Development of droplet digital Polymerase Chain Reaction assays for the detection of long-finned (*Anguilla dieffenbachii*) and short-finned (*Anguilla australis*) eels in environmental samples

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ABSTRACT

Freshwater eels are ecologically, and culturally important worldwide. The New Zealand long-finned eel (*Anguilla dieffenbachii*) and short-finned eel (*Anguilla australis*) are apex predators, playing an important role in ecosystem functioning of rivers and lakes. Recently, there has been a national decline in their populations due to habitat destruction and commercial harvest. The emergence of targeted environmental DNA detection methodologies provides an opportunity to enhance information about their past and present distributions. In this study we successfully developed species-specific droplet digital Polymerase Chain Reaction (ddPCR) assays to detect *A. dieffenbachii* and *A. australis* DNA in water and sediment samples. Assays utilized primers and probes designed for regions of the mitochondrial cytochrome b and 16S ribosomal RNA genes in *A. dieffenbachii* and *A. australis*, respectively. River water samples (n = 27) were analyzed using metabarcoding of fish taxa and were compared with the ddPCR assays. The presence of *A. dieffenbachii* and *A. australis* DNA was detected in a greater number of water samples using ddPCR in comparison to metabarcoding. There was a strong and positive correlation between gene copies (ddPCR analyses) and relative eel sequence reads (metabarcoding analyses) when compared to eel biomass. These ddPCR assays provide a new method for assessing spatial distributions of *A. dieffenbachii* and *A. australis* in a range of environments and sample types.

Subjects Aquaculture, Fisheries and Fish Science, Ecosystem Science, Molecular Biology, Zoology, Freshwater Biology
Keywords Environmental DNA, eDNA, Metabarcoding, High-throughput sequencing, *Anguilla*, Droplet digital PCR

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INTRODUCTION

Documenting changes in biodiversity is becoming increasingly important due to the exponential rise in species losses at local, regional, and global scales (*e.g.*, *Butchart et*

al., 2010; Dirzo & Raven, 2003; He et al., 2017). In fresh-water ecosystems, traditional surveillance for fish uses nets, carrion-baited traps, visual surveys, or electrofishing to obtain an overview of the existing community (Joy, David & Lake, 2013). These techniques are costly, labor and time intensive, and the detection of rare species requires a high sampling effort. Traditional methods can be environmentally invasive, often resulting in bycatch and require direct handling of target organisms (Portt et al., 2006; Reynolds & Holliman, 2004).

The application of molecular techniques to detect environmental DNA (eDNA) in a range of sample types forgoes many limitations of traditional surveying (reviewed by Senapati et al., 2019). The concept of species detection based on eDNA relies on the assumption that all organisms release their DNA (*i.e.,* through decomposition, skin cell shedding, waste production) to a collective pool of DNA that exists in the physical environment. Assays analyzing eDNA can be either designed for a specific target, such as a single species (or taxa) or non-targeted to assess an entire biological community.

Target-specific eDNA detection techniques have been applied to a range of aquatic vertebrates and invertebrates, including abundant, rare, invasive, and endangered taxa. Detecting eDNA of specific species can be more sensitive than traditional practices, especially when the organisms are at low densities, *i.e.,* rare species in large water bodies (*e.g.*, Jerde et al., 2011; Sigsgaard et al., 2015; Takahara, Minamoto & Doi, 2013; Wilcox et al., 2016). Quantitative real-time PCR (qPCR) assays have been shown to be a sensitive and quantitative approach to detect aquatic organisms, *i.e.,* fish (*e.g.*, Atkinson et al., 2018; Laramie, Pilliod & Goldberg, 2015; Olsen et al., 2015; Piggott, 2017; Sigsgaard et al., 2015; Takahara, Minamoto & Doi, 2013; Turner et al., 2014; Wilcox et al., 2013), invertebrates (*e.g.*, Goldberg et al., 2013; Mauvisseau et al., 2018; Tréguier et al., 2014) and amphibians (*e.g.*, Pilliod et al., 2013; Secondi et al., 2016; Smart et al., 2015). Recently, the development of droplet digital PCR (ddPCR), which measures absolute DNA copy numbers, has further increased assay sensitivity, especially in the presence of PCR inhibitors (Doi et al., 2015a; Doi et al., 2015b; Mauvisseau et al., 2019b; Simmons et al., 2015). Some studies that use quantitative methods (*i.e.,* qPCR and ddPCR) have shown positive correlations of PCR copy numbers to the abundance and/or biomass of the target organism in a waterbody (Hinlo et al., 2017; Klobucar, Rodgers & Budy, 2017; Mizumoto et al., 2018).

Metabarcoding is increasingly being used to characterize the species diversity of aquatic communities (Blackman et al., 2017; Hänfling et al., 2016; Klymus, Marshall & Stepien, 2017; Shaw et al., 2016; Valentini et al., 2016). In contrast to target-specific approaches such as ddPCR, metabarcoding enables the simultaneous identification of many species and thus the community composition of groups of organisms, *e.g.* eukaryotes. However, many studies recognize the various challenges associated with the amplification of multi-template sequences. For example, primers are often not conserved across the entire community of interest and therefore not universal, primers can also be biased to certain organisms or species and reference databases remain inaccurate and often incomplete leading to incorrect or incomplete taxonomic assignment of sequences (Clarke et al., 2014; Deagle et al., 2014; Dowle et al., 2016; Wangensteen et al., 2018). Recently, universal primer sets and PCR assays for metabarcoding fishes have been developed (MiFish-U/E and Teleo-F/R; Miya et al.,

2015; Valentini et al., 2016). Each primer set targets a section within the 12S rRNA gene; Mifish-U-F and Mifish-U-R (Miya et al., 2015) targets a region of approximately 220 base pairs (bp), while Teleo-F and Teleo-R (Valentini et al., 2016) targets a different region of approximately 100 bp. Both primer sets have sufficient coverage to detect a wide range of fishes in various habitats; marine (Miya et al., 2015), lakes (Fujii et al., 2019) and rivers (Doi et al., 2019; Valentini et al., 2016). However, *in silico* and *in vitro* trials of the two primer pairs have previously highlighted differences in their ability to distinguish New Zealand freshwater fish species (Banks, Kelly & Clapcott, 2020). Only a limited number of studies have compared detection rates between qPCR or ddPCR and metabarcoding approaches, with most recommending application of a targeted approach when sensitive detection of a specific species is paramount (*e.g.*, Wood et al., 2019). A further significant advantage of qPCR or ddPCR assays over metabarcoding, is that the results are instantaneous post PCR, whereas metabarcoding samples require high-throughput sequencing post PCR and bioinformatic processing. The cost of high-throughput sequencing machines is often prohibitive meaning they are sent to specialized laboratories, adding to the length of time for results to be returned.

In freshwater environments, the analysis of eDNA in water samples is commonly used in preference to sediment samples because there is generally a greater probability that eDNA from fish and other vertebrates will be detected in these samples (*e.g.*, Baldigo et al., 2017; Buxton, Groombridge & Griffiths, 2018; Shaw et al., 2016). However, some studies indicate that eDNA is found at higher, albeit more variable, concentrations and persists for longer in aquatic sediments in comparison to water (Eichmiller, Bajer & Sorensen, 2014; Sakata et al., 2020; Turner, Uy & Everhart, 2015). Sediments can also act as a sink for DNA, expanding the timescale at which eDNA can be assessed (Sakata et al., 2020). A number of studies have highlighted the potential for reconstructing historical trends in catchment use, species colonization history and aquatic community composition by eDNA analysis of terrestrial plants and animals (Giguet-Covex et al., 2014; Parducci et al., 2019; Pedersen et al., 2016), and freshwater fishes (Nelson-Chorney et al., 2019; Olajos et al., 2018) in lake sediment cores.

Freshwater eels (*Anguilla* sp.) have large economic, cultural and ecological importance worldwide, but global stocks are declining (Arai, 2014; Castonguay & Durif, 2015). Three freshwater eel species are found in New Zealand. The endemic long-finned eel (*Anguilla dieffenbachii*) and the native short-finned eel (*Anguilla australis*) are widespread throughout rivers and lakes. The Australian speckled long-finned eel (*A. reinhardtii*) inhabits a small western region of the North Island (Jellyman et al., 1996). In freshwater food webs, eels are the apex predator, and they play an important role in ecosystem functioning (*e.g.*, Kelly & Jellyman, 2007). Additionally, *A. dieffenbachii* and *A. australis* support important traditional and commercial fisheries in New Zealand (Jellyman, 2007). These eels (or tuna, as the are known by Māori, the indigenous people of New Zealand) are harvested by Māori and represent an important part of their cultural history, often featuring in their mythology (Doole, 2005; Jellyman, 2007). Although still common, there has been a national decline in eel populations, especially *A. dieffenbachii* due to habitat destruction (*i.e.,* installation of dams, weirs and wetland loss) and commercial take (Beentjes, Jellyman

& Kim, 2006; *Boubee et al., 2003*; *Doole, 2005*; *Hoyle & Jellyman, 2002*; *Jellyman, 2007*; *Jellyman et al., 2000*). Climate change has also been implicated as a future risk to eel recruitment in New Zealand (*August & Hicks, 2008*). Eel populations in New Zealand are dependent on a successful reproductive life cycle, characterized by long-distance migrations between fresh- and seawater environments where feeding and growth, and spawning occur, respectively. The life histories of both New Zealand eels remain enigmatic, with the exact location of spawning grounds in the Pacific Ocean not yet known (*Jellyman & Tsukamoto, 2002*).

Historically, eel population size and distribution have been determined *via* a range of different capture methods, *e.g.*, baited or unbaited traps, fyke netting, beam trawls or electrofishing (*e.g.*, *Beentjes, Jellyman & Kim, 2006*; *Jellyman, 1996*; *Jellyman & Chisnall, 1999*; *Jellyman & Graynoth, 2005*). Species-specific probe-based qPCR assays have been successfully developed for a range of freshwater eels globally, namely the European eel, *Anguilla anguilla* (*Weldon et al., 2020*), giant mottled eel *Anguilla maramorata* (*Itakura et al., 2020*), and Japanese eel *Anguilla japonica* (*Watanabe et al., 2005*). These *Anguilla* sp. specific assays have been used successfully in rivers (*Itakura et al., 2020*; *Itakura et al., 2019*), lakes (*Weldon et al., 2020*), and experimental tanks. Comparisons between quantitative eDNA methods and fishing surveys have highlighted the reliability and sensitivity of these eDNA methods and there are weak correlations between eDNA concentration with the abundance and biomass of eels (*Itakura et al., 2020*; *Itakura et al., 2019*; *Weldon et al., 2020*). This study aimed to develop species-specific molecular assays that could be used for the detection of *A. dieffenbachii* and *A. australis* in environmental water and sediment samples.

