ISSN 2350-3432

Comparison of the efficiency of three methods of isolating fish DNA from environmental samples

2014

ERI report number 45

Client report prepared for Lake Ecosystem Restoration New Zealand

By Jonathan C. Banks, Nathan Watson, Ian D. Hogg

Environmental Research Institute

Faculty of Science and Engineering

University of Waikato, Private Bag 3105

Hamilton 3240, New Zealand

Cite report as:

Banks JC, Watson N, Hogg ID, 2014. Comparison of the efficiency of three methods of isolating fish DNA from environmental samples. *Environmental Research Institute Report No. 45*. Client report prepared for Lake Ecosystem Restoration New Zealand. Environmental Research Institute, Faculty of Science and Engineering, The University of Waikato, Hamilton. 17 pp.

Reviewed by:

S. Craig Cary

Professor

Environmental Research Institute

University of Waikato

Approved for release by

John Tyrrell

Environmental Research Institute

University of Waikato

Executive summary

The Lake Ecosystem Restoration New Zealand (LERNZ) project was initiated to restore indigenous biodiversity in lakes in part by developing new pest fish management and control technologies. One objective of the management of pest fish was to develop genetic based assays to detect pest fish from environmental samples such as water, and to assess the feasibility of using these methods in the field.

This report compares three methods (the Piscitron II, Piscitron III and a pH adjusted method) of isolating DNA for two species of exotic fish from water samples. We compared DNA isolation efficiency using newly developed qPCR assays for koi carp, *Cyprinus carpio* and goldfish, *Carassius auratus,* and present the primer and hydrolysis probe sequences for the assays, and assay thermal conditions. Minimum levels of detectable fish biomass are reported for koi carp water collected from a 6800 L tank at ambient temperature containing a 1.6 kg koi carp. The koi carp and goldfish assays were also tested on Waikato River water collected next to Hammond Park, Hamilton, New Zealand, an area of river known to contain koi carp and goldfish.

We found that the Piscitron II and Piscitron III methods, which filtered water through glass fibre filters or glass wool aquarium filters, respectively, performed equally well in terms of DNA extraction but considerably better than the pH adjusted method. Minimum detectable amounts of koi carp DNA were calculated by producing serial dilutions of the tank water containing 2.35×10^{-4} kg of koi carp/L. Because fish densities tend to be reported as kg/m^2 of lake surface, we converted our minimum detectable amount from a weight per volume to a weight per area by assuming an average lake depth of 1.5 m (a typical Waikato lake depth) which resulted in a minimum detectable amount of 0.35 kg carp/m^2 of water surface area. We also produced serial dilutions of DNA extracted from the koi carp tank and the minimum detectable amount of koi carp biomass was much lower at 0.0035 kg/m^2 which is less than the range of carp densities (0.005 - 0.0768 kg/m^2) reported for Waikato lakes and rivers.

Table of contents

List of tables

List of figures

Introduction

The Lake Ecosystem Restoration New Zealand (LERNZ) project was initiated to restore indigenous biodiversity in lakes by developing new models and technologies to manage harmful algal blooms, and to develop new pest fish management and control technologies. One objective of the pest fish component was to develop genetic-based assays to detect pest fish using environmental water samples and to assess the more widespread application of these methods.

Determining the distribution and presence of exotic taxa within a defined area and effective evaluation of eradication efforts are significant challenges for restoration ecology. One option is to detect the presence of such introduced species using environmental DNA (eDNA) - DNA that is collected indirectly from shed skin cells or other cellular secretions which are obtained from water or sediment samples (Ficetola et al. 2008; Jerde et al. 2011; Dejean et al. 2012; Mahon et al. 2013). Previous examples of sample types examined for eDNA have included soils (Hofreiter et al. 2003), ice cores (Willerslev et al. 2007) and faeces (Banks et al. 2009). For freshwater vertebrates, eDNA has been used to infer the range of introduced Asian carp (*Hypophthalmichthys molitrix*), bighead carp (*H. nobilis*) (Jerde et al. 2011), frogs and salamanders (Ficetola et al. 2008; Goldberg et al. 2011), six species of fish in the Yura River, Japan (Minamoto et al. 2012) and to identify species of fish imported for the ornamental fish market (Collins et al. 2012).

