapia surveillance tool. However, perhaps eDNA should be used in addition to traditional methods like electrofishing, as the two methods should be seen as complementary rather than competitive. The main benefits of eDNA are that it is more sensitive than electrofishing in detecting tilapia and can be carried out very efficiently in the field allowing more water bodies to be surveyed in an equivalent field program. Although electrofishing is more labour intensive in the field which means only a few sites can be sampled in a day, it can provide more detailed information on the invasion, such as sexual maturity of fish and abundance relative to other species. In this sense eDNA may be used to conduct broad-scale surveys of areas like invasion fronts and the results used to guide electrofishing so that fishing effort can be targeted to critical areas where tilapia have been identified by eDNA methods.
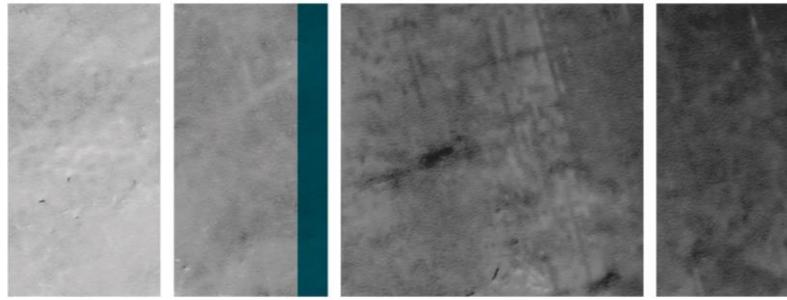
8. Conclusions

A critical component in controlling the spread of invasive species is early detection. Environmental DNA has proven useful in early detection and detecting species at low densities, however, current knowledge on eDNA technology stems primarily from temperate freshwater environments. In the tropics there are many differences in environmental conditions which may have an effect on the utility of eDNA for detecting invasive animals in these areas. In tropical north-eastern Australia, where tilapia (*Oreochromis* spp. and *Tilapia mariae*) have become widespread, this project has successfully implemented the eDNA technology to detect tilapia across a range of freshwater habitats. The utility of eDNA as an effective method in tilapia surveillance has been proven and will be highly beneficial in aiding programs to reduce further spread of these two species.

The first stage of developing eDNA for tilapia detection was finding suitable DNA markers capable of discriminating tilapia from other organisms in a mixed environmental sample. The available DNA markers resulted in amplification of multiple other freshwater fish species when tested against genomic DNA samples of these fish. Therefore, development of new and novel DNA primers that would be suitable eDNA markers for tilapia detection was required. The new markers developed were diagnostic for tilapia, particularly in the early stages of testing where the specificity was tested against the native fish DNA collection and in tilapia-infested sites around Townsville. As the project progressed and more and more environments were tested and PCR conditions become further optimised, non-specific binding using these primers started occurring. However, the non-specific PCR products were easily recognisable when checked on an agarose gel or melt curve analysis when using qPCR, thus limiting the chances of false positive detections due to non-specificity.

Throughout this project, continual developments to original and available eDNA protocols were made so that the eventual protocol was more suited to eDNA surveys in tropical environments. The main developments included using large pore-filter papers so that the highly turbid water samples often experienced could be filtered efficiently. Moving to larger sized filters did not impact the ability to detect tilapia eDNA. Further, the use of real-time quantitative PCR instead of conventional end-point PCR has helped to make the process of eDNA analysis more efficient by having the PCR and post PCR fragment size identification all in one analysis. Also, qPCR has eliminated the need to handle post PCR product and therefore reduces the risks of contamination.

The two controlled experiments undertaken provided insight into the effects of typical tropical temperatures on eDNA detection and the level of detection sensitivity using the eDNA methods developed during this project. When carrying out the eDNA persistence experiment, temperature was found to have a huge effect on eDNA shedding rates, but only when the animal was at its physiological threshold. The ability to detect tilapia eDNA once removed did generally decrease over the experimental time period, however, when there was a huge amount of eDNA in the environment, such as when tilapia were held at 35 °C, detection rate stayed very high. Previous studies found temperature did not affect eDNA shedding rates, the opposite of what was found when tilapia were near their physiological limits. The second experiment demonstrated the methods developed were very sensitive and capable of detecting tilapia DNA from as little as one fish in 400,000 L of water. When fish density was higher the detectability increased significantly and was much more consistent. When eDNA was compared to electrofishing for tilapia surveillance, the eDNA method performed very well. The results of this study suggest eDNA may be more sensitive than the traditional method of tilapia surveillance, electrofishing. However, the two methods should be used in
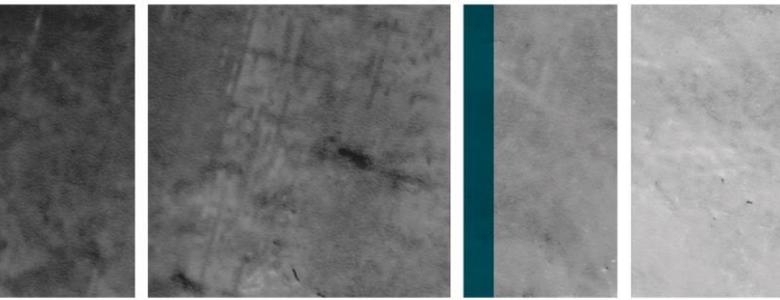
conjunction with each other in such a way that the greater sampling efficiencies and sensitivity of eDNA can be used to conduct initial broad surveys and electrofishing can then target the areas identified with tilapia presence. By surveying tilapia in this way, monitoring of their spread can be undertaken more efficiently and effectively.

The eDNA techniques to detect tilapia developed throughout the project has resulted in a high quality service that will be beneficial to many organisations and associations to help stop the spread of invasive tilapia in Australia. Already, the uptake of eDNA as a method of tilapia surveillance and monitoring is occurring. The Fitzroy Basin Association and Catchment Solutions have employed the eDNA services developed from this project to survey the recent invasion of tilapia in central Queensland.
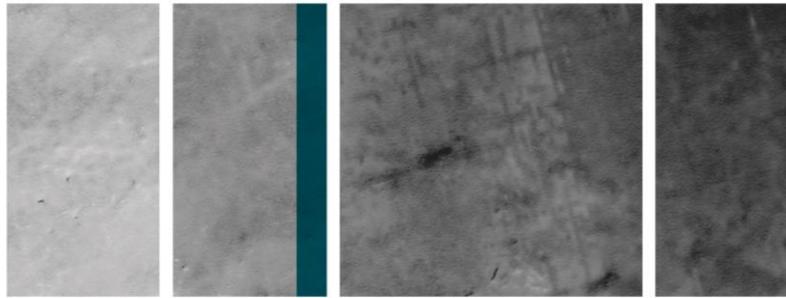
List of references

Adamkewicz SL, Harasewych MG (1996) Systematics and biogeography of the genus *Donax* (bivalvia: Donacidae) in eastern North America. American Malacological Bulletin 13:97-103

Bastien P, Procop GW, Reischl U (2008) Quantitative real-time PCR is not more sensitive than "conventional" PCR. Journal of Clinical Microbiology 46:1897-1900

Bott NJ, Ophel-Keller KM, Sierp MT, Herdina and others (2010) Toward routine, DNA-based detection methods for marine pests. Biotechnology Advances 28:706-714

Burrows DW (2009) Final report: Preventing tilapia (and other exotic fish) from taking over northern gulf waterways, The Northern Gulf Resource Management Group, Georgetown, Qld

Burrows DW, Butler B (2012) Preliminary studies of temperature regimes and temperature tolerance of aquatic fauna in freshwater habitats of northern Australia. Australian Centre for Tropical Freshwater Research, Townsville, Australia, 49 p.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., Miaud, C. (2011) Persistence of environmental DNA in freshwater ecosystems. PLoS One, 6, e23398.

Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C (2012) Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bull frog *Lithobates catesbeianus*. Journal of Applied Ecology 49:953-959

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biology Letters 4:423-425

Goldberg CS, Pilliod DS, Arkle RS, Waits LP (2011) Molecular detection of vertebrates in stream water: A demonstration using rocky mountain tailed frogs and idaho giant salamanders. PLoS ONE 6: e22746

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP (2013) Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science 32:792-800

Guy RA, Payment P, Krull UJ, Horgen PA (2003) Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Applied and Environmental Microbiology 69:5178-5185

Jane SF, Wilcox TM, McKelvey KS, Young MKand others (2014) Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources 15: 216-227

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4:150-157

Jerde CL, Chadderton WL, Mahon AR, Renshaw MA and others (2013) Detection of asian carp DNA as part of a great lakes basin-wide surveillance program. Canadian Journal of Fisheries and Aquatic Sciences 70:522-526

Jones R, Gilliver R, Robson S, Edwards W (2014) S-Plus for the Analysis of Biological Data.

Klymus K, Richter C, Chapman D, Paukert C (2013) ECALS: Loading studies interim report July 2013. Asian Carp Regional Coordinating Committee, U.S.A., 11p.
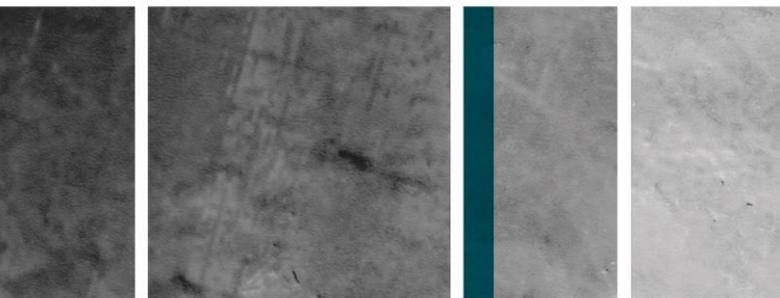
Lodge DM, Turner CR, Jerde CL, Barnes MAand others (2012) Conservation in a cup of water: Estimating biodiversity and population abundance from environmental DNA. Molecular Ecology 21:2555-2558

Meyer BS, Salzburger W (2012) A novel primer set for multilocus phylogenetic inference in east african cichlid fishes. Molecular Ecology Resources 12:1097-1104

Minamoto T, Yamanaka H, Takahara T, Honjo MN, Kawabata Zi (2011) Surveillance of fish species composition using environmental DNA. Limnology 13:193-197

Ovenden JR, Macbeth MG, Pope LC, Thuesen P, Street R, Broderick D (2014) Translocation between freshwater catchments has facilitated the spread of tilapia in eastern Australia. Biological Invasions 17: 637-650

Pearce MG, Perna C, Hedge S (2009) Survey of Eureka Creek and Walsh River fish community following rhe removal of tilapia using rotenone. Queensland Primary Industries and Fisheries, Brisbane

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013a) Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources 14: 109-116

Pilliod DS, Goldberg CS, Arkle RS, Waits LP, Richardson J (2013b) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences 70:1123-1130

Russell DJ, Thuesen PA, Small FE (2010) Tilapia in Australia: Development of management strategies for the control and eradication of feral tilapia populations in Australia, PestSmart Toolkit publication, Invasive Animals Cooperative Research Centre, Canberra, Australia

Russell DJ, Thuesen PA, Thomson FE (2012) A review of the biology, ecology, distribution and control of mozambique tilapia, *Oreochromis mossambicus* (peters 1852) (Pisces: Cichlidae) with particular emphasis on invasive Australian populations. Reviews in Fish Biology and Fisheries 22:533-554

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of fish biomass using environmental DNA. PLoS One 7:e35868

Thomsen PF, Kielgast J, Iversen LL, Wiuf C and others (2012) Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology 21:2565-2573

Thuesen PA, Russell DJ, Thomsen FE, Pearce MG, Vallance TD, Hogan AE (2011) An evaluation of electrofishing as a control measure for an invasive tilapia (*Oreochromis mossambicus*) population in northern Australia. Marine and Freshwater Research 62:110-118

Tréguier A, Paillisson J-M, Dejean T, Valentini A, Schlaepfer MA, Roussel J-M, Crispo E (2014) Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. Journal of Applied Ecology 51:871-879

Tringe SG, Rubin EM (2005) Metagenomics: DNA sequencing of environmental samples. Nature Reviews Genetics 6:805-814

Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, Schwartz MK (2013) Robust detection of rare species using environmental DNA: The importance of primer specificity. PLoS One 8:e59520

Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution, 1:3-14
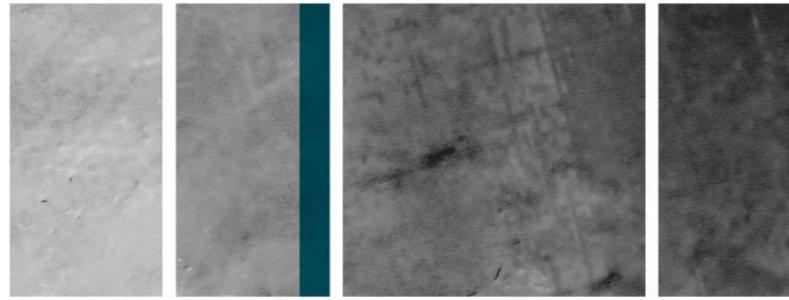
Appendix A

<u>Laboratory Manual</u>

Environmental DNA (eDNA) analyses for tilapia surveillance

and monitoring

Centre for Sustainable Tropical Fisheries and Aquaculture

College of Marine and Environmental Sciences

James Cook University

Prepared by:
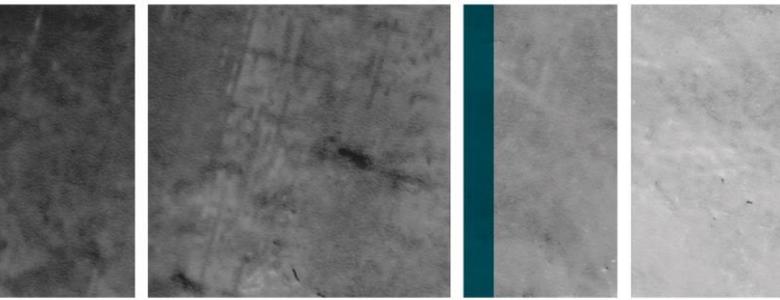
Field sampling of water for eDNA analyses

JCU WHS RiskWare ID: 1706

This manual uses 2 L water samples collected from various water bodies (e.g. streams, rivers, ponds or lagoons) to screen for tilapia eDNA as an indicator of tilapia presence at the site. It is recommended at each site at least five replicate 2 L water samples are collected. For large water bodies or flowing streams and rivers it may be necessary to increase replication at the site. Due to the extremely high sensitivity of eDNA analyses, the highest care must be taken to avoid cross contamination between samples and sites. We recommend sampling the water from the bank rather than on board a boat and if a boat is part of the survey then eDNA sampling at each site should take place before the vessel enters the water. When samples are unable to be collected from the bank it is crucial to decontaminate the boat both inside and out by washing/wiping with 10% bleach solution to avoid cross contamination between sites.