MATERIALS & METHODS

Primer/probe design and *in silico* specificity

Species-specific assays were designed *in silico* for *A. australis* and *A. dieffenbachii*. The *A. australis* assay targeted the mitochondrial 16S ribosomal RNA (16S rRNA) gene and the *A. dieffenbachii* assay targeted the mitochondrial *cytochrome b* (*cytb*) gene. Nucleotide sequences of *A. australis* and *A. dieffenbachii* (16S *rRNA* and *cytochrome b* genes) were sourced from the National Centre for Biotechnology Information nucleotide database (NCBI; https://www.ncbi.nlm.nih.gov/; Tables S1 and S2). Primers and probes were designed using Primer3 (*Untergasser et al., 2012*) from a consensus alignment of multiple sequences (Tables S1 and S2) to reduce potential intraspecific variability. In addition, target amplicons were aligned *in silico* with a wider range of *Anguilla* spp. (Tables S1 and S2) to determine percent similarity of sequences and to check for interspecific cross-reactivity. Target amplicons were also blasted against a wider database (Blastn; NCBI) to further check that no cross-reactivity would occur with other fish species. Primetime TaqMan probes and molecular beacon probes (IDT) were used for *A. australis* and *A. dieffenbachii,* respectively. Both probes are oligonucleotides that hybridize to an internal region of the PCR product and release fluorescence during PCR, but unlike TaqMan probes that release fluorescence during replication through cleavage, molecular beacons use changes in

structure to cause fluorescence and therefore remain intact during PCR and must rebind to the target in every cycle, which makes the probes more sensitive to single-base mismatches. To maximize assay specificity, primers and probes were designed in regions of the genes exhibiting the most interspecific variability among all eel species found in New Zealand (*A. australis*, *A. dieffenbachii* and *A. reinhardhtii*; Tables S1 and S2). The design specifically focused on identifying nucleotide mismatches among species at the 3′end of the primer.

Sample collection

Tissue samples

The specificity of both assays was tested on DNA extracted from tissue from *A. dieffenbachii* and *A. australis*, as well as a range of freshwater fish species commonly found in New Zealand. Tissue samples from morphologically identified *A. australis* and *A. dieffenbachii* specimens were provided from other projects. The samples were collected from Whakaki Lagoon (39°02′45″S, 177°32′50″E) or Te Waihora/Lake Ellesmere (43°47′21″S, 172°27′19″E), and the Maitai River (41°16′49″S, 173°19′47″E), respectively. All tissue samples were collected under the specifications of Special Permit 651 from the New Zealand government agency Ministry for Primary Industries.

The specificity of the assays was tested using DNA from other New Zealand freshwater/brackish fish species which were collected as described in *Brjkic & Lear (2017)*. Species tested included giant kokopu (*Galaxias argenteus*), black mudfish (*Neochanna diversus*), estuarine triplefin (*Forsterygion nigripenne*), Cran's bully (*Gobiomorphus basalis*), upland bully (*Gobiomorphus breviceps*), giant bully (*Gobiomorphus gobioides*), bluegill bully (*Gobiomorphus hubbsi*), redfin bully (*Gobiomorphus huttoni*) and shortjaw kokupu (*Galaxias postvectis*).

Environmental samples

Water samples were collected from 11 rivers across New Zealand (Table S3). Water samples ($n = 1$ to 5 per site) were collected to compare metabarcoding and ddPCR methods across a range of rivers and sites. For a subset of these sites (W9–W13), water samples were collected in triplicate to compare metabarcoding and ddPCR with eel biomass.

Single point water samples (0.25–10 L) were collected mid-river using a Smith-Root eDNA backpack sampler (ANDe^{TM} system; *Thomas et al., 2018*) or Geotech pump system and filtered using Polyethersulfone (PES) membrane filters (1.2 μm or 5 μm; Table S3). One liter of sterile water was filtered in the field as a control for onsite contamination (sample W11). Filters were transferred to sterile tubes and stored at −20 °C (<3 weeks) before DNA extraction and subsequent ddPCR and high-throughput sequencing (HTS).

Within one day of water sample collection at sites W9–W13, fish biomass assessments were also carried out. In a 150 m stretch of river, fish were caught by electrofishing as per *Joy, David & Lake (2013)*, taxonomically identified, counted and length measured. Fish weight in grams (W) was calculated by $W = aL^b$, where L is fish length (cm), a is the intercept and b is the slop value estimated from a linear regression of log-transformed length-weight data (*Jellyman et al., 2013*). Total fish biomass was calculated at each site and used to determine both total and relative biomass of *A. dieffenbachii* and *A. australis*. Eels that could not be identified to species level were classified as unidentified *Anguilla*. Two

of these sites, W9 and W10, had no eel biomass recorded and were therefore considered negative field control sites.

Surface sediment samples were collected from three sites in the upper South Island: Lake Rotoiti, Maitai River and Tasman Valley Stream (Table S4). These locations were chosen due to previous knowledge and observations of high concentrations of *A. dieffenbachii* and/or *A. australis*. At each location, a combination of surface sediment (<2 cm depth) and biofilm (removed from rock surfaces) samples ($n = 5$ to 7) were collected using a sterile spatula and stored in sterile tubes at −20 °C (<1 week) before DNA extraction and subsequent ddPCR analysis (Table S4)

DNA extraction

All molecular analyses (DNA extractions and PCRs) were conducted in sterile laboratories, with separate and sequential workflow to reduce cross-contamination. Benchtop UV sterilisation (>15 min) was undertaken before DNA extractions and PCR set-up. PCR set-up was done in laminar flow cabinets with HEPA filtration.

DNA was extracted from tissue samples using the DNeasy® Blood and Tissue Kit (QIAGEN, USA) following the manufacturer's instructions for tissue samples. DNA was extracted from the PES filters using the Zymo Blood and Tissue Kit according to the manufacturer's directions. As preliminary experiments indicated that inhibition was present in most samples, all DNA samples were diluted 1 in 10 prior to downstream analysis. DNA was extracted from sediment samples using the DNeasy PowerSoil® DNA Isolation Kit (QIAGEN, USA). A subsample of surface sediment was weighed directly into the first tube of the kit and the extraction performed following the manufacturer's protocol. A blank extraction without a sample was undertaken using only extraction kit buffers for all sample types.

Droplet digital PCR

Absolute concentrations of the mitochondrial 16S rRNA and *cytb* genes for *A. australis* and *A. dieffenbachii* respectively, were measured in tissue and environmental samples using a BioRad QX200 ddPCR system. Each ddPCR reaction had a total volume of 22 μL and included primers (forward and reverse; 454 nM), probe (454 nM), 1× BioRad ddPCR Supermix for probes (no dUTP), 1–3 μL DNA, and sterile water. The ddPCR reaction mixture (20 μL) was combined with 70 μL of BioRad droplet oil for probes and partitioned into nanodroplets by the BioRad QX200 droplet generator. The nanodroplet emulsion (40 μL) was transferred to and amplified in a PCR plate using the following cycling protocol; 95 °C for 10 min for initial denaturation, 45 cycles of 94 °C (30 s) and 59 °C (1 min; selected after testing different annealing temperatures), and a final step of 98 °C, 10 min for enzyme deactivation. The QX200 droplet reader (BioRad) was then used to analyze the plate. For each ddPCR assay, at least one negative methodological control (RNA/DNA-free water Life Technologies), one negative biological control (1 ng μL^{-1} tissue DNA extracted from non-target eel species) and one positive control (1 ng μL^{-1} tissue DNA extracted from target eel species) were included.

Fluorescence amplitude thresholds for positive droplets were determined separately for each assay (10,000 and 2,000 amplitude for *A. dieffenbachii* and *A. australis* assays,

respectively) based on the amplitude of negative droplets across both methodological and biological negative controls. For quality control, no positive droplets were allowed in either negative control for assay results to be accepted. When a single positive droplet occurred in a well, the sample was run twice more to confirm if the sample was positive (droplet in two of the triplicates) or negative (droplet only in one of the triplicates).

Estimation of assay limit of detection and quantification

Synthetic sections of target DNA (gblocks; manufacturer requirement to be >125 bp, Integrated DNA Technologies) were designed to match the *A. australis* 16S rRNA gene amplicon sequence (126 bp; including 21 additional bases on each end of the amplicon; Table S5) and the *A. dieffenbachii cytb* gene amplicon sequence (138 bp; including 6 additional bases on each end of the amplicon; Table S5). The highest concentrations of gblocks and target tissue DNA were quantified (ng μL^{-1}) using a Qubit (ThermoFisher Scientific, USA).

The ddPCR assay limits of detection (LOD) and quantification (LOQ) for tissue DNA were estimated using a ten-fold dilution series (in duplicate) of target tissue DNA ranging from 1 ng μL^{-1} to 0.1 fg μL^{-1}. The LOD was defined as the last standard dilution at which the targeted DNA was detected and quantified in at least two out of three replicates. The LOQ was defined as the last standard dilution in which the targeted DNA was detected and quantified in all replicates.

Assay accuracy was tested by calculating % yield from gblocks (formula below) using ten-fold dilution series ranging from 6000–0.0006 copies uL^{-1} and 10,000–0.001 copies uL^{-1} for *A. australis* and *A. dieffenbachia*, respectively. Copies per well (of gblocks) was calculated from a known concentration (ng) using the molecular weights (provided by manufacturer) of the target amplicons for *A. australis* (77,716 g mol^{-1}) and *A. dieffenbachii* (85,134 g mol^{-1}).

$$\%\ yield\ from\ gblocks = \frac{number\ of\ copies\ (per\ well)\ measured}{number\ of\ copies\ (per\ well)\ expected} \times 100.$$

Sanger sequencing

Amplicon sequence confirmation was carried out on DNA from *A. australis* and *A. dieffenbachii* tissue samples, as well as environmental samples to confirm assay specificity (see Table 1). For sequencing preparation, ddPCR product was pooled and cleaned based on the manufacturer suggested protocol (Droplet Digital Application Guide; "Amplicon Recovery from Droplets"; http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf; BioRad). Briefly, ddPCR reactions were carried out for each assay separately as previously described. Samples were assayed using 2 to 10 times dilutions depending on the amplicon concentration. Droplets for one replicate were read as per the normal protocol to confirm the successful amplification of ddPCR product. Prior to droplet analysis, the full well volume (40 μL) of all other replicate samples were transferred to a new tube.

Table 1 Droplet digital PCR and high-throughput sequencing of *Anguilla* DNA extracted from river water. Droplet digital PCR (ddPCR) amplification with *Anguilla australis* and *Anguilla dieffenbachii* specific assays and high-throughput sequencing (HTS) of DNA extracted from river water samples.