Although eDNA has been developed for several groups of animals, there is no consensus on the most appropriate methods to isolate DNA from environmental samples and it is likely that minimum detectable biomass varies among species and among habitats. Here, we compare the effectiveness of three methods of extracting DNA from water samples and determine the minimum detectable amounts of koi carp biomass for each method using water collected from a tank containing a single koi carp. We also tested these methods on water collected from the Waikato River near Hamilton Gardens, within the city of Hamilton, New Zealand to assess the utility of these methods in a field setting.

Methods

Comparing methods for extracting eDNA

Three methods of DNA isolation were tested by extracting DNA from water sampled from a tank containing a single koi carp on 12 March 2013. A single koi carp (collected from Waikato river, mass = 1.616 kg) was placed in a tank of de-chlorinated city water at ambient temperature filled to a volume of 6800 L giving a fish biomass of 2×10^{-4} kg/L and left for 24 hours. The carp was fed 5 mm trout pellets (Reliance Stock Foods, Dunedin, New Zealand) and housed under natural daylight conditions at ambient temperature.

The three methods of DNA isolation were also used on water from the Waikato River adjacent to Hammond Park, Hamilton (37.806617° S 175.315394° E). River water was collected after rinsing the high-density polyethylene (H.D.P.E.) sample containers (Model D 2.2, New Zealand Blow Moulders, Auckland) several times with river water and returned to the lab for filtering (Piscitron II and pH adjusted filtration) or filtered in the field (Piscitron III) on 9 March 2013.

Sampling Method 1 (the Piscitron II):

The first isolation method (Figure 1), used an inverted 3 L glass volumetric flask, with the base removed, and placed on a 90 mm diameter perspex filter platform (Technical Glass Products, Dunedin). The volumetric flask and filter platform were held on a 90 mm diameter glass funnel using 25 mm tool clips. The funnel ran through a stopper which sat on a 2 L Büchner flask. We cut a 150 mm diameter GF/A glass fibre filter (pore size 1.6 μm, Whatman, Maidstone, Catalogue number 1820 150) to fit the filter platform. We connected a vacuum pump (Cole Parmer, Vernon Hills, Model number EW 79200-05) to produce approximately 40 mm Hg of negative pressure on the Büchner flask to increase the filtration rate. Filters containing the filtrate were individually placed in 50 mL conical tubes (Corning, Corning) and frozen at -20^{o}C until the DNA could be extracted from the filters. We filtered 2 L of distilled, deionised water as negative controls. Equipment was sterilised after each extraction by immersion in a 10% bleach solution (final concentration of sodium hypochlorite = 0.4% w/v) for 20 minutes followed by two washes in distilled, deionised water.

