Detection of non-native freshwater fishes using environmental DNA

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Abstract

Invasive non-native species are a serious conservation threat, and management to mitigate their impact depends on accurate survey methods. Early detection enables a rapid response while an invader is still localised. Recently, environmental DNA (eDNA) techniques, based on detection from DNA present in water samples, have been applied to search for a range of organisms, from invertebrates in small ponds to cetaceans at sea. The aim of this thesis was to investigate the use of eDNA methods to detect non-native freshwater fish.

Sampling methods and species-specific primers were developed for pumpkinseed *Lepomis gibbosus*, topmouth gudgeon *Pseudorasbora parva*, sunbleak *Leucaspius delineatus*, and fathead minnow *Pimephales promelas*. All four species were detected within 24 hours of introduction into aquarium tanks, and DNA of *L. gibbosus* was detected within 6-12 hours of their release into experimental 5m x 5m ponds. Further validation of the techniques was obtained in a survey of ponds for *P. parva* on a site where the species was known to occur.

The methods were then applied to a real-world scenario, mapping the distribution of *P. parva* on a complex of fishing ponds where an eradication attempt was in progress. The eDNA survey proved the persistence of a small population in one lake, and provided evidence for the patchy distribution of eDNA in a still water body. A laboratory protocol based on nested PCR was developed, and determined in laboratory trials to be more sensitive than conventional or quantitative PCRs. This more sensitive technique was then applied to a repeat sampling at the fishing pond complex, confirming the previous results in only providing positive detections at the one pond. The nested PCR protocol was used to assess a second eradication attempt at this site.

Moving the method development from still to flowing water, eDNA surveys were conducted over three days to map the distributions of *Lepomis gibbosus* and *Leucaspius delineatus* in two river catchments in southern England (Sussex Ouse and Hampshire Test). This demonstrated the utility of the method for rapidly assessing distribution, with the results broadly matching previous knowledge gained through conventional survey methods, but with the addition of the discovery of *L. delineatus* detections upstream of the ponds containing a known source population in the River Ouse.

Successful management of non-native species often depends on accurate detection of small populations (or reliable interpretation of negative results), and environmental managers need to know the sampling effort (and therefore allocation of financial resources) required to attain the necessary level of sensitivity. To this end, a field trial was conducted in which a known low density (100 fish per hectare) of two species (rainbow trout *Oncorhynchus mykiss* and barbel *Barbus barbus*) was stocked into six fishing ponds, and electrofishing compared directly with trapping and electrofishing. Different detectability was observed in the two species: the eDNA sampling proved the only effective method for detecting *O. mykiss* (detected on all six ponds), but *B. barbus* was not detected in all ponds, by any method.

This trial, and the project as a whole, demonstrate that eDNA surveying is an effective method for detection of non-native fishes to inform management decisions, but that surveys need to be tailored to the ecology of the target species. The DNA, particularly of rare species, has been shown to be patchily distributed within the water body (at detectable levels), and a high level of sampling effort may be required to detect low density populations.

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Acknowledgements

Author's declaration

I confirm that the work presented in this thesis is my own work, with the following exceptions:

Chapter 2 to Chapter 6 are based on papers produced in collaboration with the named co-authors:

Davison, P.I., Créach, V., Liang, W.-J., Andreou, D., Britton, J.R. and Copp, G.H., 2016. Laboratory and field validation of a simple method for detecting four species of non-native freshwater fish using eDNA. *Journal of Fish Biology*, **89, 1782-1793. [Chapter 2]**

PID, VC, WJ-L, DA, JRB and GHC designed the project; PID and GHC carried out fieldwork; PID carried out laboratory analysis; PID, JRB and GHC analysed the data; PID and all co-authors wrote the paper.

Davison, P.I., Copp, G.H., Créach, V., Vilizzi, L. and Britton, J.R., 2017. Applications of environmental DNA analysis to inform invasive fish eradication operations. *The Science of Nature*, **104, 35. [Chapter 3]**

PID, GHC and VC designed the project, with statistical advice from LV; PID and GC carried out fieldwork; PID carried out laboratory analysis; PID, GHC and LV analysed the data; PID and all co-authors wrote the paper.

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PID and GHC designed the project, with statistical advice from LV and JRB; PID and KW conducted fieldwork; PID conducted laboratory analysis; PID, GHC and LV analysed the data; PID and all co-authors wrote the paper.

1. Introduction

This thesis aims to study how a relatively new survey method, the detection of aquatic organisms from environmental DNA, can be used to assist the management of non-native species. It uses several freshwater fishes as the model species, and the UK as the study area, although the methods developed are applicable to other taxonomic groups and regions.

1.1 Literature review

Non-native species introductions are increasing worldwide (Seebens et al. 2020), with the rate of freshwater fish introductions worldwide doubling over the last 30 years (Gozlan et al. 2010b). The subset of non-native species which cause significant environmental or economic impacts are defined as invasive (Copp et al. 2005a). Invasive species are thought to represent one of the greatest threats to global biodiversity (McNeely et al. 2001; Pyšek et al. 2020), and freshwater ecosystems are considered to be particularly susceptible to biological invasions and resulting negative effects (Lodge et al. 1998; Gallardo et al. 2016). Invasive species can also have serious economic impacts. For example, the annual cost of invasive non-native species to the British economy was recently estimated to be £1.7 billion (Williams et al. 2010), and recent studies have demonstrated that it is cheaper to prevent biological invasions than to manage (eradicate, control, contain) the species once they have established (Finnoff et al. 2007; Mehta et al. 2007; Epanchin-Niell 2017), when complete eradication may be impractical (Gozlan et al. 2010b). Therefore, early detection, while the species is present in low densities at a limited number of sites, is of great importance to enable management action to prevent further spread or extirpate the species entirely (Mehta et al. 2007; Trebitz et al. 2017).

There are several aquatic examples where early detection has enabled successful eradication at recently colonised sites, including zebra mussels *Dreissena polymorpha* in Lake George, New York (Wimbush et al. 2009) and the marine alga *Caulerpa taxifolia* in California (Anderson 2005). Eradication programmes for freshwater fishes

have frequently achieved success (Rytwinski et al. 2019; Simberloff 2020). Recognising the importance of early detection, the development of monitoring and surveillance programmes for non-native species was one of the key recommendations of the GB Non-native Species Framework Strategy (Defra 2008) and of current UK government policy (Defra 2011).

During the early stages after a non-native species has been introduced, while it is present at low population densities, detection depends on the sensitivity of the monitoring/surveillance methods used. Aquatic species in particular can be difficult or costly to monitor using conventional survey methods. One field which has rapidly developed and shown great potential for surveillance of both marine and fresh waters for the presence of target species is environmental DNA analysis, whether for species of conservation interest or for non-native species. Environmental DNA (eDNA) consists of nuclear or mitochondrial DNA that has been shed by an organism into the environment via urine, faeces, mucus and epidermal cells (Bohmann et al. 2014). Early applications of this methodological technique were developed to estimate species richness of micro-organisms in soil or water (Blanchet 2012), and eDNA analysis has since been used to identify species found in soil, ancient sediments, permafrost and ice cores (Torsvik and Øvreås 2002; Simon et al. 2009; Nagler et al., 2018), in addition to applications in marine and freshwater environments.

In water, eDNA is present at dilute concentrations and is subject to dispersal and mixing by water currents (Ficetola et al. 2008; Shogren et al. 2017). The concentration of eDNA is a function of the rate of release from the organism and the rate of breakdown or degradation, which itself is a function of physical factors (e.g. ultraviolet radiation, temperature) and biological processes, including endogenous nucleases and the actions of bacteria and fungi (Barnes and Turner 2016; Harrison et al. 2019).

Early studies of fish eDNA persistence in experimental ponds found eDNA fragments (target fragment lengths of 98 base pairs) to be detectable for up to 21 days at 8–11°C after removal of the fish (Dejean et al. 2011). Persistence times however seem to be

variable; a review (Collins et al. 2018) showed that half-lives of detectability measured in freshwater environments ranged from 0.7 hrs in acidic streams to 47.5 hrs in a eutrophic lake, with temperature having a strong effect. Persistence time of eDNA is particularly important when applying the technique to survey streams and rivers, where downstream transport needs to be considered. In one example, Deiner and Altermatt (2014) detected eDNA of target crustacean and mollusc species 12.3 km and 9.1 km downstream of their respective populations. .

The marine environment provides challenges to environmental DNA detection methods, especially in open waters, due to the large volume of water and the dilution and dispersal effects of tidal current processes (Foote et al. 2012). In sea water, eDNA typically remains above detection thresholds for about 48 hours (Collins et al. 2018). Nonetheless, eDNA techniques have been successfully applied to the detection of large pelagic open-water vertebrates at sea (Bakker et al. 2017, Boussarie et al. 2018, Huveneers et al. 2018) and also to the detection of the planktonic life stages of invasive tunicates (Simpson et al. 2017).

Studies have shown that the eDNA approach is applicable for detecting almost any aquatic taxa in a wide range of environments. Many studies have focussed on amphibians, following from early work on non-native American bullfrog *Lithobates catesbeianus* in European ponds (Dejean et al. 2012), which demonstrated an improved detection rate in comparison to conventional methods. A considerable degree of method development has been conducted using great crested newts *Triturus cristatus* in lowland ponds (Biggs et al. 2015; Buxton et al. 2017; Rees et al. 2017; Harper et al. 2018), and stream-dwelling salamanders (Olson et al. 2012; Pilliod et al. 2013, 2014; Takahashi et al. 2018; Wineland et al. 2019). Mammals have been targeted in both freshwater (otters; Thomsen et al. 2012b; Padgett-Stewart et al. 2016) and marine environments (cetaceans; Foote et al. 2012; Baker et al. 2018), whilst eDNA metabarcoding of water samples from ponds and rivers is increasingly proving a useful method for surveying terrestrial mammals (Ushio et al. 2017; Sales et al. 2020). Other studies have targeted non-native or conservation-dependent reptiles (Hunter et al. 2015; Adams et al. 2019a). These methods have been used to detect

invertebrates, with applications to invasive species monitoring including the detection of New Zealand mudsnails *Potamopyrgus antipodarum* (Goldberg et al. 2013), several species of crayfishes (Tréguier et al. 2014; Dougherty et al. 2016; Geerts et al. 2018), dreissenid mussels (Ardura et al. 2017; De Ventura et al. 2017; Gingera et al. 2017; Amberg et al. 2019), clams *Corbicula* spp. (Cowart et al. 2018), and mosquitoes (Schneider et al. 2016).