Site ID	Sample size	Species	HTS relative abundance (% of total fish community)		ddPCR (copies μL^{-1})	
			Average (±se)	Positive detection/ samples analyzed	Average (±se)	Positive detection/ samples analyzed
W1	$n = 1$	*A. australis*	44%	1/1	0.75	1/1
		A. dieffenbachii	55%	1/1	0.24	1/1
W2	$n = 1$	*A. australis*	17%	1/1	0.085	1/1
		A. dieffenbachii	–	0/1[b]	0.2	1/1[b]
W3	$n = 1$	*A. australis*	62%	1/1	0.51	1/1
		A. dieffenbachii	–	0/1	–	0/1
W4	$n = 1$	*A. australis*	60%	1/1	1.03	1/1
		A. dieffenbachii	21%	1/1	0.06	1/1
W5	$n = 1$	*A. australis*[a]	14%	1/1	2.6	1/1
		A. dieffenbachii[a]	9%	1/1	4.33	1/1
W6	$n = 1$	*A. australis*[a]	7%	1/1	5.21	1/1
		A. dieffenbachii	2%	1/1	0.34	1/1
W7	$n = 1$	*A. australis*	1%	1/1	0.02	1/1
		A. dieffenbachii	29%	1/1	0.38	1/1
W8	$n = 3$	*A. australis*	–	0/3[b]	0.12	1/3[b]
		A. dieffenbachii	75 ± 11.3%	2/3[b]	0.51 ± 0.21	3/3[b]
W9	$n = 3$	*A. australis*	–	0/3	–	0/3
		A. dieffenbachii	12.6%	1/2[b]	0.56 ± 0.36	3/3[b]
W10	$n = 3$	*A. australis*	–	0/3	–	0/3
		A. dieffenbachii	–	0/3[b]	0.07	1/3[b]
W11	$n = 5$	*A. australis*	13.0 ± 2.64%	5/5	4.69 ± 0.47	5/5
		A. dieffenbachii	52.9 ± 2.00%	5/5	12.62 ± 0.93	5/5
W12	$n = 3$	*A. australis*	77.7 ± 3.91%	3/3	4.82 ± 0.44	3/3
		A. dieffenbachii	16.9 ± 2.75%	3/3	1.26 ± 0.37	3/3
W13	$n = 3$	*A. australis*	15.0%	1/3[b]	1.01 ± 0.16	3/3[b]
		A. dieffenbachii	10.6 ± 0.36%	2/3[b]	1.74 ± 0.31	3/3[b]
W14 (control)	$n = 1$	*A. australis*	–	0/1	–	0/1
		A. dieffenbachii	–	0/1	–	0/1

Notes.
[a]ddPCR products were sequenced and confirmed amplification of correct sequence.
[b]samples with different number of positive detections between HTS and ddPCR methods.

After all droplets floated to the top, the floating droplet phase was retained from the ddPCR product mix and the bottom oil phase discarded. An aliquot of TE buffer (20 µL for 1× ddPCR well, total 40 µL > 1 ddPCR well) was added to the droplet phase, followed by chloroform (70 µL for 1× ddPCR well, total 140 µL > 1 ddPCR well). The mix was vortexed (1 min) and centrifuged (15,500× g, 10 min). The ddPCR amplicon (upper aqueous layer) was retained, quantified (Qubit) and kept at −4 °C prior to sequencing. Bi-directional

sequencing was undertaken using the BigDye Terminator v3.1 Cycle Sequencing Kit at the Genetic Analysis Services, University of Otago (Applied Biosystems, CA, USA).

High-throughput sequencing and bioinformatics

For water samples (W1–W8, W14), regions of the mitochondrial 12S rRNA gene were amplified using two previously published primer sets with illumina tags: MiFish-UF and MiFish-UR (*Miya et al., 2015*) and Teleo-R and Teleo-F (*Valentini et al., 2016*). For samples W9–W13, only the MiFish primer set was used. Each PCR reaction consisted of 10 μL of 2× MiFi Taq Mastermix (Bioline, London, UK), 1 μL of the relevant forward and reverse primer, 6 μL of DNAse free sterile water (Invitrogen, Carlsbad, CA, USA) and 2 μL of template DNA. Each PCR run included a positive control (DNA extracted from the tissue of *G. argenteus*) and a no template control. Cycling conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min. Each PCR was conducted in triplicate to minimize the impact of PCR biases and the PCR product pooled for visualization on a 1% agarose gel. The pooled PCR product was purified and normalized using SequelPrep Normalization plates (Applied Biosystems, Foster City, CA, USA), resulting in a concentration of ~1 ng mL^{-1}. The cleaned samples were sent to Auckland Genomics Facility for paired-end sequencing on an Illumina Miseq™ platform (2 × 250 bp and 1 × 150 bp for MiFish and Teleo assays, respectively). The concentration and quality of the library was quantified using a bioanalyzer. The library was diluted to 4 nM, denatured and a 15% PhiX spike added. The library was further diluted to a final loading concentration of 7 pM. Raw sequence reads are deposited in the NCBI short read archive (SRP319777).

Primers were removed from the raw reads with the program Cutadapt (*Martin, 2011*) allowing one mismatch. Sequences without primer sequences were discarded. Remaining sequences were processed with DADA2 (*Callahan et al., 2016*) within the R framework (*R Core Team, 2016*). Sequences were filtered and trimmed to 150 bp for the MiFish primer set and 85 bp for the Teleo primer set, with a maximum expected error of two for forward reads and four for reverse reads. Error profiles for both forward and reverse reads were estimated with DADA2 using 10^8 bases. Sequences were then dereplicated and sample inference undertaken for each sample. Forward and reverse reads were merged with a maximum of one mismatch and a minimum overlap of 50 bp for the MiFish sequences and 40 bp for the Teleo sequences. Sequences were size-selected (160–240 nucleotides for MiFish and 90–140 nucleotides for Teleo), and chimeras were removed using the removeBimeraDenovo command in DADA2. A reference database was constructed using 12S rRNA sequences of chordates downloaded from GenBank and supplemented with 12S rRNA sequences of New Zealand native fishes (Table S6; *Banks, Kelly & Clapcott, 2020*). Taxonomic assignment was undertaken using DADA2 and the assign Taxonomy command with bootstrapping increased to 90. This was undertaken due to the closely related nature of many of New Zealand's freshwater species to reduce the risk of spurious species assignments. The number of reads for amplicon sequence variants (ASVs) present in the negative controls was subtracted from all samples. The resulting ASVs with the

corresponding taxonomic assignment were filtered to exclude non-fish sequences. Samples with <100 reads were removed. Read abundance tables for *Anguilla* spp. were constructed from the data using the Phyloseq package (*McMurdie & Holmes, 2013*) in Rstudio (*R Studio Team, 2015*). Read numbers were converted into relative read abundance (% of total fish abundance) and when both primer sets were used, results were averaged from Teleo and MiFish primer sets.

Data analysis

Data distributions were evaluated with exploratory histograms and boxplots to ensure assumptions of normality and homogeneity of variance (Levene's test) were met. DNA concentrations (from eel tissue and gblocks), copy numbers of amplicons, relative eel reads from metabarcoding and eel biomass parameters were log-transformed prior to analysis to normalize the data. Simple linear regression was undertaken to determine standard curve correlations between dilution series of a known amount of DNA (from eel tissue or gblocks) and copy numbers of amplicons. For environmental data, simple linear regressions were used to determine relationships between log-transformed biomass parameters (relative and total), relative eel reads from metabarcoding and copy numbers of amplicons in a subset of water samples (W11–W13) with sufficient biological replication ($n = 3$ or $n = 5$). Data from negative sites W9 and W10 were predominantly zero values and these were excluded from linear regression analyses. Statistical analyses were conducted using R software (*R Core Team, 2016*; *R Studio Team, 2015*) with ggplot2 (*Wickham, 2016*) and heplots (*Fox, Friendly & Monette, 2018*).

RESULTS

Primer/probe design and *in silico* specificity

The *A. australis* assay amplified a 126 bp region of the 16S rRNA mitochondrial gene using the forward primer (A.aust16S-F: 5′–CCC AAA AGC AGC CAC CTG –3′), reverse primer (A.aust16S-R: 5′–AGG GGG TGG GGA GTT TAT TA –3′) and primetime probe (A.aust16S-P: 5′–/56-FAM/AAA GAA AGC/ZEN/GTT AAA GCT CCG A/3IABkFQ/ –3′; Fig. 1A). The *A. dieffenbachii* assay amplified a 138 bp region of the *cytb* mitochondrial gene using the forward primer (A.dieffCytB-F: 5′–GAT TCT TCG CAT TCC ACT TCT TA –3′), reverse primer (A.dieffCytB-R: 5′–GGA CTT TGT CTG CGT CAG AGT TT –3′) and molecular beacon probe (A.dieffCytB-P: 5′–/56-FAM/TCC TAC ATG AAA CAG GAT CAA GCA ATC CA/3IABkFQ/ –3′; Fig. 1B). The sequence similarity of *A. dieffenbachii* and *A. australis* for the whole 16S rRNA and *cytb* genes, was 97% and 94% respectively. Sequence similarity of amplified products between the two species was 86% and 89% for 16S rRNA and *cytb*, respectively (Tables S1 and S2).

In silico specificity was validated by nucleotide mismatches between species-specific primer and probes and non-target *Anguilla* sp. Specifically, there were 10 base pair mismatches (across both primers and probe) between the *A. australis* assay and the *A. dieffenbachii* gene sequence (Fig. 1A) and similarly there were 11 base pair mismatches between the *A. dieffenbachii* assay and the *A. australis* gene sequence (Fig. 1B). In addition,

Figure 1 Target species-specific primer and probe sequences used for amplification of *Anguilla australis* or *Anguilla dieffenbachii* DNA by droplet digital PCR. Target species-specific sections of; (A) 16S ribosomal RNA gene, and (B) *Cytochrome b* mitochondrial gene for species-specific amplification by droplet digital PCR (ddPCR) for; (A) *Anguilla australis*, and (B) *Anguilla dieffenbachii*. Species-specific primer and probe positions are indicated by red and blue text, respectively. Interspecific sequence mismatches between New Zealand *Anguilla* spp. are shown.

Full-size DOI: 10.7717/peerj.12157/fig-1

in silico testing identified six and seven base pair mismatches between the *A. reinhardtii* gene sequence and the *A. australis* and *A. dieffenbachii* assays, respectively.

Assay validations using ddPCR

Assay specificity

Anguilla australis and *A. dieffenbachii* ddPCR assays successfully amplified tissue DNA from morphologically identified *A. australis* and *A. dieffenbachii* specimens (Table 1). There was a distinctive division between positive and negative droplets in both ddPCR assays (Fig. S1). There was no cross-reactivity between assays for each eel species and tissue DNA from the non-target eel species at the maximum DNA concentrations tested (1 ng μL^{-1}; Fig. S1). Sequencing confirmed the correct amplification of either the *A. australis* 16S rRNA gene or *A. dieffenbachii cytb* gene in all ddPCR products that were sent for sequencing. Neither eel ddPCR assay cross-reacted with any of the non-target freshwater fish species assessed.