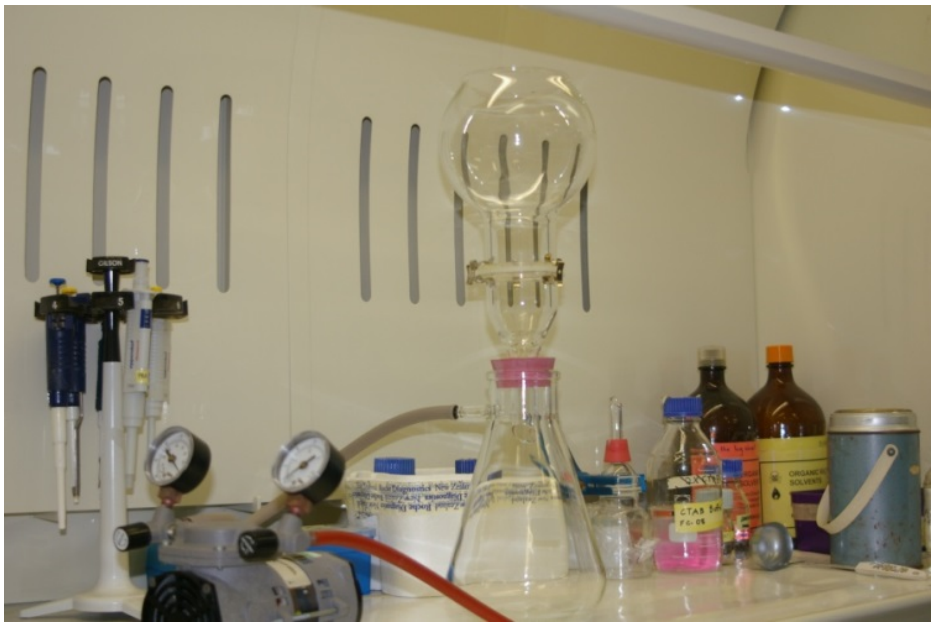

Figure 1 The Piscitron II showing the arrangement of the inverted volumetric flask sitting on top of the glass fibre filter.

We also prepared a series of dilutions by mixing 200 mL of the water containing the carp with 1800 mL of distilled water in the D 2.2 H.D.P.E containers to reduce the concentration of DNA by a factor of 10. The dilutions were repeated serially to produce a dilution series down to 10^{-6} of the original concentration. These water samples were then filtered using the Piscitron II as described above. A "no template" (blank) extraction was performed at the same time as the template extractions.

DNA was extracted from the filters using Power Water kits (MoBio Laboratories, Carlsbad). The manufacturer's directions for extracting DNA were followed except that we halved each filter, because of each filter's size, with scissors that had been dipped in 100% ethanol and flamed to remove any residual DNA. We also increased the bead beating step from the manufacturer's recommended five minutes to 10 minutes to break up the larger filters, and we centrifuged the broken up filters for six minutes rather than the manufacturer's recommended one minute to release the extraction buffer from the homogenised filters. To ensure buffer concentrations were maintained for the DNA extractions, the two aliquots of

extraction buffer were processed separately until the DNA was loaded on to the spin column at which point the two aliquots of solubilised DNA from each water sample were recombined and filtered through a single column. DNA was eluted from the column using 100 µL of the manufacturer's elution buffer.

Sampling Method 2 (the Piscitron III)

The second method of isolating DNA from water filtered 20 L of water through glass wool aquarium filter pads (2500 1W, Aqua One, Ingelburn). Filters were cut to size and sterilised by exposure to UV light for 20 minutes on each side and then mounted in plastic housings. The housing containing the filters was connected to a 12V battery powered pump (Propumps®, Model FLO-2202, New York City, USA) using 10 mm diameter hose (Figure 2). Two replicate samples ("A" and "B") were collected by pumping 20 L of koi tank water through the filters that were then returned to the lab and DNA extracted using a Mo-Bio Power Water kit (MoBio Laboratories, Carlsland, USA) according to the manufacturer's protocol. DNA was re-suspended in 100 µL of DNA extraction buffer. A **"**no template**"** (blank) extraction on an unused filter was performed at the same time as the template extractions.