1. Decontaminate transport esky and nally bins used for field sampling with 10% bleach solution ensuring the bleach solution is left to stand for a few minutes on the surface.

2. Organise all equipment needed for field sampling; this will include the transport esky, nally bin, 2 L sample bottles, 2 L negative sampling controls filled with deionised laboratory water, gloves, marker for labelling bottles and rubbish bags for used gloves.

3. Once at a sampling site, put on examination gloves and begin filling 2 L sample bottles from just under water surface. Change gloves between sample collections. It is important to collect water samples from areas that are likely to pool/concentrate eDNA, such as eddies, stream blockages and shady areas. Try not to get shoes or clothes wet to avoid cross contamination between sites.

4. For the negative sampling control, wearing fresh gloves, remove the bottle cap then reseal and submerge the bottle in the field water before placing in the transport esky.

5. Once all samples have been collected, cover bottles with ice as soon as possible to ensure samples are kept cool on return to the laboratory.

6. Back in the laboratory, remove samples from transport esky and rinse sample bottles under running tap water to remove any residual field water and/or biological material to avoid cross contamination.

7. Store samples in fridge (4 °C) in a clean container until processing. Samples should be processed within 24 hours after collection.

8. Before beginning filtration, personnel should change from field clothing and shoes to reduce risk of contamination.
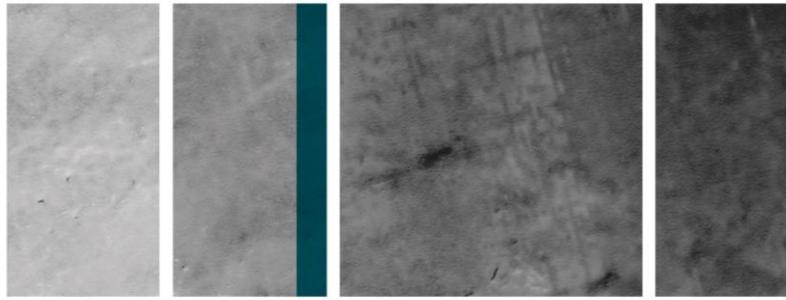
Filtration of water samples for eDNA analyses

JCU WHS RiskWare ID: 2316

Water samples collected from the field need to be filtered, the purpose of this is to capture the eDNA in the water onto a filter paper so that the DNA can then be extracted and analysed for presence of tilapia DNA. It is important to filter the water samples as soon as possible after collection so that the eDNA in the water does not degrade. All water samples should be filtered within 24 hours of collection.

Laboratory preparation:

Photo: Laboratory set of filtration equipment ready for filtering eDNA water samples.

1. Decontaminate low copy DNA room by mopping floor and wiping all surfaces with 10% bleach solution.

2. Prepare two tubs or nally bins with 10% bleach solution, one for soaking equipment (~50 L total volume) and the other for running bleach through filtering apparatus (1 L per sample).

3. Soak all filtering and laboratory equipment to be used (filter funnels, hosing, collection carboy, forceps, scissors, beakers, drainer etc) in the 10% bleach solution for 10 minutes.

4. Rinse equipment thoroughly under running tap water (20 seconds each).
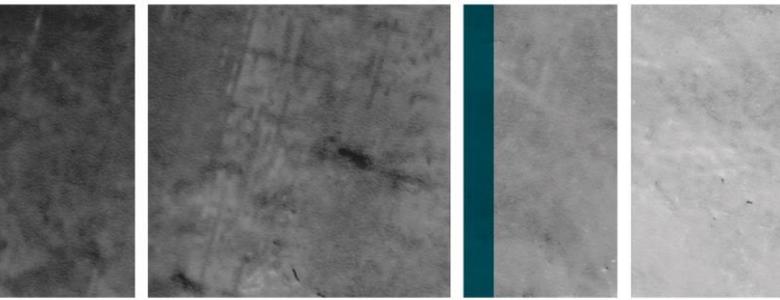
5. Set up filtering apparatus by connecting rubber tubing from the filter funnel manifold (Pall Filter Funnel Manifold, item no. 15402) to the collection carboy, this is where the waste water passes through. Attach another piece of tubing to connect the vacuum (either in line vacuum or vacuum pump) to the collection carboy. Ensure the vacuum tubing is higher than the level of waste water collected in the carboy when full so no water is drawn back up into the vacuum.

6. Set up a station for the filter papers, this station should be kept as clean as possible with paper towel laid down, designated clean forceps for only handling clean filter papers and clean gloves should be worn.

7. Label tubes for storing filter papers (at least four tubes per sample, two for equipment control and two for the sample, filter papers are cut in half with one half for further processing and the other half stored); label each tube with site ID, sample number and date. Label two of the tubes with an additional "EC" after sample number to indicate this is the equipment control for the corresponding sample.

8. Set up workstation for filtering sample – At each station place a clean paper towel with:
   1x forceps
   1x scissors
   4x tubes (Axygen 2 ml microcentrifuge tubes) for storing filter papers. (See below for labelling)
   1x filter funnel (Pall Magnetic Filter Funnel, item no. 4238 or Pall MicroFunnel disposable filter funnels, item no. 4819)

9. Place bottom half of filter funnel on manifold leaving the top half on the bench on the paper towel.

Filtering the equipment control (EC):

10. Put on clean examination gloves before each sample to be filtered.
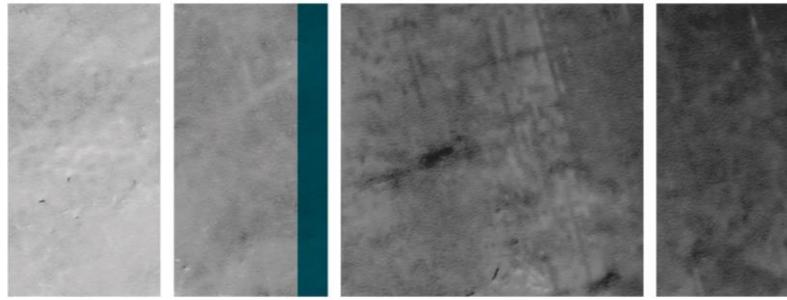
11. Using forceps place a new filter paper (Merck Millipore Nylon Net Filters, Mesh Opening 20 μm, Filter Diameter 47 mm, item no. NY2004700) on bottom half of filter funnel and ensure these forceps are returned to the filter paper station. Ensure not to touch any part of the forceps to the funnel apparatus. If contamination does occur, decontaminate the forceps by soaking in bleach solution for 10 minutes.

12. Secure the top half of the filter funnel. Using a clean beaker pour 500 ml of de-ionised water into the filter funnel making sure the funnel is filled to the top.