Assay sensitivity and percentage yield

Serial dilutions of tissue DNA and synthetic DNA (gblocks) had a strong and significant correlation ($r^2 > 0.96$, $p < 0.001$) to the amplicon copies per well determined by ddPCR fluorescence (Fig. 2). Using synthetic gblocks as template, the assays were linear with positive detections in range from 6000–0.06 copies uL^{-1} and 10,000–0.1 copies uL^{-1} for *A. australis* and *A. dieffenbachii*, respectively. Within this linear range, percentage yield of gblock DNA (number of copies measured/number of copies expected) was on average 80.07% ± 15.36% for the *A. australis* assay and 62.65% ± 7.39% for *A. dieffenbachii* assay.

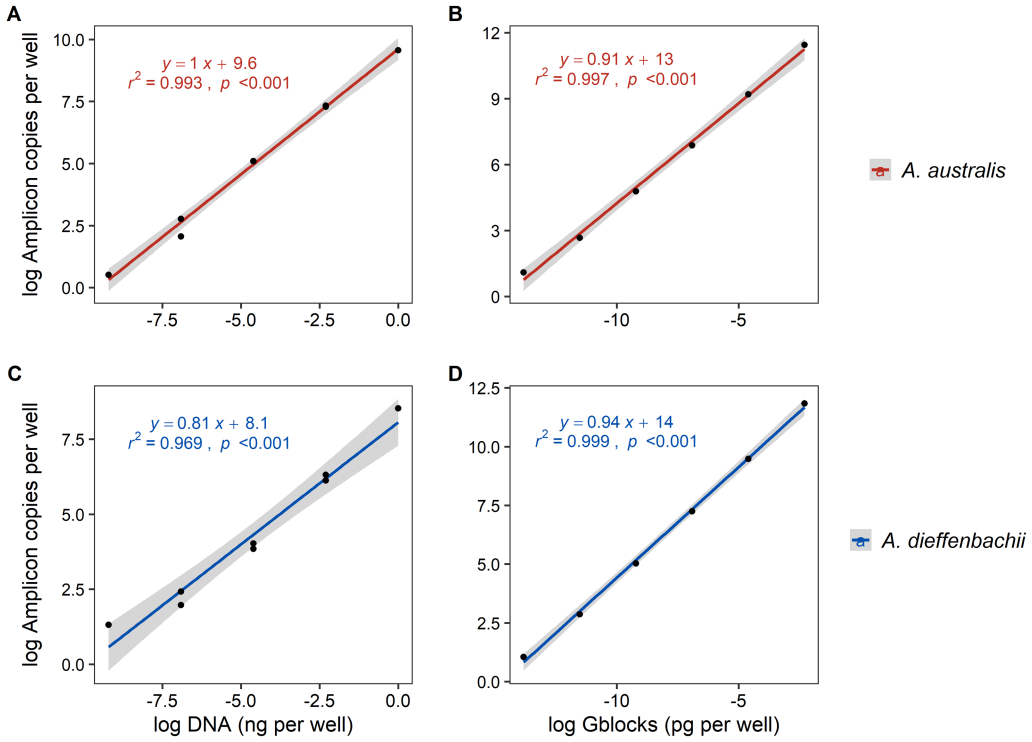

Figure 2 Linear regression analysis of droplet digital PCR copy numbers and target DNA concentrations from eel tissue DNA or synthetic amplicon sequences. The relationship between the log-transformed amplicon copies per well of droplet digital PCR (A, B) *Anguilla australis* and (C, D) *Anguilla dieffenbachii* assays and log-transformed dilution series concentration of target DNA concentrations sourced from (A, C) eel tissue DNA, and (B, D) synthetic amplicon sequence (gblocks). Results from linear regression analysis are shown.

Full-size DOI: 10.7717/peerj.12157/fig-2

Using tissue DNA as template, the assays were linear with positive detections in range from 1–0.001 ng μL^{-1} for *A. australis* and *A. dieffenbachii* (Fig. 2.). The LOQ and LOD of tissue DNA amplification was 0.001 and 0.0001 ng μL^{-1}, respectively, for both *A. australis* and *A. dieffenbachii* assays.

Environmental sample assessment

Water samples

Of the 27 filtered river water samples tested, 16 were positive for *A. australis* and 18 for *A. dieffenbachii* in the metabarcoding and ddPCR analysis (Table 1). In addition, the ddPCR assays detected *A. australis* and *A. dieffenbachii* DNA in three and six samples, respectively in which there was no positive detection in the metabarcoding analysis. At sites with biomass assessment ($n = 5$), sites with eel biomass (W11–W13) also had positive detection of eel DNA by metabarcoding or ddPCR for both species. In addition, for W9 and W10, ddPCR and metabarcoding detected *A. dieffenbachii*, despite no eel biomass recorded. Sequencing confirmed the correct ddPCR amplification of both *A. australis* and *A. dieffenbachii* DNA in sample W5 as well as additional confirmation of *A. australis* DNA in sample W6 (Table 1).

Eel biomass at sites differed between species, ranging from 0–901 g and 0–7130 g for *A. australis* and *A. dieffenbachii*, respectively. There was a significant positive relationship ($p < 0.001$) between eel biomass (g) in the river and ddPCR copy numbers per mL of river water filtered for both *A. australis* and *A. dieffenbachii* (Fig. 3). Goodness of fit of models were strong, with high r^2 values for the *A. australis* ($r^2 = 0.92$) and *A. dieffenbachia* ($r^2 = 0.91$). For *A. australis*, a positive relationship ($p < 0.007$) but with a lower goodness of fit ($r^2 = 0.66$) was also identified between metabarcoding relative eel reads (%) and relative biomass, however a contrasting negative relationship was identified when % reads was compared to absolute *A. australis* biomass (g; Fig. 3). In contrast, a significant positive relationship was identified between metabarcoding relative eel reads (%) and both total and relative biomass for *A. dieffenbachia*, with r^2 values improved when relative eel reads were compared to relative biomass ($r^2 = 0.74$) in comparison to absolute biomass ($r^2 = 0.95$; Fig. 3). Despite significant relationships existing between eel DNA proxies (ddPCR and metabarcoding relative reads) and eel biomass, there was only a positive relationship between ddPCR copies and relative metabarcoding reads for *A. dieffenbachii* ($r^2 = 0.69$, $p = 0.003$) with an opposing negative relationship for *A. australis* ($r^2 = 0.91$, $p < 0.001$; Fig. 3).

There was a proportion of eel biomass and metabarcoding reads that were identified as *Anguilla* sp. but were unable to be further classified to species level. In the metabarcoding analysis of sample W11, *Anguilla* sequences that could only be assigned to genus level accounted for a relatively small proportion of total fish community ($1.64 \pm 0.03\%$) in comparison to the proportion of *A. australis* and *A. dieffenbachii* (13% and 53%, respectively). In comparison, *Anguilla* biomass that could not be morphologically identified to species level was 0.8%, 49.2% and 26% of the total eel biomass at sites W11, W12 and W13, respectively. Analysis of species-specific relationships between DNA proxies and biomass did not include unidentified *Anguilla* biomass.

To further explore if the high proportion of unspecified *Anguilla* biomass impacted these relationships, analyses were also carried out at genus level (*i.e.,* combined species data for metabarcoding and ddPCR analyses) that also included unidentified *Anguilla* biomass in the models (Fig. 4). These produced positive, significant ($p < 0.001$) and strong ($r^2 > 0.8$) relationships between ddPCR concentrations and total biomass as well as between metabarcoding relative reads and relative biomass. No significant relationship was identified between % reads and absolute biomass ($p = 0.9$; Fig. 4). Even with species data combined, there was no significant relationship between ddPCR concentrations and relative metabarcoding relative reads ($r^2 = 0.03$, $p = 0.07$; Fig. 4).

Sediment and biofilm samples

The ddPCR assay results were positive for *A. dieffenbachii* in all samples from Lake Rotoiti, with only one detection of *A. australis* (Table 2). This was consistent with multiple *A. dieffenbachii* observed at the site during sampling. In the Maitai River, *A. dieffenbachii* was detected in only two of the five samples (M1 and M5) even though multiple *A. dieffenbachii* were visibly present at the sampling site. No *A. australis* was detected. There were three and two positive detections for *A. australis* and *A. dieffenbachii* in the Tasman Valley Stream,

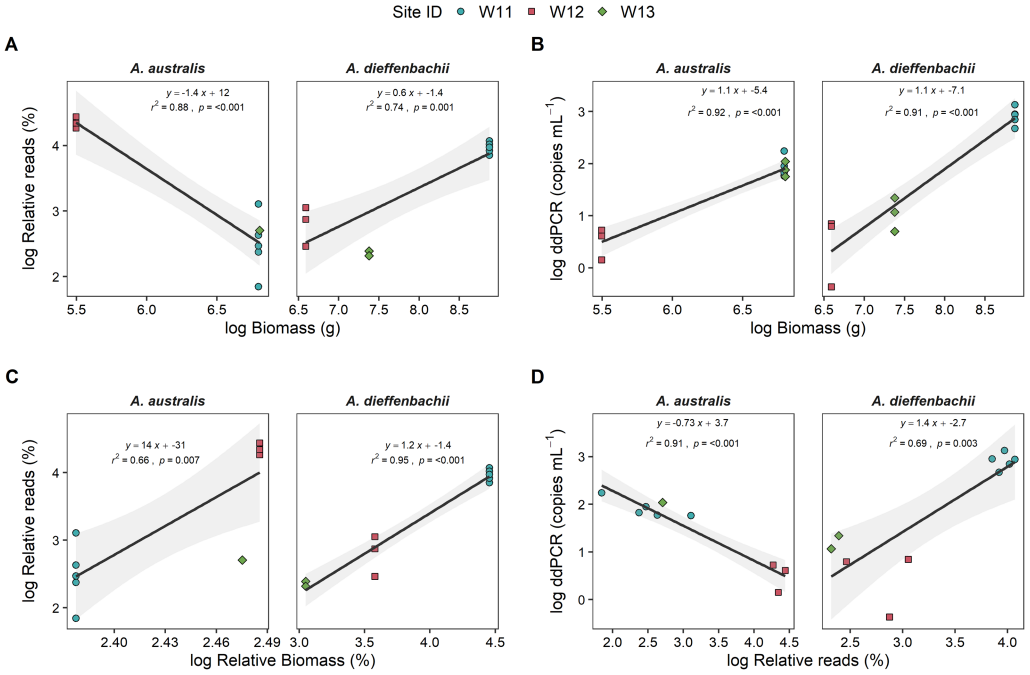

Figure 3 Targeted species-specific linear regressions between droplet digital PCR and metabarcoding analyses of eel DNA in river water and associated eel biomass. The relationships between log-transformed (A) high-throughput sequencing relative reads (%) and eel biomass (g), (B) droplet digital PCR (ddPCR) amplicon copies per mL of filtered water and eel biomass (g) (C) high-throughput relative reads (%) and eel biomass relative to total fish biomass (%) and (D) dd PCR amplicon copies per mL of filtered water and high-throughput sequencing relative reads for *Anguilla dieffenbachii* and *Anguilla australis* at three river sites ($n = 3$ DNA samples per site except for W11 with $n = 5$). Results from linear regression analysis are shown.