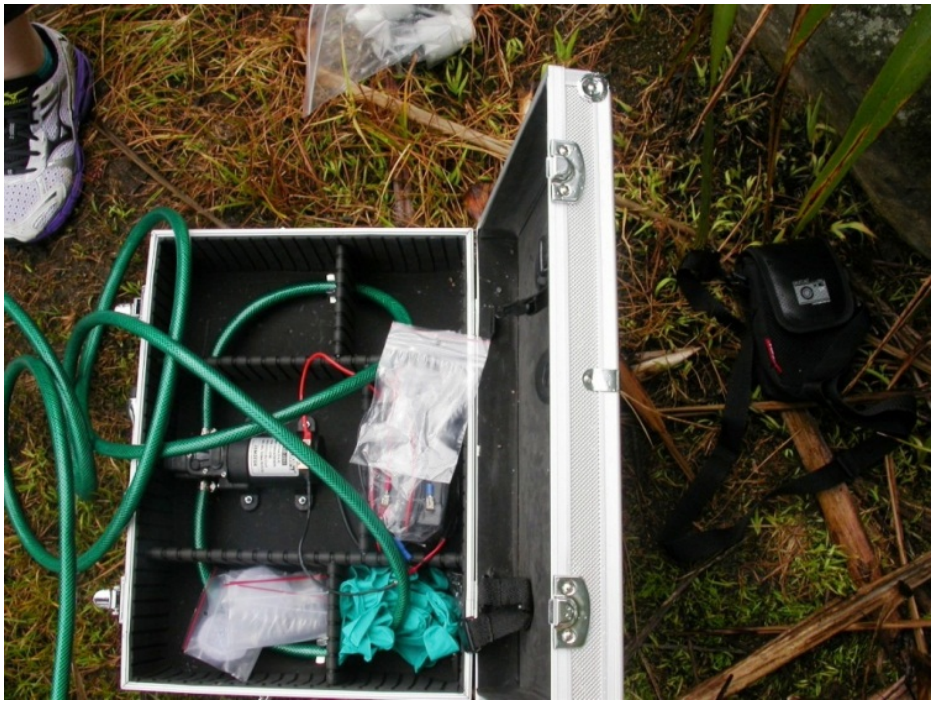

Figure 2 The Piscitron III.

Sampling Method 3 (pH adjusted filtration)

The pH of 300 mL of water was adjusted to 3.5 using hydrochloric acid 20% and then filtered through a 47 mm diameter, 0.45 µm pore nitrocellulose filter (A045H047A, Advantec, Dubbin, USA) held in a filter tower mounted on a Büchner flask. We connected a vacuum pump (Cole Parmer, Vernon Hills, Model number EW 79200-05) to produce approximately 40 mm Hg of negative pressure to the Büchner funnel to increase the filtration rate. DNA was extracted from filters using a MoBio Power Soil DNA Isolation kit (Mo Bio, Carlsbad, catalogue number 1288) following the manufacturer's protocol. DNA was re-suspended in 100 µL of extraction buffer.

We prepared a series of dilutions by mixing 3 mL of the water from the carp's tank with 297 mL of distilled water to reduce the concentration of DNA to 0.1 of the starting concentration. The dilutions were repeated serially to produce a dilution series down to 10^{-6} of the original concentration. These water samples were then extracted using the pH adjusted filtration method. A no template (blank) extraction was performed at the same time as the template extractions.

Dilutions of extracted DNA

We also produced a series of DNA dilutions from the DNA extracted using the Piscitron II and III methods by diluting 1 part extracted DNA with 99 parts DNAse RNAse free water (in contrast to dilutions produced from tank water that were then individually filtered). The final series of dilutions prepared from the PC2, PC3A, and PC3B DNA extractions produced DNA concentrations 10^{-2}, 10^{-4} and 10^{-6} of the original concentrations. Because the efficiency of the pH adjusted method was low, we did not produce a dilution series from the extracted DNA by this method.

Detecting the presence of koi and goldfish eDNA using quantitative polymerase chain reactions (qPCR)

Sequences for the mitochondrial cytochrome b gene of koi and goldfish were downloaded from GenBank. This gene was chosen as we have generally found it to show the most variation among fish species, thus increasing the probability of assay specificity. Sequences for cytochrome b were then loaded into the IDT PrimerQuest™ software (http://sg.idtdna.com/PrimerQuest/Home/Index) for designing primers and hydrolysis probes. PrimerQuest™ returned several potential primer and probe sequences for the assays. These suggested sequences were then compared with sequences from other closely related fish species known to be present in New Zealand to assess similarity to non-target DNA. The most dissimilar primer and probe sequences were selected for synthesis as IDT mini-assays.

Koi carp

Quantitative PCRs consisting of 10 µL of Kapa Probe Universal qPCR master mix 2X (Kapabiosystems, Boston, Catalogue number KK4701), 2 µL of IDT Miniassay containing the primers TCCTATCTGCCGTACCATACA (forward) and GTAGGAAGTGGAATGCGAAGAA (reverse) with the probe FAM/TGTTGCATT/ZEN/GTCTACTGAGAACCCACC/3IABkFQ Integrated DNA Technologies, Singapore), 6 µL of water and 2 µL of template DNA were run on a Corbett RG 6000 instrument (Corbett, Concorde). Reaction conditions were an initial denaturing cycle of 95°C for 3 minutes followed by 50 cycles of 95°C for 3 seconds and 65°C for 30 seconds. Negative (distilled water as a template) and positive controls (DNA extracted from koi carp muscle tissue) were run simultaneously with the test samples.