13. Open the vacuum line to filter water through and close off again once the 500 ml of water has been filtered.

14. Remove filter funnel top and using forceps and scissors roll the filter paper then cut in half. Place one half in each of the two tubes with "EC" labelled on them.

15. Place tubes in tube rack ready for processing or storage.

Filtering the sample:

16. Place a new filter paper on the same filter apparatus used to filter the equipment control and secure filter funnel top.

17. Remove first sample from fridge and place at the workstation on paper towel.

18. Invert sample bottle three times to ensure sample is well mixed before pouring into the filter funnel.

19. Open vacuum line to filter sample water through and continue pouring in the sample until all 2 L has been filtered.

20. Remove filter, cut in half and place each half in separate labelled tubes then place in tube rack.

21. If water sample exhibits excessively slow filtration (turbid samples or samples with high amounts of algae), multiple filter papers may be used to filter the entire 2 L sample. Additional filter papers are cut in half and stored in separate tubes (these are combined during the DNA isolation phase).
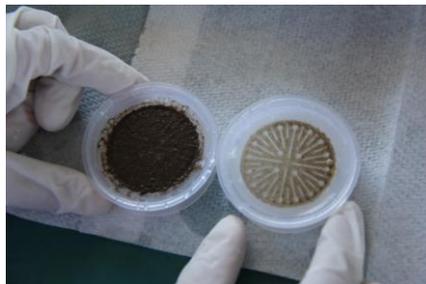
22. Store filter papers in -20 °C freezer until DNA extraction or if unable to store at -20 °C filters can be preserved in 95% ethanol solution and kept at room temperature.
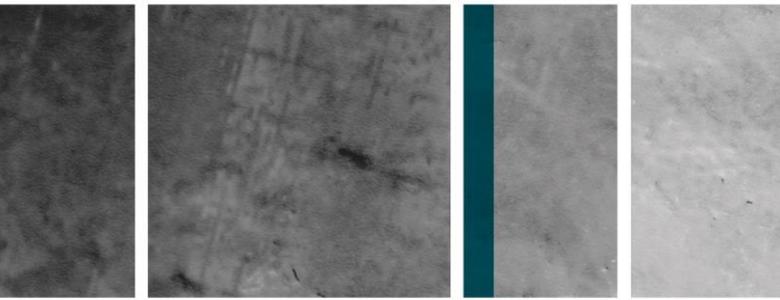
Decontamination between samples:

23. Once the sample has been filtered, run 1L of 10% bleach through filtering equipment (without filter paper).

24. Place filter funnel, rubber stopper, forceps and scissors in nally bin with 10% bleach solution for soaking equipment. Soak for 10minutes.

25. After bleaching equipment, rinse thoroughly under tap water (20 seconds) and place on clean drainer.

26. Wipe down bench with 10% bleach solution. Proceed to next sample starting at step 8.
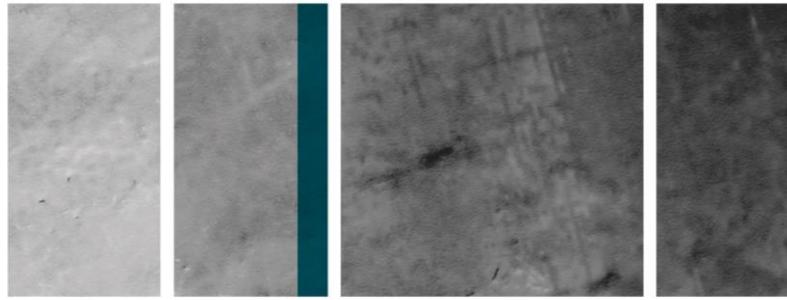
DNA extraction from filtered water samples

Once the samples have been filtered, the DNA needs to be extracted from the filter paper and isolated in order to analyse for presence of tilapia DNA. Only one half of the filter paper is processed so that the other half can be stored and used later if needed. This manual uses the Bioline ISOLATE II Genomic DNA kit (BIO-52067) for extracting eDNA samples.

Laboratory preparation:

- wipe over bench tops in low copy room with 10% bleach solution

- lay down paper towel on bench

- set incubator to 56 °C.

Sample preparation:

- remove filter samples from -20 °C freezer and organise in tube racks so equipment controls are on a separate rack to the samples

- label spin columns and tubes for extracted DNA with site ID, sample number and date.

DNA extraction:

Before opening tubes at any step pulse centrifuge samples to ensure there is no liquid around the lid that may flick on opening and contaminate other samples. Always start by opening equipment control tubes first with clean gloves, adding appropriate buffer, closing equipment control tubes then moving on to opening eDNA sample tubes.

1. Add 180 μl Lysis Buffer GL and 25 μl Proteinase K solution to each tube, if multiple filter papers were used for a sample; add the same amount of buffer and proteinase K to each additional filter paper.

2. Vortex each tube for 10 seconds.

3. Incubate at 56 °C for 10 minutes with rotation. Once completed remove and set incubator to 70 °C.

4. Pulse spin. Change gloves. Add 200 μl Lysis Buffer G3 to each tube.

5. Vortex each tube for 10 seconds and incubate at 70 °C for 10 minutes with rotation.
   Note: Also place Elution Buffer G into the oven to preheat for elution step.

6. Pulse spin. Change gloves. Add 210 μl of ethanol (96-100%) to each sample and vortex for 10 seconds each.

7. Place spin column in a 2 mL collection tube. Load 600 μl of the sample into each column and centrifuge 1 minute at 11,000 x g. Discard flow through into a covered beaker filled with water, blot top of collection tube on a clean paper towel and transfer spin column into new collection tube.
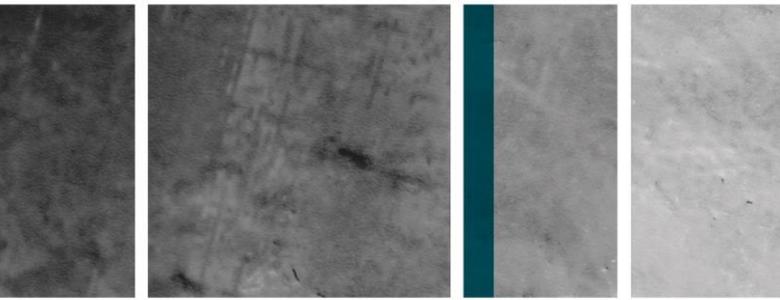
Note: If multiple filter papers were used for a single 2 L sample this is the point in which they are combined. Repeat step 5 by loading 600 μl from additional filter papers, ensuring each extra filter paper that was required is loaded into the same corresponding sample spin column.

8. Change gloves. Add 500 μl of Wash Buffer GW1 and centrifuge for 1 minute at 11,000 x g. Discard flow through, blot and reuse collection tube.

9. Change gloves. Add 600 μl of Wash Buffer GW2 and centrifuge 1 minute at 11,000 x g. Discard flow through into a covered beaker filled with water, blot top of the collection tube on a clean paper towel and reuse.