Full-size DOI: 10.7717/peerj.12157/fig-3

respectively (Table 2), consistent with the visual identification of both species. Despite both eel species being present, eels were not detected in three out of the five samples from Tasman Valley Stream.

DISCUSSION

Assay design, specificity, and sensitivity

In this study we successfully developed ddPCR assays for two closely related eel species, *A. australis* and *A. dieffenbachii*. Using these assays, eDNA from both species was detected in environmental water and sediment samples collected from lakes and rivers. There was no cross-reactivity with any of the other New Zealand freshwater fish species tested. These results corroborate many studies that highlighted the ability of probe-based qPCR or ddPCR assays to specifically detect freshwater fishes including other *Anguilla* sp. in environmental samples, even at low abundances (*e.g.*, *Atkinson et al., 2018*; *Bergman et al., 2016*; *Itakura et al., 2020*; *Itakura et al., 2019*; *Piggott, 2017*; *Simmons et al., 2015*; *Weldon et al., 2020*).

Attaining species-specific detection can be problematic when attempting to distinguish among closely related species. For example, *Wilcox et al. (2013)* and *Wilcox et al. (2015)*

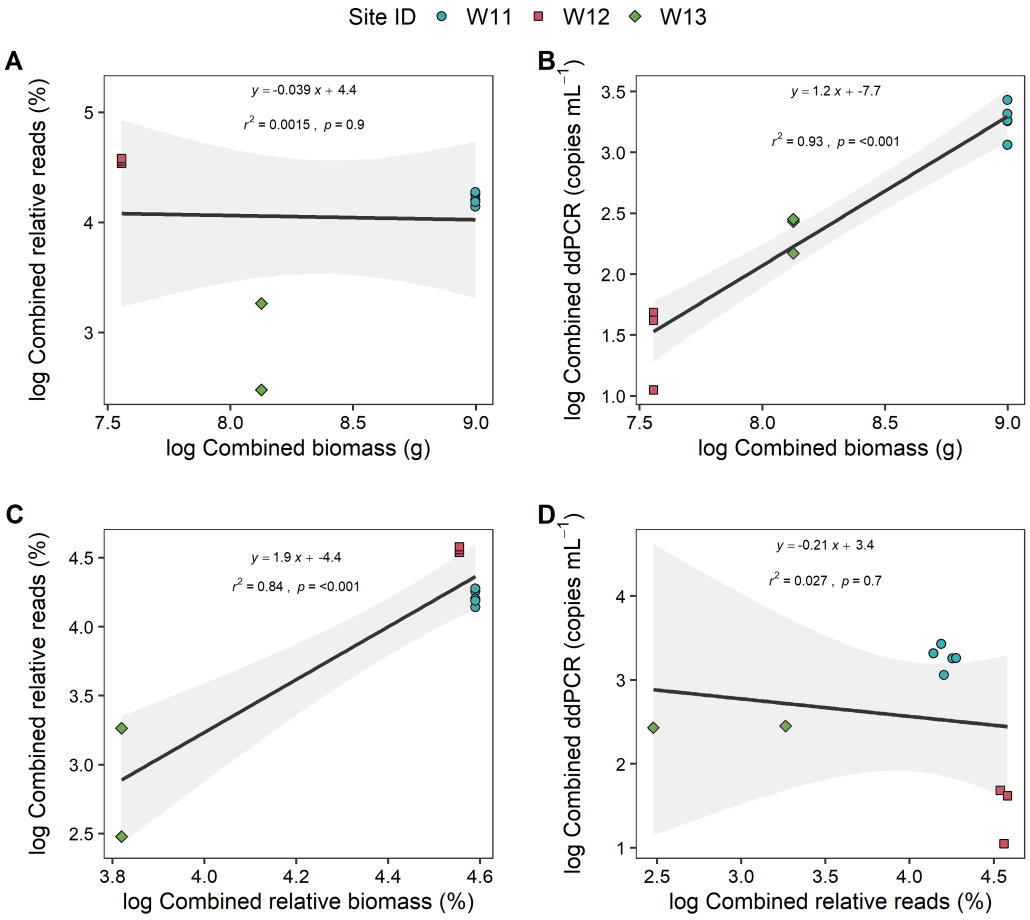

Figure 4 Linear regressions between droplet digital PCR and metabarcoding analyses of total eel DNA in river water and associated eel biomass. The relationships between log-transformed (A) high-throughput sequencing relative reads (%) and eel biomass (g), (B) droplet digital PCR (ddPCR) amplicon copies per mL of filtered water and eel biomass (g) (C) high-throughput relative reads (%) and eel biomass relative to total fish biomass (%) and (D) dd PCR amplicon copies per mL of filtered water and high-throughput sequencing relative reads for *Anguilla* (combined results from *Anguilla dieffenbachii*, *Anguilla australis* and unidentified *Anguilla*) at three river sites ($n = 3$ DNA samples per site except for W11 with $n = 5$). Results from linear regression analysis are shown.

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noted it was challenging to design species-specific assays for closely related species of char (*Salvelinus* sp.) and subspecies of trout (*Oncorhynchus* sp.), respectively. A decline in assay specificity can result in an increase of both false negative and positive target species detections (*Freeland, 2017*; *Wilcox et al., 2013*). In the present study, there was high sequence similarity between *A. dieffenbachii* and *A. australis* for both target genes and therefore careful primer and probe design was required to maximize sequence mismatches between the species. *Wilcox et al. (2013)* highlighted the importance of mismatches being in the primer in preference to the probe, and for these mismatches to be concentrated at the 3′end of the primers. We followed this approach, which restricted the flexibility of primer and probe design. Although this enabled specific assays to be developed, it is likely that assay sensitivity was slightly reduced (*i.e.,* for optimised ddPCR assay design refer to *Edwards &*

Table 2 Droplet digital PCR analysis of *Anguilla* DNA extracted from surface sediments. Droplet digital PCR (ddPCR) amplification with *Anguilla dieffenbachii* and *Anguilla australis* specific assays of DNA extracted from various surface sediment samples.

Site	Sample ID	Species identified	ddPCR (number of copies per mg wet weight of material)	Eels present at sampling (++) abundant (>10) (+) present (>0 and <10) (-) absent
Lake Rotoiti	R1	*A. australis*	0.29[b]	–
		A. dieffenbachii	2.81	++
	R2	*A. australis*	–	–
		A. dieffenbachii	0.56	++
	R3	*A. australis*	–	–
		A. dieffenbachii	3.59	++
	R4	*A. australis*	–	–
		A. dieffenbachii	1.32	++
	R5	*A. australis*	–	–
		A. dieffenbachii [a]	3.21	++
Maitai River	M1	*A. australis*	–	–
		A. dieffenbachii	0.16[b]	++
	M2	*A. australis*	–	–
		A. dieffenbachii	–	++
	M3	*A. australis*	–	–
		A. dieffenbachii	–	++
	M4	*A. australis*	–	–
		A. dieffenbachii	–	++
	M5	*A. australis*	–	–
		A. dieffenbachii	0.25[b]	++
Tasman Valley Stream	J1	*A. australis*	–	+
		A. dieffenbachii	0.13[b]	++
	J2	*A. australis*	–	+
		A. dieffenbachii	–	++
	J3	*A. australis*	–	+
		A. dieffenbachii	–	++
	J4	*A. australis*	–	+
		A. dieffenbachii	–	++
	J5	*A. australis*[a]	40.54	+
		A. dieffenbachii[a]	24.76	++
	J6	*A. australis*	1.07[b]	+
		A. dieffenbachii	6.88	++

Notes.
[a]ddPCR products were sequenced and confirmed amplification of correct sequence.
[b]single droplet samples were measured in triplicate to confirm true positives.

Logan, 2004; *Huggett et al., 2013*). The LOQ for target tissue DNA was 10^{-3} ng μL^{-1} and LOD 10^{-4} ng μL^{-1}, respectively for *A. australis* and *A. dieffenbachii*. These levels are within the ranges of LOD and LOQ reported for other targeted species assays, albeit at the lower end of sensitivity. For example, LOD for the mussel *Margaritifera margaritifera* was 10^{-4} ng or 10^{-5} ng of DNA depending on the target gene (*Mauvisseau et al., 2019a*; *Stoeckle, Kuehn*

& Geist, 2016), whereas higher sensitivity was found for the invasive crayfish *Procambarus clarkia*, and the endangered newt *Triturus cristatus* (10–8 ng μL^{-1} and 10–7 ng μL^{-1}, respectively; Buxton et al., 2017; Tréguier et al., 2014). Despite lower LODs, the LOQs for *A. australis* and *A. dieffenbachii* were similar to *P. clarkia* and *T. cristatus* (10–4 ng μL^{-1} and 10–5 ng μL^{-1}, respectively; Buxton et al., 2017; Tréguier et al., 2014).

Comparison of droplet digital PCR with metabarcoding

Positive eel DNA detection by ddPCR occurred at all sites with eel presence as determined by metabarcoding analysis. Furthermore, the new targeted ddPCR approach resulted in a slightly higher number of positives detections of *A. dieffenbachii* and *A. australis* in comparison to commonly used metabarcoding methods (MiFish-U/E and Teleo-F/R; Miya et al., 2015; Valentini et al., 2016) corroborating the results from other studies (Bylemans et al., 2019; Harper et al., 2018; Schenekar et al., 2020). Primer bias is a plausible explanation for the lower number of detections in the metabarcoding approach. Metabarcoding studies on 'mock communities' have highlighted that the detection of specific taxa within more complex communities can be markedly reduced and alluded to primer bias as a reason for this (Lee et al., 2012; Pochon et al., 2013). In a complex freshwater community matrix, as investigated here, the target gene copy numbers of other taxa in the samples may be differentially enhanced in comparison to *A. dieffenbachii* and *A. australis.* These results highlight the need for careful consideration when using metabarcoding approaches to detect specific species in environmental samples.