Goldfish

Quantitative PCRs consisting of 10 µL of Kapa Probe Universal qPCR master mix 2X (Kapabiosystems, Boston, Catalogue number KK4701), 2 µL of IDT Miniassay containing the primers TCCTTGAATGACTCAACTAAGGTTT (forward) and CCACAGTTAGTGTCCTTGATTCC (reverse) with the Probe FAM/ATGTAGTAA/ZEN/GAAACCACCAACCAGT/3IABkFQ (IDT, Singapore), 6 µL of water and 2µL of template DNA samples were run on a Corbett RG 6000 instrument. Reaction conditions were an initial denaturing cycle of 95^{o}C for 3 minutes followed by 50 cycles of 95^{o}C for 3 seconds and 65^{o}C for 30 seconds. Negative (distilled water as a template) and positive controls (DNA extracted from gold fish muscle tissue) were run simultaneously with the test samples.

Results

Comparing eDNA extraction methods

Tank water

Koi carp

Amplifiable DNA was extracted successfully from the tank water using all three methods. Amplification of DNA occurred at a lower number of PCR cycles for the Piscitron II and Piscitron III methods compared with the pH adjusted method suggesting that more DNA was extracted by the Piscitron II and III methods of DNA isolation (From the diluted samples, detectable DNA was obtained from the 1:100 dilution produced from extracted DNA and the 1:10 000 dilution of Piscitron III extract A. No amplification was detected in the lower concentrations of DNA (Table 2).

Waikato River water

Koi carp

Only one of the extractions (Piscitron II extraction B) from the Waikato River was positive using the koi carp assay (Table 3).

Goldfish

None of the water samples collected from the Waikato River was positive for goldfish DNA.

Table 1). The Piscitron III method appeared to extract slightly more DNA than the Piscitron II method (**Error! Reference source not found.**). No detectable DNA was obtained from the dilution series produced from the tank water. From the diluted samples, detectable DNA was obtained from the 1:100 dilution produced from extracted DNA and the 1:10 000 dilution of Piscitron III extract A. No amplification was detected in the lower concentrations of DNA (Table 2).

Waikato River water

Koi carp

Only one of the extractions (Piscitron II extraction B) from the Waikato River was positive using the koi carp assay (Table 3).

Goldfish

None of the water samples collected from the Waikato River was positive for goldfish DNA.

Table 1 Comparison of DNA isolation method efficiency using DNA extracted from water taken from a 6800 L tank containing a 1.6 kg koi carp.

Extraction method	Amplification cycle number
Piscitron II	27.84
Piscitron II 10^{-1} dilution of tank water	No amplification
Piscitron II 10^{-2} dilution of tank water	No amplification
Piscitron II 10^{-3} dilution of tank water	No amplification
Piscitron II 10^{-4} dilution of tank water	No amplification
Piscitron II 10^{-5} dilution of tank water	No amplification
Piscitron II 10^{-6} dilution of tank water	No amplification
Piscitron II no template extraction (negative extraction)	No amplification

Piscitron III 2500 1W extraction A	27.03
Piscitron III 2500 1W extraction B	27.58
Piscitron III no template extraction (negative extraction)	No amplification
pH adjusted method	36.01
pH adjusted method 10^{-2} of tank water	No amplification
pH adjusted method 10^{-4} of tank water	No amplification
pH adjusted method 10^{-6} of tank water	No amplification
pH adjusted method no template extraction (negative extraction)	No amplification
Positive control (Koi tissue extract 3 1:10 dilution)	18.28
No template (negative) control	No amplification

Table 2. Minimum detectable concentration of DNA from the dilution series produced by diluting DNA extracted from a 6800 L tank containing a 1.6 kg koi carp.