Environmental DNA methods might be expected to be particularly applicable to fish, and indeed the efficacy of the technique has been demonstrated in a range of environments. This approach has been used to search for freshwater species of conservation importance, e.g. European weather loach *Misgurnus fossilis* in Denmark and Belgium (Thomsen et al. 2012b; Brys et al. 2020) and juvenile European eels *Anguilla anguilla* ascending rivers (Cardás et al. 2020). These methods have proved useful for generating species inventories for native fish communities in lakes (Hänfling et al. 2016; Li et al. 2019a), rivers (Minamoto et al. 2012; Antognazza et al. 2021; Sales et al. 2021) and inshore marine waters (Thomsen et al. 2012a; Stat et al. 2019).

Surveys based on detecting eDNA have several potential advantages over traditional survey methods, although the decision of whether to use them instead of these methods, or in conjunction with them, needs to be evaluated for each application or species. Traditional methods could be more appropriate for organisms that are easy to observe or capture, but eDNA surveys can be a cost-effective alternative for detection of elusive species or those at low abundance which might require considerable effort to physically catch. Several studies have shown eDNA surveys to outperform traditional methods in such cases (e.g. Hinlo et al. 2018; Brys et al. 2020). Collection of water samples represents a non-invasive method of sampling, avoiding potential damage to the target animals or the environment which could be caused by, for example, netting surveys. Although laboratory analysis is an expert procedure, the relative ease and speed of sample collection means that samples can be collected by personnel with limited training, or 'citizen scientists' (Biggs et al. 2015), enabling a higher level of spatial or temporal sampling than might be achievable otherwise. Fewer staff might need to be deployed into the field for shorter time periods to sample the

same number of water bodies, for example in comparison to staff numbers needed to conduct electric fishing or netting surveys, providing financial cost savings – although these need to be balanced against the costs of laboratory molecular analysis.

Another advantage, of particular importance when producing species inventories of communities, is that eDNA analysis can be conducted without the need to identify species based on their morphology, which typically requires expert training and indeed may be impractical for some taxonomic groups or life stages. Some studies have shown the specific identification of invertebrate specimens by morphological methods to have a high error rate (Stribling et al. 2008; Packer et al. 2009), which molecular identification could avoid, although this is critically dependent on the quality of information in the sequence databases for the relevant species (and its relatives). Gaps in these databases are one of the current obstacles to this approach. European freshwater fish are comparatively well covered in these databases, with mitochondrial DNA sequence data for 87.9% of species, but coverage of freshwater invertebrates (64.5%) and marine invertebrates (22.1%) is less comprehensive (Weigand et al. 2019). Barcode libraries, and hence the ability to assign sequences to a species, are continually improving, with many programmes currently attempting to fill these gaps with validated sequences.

Other considerations when deciding whether to deploy eDNA surveying or conventional methods include whether the 'burden of proof' for the particular application is such that a specimen is necessary, to indisputably prove that the species is definitely present in the water, rather than just the DNA which might have entered the system by another method (see Section 1.1.2). Such proof may be needed by stakeholders in cases where the presence of a species has potentially costly management consequences (Jerde 2021), and in such cases those making decisions on whether to use eDNA surveying should consider the consequences of errors caused by both false positives and false negatives (Darling 2019). Like all ecological survey methods, eDNA detection is imperfect, and a species will very often not be detected in every sample in which it might be expected (Willoughby et al., 2016). As with trapping or netting, decisions therefore need to be made about the sampling effort

required, which can be informed by using statistical techniques such as occupancy modelling (Matter et al. 2018; Neto et al. 2020).

Although eDNA surveys have proved effective at determining presence or absence of target species, they cannot provide the information on size, condition status or maturity on which much fisheries science is based, so capture-based methods are needed to obtain such information. The use of eDNA to assess abundance is still in its infancy. Some studies have shown correlation between abundance and DNA signal (Lacoursière-Roussel et al. 2016; Di Muri et al. 2020). However, much work will be needed before a similar level of abundance information can be achieved from eDNA surveys in comparison to netting or trapping surveys.

1.1.1 Methods used in eDNA studies

A variety of methods have been trialled as eDNA studies have developed over the last 15 years, but the scientific community has often converged on common methodology for the basic steps involved. Methods of eDNA capture from environmental samples used in published work have taken two approaches. These are:

1) precipitation methods, involving the collection of a small sample (typically 15 ml) of water, which is then preserved using ethanol and sodium acetate, and immediately frozen (Ficetola et al. 2008; Dejean et al. 2012; Foote et al. 2012, Thomsen et al. 2012a). The entire water sample is subsequently defrosted and immediately centrifuged. The DNA is extracted from the resultant pellet using a commercially available extraction kit, such as the Qiagen Blood and Tissue Extraction Kit (Ficetola et al. 2008; Dejean et al. 2012; Biggs et al. 2015).

2) filtration methods, involving the filtering of a larger water volume, typically 1–2 L but in some cases up to 10 L, through a cellulose nitrate, glass fibre or carbonate filter (Goldberg et al. 2011; Jerde et al. 2011; Takahara et al. 2012; Jerde et al. 2013) using peristaltic or vacuum pumps. An alternative and

increasingly used method is to use a syringe to force water through a filter enclosed in a cartridge (Spens et al. 2017). The pore size used for filters varies amongst studies, with 0.2 µm initially recommended for optimal eDNA capture (Turner et al. 2014), although most fish eDNA studies have used pore sizes of 0.45 µm or 0.7 µm (Wang et al. 2021). Larger pore sizes will be more practical for waters containing elevated concentrations of suspended solids. For example, a 3.0 µm filter was found to be effective for sampling eDNA in a freshwater lagoon (Takahara et al. 2012). Immediately following water filtration, the filter paper is either frozen, or dehydrated using molecular grade ethanol (Hinlo et al. 2017b). DNA can subsequently be extracted in the laboratory either directly from the defrosted filter paper using commercially available isolation kits (evaluated by Hinlo et al. 2017b), or by rehydrating the sample, centrifuging, and extracting DNA from the resulting pellet using a blood-and-tissue kit (Minamoto et al. 2012; Takahara et al. 2013).

A number of comparative trials have looked at eDNA capture from different method combinations (Deiner et al. 2015, Minamoto et al. 2016; Spens et al. 2017; Muha et al. 2019). The filtration method is now generally considered to yield higher DNA quantities than the precipitation method, particularly in larger systems where it is beneficial to be able to sample larger volumes of water to detect rarer species (Spens et al. 2017; Muha et al. 2019).

Analysis of the eDNA requires amplification of DNA sequences of interest. Primers are designed which will enable selection of the DNA fragment to be amplified. Two approaches can then be used, either:

1) targeted at detection of a particular species of interest, using Polymerase Chain Reaction (PCR) methods to amplify DNA regions specific to the target organism, referred to hereafter as 'single-species targeting'. **Conventional PCR** (also known as standard PCR) requires visualisation of DNA bands on an electrophoresis gel under ultraviolet light. An alternative method is **quantitative PCR**, in which fluorescence of an attached probe is measured to determine DNA concentration, potentially enabling estimation of population size (Lodge et

al. 2012; Thomsen et al. 2012a). Less frequently used at present is **droplet digital PCR** technology, which offers increased sensitivity which has been shown to increase detection capabilities in some studies (Doi et al. 2015; Mauvisseau et al. 2019; Brys et al. 2020).

2) using degenerated primers for a group of organisms (for example, all metazoans, or all fish) to generate a list of all sequences present in a sample, which can then be matched to sequence databases to assign them to species (Sevilla et al. 2007; Minamoto et al. 2012), in the technique known as metabarcoding. This enables a more comprehensive assessment of community composition than the single-species targeted approach (Shokralla et al. 2012; Jerde et al. 2013). Such degenerated primers have been designed for use on fish, and Zhang et al. (2020) reviewed and evaluated 22 primer sets that have been used for metabarcoding of teleost fish.

Despite the quantity of information produced by the metabarcoding approach, single-species targeting still provides the most sensitive method for detecting a species of particular interest, particularly if it occurs at low density. This was demonstrated by Harper et al. (2018) who compared qPCR and metabarcoding detections of great crested newts *Triturus cristatus* in eDNA samples from 532 ponds. Targeted qPCRs detected newts in 49.8% of ponds, whereas metabarcoding using vertebrate primers produced positive detections from 34.2 % of ponds (Harper et al. 2018). One reason is because targeted primers can be optimised for a particular species, ensuring the signal is not 'drowned out' by more abundant DNA sequences or missed due to primer bias (Schenekar et al. 2020). Surveillance programmes designed for detection of invasive species, for example in ports and marinas, are likely to be most effective if they employ a combination of metabarcoding studies to detect a suite of potential invaders (Zaiko et al. 2012), combined with single-species targeting to detect key species.

Mitochondrial DNA is generally used as it is more abundant than nuclear DNA, and more sequence data are publicly available (Bohmann et al. 2014). For the majority of animals, the region most commonly used for DNA barcoding work is the cytochrome

c oxidase I gene (COI). This mitochondrial gene of 652 base pairs was proposed as the best target for bar-coding applications because it allows discrimination of closely allied species, and its suitability across a large range of animal taxa (Hebert et al. 2003; Frézal and Leblois 2008; Andújar et al. 2018). The COI gene has been shown to differentiate robustly between fish species in a number of studies (Ward et al. 2005; Hubert et al. 2008). Other genes targeted in fish eDNA studies include the 12S rRNA, 16S rRNA and cytochrome *b* genes; Zhang et al. (2020) generally detected greatest fish diversity when they used metabarcoding primers targeting the 12s rRNA gene.

DNA sequences of many species are published in online databases such as GenBank (National Center for Biotechnology Information 2013), and whilst there are currently many species for which no sequences are available in these databases (a limitation that is commonly encountered in metabarcoding studies), an ambitious project has been launched to sequence all species on the planet by 2028 (the Earth Biogenome Project; Lewin et al. 2018).