Comparison of DNA methods with biomass measurement

Positive DNA detections in the water aligned with the presence of eel biomass at sites. In addition, metabarcoding and ddPCR positively detected eel DNA in the water at a site with no eel biomass measured. This positive detection could be due to various factors, *i.e.*, DNA methods being more sensitive than electrofishing methods that are known to range in efficacy (Meador, McIntyre & Pollock, 2003) or downstream transportation of fish eDNA from above the defined fishing site (Pont et al., 2018).

Both ddPCR and metabarcoding DNA detection methods performed well at estimating *A. dieffenbachii* biomass across five river sites as determined by traditional electrofishing approaches. The ddPCR approach improved model goodness of fit and had a positive significant relationship with *A. australis* biomass in comparison to metabarcoding, suggesting that the relationship with ddPCR concentration was more reliable at a lower biomass, as found for *A. australis*. In previous studies, eDNA concentrations in water samples have been similarly correlated to eel abundance and/or biomass in rivers (Chin et al., 2021; Itakura et al., 2020; Itakura et al., 2019) and lakes (Weldon et al., 2020). Despite this, the reliability of using eDNA concentrations to quantify population abundances is under considerable debate. Some studies on a wider range of organisms have found a positive correlation among results generated using molecular techniques and biomass and abundance estimates (Klobucar, Rodgers & Budy, 2017; Klymus et al., 2015; Mizumoto et al., 2018; Takahara et al., 2012), while others note the absence of such correlation (Capo et al., 2019; Deutschmann et al., 2019; Spear et al., 2015). Many of these positive relationships

have been found in controlled laboratory set ups (*Doi et al., 2015b*; *Harper et al., 2019*; *Klobucar, Rodgers & Budy, 2017*; *Mizumoto et al., 2018*; *Takahara et al., 2012*) with limited success in the natural environment (*Capo et al., 2019*; *Yates, Fraser & Derry, 2019*). There are a number of factors such as temperature (*Lacoursière-Roussel, Rosabal & Bernatchez, 2016*; *Takahara et al., 2012*), and feeding and diet (*Klymus et al., 2015*) that influence the amount of DNA in the environment and thus the relationship between abundance or biomass and eDNA concentrations. Further caution is required as different life stages often have variable cell numbers and different amounts of DNA may be shed at each life stage. For example, *Takeuchi et al. (2019)* found that concentrations of eDNA shed from the Japanese eel differed significantly among all life stages. In fresh water, the eel life cycle encompasses elvers (ca. 6–20 cm), juveniles and adults (up to 24 kg and 3 kg for *A. dieffenbachii* and *A. australis*, respectively) with sexual dimorphism in body size (*Todd, 1980*). At each eel life stage there are also differences in habitat as well as diet (*e.g.*, *Jellyman, 1996*; *Jellyman & Chisnall, 1999*). Controlled experiments to compare the detection of eel DNA in water and sediment with known parameters such as eel abundance, sex and body size are required to address these issues and understand the future potential of using eel DNA as a proxy for abundance under different conditions. Despite these uncertainties, targeted approaches, such as the ddPCR assays developed in this study are extremely sensitive and specific. The results are obtained instantaneously after the PCR step and using the BioRad machine up to 96 samples including controls can be analyzed simultaneously allowing for high-throughput and rapid turnaround times.

Application of droplet digital PCRs on surface sediment DNA

Positive eel DNA detection in sediment samples aligned with the presence of eels at sites as determined by visual surveys. However, in contrast to the consistency of water eDNA detections, our data indicates that eDNA detections are more variable in sediment. Several sediment samples were collected at three sites (two rivers, one lake). At each site these were spatially close and taken near target species (ca. 5 m distance). Positive detections (per site) corresponded to the eel species observed at sites, but detections were variable among samples with some replicates failing to detect either eel species, highlighting the problem of false negatives. There is mixed evidence in the literature about the effectiveness of assessing eDNA in sediment. *Turner et al. (2014)* found that fish DNA persisted for longer in sediment than water and suggested that eDNA was more stable in sediment. In contrast, comparisons between water and sediment samples for targeted fish detection or metabarcoding found that detection was more effective in water column samples (*Buxton, Groombridge & Griffiths, 2018*; *Eichmiller, Bajer & Sorensen, 2014*; *Shaw et al., 2016*). *Eichmiller, Bajer & Sorensen (2014)* observed that DNA was concentrated in sediment but was highly variable and suggested this was due to differential deposition and resuspension of sediment and DNA degradation. A larger number of samples from a wider variety of habitats are required to confirm these possible explanations. Furthermore, different sampling strategies and sample replication need to be investigated to determine how sampling methods may affect the occurrence of false

negatives and therefore the likelihood of positive detection. This next step is necessary before considering the application of these eDNA assays as monitoring tools.

CONCLUSIONS

In this study we successfully developed species-specific ddPCR assays to detect *A. dieffenbachii* and *A. australis* DNA in both water and sediment samples. The ddPCR assays detected eels in a greater number of waters samples than when metabarcoding techniques were applied. Water sample analyses using ddPCR and metabarcoding methods were positively correlated with species-specific biomass. We recommend further research across a greater number and type of river sites to determine the consistency of these relationships and establish whether DNA methods are a reliable proxy of eel biomass. When analyzing surface sediment/biofilm samples, there were several false negative results that may relate to our ability to effectively extract DNA from sediment/biofilms or spatial variation in organism DNA. The successful detections of eel DNA in water by ddPCR in addition to its correlation with eel biomass coupled with the high-throughput and rapid turnaround times highlights the potential for using these assays as a monitoring tools which would enable analysis of eel population at scales and resolutions not previously possible.

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Competing Interests

Xavier Pochon is an Academic Editor in the Aquatic Biology section of PeerJ.

Author Contributions

- Georgia Thomson-Laing conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Russleigh Parai performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Laura T. Kelly conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Xavier Pochon, Rewi Newnham, Marcus J. Vandergoes and Jamie D. Howarth conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Susanna A. Wood conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All tissue samples were collected under the specifications of Special Permit 651 from the New Zealand government agency Ministry for Primary Industries.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

All tissue samples were collected under the specifications of Special Permit 651 from the New Zealand government agency Ministry for Primary Industries.

Data Availability

The following information was supplied regarding data availability:

The raw sequence reads are available in the NCBI Short Read Archive: PRJNA729802, SRP319777, SRX10876622 SRX10876641.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12157#supplemental-information.

REFERENCES

Arai T. 2014. Do we protect freshwater eels or do we drive them to extinction? *SpringerPlus* **3**:534 DOI 10.1186/2193-1801-3-534.

Atkinson S, Carlsson JEL, Ball B, Egan D, Kelly-Quinn M, Whelan K, Carlsson J. 2018. A quantitative PCR-based environmental DNA assay for detecting Atlantic salmon (*Salmo salar* L.). *Aquatic Conservation: Marine and Freshwater Ecosystems* **28**:1238–1243 DOI 10.1002/aqc.2931.

August SM, Hicks BJ. 2008. Water temperature and upstream migration of glass eels in New Zealand: implications of climate change. *Environmental Biology of Fishes* **81**:195–205 DOI 10.1007/s10641-007-9191-z.

Baldigo BP, Sporn LA, George SD, Ball JA. 2017. Efficacy of environmental DNA to detect and quantify brook trout populations in headwater streams of the Adirondack Mountains, New York. *Transactions of the American Fisheries Society* **146**:99–111 DOI 10.1080/00028487.2016.1243578.

Banks J, Kelly L, Clapcott J. 2020. Molecular tools for characterising freshwater fish communities in New Zealand. Prepared for ministry of business, innovation and the environment, Envirolink CAWX1802. Cawthron Report (3573) 66 p. plus appendices.

Beentjes MP, Jellyman DJ, Kim SW. 2006. Changing population structure of eels (*Anguilla dieffenbachii* and *Aaustralis*) from southern New Zealand. *Ecology of Freshwater Fish* **15**:428–440 DOI 10.1111/j.1600-0633.2006.00165.x.

Bergman PS, Schumer G, Blankenship S, Campbell E. 2016. Detection of adult green sturgeon using environmental DNA analysis. *PLOS ONE* **11**:e0153500 DOI 10.1371/journal.pone.0153500.

Blackman RC, Constable D, Hahn C, Sheard AM, Durkota J, Hänfling B, Handley LL. 2017. Detection of a new non-native freshwater species by DNA metabarcoding of environmental samples—first record of *Gammarus fossarum* in the UK. *Aquatic Invasions* **12**:177–189 DOI 10.3391/ai.2017.12.2.06.

Boubee J, Chisnall B, Watene E, Williams E, Roper D, Haro A. 2003. Enhancement and management of eel fisheries affected by hydroelectric dams in New Zealand. *American Fisheries Society Symposium* **2003**:191–205.

Brjkic M, Lear G. 2017. Monitoring our native New Zealand freshwater & estuarine fish species with environmental DNA. University Dissertation.

Butchart SHM, Walpole M, Collen B, Van Strien A, Scharlemann JPW, Almond REA, Baillie JEM, Bomhard B, Brown C, Bruno J, Carpenter KE, Carr GM, Chanson J, Chenery AM, Csirke J, Davidson NC, Dentener F, Foster M, Galli A, Galloway JN, Genovesi P, Gregory RD, Hockings M, Kapos V, Lamarque J-F, Leverington F, Loh J, McGeoch MA, McRae L, Minasyan A, Morcillo MH, Oldfield TEE, Pauly D, Quader S, Revenga C, Sauer JR, Skolnik B, Spear D, Stanwell-Smith D, Stuart SN, Symes A, Tierney M, Tyrrell TD, Vié J-C, Watson R. 2010. Global biodiversity: indicators of recent declines. *Science* **328**:1164 DOI 10.1126/science.1187512.

Buxton AS, Groombridge JJ, Griffiths RA. 2018. Seasonal variation in environmental DNA detection in sediment and water samples. *PLOS ONE* **13**:e0191737 DOI 10.1371/journal.pone.0191737.

Buxton AS, Groombridge JJ, Zakaria NB, Griffiths RA. 2017. Seasonal variation in environmental DNA in relation to population size and environmental factors. *Scientific Reports* **7**:46294 DOI 10.1038/srep46294.

Bylemans J, Gleeson DM, Duncan RP, Hardy CM, Furlan EM. 2019. A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environmental DNA* **1**:402–414 DOI 10.1002/edn3.41.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**:581–583 DOI 10.1038/nmeth.3869.

Capo E, Spong G, Norman S, Königsson H, Bartels P, Byström P. 2019. Droplet digital PCR assays for the quantification of brown trout (*Salmo trutta*) and Arctic char (*Salvelinus alpinus*) from environmental DNA collected in the water of mountain lakes. *PLOS ONE* **14**:e0226638 DOI 10.1371/journal.pone.0226638.