Extraction concentration	Amplification cycle number
Piscitron II	27.81
Piscitron II 10^{-2} dilution of DNA	34.42
Piscitron II 10^{-4} dilution of DNA	No amplification
Piscitron II 10^{-6} dilution of DNA	No amplification
Piscitron III extraction A	27.02
Piscitron III extraction A 10^{-2} dilution of DNA	33.40
Piscitron III extraction A 10^{-4} dilution of DNA	36.17
Piscitron III extraction A 10^{-6} dilution of DNA	No amplification
Piscitron III extraction B	27.09
Piscitron III extraction B 10^{-2} dilution of DNA	34.17
Piscitron III extraction B 10^{-4} dilution of DNA	No amplification
Piscitron III extraction B 10^{-6} dilution of DNA	No amplification
No template (negative) control	No amplification

Table 3. Comparison of DNA isolation method efficiency using the koi carp assay on DNA extracted from the Waikato River at Hammond Park, Hamilton.

Extraction method	Amplification cycle number
Piscitron II extraction A	No amplification
Piscitron II extraction B	36.09
Piscitron II extraction C	No amplification
Piscitron II no template extraction (negative extraction)	No amplification
Piscitron III glass wool 2500 1W extraction A	No amplification
Piscitron III glass wool 2500 1W extraction B	No amplification
Piscitron III glass wool 2500 1W extraction C	No amplification
Piscitron III glass wool 2500 1W no template extraction (negative extraction)	No amplification
pH adjusted extraction A	No amplification
pH adjusted extraction B	No amplification
pH adjusted extraction C	No amplification
pH adjusted no template extraction (negative extraction)	No amplification
Koi 3 1:10 (positive control)	18.27
No template (negative) control	No amplification

Discussion

Tank-water samples

The Piscitron II and III DNA isolation methods had similar extraction efficiencies as DNA extracted from the tank water amplified at 27 cycles for both methods. Both Piscitron methods were more efficient than the pH adjusted method which had an amplification threshold of 36 cycles for DNA extracted from the tank water. A future improvement may be to dry the glass wool filters of the Piscitron III DNA isolator (perhaps using pressure) as these filters retain a large volume of water which may interfere with the DNA extraction process by diluting extraction buffers.

The DNA extracted from the tank water using the Piscitron methods detected 1.6 kg of koi carp per 6800 L or 2.35 $x10^{-4}$ kg/L. As fish biomass tends to be reported as fish per hectare of lake surface rather than fish per cubic metre of water, we have converted our results to fish biomass per m^2. Assuming a typical average Waikato lake depth of 1.5 m, a square metre of lake contains 1500 L of water, thus our detection limit is equivalent to 0.35 kg/m^2 of lake area.

No isolation method amplified DNA from the diluted water samples, although diluted extracted DNA did amplify. We are unsure why we were unable to detect koi DNA from the serial dilution of water samples as we would have expected that a 10 fold reduction in template concentration should increase the amplification threshold by approximately 3.3 cycles during the exponential phase of the reaction (assuming a reaction efficiency of 100%) which would be within the range of the assay for the Piscitron II and III isolation methods. It may be that the DNA is not distributed homogenously throughout a water sample and our subsampling to produce the dilution series took water without DNA.

Alternatively, it may be that the low concentrations of target DNA in the samples were lost due to binding to the sample container. It may be that this problem did not occur initially because rinsing the containers several times with the tank water before taking the initial sample saturated the DNA adsorption sites. Conversely when a small amount of water was added to the un-rinsed plastic containers to make the serial dilutions before filtering the water, there was insufficient DNA to saturate binding sites and the DNA was retained in the container rather than being available for extraction. DNA has been shown to bind to some types of plastics (Gaillard & Strauss 1998) and although we used HDPE, a plastic material commonly used to store samples before DNA extraction, it has been suggested that polyallomer plastics are less likely to bind DNA or to denature it (Gaillard & Strauss 1998).

Although no DNA was detected at lower concentrations of DNA when a dilution series was produced from aliquots of tank water, we were able to detect carp densities of 0.0035 kg/m^2

and for one extraction (PC3A) 3.5 x 10^{-5} kg/m^2 from a dilution series produced from serial dilutions of actual DNA isolated using Piscitrons II and III. Depending on the correction factor used for electrofishing efficacy, koi carp densities in the Waikato River have been shown by electrofishing to average between 0.022 kg/m^2 to 0.0768 kg/m^2 (Hicks et al. 2005), and in the Kauri Point ponds, Waikato, carp densities ranged from 0.005 kg/m^2 to 0.018 kg/m^2 (Hicks et al. 2008). These carp densities are at the limits of detection for the assay so the reason for the failure to detect lower concentrations of DNA considerably affects our conclusions. If the assay failed to detect DNA from the dilution series produced from tank water because the DNA is distributed heterogeneously in the environment, it is likely the false negative rate (i.e., non-detection when the fish are present) of the assay will be high. However, if the non-amplification of DNA is due to DNA adsorbing to the sample containers, the choice of alternative 2 L sample containers such as those made from polypropylene which is known to have a lower affinity for DNA may increase the sensitivity of the assay to cover the range of densities of the target species.