Primer (and probe) design for specific sequences is achieved using specific software packages. Thereafter, the primers need to be tested to assess their reliability and specificity before applying them in the analysis of field samples (Bohmann et al. 2014), To provide increased confidence in the detection of the target species, it is recommended that the visualisation of products using gel electrophoresis be complemented by the sequencing of PCR product of a certain proportion of all positive samples (Darling and Mahon 2011).

1.1.2 Potential sources of error

A number of potential sources of error must be considered when developing eDNA monitoring strategies and assessing results. Several sources of both false positive (type I errors) and false negative (type II) errors have been identified (Darling and Mahon 2011). Sources of Type I errors arising from the laboratory protocols include the erroneous detection of non-target species during PCR amplification (Darling and Mahon 2011) or due to contamination. False positives could also occur if there is an

alternative explanation for target DNA presence in the water column. It has been suggested that positive e-DNA results obtained for Asian carp in the Great Lakes Basin may originate from sources other than live fish currently present in the lakes – for example dead fish on the decks of ships, excrement from birds that have recently fed on that species, or sewage outflows in regions where the fish are prepared for human consumption (Darling and Mahon 2011; Jerde et al. 2011, 2013).

False negatives are an inherent problem with most fisheries survey methods (Bayley and Peterson 2001). In eDNA studies, Type II errors can result from the presence of a substance in the water that inhibits DNA amplification, such as humic acid (Wilson 1997), or from limitations of laboratory protocols, which can be minimised with improved method sensitivity (Darling and Mahon 2011). Some studies have found quantitative PCR methods to be more sensitive to low eDNA concentrations than the conventional PCR-based genetic screening used by many studies (e.g. Wilcox et al. 2013) [but see Chapter 4 of this thesis]. For example, Wilcox et al. (2013) were able to detect eDNA of the target species, brook trout *Salvelinus fontinalis* and bull trout *S. confluentus*, at concentrations as low as 0.5 target copies per µl, which compares well with the detection limit of 7 copies per µl reported by Jerde et al. (2012) for silver carp *Hypophthalmichthys molitrix* using traditional PCR methods.

With increasing sensitivity in the laboratory methods, an adequate system of controls is essential when working with eDNA, to avoid false positive errors due to contamination. Samples should be collected using sterilised equipment, with precautions taken to ensure that DNA is not transferred from one sampling location to the next, for example on the bottom of a boat. Similarly, laboratory procedures need to be carefully planned so that contamination cannot occur at any stage. Separate rooms should be used for different stages of the process, with sample preparation undertaken in a different place to post-PCR processes (due to the greater contamination risk caused by the amplified DNA following PCR). One control against false positives due to contamination is the use of 'cooler blanks' (e.g. Jerde et al. 2013) – bottles of deionised water that are transported with the water samples taken from the field and treated in the same way as samples before testing for the target DNA.

Controls can be used at each stage of the laboratory processes, to check both for contamination (avoiding false positives) and for successful completion of each stage (avoiding false negatives). In addition to the negative controls from the field sampling discussed above, each PCR run should contain a negative control in which the sample is substituted with laboratory water, to ensure that reagents are not contaminated. To ensure that DNA in the sample is not lost during storage and that the extraction procedure has worked correctly, a non-target sequence can be added to the sample (Harper et al. 2019). Each PCR run will also include at least one positive control, using tissue-derived DNA from the target species. Another potential obstacle to successful eDNA detection is the presence of compounds within the environmental sample which inhibit PCR amplification (McKee et al. 2015; Lance and Guan 2020), which can be tested for using a spiked control (looking for difference in DNA signal when a known quantity of tissue-derived DNA is spiked into laboratory water, in comparison to when spiked into an environmental sample).

When planning a field sampling programme, it is important to consider the many factors which may result in false negatives. Foremost among these is the patchiness of eDNA, particularly in still water bodies with limited water mixing, where dispersal of DNA particles through the system has been shown to be limited, sometimes to within a few metres (Harper et al. 2019a). Sufficient spatial coverage of a water body therefore needs to be achieved before it can be considered likely that negative results represent a genuine absence of the species. Other environmental factors may need to be considered. High stream flows, for example, can dilute eDNA concentration, which in one study was considered to cause non-detection of a relatively common bivalve (Curtis et al. 2020). Seasonal effects relating to the animal's life history may affect DNA signal, particularly for crustaceans which are most reliably detected when the females are carrying eggs (Dunn et al. 2017; Crane et al. 2021). Freshwater fishes may be more active in warm weather, and therefore shed more DNA into the water column, as would also be the case at times of spawning activity.

Two elements of the laboratory protocol are particularly important for avoiding false positive or false negative errors: the primers that are deployed, and the number of PCR replicates. Primer design needs to be robust enough to ensure that all genetic variation (all haplotypes) within a species is covered, but that co-occurring closely-related species are excluded (Goldberg et al. 2013). Primers should be designed according to established guidelines (e.g. Bustin et al. 2009 for qPCR primers), and rigorously tested in the laboratory to determine their level of sensitivity, given that target eDNA in environmental samples will typically be at low concentrations. To avoid false positives due to amplification of DNA from the wrong species, specificity testing needs to be conducted. *In silico* testing should establish that there are no close matches to other species when those primers are used; a threshold mismatch of >four base pairs has been used in fish eDNA studies (e.g. Harper et al. 2019). Specificity testing should also be conducted *in vitro*, evaluating the primers against tissue-derived DNA of related species occurring at the study site. The number of PCR replicates is important due to stochasticity in the amplification process (for DNA at very low concentrations in the samples), and some studies recommend a minimum of 12 qPCR replicates to give a high probability of detection of low abundance species (e.g. Harper et al. 2018).

1.1.3 Application of eDNA methods to invasive species monitoring

The potential for detecting and mapping the distribution of non-native fish species was recognised at an early stage. Water samples taken from the North American Great Lakes basin have been used to document the spread of two Asian carp species, silver carp and bighead carp *Hypophthalmichthys nobilis* (Jerde et al. 2013), whereas Takahara et al. (2013) sampled ponds in Japan to map the distribution of the invasive bluegill sunfish *Lepomis macrochirus*.

Surveys using eDNA techniques compare favourably with other, traditional, survey methods, and this is apparent in a study of invasive American bullfrogs *Lithobates catesbeianus* in 49 ponds surveyed in France, where positive detections were obtained for 38 ponds using eDNA techniques, compared to just seven ponds using

conventional survey methods (Dejean et al. 2012). Similarly, a study using eDNA techniques detected invasive bluegill sunfish *Lepomis macrochirus* in 11 ponds where they had not been observed visually (Takahara et al. 2013).

The ability of eDNA surveys to survey a large number of water bodies rapidly under virtually any weather conditions, with no effect on the environment and at relatively low financial cost has been recognised (Dejean et al. 2012). For example, the costs of eDNA sampling for invasive gastropod molluscs were reported to be considerably lower ($35–$80 US per sample) than those for collecting and sorting conventional invertebrate samples (>$300 US per sample) (Goldberg et al. 2013). And although the laboratory analysis of eDNA samples requires specialist training, the collection of water samples is relatively simple, as demonstrated in a pilot study in 2013 to assess how easy and practicable volunteers would find the collection of samples for assessing the distribution of great-crested newts *Triturus cristatus* (Biggs et al. 2015). Volunteers collected water samples from 239 ponds where this species was known to be present, with positive detections at 91.2% of sites (Biggs et al. 2015). Such an approach could potentially allow large-scale distributions to be mapped, with the limiting factor then being the cost of laboratory analysis.

Most work to date has used environmental DNA surveys to determine presence or absence, but management decisions could be better informed with knowledge of numbers or biomass of the target organism. Takahara et al. (2012) hypothesized that eDNA concentration, as measured by qPCR methods, could be used to estimate biomass of a target species in a water body, assuming that aquatic vertebrates release eDNA into the water in proportion to their biomass. A series of trials in both laboratory tanks and experimental ponds with varying numbers of common carp *Cyprinus carpio* showed a strong correlation between biomass and eDNA concentration (Takahara et al. 2012). Other studies have taken number of positive eDNA samples to represent a measure of species abundance; Mahon et al. (2013b) found that number of positive samples in a 4.18 km stretch of river could generally be related to number of fish present.

Another application for environmental DNA techniques is in the surveillance of potential pathways for non-native species introductions. Ballast water transfer by ships is recognised as one of the main sources for non-native species (Drake and Lodge 2004), and eDNA monitoring could potentially be used to screen ballast water. An experimental survey conducted on ballast water of a ship travelling from Germany to South Africa demonstrated the ability of eDNA analysis to detect a range of taxa in ballast water comparable to visual sampling methods (Zaiko et al. 2015). Another significant pathway of introduction is shipments of live fish contaminated with misidentified or concealed species. A good example of this is topmouth gudgeon *Pseudorasbora parva*, which was accidentally introduced to countries throughout Europe, including the UK, as a contaminant of authorised freshwater fish consignments (Gozlan et al. 2010a). For import monitoring programmes, eDNA analysis of water samples provide a cost-effective, non-lethal means with which to screen fish imports for unseen contaminant species (Collins et al. 2013), especially consignments containing a number of morphologically similar species (Collins et al. 2012).

1.1.4 Future technological advances

Ongoing research is progressively increasing the efficiency (speed), sensitivity and portability of eDNA analysis (Jerde et al. 2013), and developments in next-generation sequencing technology could make it possible to bypass primer design and the sequencing of PCR products, using metagenomic methods (Ruppert et al. 2019). This increased sensitivity may enable the detection from water samples of other genes which can be used for population genetics, thereby potentially providing a more reliable source of information on population levels than is currently available from eDNA (Adams et al. 2019b).

Advances in technology have increased the rate at which specimens can be sequenced, which has enabled the initiation of programmes to sequence all fauna and

flora from entire countries (such as the Darwin Tree of Life Project in the UK; www.darwintreeoflife.org). As sequence databases continue to improve, metabarcoding will become a considerably more powerful tool for any application for which knowledge of community structure is useful, such as screening for non-native species or studying their impacts on a community.

1.1.5 Conclusions

Since the publication of the first paper on the use of eDNA to detect aquatic macro-organisms (Ficetola et al. 2008), the field has advanced considerably in both methodology and applications. However, there remain several questions regarding application of eDNA surveys; Jerde and Mahon (2015) identified a number of key issues which require further study, including resolving methodological issues (elimination of 'false negatives' by greater knowledge of the number of replicates required); increasing our knowledge of how eDNA is moved within both lentic and lotic systems; and investigating the considerable unexplained variability surrounding the relationship between target organism biomass and DNA in the system. The work undertaken for this PhD thesis addresses some of these knowledge gaps.