Castonguay M, Durif CMF. 2015. Understanding the decline in anguillid eels. *ICES Journal of Marine Science* **73**:1–4 DOI 10.1093/icesjms/fsv256.

Chin SC, Waldman J, Bednarski M, Camhi M, LaBelle J, Alter SE. 2021. Relating American eel *Anguilla rostrata* abundance to environmental DNA concentration in the Bronx River. *North American Journal of Fisheries Management* DOI 10.1002/nafm.10625.

Clarke LJ, Soubrier J, Weyrich LS, Cooper A. 2014. Environmental metabarcodes for insects: *in silico* PCR reveals potential for taxonomic bias. *Molecular Ecology Resources* **14**:1160–1170 DOI 10.1111/1755-0998.12265.

Deagle BE, Jarman SN, Coissac E, Pompanon F, Taberlet P. 2014. DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: not a perfect match. *Biology Letters* **10**:20140562 DOI 10.1098/rsbl.2014.0562.

Deutschmann B, Muller AK, Hollert H, Brinkmann M. 2019. Assessing the fate of brown trout (*Salmo trutta*) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe. *Science of the Total Environment* **655**:321–327 DOI 10.1016/j.scitotenv.2018.11.247.

Dirzo R, Raven PH. 2003. Global state of biodiversity and loss. *Annual Review of Environment and Resources* **28**:137–167 DOI 10.1146/annurev.energy.28.050302.105532.

Doi H, Inui R, Matsuoka S, Akamatsu Y, Goto M, Kono T. 2019. Evaluation of biodiversity metrics through environmental DNA metabarcoding outperforms visual and capturing surveys. *bioRxiv* 617670 DOI 10.1101/617670.

Doi H, Takahara T, Minamoto T, Matsuhashi S, Uchii K, Yamanaka H. 2015a. Droplet digital Polymerase Chain Reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. *Environmental Science & Technology* **49**:5601–5608 DOI 10.1021/acs.est.5b00253.

Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H, Minamoto T. 2015b. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLOS ONE* **10**:e0122763 DOI 10.1371/journal.pone.0122763.

Doole GJ. 2005. Optimal management of the New Zealand longfin eel (*Anguilla dieffenbachii*). *Australian Journal of Agricultural and Resource Economics* **49**:395–411 DOI 10.1111/j.1467-8489.2005.00310.x.

Dowle EJ, Pochon X, Banks J, Shearer K, Wood SA. 2016. Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: a case study using freshwater macroinvertebrates. *Molecular Ecology Resources* **16**:1240–1254 DOI 10.1111/1755-0998.12488.

Edwards KJ, Logan JM. 2004. *Performing real-time PCR. Real-time PCR an essential guide Horizon bioscience, Norfolk*. Wymondham, Norfolk: Horizon Bioscience.

Eichmiller JJ, Bajer PG, Sorensen PW. 2014. The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLOS ONE* **9**:e112611 DOI 10.1371/journal.pone.0112611.

Fox J, Friendly M, Monette G. 2018. heplots: visualizing tests in multivariate linear models. R package version 1.3-5. *Available at https://cran.r-project.org/package=heplots*.

Freeland JR. 2017. The importance of molecular markers and primer design when characterizing biodiversity from environmental DNA. *Genome* **60**:358–374 DOI 10.1139/gen-2016-0100.

Fujii K, Doi H, Matsuoka S, Nagano M, Sato H, Yamanaka H. 2019. Environmental DNA metabarcoding for fish community analysis in backwater lakes: A comparison of capture methods. *PLOS ONE* **14**:e0210357 DOI 10.1371/journal.pone.0210357.

Giguet-Covex C, Pansu J, Arnaud F, Rey P-J, Griggo C, Gielly L, Domaizon I, Coissac E, David F, Choler P. 2014. Long livestock farming history and human landscape shaping revealed by lake sediment DNA. *Nature communications* **5**:1–7.

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 DOI 10.1899/13-046.1.

Hänfling B, Lawson Handley L, Read DS, Hahn C, Li J, Nichols P, Blackman RC, Oliver A, Winfield IJ. 2016. Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology* **25**:3101–3119 DOI 10.1111/mec.13660.

Harper LR, Griffiths NP, Lawson Handley L, Sayer CD, Read DS, Harper KJ, Blackman RC, Li J, Hänfling B. 2019. Development and application of environmental DNA surveillance for the threatened crucian carp (*Carassius carassius*). *Freshwater Biology* **64**:93–107 DOI 10.1111/fwb.13197.

Harper LR, Lawson Handley L, Hahn C, Boonham N, Rees HC, Gough KC, Lewis E, Adams IP, Brotherton P, Phillips S, Hänfling B. 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution* **8**:6330–6341 DOI 10.1002/ece3.4013.

He F, Zarfl C, Bremerich V, Henshaw A, Darwall W, Tockner K, Jähnig SC. 2017. Disappearing giants: a review of threats to freshwater megafauna. *WIREs Water* **4**:e1208 DOI 10.1002/wat2.1208.

Hinlo R, Furlan E, Suitor L, Gleeson D. 2017. Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. *Management of Biological Invasions* **8**:89 DOI 10.3391/mbi.2017.8.1.09.

Hoyle SD, Jellyman DJ. 2002. Longfin eels need reserves: modelling the effects of commercial harvest on stocks of New Zealand eels. *Marine and Freshwater Research* **53**:887–895 DOI 10.1071/MF00020.

Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Hellemans J, Kubista M, Mueller RD, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer

CT, Bustin SA. 2013. The digital MIQE Guidelines: Minimum information for publication of quantitative digital PCR experiments. *Clinical Chemistry* **59**:892–902 DOI 10.1373/clinchem.2013.206375.

Itakura H, Wakiya R, Sakata MK, Hsu H-Y, Chen S-C, Yang C-C, Huang Y-C, Han Y-S, Yamamoto S, Minamoto T. 2020. Estimations of riverine distribution, abundance, and biomass of Anguillid eels in Japan and Taiwan using environmental DNA analysis. *Zoological Studies* **59**:e17.

Itakura H, Wakiya R, Yamamoto S, Kaifu K, Sato T, Minamoto T. 2019. Environmental DNA analysis reveals the spatial distribution, abundance, and biomass of Japanese eels at the river-basin scale. *Aquatic Conservation: Marine and Freshwater Ecosystems* **29**:361–373 DOI 10.1002/aqc.3058.

Jellyman DJ. 1996. Diet of longfinned eels, *Anguilla dieffenbachii*, in Lake Rotoiti, Nelson Lakes, New Zealand. *New Zealand Journal of Marine and Freshwater Research* **30**:365–369 DOI 10.1080/00288330.1996.9516723.

Jellyman DJ. 2007. Status of New Zealand fresh-water eel stocks and management initiatives. *ICES Journal of Marine Science* **64**:1379–1386 DOI 10.1093/icesjms/fsm073.

Jellyman DJ, Chisnall BL. 1999. Habitat preferences of shortfinned eels (*Anguilla australis*), in two New Zealand lowland lakes. *New Zealand Journal of Marine and Freshwater Research* **33**:233–248 DOI 10.1080/00288330.1999.9516873.

Jellyman DJ, Chisnall BL, Dijkstra LH, Boubee JAT. 1996. First record of the Australian longfinned eel, Anguilla reinhardtii, in New Zealand. *Marine and Freshwater Research* **47**:1037–1040 DOI 10.1071/MF9961037.

Jellyman DJ, Graynoth E. 2005. The use of fyke nets as a quantitative capture technique for freshwater eels (*Anguilla* spp.) in rivers. *Fisheries Management and Ecology* **12**:237–247 DOI 10.1111/j.1365-2400.2005.00445.x.

Jellyman DJ, Graynoth E, Francis RICC, Chisnall BL, Beentjes MP. 2000. A review of evidence for a decline in the abundance of longfinned eels (*Anguilla dieffenbachii*) in New Zealand. NIWA Report for Ministry of Fisheries Research Project EEL9802.

Jellyman DJ, Tsukamoto K. 2002. First use of archival transmitters to track migrating freshwater eels *Anguilla dieffenbachii* at sea. *Marine Ecology Progress Series* **233**:207–215 DOI 10.3354/meps233207.

Jellyman PG, Booker DJ, Crow SK, Bonnett ML, Jellyman DJ. 2013. Does one size fit all? An evaluation of length–weight relationships for New Zealand's freshwater fish species. *New Zealand Journal of Marine and Freshwater Research* **47**:450–468 DOI 10.1080/00288330.2013.781510.

Jerde CL, Mahon AR, Chadderton WL, Lodge DM. 2011. Sight-unseen detection of rare aquatic species using environmental DNA. *Conservation Letters* **4**:150–157 DOI 10.1111/j.1755-263X.2010.00158.x.

Joy M, David B, Lake M. 2013. *New Zealand freshwater fish sampling protocols, part 1: wadeable rivers and streams.* Massey Univerisity, Palmerston North, New Zealand: The Ecology Group - Institute of Natural Resources.

Kelly DJ, Jellyman DJ. 2007. Changes in trophic linkages to shortfin eels (*Anguilla australis*) since the collapse of submerged macrophytes in Lake Ellesmere, New Zealand. *Hydrobiologia* **579**:161–173 DOI 10.1007/s10750-006-0400-0.

Klobucar SL, Rodgers TW, Budy P. 2017. At the forefront: evidence of the applicability of using environmental DNA to quantify the abundance of fish populations in natural lentic waters with additional sampling considerations. *Canadian Journal of Fisheries and Aquatic Sciences* **74**:2030–2034 DOI 10.1139/cjfas-2017-0114.

Klymus KE, Marshall NT, Stepien CA. 2017. Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. *PLOS ONE* **12**:e0177643 DOI 10.1371/journal.pone.0177643.

Klymus KE, Richter CA, Chapman DC, Paukert C. 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation* **183**:77–84 DOI 10.1016/j.biocon.2014.11.020.

Lacoursière-Roussel A, Rosabal M, Bernatchez L. 2016. Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. *Molecular Ecology Resources* **16**:1401–1414 DOI 10.1111/1755-0998.12522.

Laramie MB, Pilliod DS, Goldberg CS. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation* **183**:29–37 DOI 10.1016/j.biocon.2014.11.025.

Lee CK, Herbold CW, Polson SW, Wommack KE, Williamson SJ, McDonald IR, Cary SC. 2012. Groundtruthing next-gen sequencing for microbial ecology-biases and errors in community structure estimates from PCR amplicon pyrosequencing. *PLOS ONE* **7**:e44224 DOI 10.1371/journal.pone.0044224.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* **17**:3 DOI 10.14806/ej.17.1.200.

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M. 2019a. Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. *Scientific Reports* **9**:1–10 DOI 10.1038/s41598-018-37001-y.

Mauvisseau Q, Coignet A, Delaunay C, Pinet F, Bouchon D, Souty-Grosset C. 2018. Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. *Hydrobiologia* **805**:163–175 DOI 10.1007/s10750-017-3288-y.