10. Centrifuge 1 minute at 11,000 x g to remove any residual ethanol.

11. Place spin column into a NEW 1.5 ml microcentrifuge tube.

12. Change gloves. Add 60 μl of preheated Elution Buffer G (70 °C) onto the centre of the silica membrane.

13. Incubate at room temperature for 5 minutes.

14. Centrifuge 1 minute at 11,000 x g. *Note: Visually check each tube before discarding spin column to ensure elution buffer was added and DNA was eluted*

15. Store eluted DNA at -20 °C.
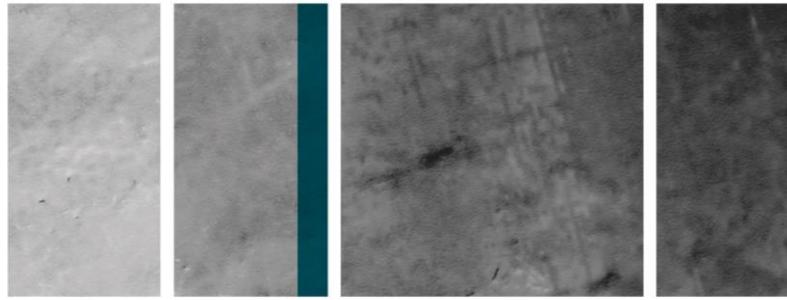
Quantitative PCR amplification of eDNA samples

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Quantitative PCR or qPCR analysis of eDNA samples is performed using a three phase analysis approach. The first phase checks each sample for PCR inhibition using an internal positive control (IPC), the second phase analyses each sample for presence or absence of tilapia DNA and phase three checks for contamination in corresponding equipment controls (EC's) for all samples that tested positive for tilapia. All three phases of the qPCR analysis utilise the same qPCR reagents (phase 1 also includes the IPC, listed below) and thermal cycling protocol. This protocol uses QIAGEN Rotor Gene Q real-time thermal cycler. A robotics system (Corbett CAS-1200) is used for pipetting the PCR master mix and eDNA samples into the qPCR analysis disc to ensure highly accurate and precise pipetting when analysing eDNA samples.

qPCR reagents:

- QIAGEN QuantiFast SYBR Green PCR kit (item no. 204054) – 2x master mix containing HotStarTaq Plus DNA polymerase, QuantiFast Sybr Green PCR buffer, dNTP mix and fluorescent dyes.
- QIAGEN RT^2 qPCR primer gDNA control or IPC (item no. 330011) – used in **phase 1** analysis **only.**
- Human genomic DNA – used in **phase 1** analysis **only**
- 16s tilapia specific primers "Oreo 16s F" – CTTCAGACGCCAGAACAG and "Tilapia 16s R" – GCTTGGAGTTGTAACTCTGG that amplify a 189 bp sequence.

qPCR reaction recipes:

Phase 1	Initial conc.	Vol. per reaction	Vol. per disc (115 reactions)	Final conc.
QuantiFast	2 X	10 µl	1150 µl	1 X
Water	-	1.4 µl	161 µl	-
IPC primer mix	10 µm	0.8 µl	92 µl	0.4 µm
Human gDNA	5 ng/µl	2 µl	230 µl	0.5 ng/µl
Oreo 16s F	10 µm	0.4 µl	46 µl	0.2 µm
Tilapia 16s R	10 µm	0.4 ul	46 µl	0.2 µm
eDNA sample	-	5 µl	-	-
Total	-	20 µl	1725 µl	-

Phase 2 & 3	Initial conc.	Vol. per reaction	Vol. per disc (115 reactions)	Final conc.
QuantiFast	2 X	10 µl	1150 µl	1 X
Water	-	4.2 µl	483 µl	-
Oreo 16s F	10 µm	0.4 µl	46 µl	0.2 µm
Tilapia 16s R	10 µm	0.4 ul	46 µl	0.2 µm
eDNA sample	-	5 µl	-	-
Total	-	20 µl	1725 µl	-
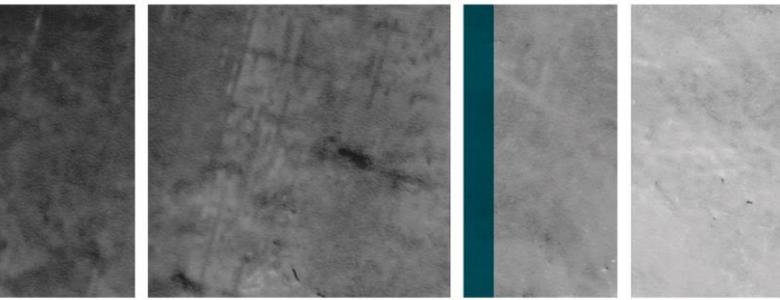
qPCR thermal cycling protocol:

Initial denaturation: 95 °C for 10minutes
Cycling: 40 cycles of 95 °C for 10 sec followed by 60 °C for 30 seconds
Melt analysis: 60 °C to 95 °C in 0.5 °C increments

Phase 1 – PCR inhibitor check using IPC:

eDNA samples are run in phase 1 without reaction replicates (i.e. singularly). Ensure at least two no template controls (NTC) are included in each run. The NTC's are used to identify eDNA samples that are inhibited by contaminants from the water. By comparing the CT values of the NTC and the eDNA sample, PCR inhibition can be identified. The eDNA sample should amplify at the same efficiency ($\pm$ SE) as the NTC for no PCR inhibition to occur, as shift in CT value (later CT value) indicates inhibition is likely occurring (Jane et al. 2014).

1. UV sterilise all equipment needed for qPCR analysis (pipettes, tips, disc and tube racks) and molecular grade water for qPCR master mix for 30 minutes. Ensure there are enough wells for each sample and controls to be screened.

2. Remove qPCR reagents and eDNA samples from freezer and allow to thaw. Vortex, pulse spin, vortex, pulse spin before use.

3. Once UV sterilisation is complete, working within the UV cabinet, prepare the qPCR master mix following recipe above for phase 1. Vortex and pulse spin master mix and keep cool until proceeding to the robot for pipetting into disc.
    Note: when preparing qPCR master mix it is important to allow at least 15% excess volume to ensure there is enough master mix for all reaction wells (included in recipe above).

4. Prepare the eDNA samples to be analysed for the robot by aliquoting samples into a plate. For each sample add 4 µl of eDNA and 6 µl of molecular grade water to the plate (this includes excess volume for pipetting error).

5. Now that the master mix and eDNA samples have been prepared, proceed to the robot to pipette mixture and samples into the qPCR analysis disc.

6. To each well add 15 µl of qPCR master mix and 5 µl of the aliquoted eDNA sample with mixing after each sample is added.

7. Once the robot has finished pipetting, seal the disc using heat sealing film and the heat sealing machine.

8. Next, place the disc into the thermal cycler and start the run (ensure the disc is in the correct position).

9. At the end of the qPCR run identify which samples were inhibited by comparing with the NTC. For samples that were identified as being inhibited continue with step 10 for inhibitor removal clean up protocol and for samples that were not inhibited proceed to phase 2 of the analysis.
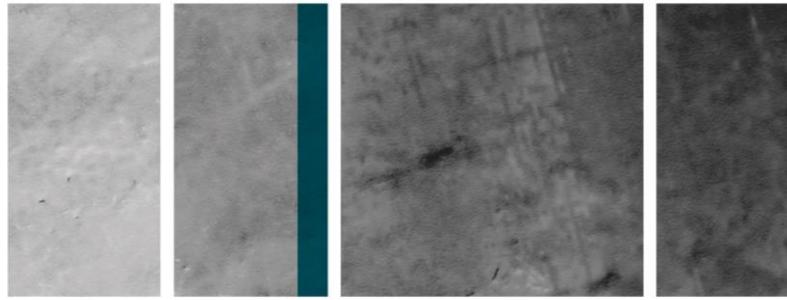
Inhibitor removal protocol:

10. Prepare inhibitor removal spin filters with green caps (Bioline ISOLATE fecal DNA kit – item. no. 52082) by snapping off the base of the spin filter, inserting into a 2 ml collection tube and centrifuging at 8,000 g for 3 minutes.