1.2 Research aims and objectives in relation to thesis structure

The research aim is to develop, validate and apply environmental DNA tools for detecting the presence of non-native fish in freshwater systems. The study area is Great Britain, although the methodology is transferrable to other locations. Correspondingly, the research develops and validates eDNA surveying protocols for non-native freshwater fishes that are relevant to British freshwaters. The research objectives (O) are outlined below, along with a brief explanation and identification of where the objective is reported in this thesis.

O1. Develop a simple method for detecting non-native freshwater fishes using eDNA via laboratory and field validation studies (Chapter 2).

This objective provided the rationale for development of conventional PCR primers and sampling methodologies for detecting four species of fish that are non-native to Great Britain: topmouth gudgeon *Pseudorasbora parva*, sunbleak *Leucaspius delineatus,* pumpkinseed *Lepomis gibbosus* and fathead minnow *Pimephales promelas*. These primers and methodologies were validated by aquarium tank studies (all four species), experimental pond studies (*L. gibbosus*) and field surveys at a known stillwater location (*P. parva*).

O2. Apply the developed eDNA tools to inform an invasive fish eradication attempt, and to assess the effectiveness of such eradication attempts from lotic and lentic sites (Chapters 3 and 4).

This objective applies the tools developed in Objective 1 for detection of *P. parva* to a real-world context. This invasive species is currently the subject of a national eradication programme. This study applies eDNA surveying to determining its continued presence in a lake and assessing its distribution in surrounding water bodies (Chapter 3). A more sensitive methodology, using a nested PCR protocol, was designed and compared directly in the laboratory against other PCR methods, to give increased confidence in negative results (Chapter 4). This methodology was then

applied to a repeat analysis of the lakes at the site in Chapter 3, confirming knowledge of *P. parva*'s distribution and thereby informing the eradication attempt (Chapter 4). These nested PCR protocols were adopted for post-eradication surveys of the one infested water body on that study site (Chapter 4).

O4. Develop a methodology for detecting non-native fishes in running waters using eDNA techniques (Chapter 5).
Here, eDNA sampling methodology was developed for detecting fishes in lotic systems, and validated through trials undertaken to map non-native fish distributions (*Lepomis gibbosus* and *Leucaspius delineatus*) in river catchments in southern England where these species are known to be present.

O5. Conduct field trials to determine the sampling regime required to detect small populations of target species, and to assess the cost-effectiveness of eDNA sampling in comparative trials against conventional sampling methods (Chapter 6)
This objective was addressed through the use of methods developed in previous chapters in a field trial in which two fish species were stocked at a known low density into six ponds. The detectability of these fish using eDNA surveys was compared directly against detection rates using conventional methods (trapping, electrofishing). The decision to use conventional PCRs for this trial was informed by the results of protocol sensitivity testing in Chapter 4.

In addition to the data chapters outlined above in relation to Objectives, this thesis contains:

Introduction (Chapter 1):
This chapter provides a survey of the current literature on eDNA sampling, focussing on the methods and applications relevant to non-native freshwater fishes but also aiming to set that work in its wider context amongst other eDNA applications. In a fast-evolving field, the majority of the cited papers have been published in the lifetime of this PhD study (2013-2019).

Discussion (Chapter 7):

This chapter summarises the main conclusions that can be drawn from the outputs of the data chapters (Chapter 7.1), in relation to the initial aims and objectives set out in Chapter 1.2. The advances in the field over the lifetime of this PhD study are also discussed, as are the key knowledge gaps remaining to be addressed before the widespread adoption of eDNA surveying as a monitoring and surveillance tool for non-native freshwater fishes (Chapter 7.2).

2. Laboratory and field validation of a simple method for detecting four species of non-native freshwater fish using environmental DNA

This chapter was published as:

2.1 Abstract

This paper presents the first phase in the development and validation of a simple and reliable environmental DNA (eDNA) method using conventional PCR to detect four species of non-native freshwater fish (pumpkinseed *Lepomis gibbosus*, sunbleak *Leucaspius delineatus*, fathead minnow *Pimephales promelas* and topmouth gudgeon *Pseudorasbora parva*). The efficacy of the approach was demonstrated in indoor (44-L tank) trials in which all four species were detected within 24 hours. Validation was through two field trials, in which *L. gibbosus* was detected 6–12 hours after its introduction into outdoor experimental ponds, and *P. parva* was successfully detected in disused fish rearing ponds where the species was known to exist. Thus, the filtration of small (30 mL) volumes of pond water was sufficient to capture fish eDNA and the approach emphasised the importance of taking multiple water samples of sufficient spatial coverage for detecting species of random or patchy distribution.

2.2 Introduction

The most economical and effective means of preventing biological invasions is early detection and eradication (Hulme 2009; Gozlan et al. 2010a). In recent years, a very promising molecular approach for early detection of non-native species has been developed (Ficetola et al. 2008), based on analysis of DNA shed by an organism into

the environment (eDNA), e.g. via urine, faeces, mucus and epidermal cells (Bohmann et al. 2014). Recent studies have shown that eDNA approaches are applicable for detecting a range of aquatic taxa (Thomsen et al. 2012b), including amphibians (e.g. Dejean et al. 2012; Pilliod et al. 2014; Rees et al. 2014a; Biggs et al. 2015), mammals (e.g. Foote et al. 2012), invertebrates (e.g. Goldberg et al. 2013; Deiner and Altermatt 2014), and fishes (e.g. Keskin 2014; Gustavson et al. 2015). Indeed, eDNA methods are particularly applicable to fishes, such as for detecting species' range expansions (e.g. Jerde et al. 2011, 2013), establishing species distributions (e.g. Takahara et al. 2013; Keskin 2014) and inventories for fish assemblages in rivers (e.g. Minamoto et al. 2012) and inshore marine waters (Thomsen et al. 2012a).

Surveys that employ eDNA techniques can complement conventional sampling methods, usually proving to be more effective for detecting elusive and/or rare species (e.g. Janosik and Johnston 2015; Sigsgaard et al. 2015). In some cases, such as surveys of invasive American bullfrogs *Lithobates catesbeianus* and native great crested newts *Triturus cristatus*, detection rates in ponds were higher using eDNA methods (Dejean et al. 2012; Biggs et al. 2015), and the financial cost was approximately 10× cheaper than with conventional methods (Biggs et al. 2015). Therefore, eDNA analysis shows great potential as a relatively rapid means of surveying a large number of water bodies with no adverse effect on the environment, with fewer restrictions caused by weather conditions, and at relatively low financial cost (Rees et al. 2014b). The information obtained from such surveys can be applied to facilitate effective management of non-native species, for example by detecting newly-arrived invaders and enabling a rapid response (Vander Zanden et al. 2010); by accurately mapping the distributions of established species (Dejean et al. 2012; Jerde et al. 2013); and by providing a means to assess the success of eradication programmes (Rout et al. 2009).

The aim of the present study was to develop and validate a simple, relatively low-cost eDNA protocol for surveying four species of freshwater fish that are non-native to the UK, in order to inform management decisions. Topmouth gudgeon *Pseudorasbora parva*, native to Asia, was accidentally introduced to the UK in the mid-1980s as a

contaminant of ornamental fish, and subsequently spread to form scattered populations throughout England and Wales (Gozlan et al. 2002). As an invasive species known to have a detrimental effect on native fish communities, all 23 known populations were targeted for eradication from the UK (GBNNSS 2015). Sunbleak *Leucaspius delineatus*, native to continental Europe, is also considered invasive in the UK, and has spread to a number of locations in southern England following its introduction in the mid-1980s with ornamental fish (Zięba et al. 2010a). The other two species, pumpkinseed *Lepomis gibbosus* and fathead minnow *Pimephales promelas*, both native to North America, have established populations in the UK without yet becoming invasive. However, they are predicted to become invasive in the UK under warmer climatic conditions (Britton et al. 2010a). *Lepomis gibbosus* was first introduced to the UK as an ornamental fish during the 1890s or early 1900s (Lever 1977), and now occurs at a number of locations in southern England (Villeneuve et al. 2005). No populations of *P. promelas* are currently known from the UK, but this species was previously established in two adjacent ponds in northern England, probably arriving as a contaminant of ornamental fish in 1996 and persisting until eradication in 2010 (Zięba et al. 2010a).

These four species were therefore selected for study primarily because of their interest to agencies managing non-native species: *P. parva* as the fish species currently considered of highest priority for national eradication (see Chapters 3 and 4); *P. promelas* as a species which has in the past been prioritised for eradication; and *L. gibbosus* and *L. delineatus* as two species which are likely to continue to spread in the UK. Although there is limited evidence for impacts of these last two species on native species or ecosystems in the UK, experimental pond trials have demonstrated that competition with native cyprinids could occur, with some species showing altered isotopic niches in the presence of *L. gibbosus* (Copp et al. 2017) or *L. delineatus* (Bašić et al. 2019).

The specific objectives were to: 1) develop specific primers for the four fish species; 2) test the primers and sampling methodology in aquarium trials, using these to determine time between the introduction of fish and the detection of eDNA, for a known

fish biomass; 3) test the methodology in experimental pond trials, again to determine time between introduction of fish and the detection of eDNA, for a known biomass of one of the species (*L. gibbosus*); and 4) conduct a field survey to validate the protocol for one of the species (*P. parva*) in small ponds where the species is known to be present.

2.3 Methods

Specimens of three fish species were sourced from established non-native populations in southern England: *L. gibbosus* and *L. delineatus* from a commercial angling venue in East Sussex, England (51.018333° N; 0.013056° E), and *P. parva* from ponds of a decommissioned ornamental fish farm in Hampshire, England (51.000556° N; 1.452778° W). Wild populations of *P. promelas* in the UK were not available, the only known population having been eradicated three years prior to start of the present study (Britton et al. 2011a), so laboratory-reared specimens were obtained from a captive source (AstraZeneca Environmental Laboratory, Brixham, UK).