River-water samples

No method was able to detect koi or goldfish DNA from the river water samples. This is unlikely to be due to the absence of koi carp from the Waikato River as koi have been collected on the Waikato River (37.80490052^o S 175.31169141^o E) less than 100 m from the sample site (New Zealand Freshwater Fish Database). Likewise goldfish have been collected at Hamilton Gardens, 37.80695128^o S 175.30497662^o E, (New Zealand Freshwater Fish Database) which is a few hundred metres downstream from the water sample site with no obvious barriers to goldfish movement up stream to the collection site. It may be that current flow at the collection site results in mixing and dilution of the DNA in the water column to concentrations below the detectable amount.

One approach that may improve the detectability of environmental DNA would be to target areas in rivers such as eddies where DNA may accumulate. A targeted approach was taken by Jerde et al. (2013) to detect big head (*Hypophthalmichthys nobilis*) and silver (*Hypophthalmichthys molitrix*) carp. Areas targeted included high food availability areas, and likely spawning areas in rivers and streams during the breeding season. However, while a

targeted sampling regime would increase the detectability of carp, it would also create problems for estimating abundances and assessing the extent of carp distributions.

Future work should focus on improving the yield of DNA from the water samples. We have developed robust assays for koi carp and goldfish which will allow different collection containers and DNA isolation methods to be trialled. The Piscitron III allows efficient filtering of larger volumes of water compared with the other methods discussed here so another trial would be to look at the effect of filtering volumes larger than the 20 L used in this study. Additionally, using the assays to identify areas of high koi densities such as spawning areas would reduce the rate of false negatives (fish present but not detected) and may identify areas that should be targeted for fish control.

Acknowledgements

This research was funded in part by FRST grants UOWX0501 and UOWX0505 as part of the Lake Ecosystem Restoration New Zealand programme.

References

Banks JC, Hogg ID, Cary SC 2009. The phylogeography of Adelie penguin faecal bacteria. Environmental Microbiology 11: 577-588.

Collins R, Armstrong K, Holyoake A, Keeling S 2012. Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. Biological Invasions: 1-7.

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 bullfrog *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.

Gaillard C, Strauss F 1998. Avoiding adsorption of DNA to polypropylene tubes and denaturation of short DNA fragments. Technical Tips Online T01392.

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.

Hicks BJ, Brijs J, Heaphy J, Bell D 2008. The use of boat electrofishing for koi carp (*Cyprinus carpio*) removal in the Kauri Point catchment. CBER Contract Report 69 16 p.

Hicks BJ, Ling N, Osborne MW, Bell DG, Ring CA 2005. Boat electrofishing survey of the lower Waikato River and its tributaries. CBER Contract Report 39 17 p.

Hofreiter M, Mead JI, Martin P, Poinar HN 2003. Molecular caving. Current Biology 13: R693-R695.

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, Corush J, Budny ML, Mysorekar S, Lodge DM 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.

Mahon AR, Jerde CL, Galaska M, Bergner JL, Chadderton WL, Lodge DM, Hunter ME, Nico LG 2013. Validation of eDNA Surveillance Sensitivity for Detection of Asian Carps in Controlled and Field Experiments. PLoS ONE 8: e58316.

Minamoto T, Yamanaka H, Takahara T, Honjo M, Kawabata Z 2012. Surveillance of fish species composition using environmental DNA. Limnology 13: 193-197.

Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, Brand TB, Hofreiter M, Bunce M, Poinar HN, Dahl-Jensen D and others 2007. Ancient biomolecules from deep ice cores reveal a forested Southern Greenland. Science 317: 111-114.