11. Transfer the spin filter into a new labelled 1.5 ml microcentrifuge tube.

12. Add the entire remaining volume of the eDNA sample to the centre of the spin filter.

13. Centrifuge at 8,000 g for 1 minute.

14. Repeat steps 1-9 of phase 1 to ensure sample is no longer inhibited. If sample is still inhibited a 1:10 dilution of the DNA may be needed.

Phase 2 – presence/absence analysis for tilapia DNA:

Positive standard controls of genomic DNA of known concentrations should be included in each run of qPCR. These can be used to relatively quantify unknown eDNA samples and give an indication of detection limits of the qPCR analysis. The positive standards are made from genomic DNA extracted from tilapia (*Oreochromis mossambicus*) fin tissue using the same DNA extraction methods described above for eDNA samples.

Preparing the positive standard controls

1. Quantify tilapia gDNA for the positive standards using a spectrophotometer and dilute DNA to a final concentration of 5 ng/ul, this will be standard 1.

2. Make a serial dilution of the gDNA, starting with standard 1 (5 ng/µl conc.) and diluting 1:20 at each stage. (DNA concentration of the standards will range from 5 ng/µl to 0.00003 ng/µl).

3. Transfer positive standards into single use aliquots (10 µl of DNA and 15 µl of molecular grade water) and keep at -80 °C until use.

qPCR preparation

1. UV sterilise all equipment needed for qPCR analysis (pipettes, tips, disc and tube racks) and molecular grade water for qPCR master mix for 30 minutes. Ensure there are enough wells for each sample (screened in replicates of 5, total 17 samples per disc), positive standard controls (analysed in triplicate) and NTC (5 replicates).

2. Remove qPCR reagents and eDNA samples from freezer and allow to thaw. Vortex, pulse spin, vortex, pulse spin before use.

3. Once UV sterilisation is complete, working within the UV cabinet, prepare the qPCR master mix following the recipe for phase 2 above. Vortex and pulse spin master mix and keep cool until proceeding to the robot for pipetting into disc.
    Note: when preparing qPCR master mix it is important to allow at least 15% excess volume to ensure there is enough master mix for all reaction wells (included in recipe).

4. Prepare the eDNA samples to be analysed for the robot by aliquoting samples into a plate. For each sample add 14 µl of eDNA and 21 µl of molecular grade water to the plate (this includes excess volume for pipetting error).

5. Now that the master mix and eDNA samples have been prepared, proceed to the robot to pipette mixture and samples into the qPCR analysis disc.

6. To each well add 15 µl of qPCR master mix, then start by adding the samples to the disc starting with 5 µl of the positive standard controls (in triplicate), 5 µl for the NTC and 5 µl of the aliquoted eDNA samples (in replicates of 5), mixing three times when DNA is added.

7. Once the robot has finished pipetting the master mix and samples into the disc, seal the disc using heat sealing films and the heat sealing machine.

8. Next, place the disc into the thermal cycler and start the run (ensure the disc is in the correct position).

9. Once the qPCR has completed, the samples may be stored at 4 °C for any downstream analyses if needed (e.g. sequencing).

Results:

10. To analyse the samples for presence/absence of tilapia first check the control samples. The positive standard controls should all have positive amplification curves and distinct peaks at the appropriate melting temperature (Fig. 1). The NTC should have no amplification curve and no peak in the melt analysis.
    Note: Sometimes primer dimer may form in the NTC which will produce a positive amplification curve and a peak in the melt analysis, however, the melting temperature in the melt analysis will be notably different (lower melting temperature) to the peak specific to tilapia (Fig. 2). If this occurs the NTC is still deemed negative or clear of contamination.
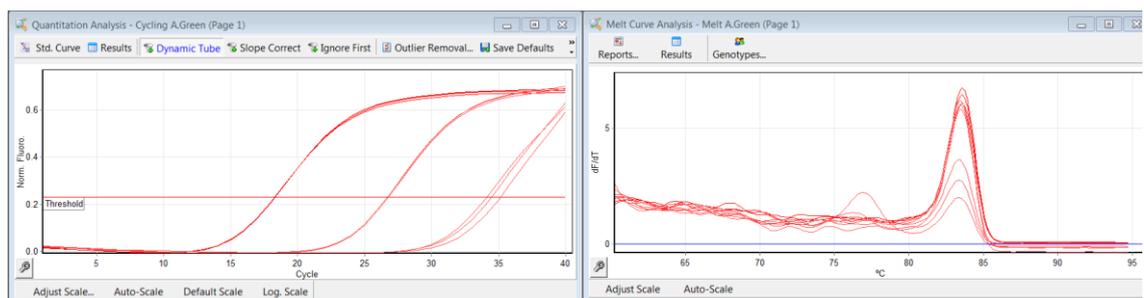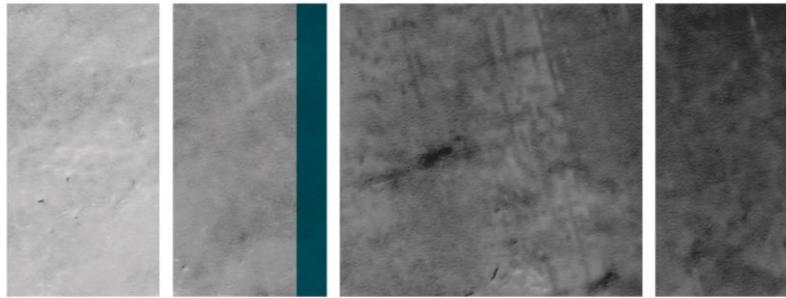
Figure 1: qPCR analysis showing the results of the positive standard controls only. On the left side is the quantitation analysis and the melt curve analysis is on the right side. All samples show positive amplification curves in the quantitation analysis as well as distinct peaks in the melt analysis. One of the lowest concentration standards exhibits some non-specific amplification seen in the melt analysis (a peak with different melting temperature), this is likely to be primer dimer.
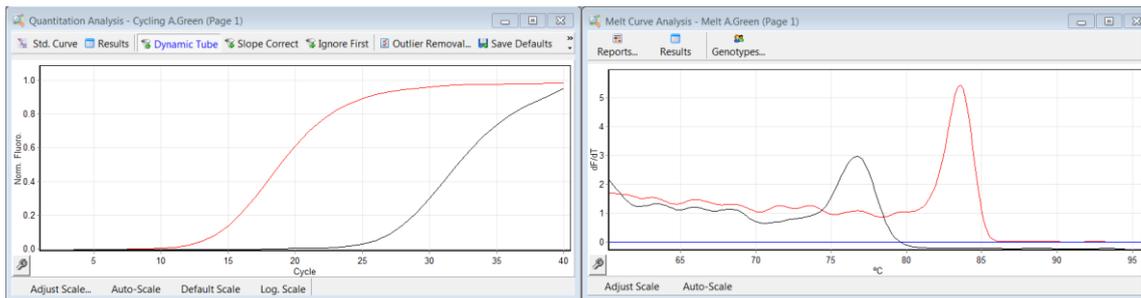

Figure 2: qPCR analysis showing the results of one positive standard control (red) and the NTC (black). On the left side is the quantitation analysis and the melt curve analysis on the right side. Both the positive standard control and NTC show positive amplification curves in the quantitation analysis as well as distinct peaks in the melt analysis, however, the melt temperature of the NTC peak is much lower than the positive standard control and likely a result of primer dimer. This NTC shown above is still considered negative or clear of tilapia contamination.