Mauvisseau Q, Davy-Bowker J, Bulling M, Brys R, Neyrinck S, Troth C, Sweet M. 2019b. Improving detection capabilities of a critically endangered freshwater invertebrate with environmental DNA using digital droplet PCR. *bioRxiv* 661447 DOI 10.1101/661447.

McMurdie PJ, Holmes S. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE* **8**:e61217 DOI 10.1371/journal.pone.0061217.

Meador MR, McIntyre JP, Pollock KH. 2003. Assessing the efficacy of single-pass backpack electrofishing to characterize fish community structure. *Transactions of the*

American Fisheries Society **132**:39–46
DOI 10.1577/1548-8659(2003)132<0039:ATEOSP>2.0.CO;2.

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society Open Science* **2**:150088 DOI 10.1098/rsos.150088.

Mizumoto H, Urabe H, Kanbe T, Fukushima M, Araki H. 2018. Establishing an environmental DNA method to detect and estimate the biomass of *Sakhalin taimen*, a critically endangered Asian salmonid. *Limnology* **19**:219–227 DOI 10.1007/s10201-017-0535-x.

Nelson-Chorney HT, Davis CS, Poesch MS, Vinebrooke RD, Carli CM, Taylor MK. 2019. Environmental DNA in lake sediment reveals biogeography of native genetic diversity. *Frontiers in Ecology and the Environment* **17**:313–318 DOI 10.1002/fee.2073.

Olajos F, Bokma F, Bartels P, Myrstener E, Rydberg J, Öhlund G, Bindler R, Wang X-R, Zale R, Englund G. 2018. Estimating species colonization dates using DNA in lake sediment. *Methods in Ecology and Evolution* **9**:535–543 DOI 10.1111/2041-210x.12890.

Olsen JB, Lewis CJ, Massengill RL, Dunker KJ, Wenburg JK. 2015. An evaluation of target specificity and sensitivity of three qPCR assays for detecting environmental DNA from Northern Pike (*Esox lucius*). *Conservation Genetics Resources* **7**:615–617 DOI 10.1007/s12686-015-0459.

Parducci L, Alsos IG, Unneberg P, Pedersen MW, Han L, Lammers Y, Salonen JS, Väliranta MM, Slotte T, Wohlfarth B. 2019. Shotgun environmental DNA, pollen, and macrofossil analysis of lateglacial lake sediments from southern Sweden. *Frontiers in Ecology and Evolution* **7**:189 DOI 10.3389/fevo.2019.00189.

Pedersen MW, Ruter A, Schweger C, Friebe H, Staff RA, Kjeldsen KK, Mendoza MLZ, Beaudoin AB, Zutter C, Larsen NK, Potter BA, Nielsen R, Rainville RA, Orlando L, Meltzer DJ, Kjær KH, Willerslev E. 2016. Postglacial viability and colonization in North America's ice-free corridor. *Nature* **537**:45–49 DOI 10.1038/nature19085.

Piggott MP. 2017. An environmental DNA assay for detecting Macquarie perch, Macquaria australasica. *Conservation Genetics Resources* **9**:257–259 DOI 10.1007/s12686-016-0666-0.

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences* **70**:1123–1130 DOI 10.1139/cjfas-2013-0047.

Pochon X, Bott NJ, Smith KF, Wood SA. 2013. Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests. *PLOS ONE* **8**:e73935 DOI 10.1371/journal.pone.0073935.

Pont D, Rocle M, Valentini A, Civade R, Jean P, Maire A, Roset N, Schabuss M, Zornig H, Dejean T. 2018. Environmental DNA reveals quantitative patterns of fish

biodiversity in large rivers despite its downstream transportation. *Scientific Reports* **8**:10361 DOI 10.1038/s41598-018-28424-8.

Portt C, Coker G, Ming D, Randall R. 2006. A review of fish sampling methods commonly used in Canadian freshwater habitats. *Canadian Technical Report of Fisheries and Aquatic Sciences* **2604**:51.

R Core Team. 2016. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

R Studio Team. 2015. RStudio: integrated development for R. Boston, MA: RStudio, Inc.

Reynolds JB, Holliman FM. 2004. Injury of American eels captured by electrofishing and trap-netting. *North American Journal of Fisheries Management* **24**:686–689 DOI 10.1577/M03-027.1.

Sakata MK, Yamamoto S, Gotoh RO, Miya M, Yamanaka H, Minamoto T. 2020. Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. *Environmental DNA* **2**:505–528 DOI 10.1002/edn3.75.

Schenekar T, Schletterer M, Lecaudey LA, Weiss SJ. 2020. Reference databases, primer choice, and assay sensitivity for environmental metabarcoding: lessons learnt from a re-evaluation of an eDNA fish assessment in the Volga headwaters. *River Research and Applications* **36**:1004–1013 DOI 10.1002/rra.3610.

Secondi J, Dejean T, Valentini A, Audebaud B, Miaud C. 2016. Detection of a global aquatic invasive amphibian, *Xenopus laevis*, using environmental DNA. *Amphibia-Reptilia* **37**:131–136 DOI 10.1163/15685381-00003036.

Senapati D, Bhattacharya M, Kar A, Chini DS, Das BK, Patra BC. 2019. Environmental DNA (eDNA): a promising biological survey tool for aquatic species detection. *Proceedings of the Zoological Society* **72**:211–228 DOI 10.1007/s12595-018-0268-9.

Shaw JLA, Clarke LJ, Wedderburn SD, Barnes TC, Weyrich LS, Cooper A. 2016. Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation* **197**:131–138 DOI 10.1016/j.biocon.2016.03.010.

Sigsgaard EE, Carl H, Møller PR, Thomsen PF. 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation* **183**:46–52 DOI 10.1016/j.biocon.2014.11.023.

Simmons M, Tucker A, Chadderton WL, Jerde CL, Mahon AR. 2015. Active and passive environmental DNA surveillance of aquatic invasive species. *Canadian Journal of Fisheries and Aquatic Sciences* **73**:76–83 DOI 10.1139/cjfas-2015-0262.

Smart AS, Tingley R, Weeks AR, Van Rooyen AR, McCarthy MA. 2015. Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications* **25**:1944–1952 DOI 10.1890/14-1751.1.

Spear SF, Groves JD, Williams LA, Waits LP. 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation* **183**:38–45 DOI 10.1016/j.biocon.2014.11.016.

Stoeckle BC, Kuehn R, Geist J. 2016. Environmental DNA as a monitoring tool for the endangered freshwater pearl mussel (*Margaritifera margaritifera* L.): a substitute

for classical monitoring approaches? *Aquatic Conservation: Marine and Freshwater Ecosystems* **26**:1120–1129 DOI 10.1002/aqc.2611.

Takahara T, Minamoto T, Doi H. 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLOS ONE* **8**:e56584 DOI 10.1371/journal.pone.0056584.

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z. 2012. Estimation of fish biomass using environmental DNA. *PLOS ONE* **7**:e35868 DOI 10.1371/journal.pone.0035868.

Takeuchi A, Iijima T, Kakuzen W, Watanabe S, Yamada Y, Okamura A, Horie N, Mikawa N, Miller MJ, Kojima T, Tsukamoto K. 2019. Release of eDNA by different life history stages and during spawning activities of laboratory-reared Japanese eels for interpretation of oceanic survey data. *Scientific Reports* **9**:6074 DOI 10.1038/s41598-019-42641-9.

Thomas AC, Howard J, Nguyen PL, Seimon TA, Goldberg CS. 2018. eDNA Sampler: A fully integrated environmental DNA sampling system. *Methods in Ecology and Evolution* **9**:1379–1385 DOI 10.1111/2041-210x.12994.

Todd PR. 1980. Size and age of migrating New Zealand freshwater eels (*Anguilla* spp.). *New Zealand Journal of Marine and Freshwater Research* **14**:283–293 DOI 10.1080/00288330.1980.9515871.

Tréguier A, Paillisson J-M, Dejean T, Valentini A, Schlaepfer MA, Roussel J-M. 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 DOI 10.1111/1365-2664.12262.

Turner CR, Miller DJ, Coyne KJ, Corush J. 2014. Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys* spp.). *PLOS ONE* **9**:e114329 DOI 10.1371/journal.pone.0114329.

Turner CR, Uy KL, Everhart RC. 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation* **183**:93–102 DOI 10.1016/j.biocon.2014.11.017.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Research* **40**:e115 DOI 10.1093/nar/gks596.

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F, Gaboriaud C, Jean P, Poulet N, Roset N, Copp GH, Geniez P, Pont D, Argillier C, Baudoin J-M, Peroux T, Crivelli AJ, Olivier A, Acqueberge M, Le Brun M, Møller PR, Willerslev E, Dejean T. 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology* **25**:929–942 DOI 10.1111/mec.13428.

Wangensteen OS, Palacín C, Guardiola M, Turon X. 2018. DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. *PeerJ* **6**:e4705 DOI 10.7717/peerj.4705.

Watanabe S, Minegishi Y, Yoshinaga T, Aoyama J, Tsukamoto KJMB. 2005. A quick method for species identification of Japanese eel (*Anguilla japonica*) using real-time

PCR: An onboard application for use during sampling surveys. *Marine Biotechnology* **6**:566–574 DOI 10.1007/s10126-004-1000-5.

Weldon L, O'Leary C, Steer M, Newton L, Macdonald H, Sargeant SL. 2020. A comparison of European eel *Anguilla anguilla* eDNA concentrations to fyke net catches in five Irish lakes. *Environmental DNA* **2**:587–600 DOI 10.1002/edn3.91.

Wickham H. 2016. ggplot2: elegant graphics for data analysis. New York: Springer-Verlag.

Wilcox TM, Carim KJ, McKelvey KS, Young MK, Schwartz MK. 2015. The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of *Oncorhynchus*. *PLOS ONE* **10**:e0142008 DOI 10.1371/journal.pone.0142008.

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 DOI 10.1371/journal.pone.0059520.

Wilcox TM, McKelvey KS, Young MK, Sepulveda AJ, Shepard BB, Jane SF, Whiteley AR, Lowe WH, Schwartz MK. 2016. Understanding environmental DNA detection probabilities: a case study using a stream-dwelling char *Salvelinus fontinalis*. *Biological Conservation* **194**:209–216 DOI 10.1016/j.biocon.2015.12.023.

Wood SA, Pochon X, Laroche O, Von Ammon U, Adamson J, Zaiko A. 2019. A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. *Molecular Ecology Resources* **19**:1407–1419 DOI 10.1111/1755-0998.13055.

Yates MC, Fraser DJ, Derry AM. 2019. Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environmental DNA* **1**:5–13 DOI 10.1002/edn3.7.