2.3.1 Development of specific primers

Specific primers for the four species (Table 2.1), targeting the mitochondrial gene encoding cytochrome *c* oxidase subunit 1 (COI), were designed using the NCBI Primer-BLAST software (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), using sequences available from an open source database (GenBank, http://www.ncbi.nlm.nih.gov/genbank/). To test the efficacy and specificity of the primers, tissue samples were taken from the dorsal muscle of each fish species and the mitochondrial DNA was extracted using a tissue extraction kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany). Amplified samples were purified (Nucleospin Gel and PCR Clean-up, Machery-Nagel, Düren, Germany) and sequenced by a commercial service (Source Bioscience, Cambridge, UK). The sequences have been deposited in Genbank with the following accession numbers: *L. gibbosus* KR092382; *L. delineatus* KR092383; *P. promelas* KR092384; *P. parva* KR092385.

Table 2.1. Species-specific primers designed for the four targeted non-native freshwater fishes in the UK: topmouth gudgeon *Pseudorasbora parva* (Pv), sunbleak *Leucaspius delineatus* (Ld), pumpkinseed *Lepomis gibbous* (Lg) and fathead minnow *Pimephales promelas* (Pp)*.* Fragment size = bp (base pairs)

	Forward Primer (5'-3)	Reverse Primer (5'-3')	bp
Pv	CCTCTTCCGGAGTAGAGGCT	TAGGATTGGGTCTCCTCCCC	350
Ld	TTCGAGCCGAACTAAGCCAR	GGCCTCAACCCCAGAAGAAG	251
Lg	CTAATAATTGGCGCCCCCGA	CGGACCAGACAAACAGTGGT	310
Pp	CCTCTAATAATCGGAGCACCTG	GCAGAAGCACAGCAGTTACAAG	337

The PCRs were performed in 20 μL reaction mixtures (2 μL of DNA template, 0.5 μM of each primer, 10 μL (= 50 units) HotStar Taq Plus DNA polymerase (Qiagen Fast Cycling PCR Kit) and 2 μL CoralLoad Fast Cycling Dye (Qiagen)). Tests of temperature gradients at the annealing stage indicated that the same optimum PCR programme could be used for all four species. The optimal temperature for detection of both *L. gibbosus* and *P. promelas* was 61.0° C, with the other two species showing equally distinct bands across the temperature range of 58–62° C. The cycling conditions were 95° C for 5 min, followed by 32 cycles of 96° C for 5 sec, 61° C for 5 sec and 68° C for 12 sec, with a final extension at 72° C for 1 min. PCR products were visualised using electrophoresis on 2% agarose gel, stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK).

Sensitivity of the primers was tested by measuring the quantity of DNA extracted from the dorsal muscle tissue using a Nanodrop ND-1000 spectrophotometer (Nanodrop Instruments, Wilmington, DE, USA), and by producing a dilution series from 0.03 ng

μL^{-1} to 1.8×10^{-6} ng μL^{-1}. Three replicate dilution series were conducted for each species.

Primers were designed to amplify relatively long sections of DNA, targeting regions of 251–350 base pairs (Table 2.1) to improve specificity. In addition to *in silico* tests for primer specificity using the Primer Blast software, all four primer pairs were tested against tissue samples from all four species in this study, with no false positives occurring from cross-species amplification.

2.3.2 Aquarium trials

The aquarium trials were conducted in 44-L tanks of de-fluorinated tap water, with no flow-through, within an indoor facility at the Cefas Lowestoft Laboratory. The water in these tanks was maintained at a similar temperature to the outside holding facilities used for the fish prior to the experiment, and was exposed to a natural photoperiod. Trials were conducted in January 2014 (*L. delineatus* and *L. gibbosus*) and March 2014 (*P. parva* and *P. promelas*). Water temperature measurements were recorded every 10 min using Tinytag Aquatic 2 temperature loggers (Gemini Data Loggers UK Ltd, Chichester, UK). Mean, minimum and maximum (mean; min–max) temperatures (° C) over the five-day experiment were as follows: *P. parva* (6.8°C; 6.5–9.9), *P. promelas* (6.3°C; 5.9–9.0), *L. delineatus* (2.1°C; 1.9–2.3), *L. gibbosus* (2.3°C; 2.2–2.5).

For each species, tanks were stocked with one, five or ten fish to achieve a range of fish biomass, with three replicate tanks for each biomass (Table 2.2). The exception was for one replicate of the high *L. gibbosus* biomass, where a single large specimen of equivalent biomass was used in lieu of smaller specimens due to limited fish availability. Fish were not fed for the duration of the experiment. On completion of the experiment, each fish was measured for total length (L_T) and mass (Table 2.2).

Table 2.2 Mean body lengths and weights of fish used in trials of eDNA detection (Detect, +) of non-native species at 24 h after release of fish into the aquaria containing one, five or ten specimens (* indicates use of one large fish in place of 10 smaller individuals due to their limited availability). n = number of fish in each treatment, in all three replicates combined. n' = number of tanks in each treatment.

Species		Total length (mm)				Fish biomass (g)			
Treatment	n	Mean	Min.	Max.	n'	Mean	Min.	Max.	Detect
L. gibbosus									
1 fish	3	41.0	39	44	3	0.9	0.7	1.1	+
5 fish	15	38.6	33	43	3	4.1	3.8	4.4	+
10 fish	20	40.7	36	46	2	9.7	9.7	9.7	+
10 fish*	1	83.0	–	–	1	8.7	–	–	+
L. delineatus									
1 fish	3	52.7	50	57	3	1.1	0.9	1.4	+
5 fish	15	44.7	33	56	3	2.6	1.6	4.0	+
P. promelas									
1 fish	3	60.3	57	65	3	2.6	2.1	3.4	+
5 fish	15	57.6	51	69	3	11.3	10.5	12.1	+
10 fish	30	56.5	50	63	3	20.7	19.9	21.1	+
P. parva									
1 fish	3	50.7	47	55	3	1.0	0.8	1.3	+
5 fish	15	49.7	39	60	3	5.3	4.8	5.6	+
10 fish	30	44.9	33	63	3	8.0	7.9	8.1	+

Water samples (1 L) were collected by submerging a sterilized plastic bottle with a gloved hand into the aquarium. Samples were collected from each tank before the addition of the fish (day 0), and at 24-h intervals following stocking, for five days. Immediately following sample collection, water samples were filtered through a 0.4 μm pore size polycarbonate filter of diameter 47 mm (Isopore, EMD Millipore, Darmstadt, Germany) using a vacuum pump (EMD Millipore). The filter was immediately frozen at –80° C. At six points during filtration, de-ionised water was run through the filtration system and filtered as above, with these samples analysed to detect any potential cross-contamination. Within six months from initial sampling, the DNA was extracted using a PowerWater DNA Isolation Kit (MO BIO, Carlsbad, CA, USA). PCR amplification was performed using the reaction mixtures and cycling conditions described previously, with three replicates for each sample, and including both a negative (de-ionised water) and positive (DNA standard) control. Filtrations, extractions and PCRs were each conducted in separate laboratories to reduce risk of contamination.

2.3.3 Experimental pond trial

A field trial was conducted in six artificial outdoor ponds (Figure 2.1) constructed previously for experimental studies (Zięba et al. 2010b; Fobert et al. 2011) on a fishery site in East Sussex, UK (51.018333° N; 0.013056° E). Each pond was 25 m^2 (5 m $\times$ 5 m), configured with a 1 m wide, 0.2–0.5 m deep shelf on one side, with the remainder being ≈ 1.2 m deep (Zięba et al. 2010b), with an approximate volume of 25750 L. Each pond was fitted with a recirculation system (a maximum rate of 2400 L h^{-1}) whereby the water was pumped into a 227-L cistern containing Canterbury spar gravel through which it filtered before returning to the pond via an overflow pipe. The ponds were enclosed within a netting cage to prevent mammalian or avian predation during the experiment. Pond temperatures were measured every 10 min using Gemini Tinytag Aquatic 2 data loggers. Mean temperature during the 48 h of the experiment was 21.2° C (min–max = 18.2–24.6).

Figure 2.1 Photograph of experimental ponds used for *Lepomis gibbosus* eDNA detection study.

Prior to commencing, the ponds were drained to ensure the absence of fish, and then left to re-fill with rain water. A semi-natural and representative fish community was then created by stocking three species of native fish (15 roach *Rutilus rutilus*, nine rudd *Scardinius erythrophthalmus*, and 10 tench *Tinca tinca*) into the ponds three months prior to *L. gibbosus* introduction. The mean, minimum and maximum (mean ± SE; min–max) biomasses of native fish in each pond, calculated from fish L_T at time of stocking using published length-weight equations (Britton and Shepherd 2005; Verreycken et al. 2011), were: *R. rutilus* (46.2 ±0.6 g, 44.1–48.5); *S. erythrophthalmus* (40.1 ±2.3 g, 36.3–49.4); *T. tinca* (56.1 ±5.3 g, 38.2–71.2).

Lepomis gibbosus were captured using electro-fishing and minnow traps from the fishery's commercial angling ponds, and 30 specimens were stocked into three of the six experimental ponds in June 2014. The mean, minimum and maximum (mean ±SE, min–max) values of L_T for *L. gibbosus* stocked into the three ponds were: Pond 1 (55.4 ±3.8 mm, 42–131); Pond 2 (57.8 ±4.0 mm, 44–123); Pond 3 (58.3 ±4.4 mm, 43–136). Fish biomass in each experimental pond was calculated from L_T using a length-weight equation derived from *L. gibbosus* measurements from the same commercial angling lakes (Villeneuve et al. 2005): Pond 1 = 71.2 g; Pond 2 = 79.3 g; Pond 3 = 89.3 g.

Before introduction of *L. gibbosus*, eight 125 mL water samples were collected from each pond at eight equidistant locations around the pond's banks using a 183 cm

sampling pole with a polypropylene sampling cup attached (Camlab Ltd, Cambridge, UK). The ponds were re-sampled in the same manner at 1, 3, 6, 12, 24 and 48 h intervals after introduction of *L. gibbosus* (these sampling intervals informed by the results of the previous tank trials). The sampling cup was moved in a standardised manner from the bank to the greatest extent reached by the pole, and vertically through the water in such a way as to obtain a sample representative of the entire water column. The water samples from a pond were combined in a sterilized 1 L plastic bottle. Between samples, the sampling pole and cup were disinfected using Microsol 3+ (Anachem Ltd, Luton, UK) and washed with de-ionised water. A plastic bottle of de-ionised water was taken into the field and transported identically to the pond samples, to act as a contamination control ('field blank').