11. Each eDNA reaction is scored for presence/absence of tilapia DNA. Tilapia DNA is present when there is a positive amplification curve and a distinct peak of the correct size (using the positive standard controls) observed in the melt analysis.

12. An eDNA sample is classified as positive when at least one of the five reaction replicates indicates presence of tilapia DNA. Samples with low detection rates (i.e. one out of five replicates was positive) should be rerun to confirm the positive detection if all quality controls are clear.

Phase 3 – checking equipment controls of positive eDNA for contamination:

Phase 3 is only performed on eDNA samples that returned positive results for presence of tilapia DNA in phase 2. All other samples that were negative are not needed to be further analysed.

1. UV sterilise all equipment needed for qPCR analysis (pipettes, tips, disc and tube racks) and molecular grade water for qPCR master mix for 30 minutes. Ensure there are enough wells for each EC sample (screened in triplicate), positive standard controls (analysed in triplicate) and NTC.

2. Remove qPCR reagents and EC samples from freezer and allow to thaw. Vortex, pulse spin, vortex, pulse spin before use.

3. Once UV sterilisation is complete, working within the UV cabinet, prepare the qPCR master mix following the recipe for phase 2 above. Vortex and pulse spin master mix and keep cool until proceeding to the robot for pipetting into disc.
Note: when preparing qPCR master mix it is important to allow at least 15% excess volume to ensure there is enough master mix for all reaction wells (included in recipe).
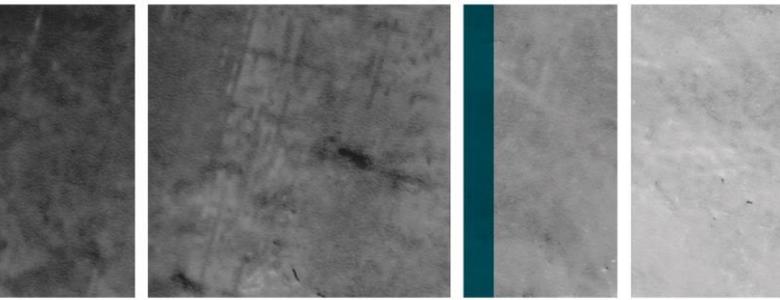
4. Prepare the EC samples to be analysed for the robot by aliquoting samples into a plate. For each sample add 8 µl of the EC sample and 12 µl of molecular grade water to the plate (this includes excess volume for pipetting error).

5. Now that the master mix and EC samples have been prepared, proceed to the robot to pipette mixture and samples into the qPCR analysis disc.

6. To each well add 15 µl of qPCR master mix, then start by adding the samples to the disc starting with 5 µl of the positive standard controls (in triplicate), 5 µl for the NTC and 5 µl of the EC samples (in triplicate), mixing three times when the samples are added.

7. Once the robot has finished pipetting the master mix and samples into the disc, seal the disc using heat sealing films and the heat sealing machine.

8. Next, place the disc into the thermal cycler and start the run (ensure the disc is in the correct position).

9. Once the qPCR has completed, check the EC samples for contamination.

Results:

10. First check the control samples, positive standard controls are positive and NTC is negative.

11. For each EC reaction check for contamination of tilapia DNA using the same guidelines given in phase 2 for eDNA samples.

12. If the EC sample is clear of contamination the corresponding eDNA samples can be confirmed a positive.

13. A subset of positive eDNA samples that passed all quality controls should be sequence analysed to confirm the PCR product is specific for tilapia.
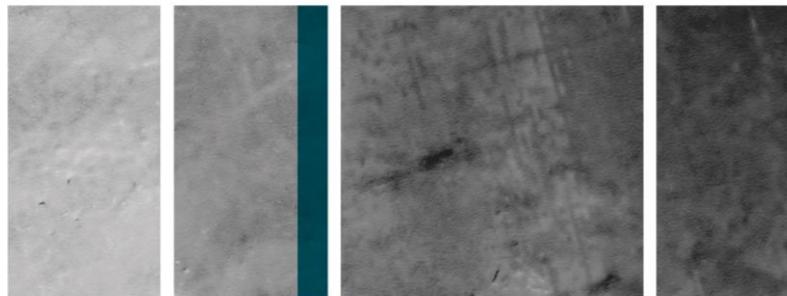
Appendix B

PCR recipes used during endpoint PCR optimisation for field testing eDNA tilapia technology

Recipe 1		
Bioline BIOTAQ	**[Initial]**	**Vol./rxn**
Taq polymerase	5 u/μL	0.1 μL
NH$_4$ reaction buffer	10 x	1 μL
MgCl$_2$	50 mM	0.3 μL
dNTP's	10 mM	0.2 μL
Forward primer	10 mM	0.2 μL
Reverse primer	10 mM	0.2 μL
H$_2$O	-	7 μL
DNA template	Variable	1 μL
Total		10 μL

Recipe 2		
Bioline BIOTAQ + Q-solution	**[Initial]**	**Vol./rxn**
Taq polymerase	5 u/μL	0.1 μL
NH$_4$ reaction buffer	10 x	1 μL
MgCl$_2$	50 mM	0.3 μL
dNTP's	10 mM	0.2 μL
Forward primer	10 mM	0.2 μL
Reverse primer	10 mM	0.2 μL
H$_2$O	-	5 μL
Q-Solution	5 x	2 μL
DNA template	Variable	1 μL
Total		10 μL

Recipe 3		
Thermo Fisher Phire Hot Start II	**[Initial]**	**Vol./rxn**
Taq polymerase	5 u/μL	0.4 μL
Reaction buffer	5 x	4 μL
dNTP's	10 mM	0.4 μL
Forward primer	10 mM	0.4 μL
Reverse primer	10 mM	0.4 μL
H$_2$O	-	12.4 μL
DNA template	Variable	2 μL
Total		20 μL