The water samples were immediately refrigerated at 4° C, and filtered as soon as practicably possible, within 72 h of collection. The pond water was pre-filtered through a 200 μm filter to remove coarse material, and then two sub-samples of 15 mL were filtered through polycarbonate filters of 0.4 μm pore size (Isopore, EMD Millipore, Darmstadt, Germany). This sample volume was determined by the amount of water which could practicably be filtered before clogging occurred. The 'field blank' was also filtered in the same way, using the same equipment immediately following filtration of the field samples, to test for contamination from the filtering process. The two filters were placed in a 2 mL tube and the DNA was extracted using the MO BIO PowerWater DNA Isolation Kit. PCR amplification was as described previously, with the exception of the use of 6 μL of sample template.

2.3.4 Field survey

For the detection of *P. parva* in natural conditions, water samples were collected from three ponds on a decommissioned ornamental fish farm, at the original introduction site of the species to the UK. (Gozlan et al. 2002). Surveys using conventional sampling techniques (e.g. fish traps) had previously shown these ponds to contain *P. parva* populations at different densities (Pond 1, low density, 52 m × 7 m; Pond 2, intermediate density, 65 m × 15 m; Pond 3, high density, 52 m × 7 m). Fish community

composition (non-target species) varied in these ponds, with no other species known from Pond 1; *T. tinca*, common carp *Cyprinus carpio*, ide *Leuciscus idus* and three-spined sticklebacks *Gasterosteus aculeatus* in Pond 2; and *G. aculeatus* in Pond 3.

From each pond, six 1-L water samples were collected from equidistant points around the pond's bank. Samples were transported to the laboratory on ice (in addition to a plastic bottle of de-ionised water transported identically to act as a control against contamination), refrigerated at 4° C and then filtered as soon as possible, within 24 h of their collection. The bottle of de-ionised water was also filtered at the end of the filtering run, to check for contamination. Water was pre-filtered as described above, then an 80 mL sub-sample was filtered, the DNA extracted and PCR-amplified as described above.

2.4 Results

The tests of primer sensitivity, on DNA extracted from fish tissue, revealed that the lower limit of detection varied between the four species. The lowest DNA quantity at which each species was reliably detected (i.e. producing a distinct gel electrophoresis band on all three PCR replicates), was: *L. gibbosus* = 4.6×10^{-4} ng; *L. delineatus* = 4.6×10^{-4} ng; *P. promelas* = 3×10^{-2} ng; *P. parva* = 1.5×10^{-2} ng.

In the aquarium trials, the eDNA of all four species, at all three levels of fish biomass, was detectable at the first sampling interval, i.e. 24 h following the addition of fish to the tanks (Table 2.2), in all three PCR replicates. All control samples, collected before introduction of the fish, were negative for DNA of the target species, as were the de-ionised samples run through the equipment during filtering to test for contamination. The eDNA remained detectable for the remainder of the five-day experiment.

In the experimental pond trials, *L. gibbosus* eDNA was first detected in two of the three ponds 6 h after introduction of the fish. In the third pond, eDNA was first detected at

12 h following *L. gibbosus* introduction. In all three ponds, this result for time of first detection was confirmed by all three PCR replicates undertaken. *Lepomis gibbosus* eDNA was detected in all subsequent samples from all ponds for the remainder of the 48-h trial. All control samples collected before fish introduction proved negative for *L. gibbosus* DNA, as did the de-ionised water taken into the field as a contamination control.

In the field survey, *P. parva* eDNA was detected in all three of the decommissioned fish farm ponds where it was known to occur. However, the distribution of eDNA in all three ponds was spatially heterogeneous (Table 2.3), with DNA detected at one location only in survey Ponds 1 and 2 (with low and intermediate *P. parva* densities, as indicated by previous trapping surveys) and at four locations in survey Pond 3 (high *P. parva* density detected in previous trapping surveys). No features of the pond were observed that were likely to account for the differences between sites of positive and negative detections. The de-ionised water taken into the field to act as a contamination control proved negative for *P. parva* DNA.

Table 2.3 Number of detections of *P. parva* eDNA from three replicate PCRs, from an 80 ml water sample collected at each of six locations in former fish rearing ponds at a decommissioned fish farm in Hampshire (England). Categorisation of fish density (low, medium, high) is based on previous trapping surveys.

Rearing pond	Sampling location in pond (1-6)					
	1	2	3	4	5	6
1 (low *P. parva* density)	0	0	3	0	0	0
2 (intermediate *P. parva* density)	0	0	3	0	0	0
3 (high *P. parva* density)	3	3	3	3	0	0

2.5 Discussion

The present study demonstrates that conventional PCR methods can reliably detect the presence of non-native fishes in water samples (Tables 2.2 and 2.3), providing an effective means with which environmental managers can map non-native species' distributions and thus inform management decisions. This enables surveys to be undertaken in circumstances where quantitative PCR (qPCR) facilities are not available or are financially prohibitive. Compared to conventional PCR, use of qPCR methods increases the costs of materials required for sample processing ($\times$ 1.5–2) and the cost of initial machine purchase ($\times$ 4).

Whilst conventional PCR methods have been used successfully to survey aquatic organisms (e.g. Deiner and Altermatt 2014; Janosik and Johnston 2015), many recent eDNA trials (e.g. Barnes et al. 2014; Pilliod et al. 2014) and field surveys (e.g. Eichmiller et al. 2014; Tréguier et al. 2014; Gustavson et al. 2015) use qPCR. One reason for considering use of quantitative PCR would be the increased sensitivity reported in published studies, compared to that achieved in the present study. Examples include detections of red swamp crayfish *Procambarus clarkii* DNA at concentrations of 10^{-8} ng μL^{-1} (Tréguier et al. 2014), and newt DNA at quantities of 3×10^{-9} ng (Biggs et al. 2015). The present results demonstrated reliable detection at DNA quantities of 10^{-2}–10^{-4} ng (DNA extracted from tissue). Importantly, detection at these DNA quantities proved sufficient to detect the target species in the present study, at relatively low densities (in the *L. gibbosus* pond trials, a mean biomass of 80 g in a pond of $\approx$ 25750 L). A study comparing conventional PCR and qPCR found no difference in ability to detect DNA presence at low target organism densities in mesocosm experiments (Nathan et al. 2014), although further work would be needed to determine how the detectability of target eDNA in field surveys in different environments relates to the sensitivity of the PCR protocol used.

These results emphasise the need to adapt sampling methodologies to the study site and the target species; this is to ensure sufficient spatial coverage of the water body

to detect species of random or patchy distribution. Positive detections at only some of the sampling points in the field survey (Table 2.3) suggested that the distribution of *P. parva* in these narrow ponds was relatively heterogeneous, as is often observed both temporally and spatially in field studies using fish microhabitat-orientated sampling approaches (Copp 2010). Indeed, raw data collected for the purposes of another study revealed that in four size-classes of *P. parva* inhabiting the stream downstream of the disused fish farm (Beyer et al. 2007), their dispersion index values were 0.004 to 0.021, indicating random distribution. In addition, experiments with *P. parva* in artificial streams have also indicated that native populations exhibit clumped/patchy distributions in response to available food and predator presence (Sunardi and Manatunge 2005). Similar patterns of patchy eDNA distribution have been reported for lake-dwelling common carp *Cyprinus carpio* (Eichmiller et al. 2014). This patchiness in eDNA distribution emphasises the need for statistically robust sampling protocols when attempting to determine presence/absence, taking into account both spatial heterogeneity due to habitat features (e.g. sediment, vegetation structure) and temporal aspects (e.g. seasonal movements of fish in relation to water temperature).

The quantity of water filtered in the current study, in both the pond trials (30 mL) and field survey (80 mL), was lower than used in many eDNA studies (reviewed by Rees et al. 2014b), and was selected as being the maximum water volume that could be filtered before the membrane became clogged. Previous studies to determine the presence of non-native fish in still water bodies have filtered 1–2 L (Takahara et al. 2012, 2013; Moyer et al. 2014), or 200 mL (Eichmiller et al. 2014), whereas those studies that sampled quantities of water similar to those in the present study (e.g. 15 mL) applied precipitation methods instead of filtration (Ficetola et al. 2008; Dejean et al. 2012). These two methods (filtration and precipitation) may yield different results in relation to detectability of eDNA, and indeed a recent comparative study found a higher diversity of eukaryotic eDNA in 15 mL samples of lake and river water using a filtration protocol than when using precipitation (Deiner et al. 2015). The present results demonstrated that filtration of small sub-sampled volumes of water can provide sufficient amounts of eDNA to detect the target fish species at the population densities and waterbody sizes employed or encountered during the current study. Further experimental studies would be needed to determine whether the variable rate of

detection at locations within ponds in the field survey would have been resolved (i.e. more positive detections) with filtration of larger volumes.

In field studies, the volume of water that can be filtered before clogging of the filter occurs will depend on water character and filter pore size. The 0.4 μm pore size used in the present study was similar to that used in many eDNA studies (Rees et al. 2014b) but smaller than that used in studies of non-native freshwater fishes in still waters, i.e. 3.0 μm (Takahara et al. 2012, 2013, 2015) and 1.5 μm (Eichmiller et al. 2014). A recent study found that common carp eDNA occurred in particles ranging from >180 μm to <0·2 μm, with eDNA most abundant in particles of 1–10 μm, and recommended a filter of 0.2 μm pore size to optimise collection of target DNA relative to total DNA (Turner et al. 2014). In the present study, use of 0.4 μm pore filters proved effective in capturing enough DNA for species detection.

Detection of fish in the present study was rapid, with successful detection within 6–12 h after *L. gibbosus* were released into the experimental ponds, and in the initial samples (at 24 h post release) in the tank experiments (Table 2.2). Given the much smaller volumes of water in the aquaria, it is likely that detection would have been considerably earlier if sampling had been initiated prior to the 24 h interval. When a fish is introduced into a new environment (tank or pond), it may be expected to release more DNA initially as a consequence of elevated stress responses (Takahara et al. 2012). Laboratory trials with Idaho giant salamanders *Dicamptodon aterrimus* found an increased rate of eDNA production in the first 2 h of aquarium occupancy, which the authors attributed to physiological stress (Pilliod et al. 2014). Environmental DNA released immediately upon fish introduction in both tank and pond trials would have been expected to persist for the length of the experiments, as previous studies have shown eDNA persistence (>5% probability of detection) for 25 days in tanks and 17 days in ponds (Dejean et al. 2011).

In the tank experiments, positive detections were recorded at 24 h post release regardless of fish density (one, five or ten fish), whereas the field survey yielded more

detections in the pond containing the higher density of the target species. Most work to date has used environmental DNA surveys to determine the presence or absence of species, but management decisions could be better informed with information on the biomass of the target species. A series of trials in both laboratory tanks and experimental ponds with varying numbers of *C. carpio* did reveal a strong correlation between fish biomass and eDNA concentration (Takahara et al. 2012), although some field surveys have found weak relationships between target species abundance (assessed using traditional survey methods) and eDNA concentration (Biggs et al. 2015; Spear et al. 2015), perhaps due to highly variable rates in the shedding of DNA within-species, and even by the same individual (Klymus et al. 2015). Thus, further work is required before eDNA can be used reliably to determine the biomass of target species in field surveys.

3. Applications of environmental DNA analysis to inform invasive fish eradication operations

This chapter has been published as:

3.1 Abstract

Environmental DNA (eDNA) detection of non-native species has considerable potential to inform management decisions, including identifying the need for population control and/or eradication. An invasive species of European concern is the Asian cyprinid fish, topmouth gudgeon *Pseudorasbora parva.* Here, eDNA analyses were applied at a fishery site in southern England to inform operations aiming to eradicate *P. parva*, which had only ever been observed in one of the venue's seven unconnected angling ponds. Eradication of *P. parva* was initially attempted by repeated depletion of the population using fish traps (crayfish traps fitted with 5 mm mesh netting) and the introduction of native predators over a four-year period. The very low number of *P. parva* captured following these eradication efforts suggested a possible population crash. Conventional PCR analysis of water samples using species-specific primers was applied to all seven ponds to confirm that *P. parva* was present in only one pond, that the eradication attempt had indeed failed and that the species' distribution in the pond appeared to be restricted to three bankside locations. The continued presence of *P. parva* at these locations was confirmed by subsequent trapping. Water samples from an adjacent, unconnected stream were also analysed using the eDNA methodology but no DNA of *P. parva* was detected. The results suggest that further management action to eradicate *P. parva* be focused on the pond shown to contain the isolated *P. parva* population and thereby eliminate the risk of further dispersal. This study is the first to apply eDNA analysis to assess the efficacy of an eradication attempt and to provide evidence that the species was unlikely to be present in the other ponds, thus reducing the resources needed to control the species.

3.2 Introduction

Surveys based on the detection of environmental DNA (eDNA) are increasingly used to detect the presence of a broad range of taxonomic groups in aquatic environments, with particular applications to species of conservation concern and non-native species (Rees et al. 2014b; Thomsen and Willerslev 2015). This is because eDNA-based surveys; which collect DNA shed by an organism via urine, faeces, mucus and epidermal cells into the water; tend to have greater power to detect elusive and/or rare organisms than conventional sampling approaches, e.g. bluegill sunfish *Lepomis macrochirus* (Takahara et al. 2013). This increased effectiveness, combined with relatively low financial costs and reduced impact on the environment, demonstrates that eDNA methodologies have high potential for enhancing the management of invasive fish species (Rees et al. 2014b, Bylemans et al. 2016). Applications so far have included distribution assessments (Takahara et al. 2013; Keskin 2014), monitoring surveys on invasion fronts (Jerde et al. 2013; Adrian-Kalchhauser and Burkhardt-Holm 2016), and the evaluation of population eradication attempts (Dunker et al. 2016).

Eradication of potentially harmful non-native species is considered a key component of invasive species management, particularly in rapid response scenarios (Defra 2008; Britton et al. 2011a; Genovesi et al. 2015). Attempts to eradicate non-native fish species often involve application of a piscicide, such as rotenone (Allen et al. 2006; Britton et al. 2008), even though this practice can have substantial impacts on non-target fauna (e.g. Finlayson et al. 2010; Billman et al. 2011). In some circumstances, such as isolated water bodies, it may be possible to eradicate a fish species through a drain-down and liming of the water body (Britton et al. 2008). Other options for controlling invasive fish populations include repeated cropping by netting, trapping or electric fishing, and biological control by stocking predators (Britton et al. 2008).

Topmouth gudgeon *Pseudorasbora parva*, a native species in eastern Asia, is one of the most invasive freshwater fish species in Europe, having spread across most of the continent within decades of its accidental introduction to Romania in the 1960s as a contaminant of Asian carp consignments (Gozlan et al. 2010a). It arrived in England by this introduction vector in the mid-1980s (Gozlan et al. 2002). Such is the threat

posed by *P. parva*, in particular its role as a healthy host of the rosette agent *Sphaerothecum destruens* (Gozlan et al. 2005), that it is the target of a national eradication campaign, which aimed to remove all 23 known UK populations by the end of 2017 (Environment Agency 2014; GBNNSS 2015). *Pseudorasbora parva* is one of just two fish species currently listed as being of European Union concern under Regulation (EU) No. 1143/2014, requiring EU member states to implement management and control measures (European Union 2014). Methods which have been successfully used to eradicate local topmouth gudgeon populations include rotenone treatments and repeated removals (Copp et al. 2007). Also, there are instances elsewhere in Europe where *P. parva* have established a population in a water body, persisted for a short period (<10 years) and then disappeared entirely (Copp et al. 2007). This suggests that the species may be susceptible to recruitment failure and local extirpation where their population numbers are dramatically reduced by either natural or human assisted means.

To facilitate this management programme, an attempt to eradicate a *P. parva* population from a pond on a commercial recreational angling venue in southern England was undertaken between 2011 and 2016 using depletion and biocontrol methods. Given the requirement of such eradication attempts to undergo thorough post-operation evaluations to measure their efficacy (Britton et al. 2011a), the development of effective survey methodologies for this species is considered a priority to assist environmental managers. The aim of this study was to demonstrate the potential use of eDNA analysis as a complement to conventional sampling methodologies for assessing the efficacy of fish eradication attempts. The specific objectives were to: (1) develop a statistically-robust eDNA sampling protocol for evaluating the *P. parva* eradication attempt; (2) assess the efficacy of the eradication attempt using conventional and eDNA methods; and (3) determine whether or not *P. parva* was likely, based on eDNA analysis results, to be present in any other water bodies at the site.

3.3 Methods

3.3.1 Primer design and testing

Species-specific primers for *P. parva* were designed to amplify a 350 base-pair region of the mitochondrial gene encoding cytochrome *c* oxidase subunit 1 (COI): forward primer (5′-3) CCTCTTCCGGAGTAGAGGCT and reverse primer (5′-3) TAGGATTGGGTCTCCTCCCC (Davison et al. 2016). Primer specificity was tested *in silico* against sequences of all UK freshwater fishes, using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were also tested experimentally in conventional PCRs against DNA extracts (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) from fish species from the same family (Cyprinidae) which are likely to occur at the study site: common carp *Cyprinus carpio*, common bream *Abramis brama*, roach *Rutilus rutilus* and rudd *Scardinius erythrophthalmus*. Conventional PCRs were conducted using 0.1 ng of genomic DNA and none of the triplicate PCRs showed amplification for any of these species.

Testing of primer efficiency and optimisation of the PCR protocol was undertaken using DNA extracted from dorsal muscle tissue samples of *P. parva*. These tests showed that the primers reliably amplified *P. parva* DNA at a quantity of 1.5×10^{-2} ng. The ability of the primers to detect *P. parva* DNA reliably from water samples was confirmed in aquarium trials (1 fish in 44-litre tanks) and in a field survey conducted in ponds where the species was known to occur (Davison et al. 2016).

3.3.2 Study site and field sampling protocol

The recreational angling venue, which was located in Kent, South-east England (latitude 51°N, longitude 0°E), has no direct hydrological connections with an adjacent stream nor are any of the seven angling ponds connected (Figure 3.2). A single specimen of *Pseudorasbora parva* was first captured in one of the angling ponds (Figure 3.1; area = 1.4 ha) in April 2004 but reported in the angling press to be a young grass carp *Ctenopharyngodon idella* (fishery owners, pers. comm.). An attempt to eradicate *P. parva* from this pond began in 2011 under the guidance of an independent

fisheries consultant (commissioned by the fishery owners). From 2011 to July 2016, this consisted of intensive depletion using cylindrical fish traps (i.e. 60 by 30 cm crayfish traps with conical funnel entrance and fitted with 5 mm mesh netting). The depletion trapping was complemented by repeated, high density (116 kg·ha^{-1}) stocking of a native predatory fish, Eurasian perch *Perca fluviatilis* – a biocontrol method that has been demonstrated to exert a top-down effect on *P. parva* abundance (Davies and Britton 2015; Verhelst et al. 2016). Initial reports received by the authors indicated that by 2014 *P. parva* were no longer being captured, however trapping data recently acquired from the venue's owners revealed persistence of a very small number of *P. parva*, with the lowest capture densities occurring after predator releases (Figure 3.3).