Recipe 4		
Thermo Fisher Phire Hot Start II + Q-Solution	**[Initial]**	**Vol./rxn**
Taq polymerase	5 u/μL	0.4 μL
Reaction buffer	5 x	4 μL
dNTP's	10 mM	0.4 μL
Forward primer	10 mM	0.4 μL
Reverse primer	10 mM	0.4 μL
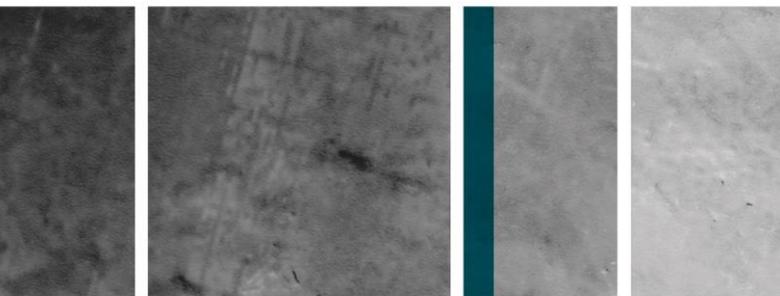
H₂O	-	8.4 μL
Q-Solution	5 x	4 μL
DNA template	Variable	2 μL
Total		20 μL

Recipe 5		
Thermo Fisher Phire Hot Start II + Bovine serum albumin (BSA)	**[Initial]**	**Vol./rxn**
Taq polymerase	5 u/μL	0.4 μL
Reaction buffer	5 x	4 μL
dNTP's	10 mM	0.4 μL
Forward primer	10 mM	0.4 μL
Reverse primer	10 mM	0.4 μL
H₂O	-	11.4 μL
BSA	4000 μg/ml	1 μL
DNA template	Variable	2 μL
Total		20 μL

Recipe 6		
QIAGEN Type-It	**[Initial]**	**Vol./rxn**
Master mix	2 x	10 μL
Forward primer	10 mM	0.4 μL
Reverse primer	10 mM	0.4 μL
H₂O	-	7.2 μL
DNA template	Variable	2 μL
Total		20 μL

PCR thermal cycling conditions for the three chemistries trialled, Bioline BIOTAQ, Thermo Fisher Phire Hot Start II and QIAGEN Type-It.

	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final extension
Bioline BIOTAQ	95 °C 120 sec	95 °C 30 sec	60 °C 30 sec	72 °C 30 sec	30	72 °C 300 sec
Thermo Fisher Phire Hot Start II	98 °C 30 sec	98 °C 5 sec	58 °C 5 sec	72 °C 10 sec	30	72 °C 60 sec
QIAGEN Type-It	95 °C 300 sec	95 °C 30 sec	60 °C 90 sec	72 °C 30 sec	30	72 °C 600 sec
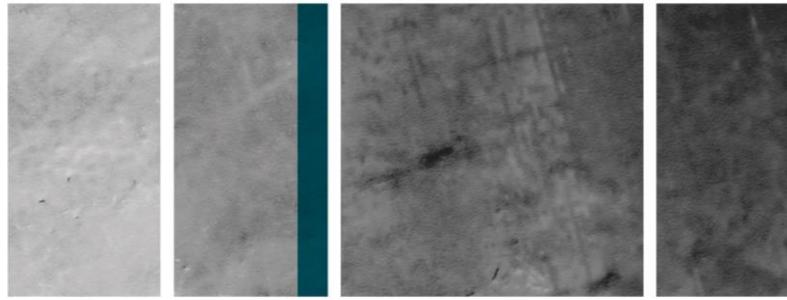
Appendix C

Real-time qPCR recipes trialled during optimisation.

Recipe 1		
Bio Rad SsoFast EvaGreen	**[Initial]**	**Vol./rxn**
Master mix	2 x	7.5 μL
Forward primer	10 mM	0.6 μL
Reverse primer	10 mM	0.6 μL
H_2O	-	4.3 μL
DNA template	Variable	2 μL
Total		15 μL

Recipe 2		
Life Technologies Power SYBR	**[Initial]**	**Vol./rxn**
Master mix	2 x	10 μL
Forward primer	10 mM	0.8 μL
Reverse primer	10 mM	0.8 μL
H_2O	-	6.4 μL
DNA template	Variable	2 μL
Total		20 μL

Recipe 3		
Bioline SensiFAST SYBR	**[Initial]**	**Vol./rxn**
Master mix	2 x	10 μL
Forward primer	10 mM	0.8 μL
Reverse primer	10 mM	0.8 μL
H_2O	-	6.4 μL
DNA template	Variable	2 μL
Total		20 μL

Recipe 4		
QIAGEN QuantiNova SYBR	**[Initial]**	**Vol./rxn**
Master mix	2 x	10 μL
Forward primer	10 mM	0.8 μL
Reverse primer	10 mM	0.8 μL
H_2O	-	6.4 μL
DNA template	Variable	2 μL
Total		20 μL
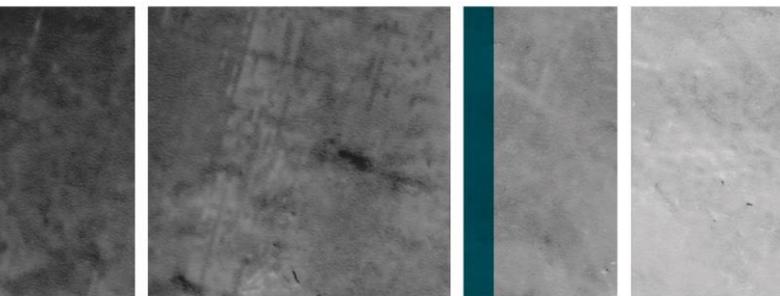
	Recipe 5		
QIAGEN Type-It + Pico Green		**[Initial]**	**Vol./rxn**
Master mix		2 x	10 μL
Forward primer		10 mM	0.4 μL
Reverse primer		10 mM	0.4 μL
H_2O		-	5.2 μL
Pico Green diluted in H_2O		1:200	2 μL
DNA template		Variable	2 μL
Total			20 μL

	Recipe 6		
QIAGEN QuantiFast SYBR		**[Initial]**	**Vol./rxn**
Master mix		2 x	10 μL
Forward primer		10 mM	0.8 μL
Reverse primer		10 mM	0.8 μL
H_2O		-	6.4 μL
DNA template		Variable	2 μL
Total			20 μL

Real-time qPCR thermal cycling conditions for each chemistries trialled. Quantitative PCR analysis for all chemistries included an initial quantitation analysis followed by a melt analysis.

	Initial denaturation	Denaturation	Annealing/ Extension		No. of cycles	Melt Analysis
Bio Rad SsoFast EvaGreen	98 °C 2 min	98 °C 5 sec	60 °C 20 sec		40	60 - 98 °C 0.5 °C incre.
Life Technologies Power SYBR	95 °C 10 min	95 °C 15 sec	60 °C 60 sec		30	60 - 95 °C 0.5 °C incre.
Bioline SensiFAST SYBR	95 °C 3 min	95 °C 5 sec	60 °C 20 sec		40	60 - 95 °C 0.5 °C incre.
QIAGEN QuantiNova SYBR	95 °C 3 min	95 °C 5 sec	60 °C 20 sec		40	60 - 95 °C 0.5 °C incre.
QIAGEN Type-It + Pico Green	95 °C 10 min	95 °C 30 sec	60 °C 90 sec	72 °C 30 sec	40	60 - 98 °C 0.5 °C incre.
QIAGEN QuantiFast SYBR	95 °C 5 min	95 °C 10 sec	60 °C 30 sec		40	60 - 95 °C 0.5 °C incre.