Figure 3.1 Photograph of the 1.4 ha angling pond in which topmouth gudgeon *Pseudorasbora parva* was detected.
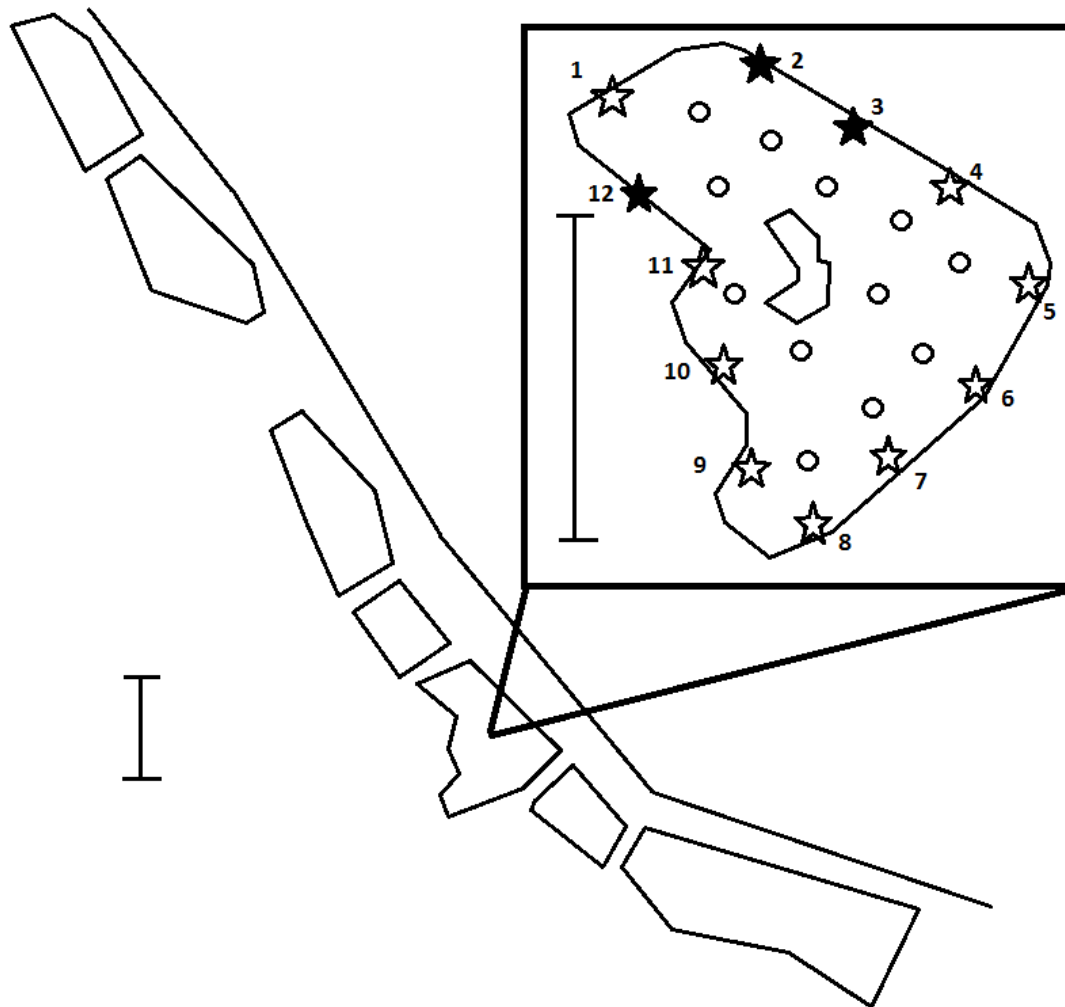
Figure 3.2 Schematic map (scale bars = 100 m) of the study site in the English county of Kent, showing location of the seven ponds and adjacent stream. In the infested lake (inset), pelagic sampling locations are indicated with small, open circles, whereas littoral sampling locations (open squares) are numbered (see Table 3.1), the filled squares indicating locations where positive detections of *P. parva* DNA occurred in the initial sampling survey (September 2014). Locations 1 and 10 also produced positive results in November 2014 (see Table 3.1).

As little published information was available to determine the minimum number of samples required from a given area of water to ensure a statistically robust eDNA sampling protocol, a protocol was developed that involved water collection from 24 sampling locations (12 littoral and 12 pelagic). At each sampling location, four sub-samples were taken for analysis, and at least two PCR amplifications should be performed for each sub-sample.

Accordingly, post-eradication assessment using eDNA analysis consisted of three sampling steps (Sept 2014; Nov 2014; Feb–Mar 2015). Firstly, 24 1-L water samples were collected on 16 Sept 2014 in the infested pond, namely from 12 littoral zone locations spread equidistantly (40 m apart) around the pond shore and 12 from pelagic zone locations spaced around the water body (Figure 3.1). Secondly, water sampling was undertaken during a return visit on 12 Nov 2014 at the six littoral sampling points in the infested water body closest to those where eDNA of *P. parva* was detected during step one. No *P. parva* DNA was detected in any of the 12 pelagic (mid-water) samples, so these pelagic sample locations were not considered further in the eDNA analysis. The water sampling on 12 Nov 2014 was complemented by intensive sampling, using the fish traps described above. Traps were deployed for five days in late December 2014, ten days in early February and six days in early April 2015 (Figure 3.3). Thirdly, water samples (1 L) were collected in 2015 on 17 Feb, 19 Feb and 5 March from 12 littoral zone locations in each of the other six ponds (areas of 0.5 to 2.4 ha), as well as at eight locations along the longitudinal course (1.5 km) of the small stream that runs adjacent to the ponds. Pelagic samples were not collected from the other six ponds, as this would have required movement of the boat between the water bodies, thus increasing the risk of cross contamination.

In all cases, water samples were collected using a 183 cm sampling pole with a 500 mL polypropylene sampling cup attached (Camlab Ltd, Cambridge, UK). The sampling cup was moved in a standardised manner from the bank (littoral samples) or boat (pelagic samples) to the greatest extent reached by the pole, ensuring no contact with the bottom sediment. At each sampling location, 1 L of water obtained using the sampling cup was poured into a sterilized plastic bottle. Samples were then placed in individual plastic bags and immediately refrigerated (4°C) for transportation back to the laboratory. On each sampling day, two identical 'field blank samples' (new sterilized bottles of de-ionised water from the laboratory) were opened briefly in the field, and then transported in the same manner as the pond samples. Between samples, the sampling pole and cup were disinfected using Microsol 3+ (Anachem Ltd, Luton, UK) and washed with de-ionised water.

3.3.3 Laboratory protocol

Within 24 hours of collection, the water samples were filtered through a 0.4 µm pore size polycarbonate filter of diameter 47 mm (Isopore, EMD Millipore, Darmstadt, Germany) using a vacuum pump (EMD Millipore). From each sampling location, four sub-samples of 100 mL were filtered. Between filtration of samples from each location, the filtering equipment was sterilized using Microsol 3+, and washed with de-ionised water, and at regular intervals during filtration, de-ionised water was run through the filtration system, with these samples analysed to detect any potential cross-contamination (in addition to the 'field blank samples' which were also filtered in the same manner). The filters were immediately frozen at –80°C. DNA extraction from the filters took place within three months from initial sampling using a PowerWater DNA Isolation Kit (MO BIO, Carlsbad, CA, USA).

Conventional PCR amplifications were performed in 20 µL reaction mixtures, containing 6 µL of DNA template, 0.5 µM of each primer, 10 µL (= 50 units) HotStar Taq Plus DNA polymerase (Qiagen Fast Cycling PCR Kit) and 2 µL CoralLoad Fast Cycling Dye (Qiagen). The cycling conditions employed were an initial denaturation step at 95°C for 5 min, followed by 32 cycles of denaturation (96°C; 5 s), annealing (61°C; 5 s) and extension (68°C; 12 s), with a final extension at 72°C for 1 min. Amplified PCR products were visualised using electrophoresis on 2% agarose gel, stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK). Three replicate PCRs were conducted for each 100 mL sub-sample, with each one including a negative control (de-ionised water) and a positive control (tissue-extracted *P. parva* DNA). To confirm the identity of sequences amplified from the pond samples, PCR products from the positive sampling points were purified (Nucleospin Gel and PCR Cleanup) and sequenced by a commercial service (Eurofins Genomic Services Ltd, Wolverhampton, UK).

To confirm that negative results were not detection errors ('false negatives') caused by PCR inhibition, additional PCRs were conducted using the PCR protocol described previously (Jane et al. 2015; Adrian-Kalchhauser and Burkhardt-Holm 2016). PCRs were performed using an eDNA sample (6 µL) from a single location within each pond that was spiked with 2 µL of genomic *P. parva* DNA (0.01 ng/µL). The strength of the

resultant electrophoresis gel band was compared visually with that from the same quantity of *P. parva* DNA amplified in deionised water alone (i.e. without sample). As these PCRs indicated the presence of inhibition, a further set of PCRs were undertaken in which the extracted samples (one sub-sample from each sampling location) were re-analysed following a 1:5 dilution in deionised water, a technique used to combat inhibition by diluting the inhibitory compounds (McKee et al. 2015). Three replicate PCRs were conducted on these diluted samples. To assess whether inhibition was still occurring following the 1:5 dilution, three replicate PCRs per pond were conducted in which a spike of 0.02 ng of tissue-extracted *P. parva* DNA was added.

Filtration, extraction, PCR preparation and post-PCR analysis were undertaken in separate rooms of a laboratory dedicated to molecular biology, observing strict anti-contamination procedures (no transfer of equipment between rooms; changing of labcoats when moving between rooms; thorough cleaning of all equipment and surfaces before and after use).

3.4 Results

In the initial sampling step, of the infested water body only, *P. parva* DNA was detected at three of the 12 littoral zone locations (Table 3.1). These sampling locations came from adjacent locations at one end of the pond (Figure 3.1). DNA of *P. parva* was not detected in any of the 100 mL sample replicates collected from the pelagic zone. Spiking tests indicated a small level of inhibition occurring in pelagic and littoral samples. Two samples contained the minimum quantity of DNA required for sequencing, which confirmed the identity of the eDNA as that of *P. parva*. Both sequences showed a 100 % match with 34 sequences of *P. parva* registered in the Genbank database (e.g. accession number HQ960448). No detection was noted in the negative control samples ('field blanks' or filtration controls).

Table 3.1 Positive (+) and negative (–) detection of *P. parva* eDNA in a field survey of the infested pond at a fishery in Kent, England, with number of positive detections from three PCR replicates shown for positive detections. Sampling location 1 is located in north-west corner of the pond; locations are numbered consecutively in an anti-clockwise direction around the pond shore (spacing = 40 m).

Sampling location in pond	100 mL subsample no.			
	1	2	3	4
1	–	–	–	–
2	–	–	+(3)	+(3)
3	–	–	–	–
4	–	–	–	–
5	–	–	–	–
6	–	–	–	–
7	–	–	–	–
8	–	–	–	–
9	–	–	–	–
10	–	–	–	–
11	–	–	+(3)	–
12	–	–	+(3)	+(3)

Subsample number denotes order in which 100 mL subsamples (from 1-L collected sample) were analysed.

In the second sampling step, repeat sampling and eDNA analysis of water from the locations where *P. parva* eDNA had been detected in step one provided further confirmation of the species' presence. This corroborated the trapping data recently acquired from the venue's owners (Figure 3.3).
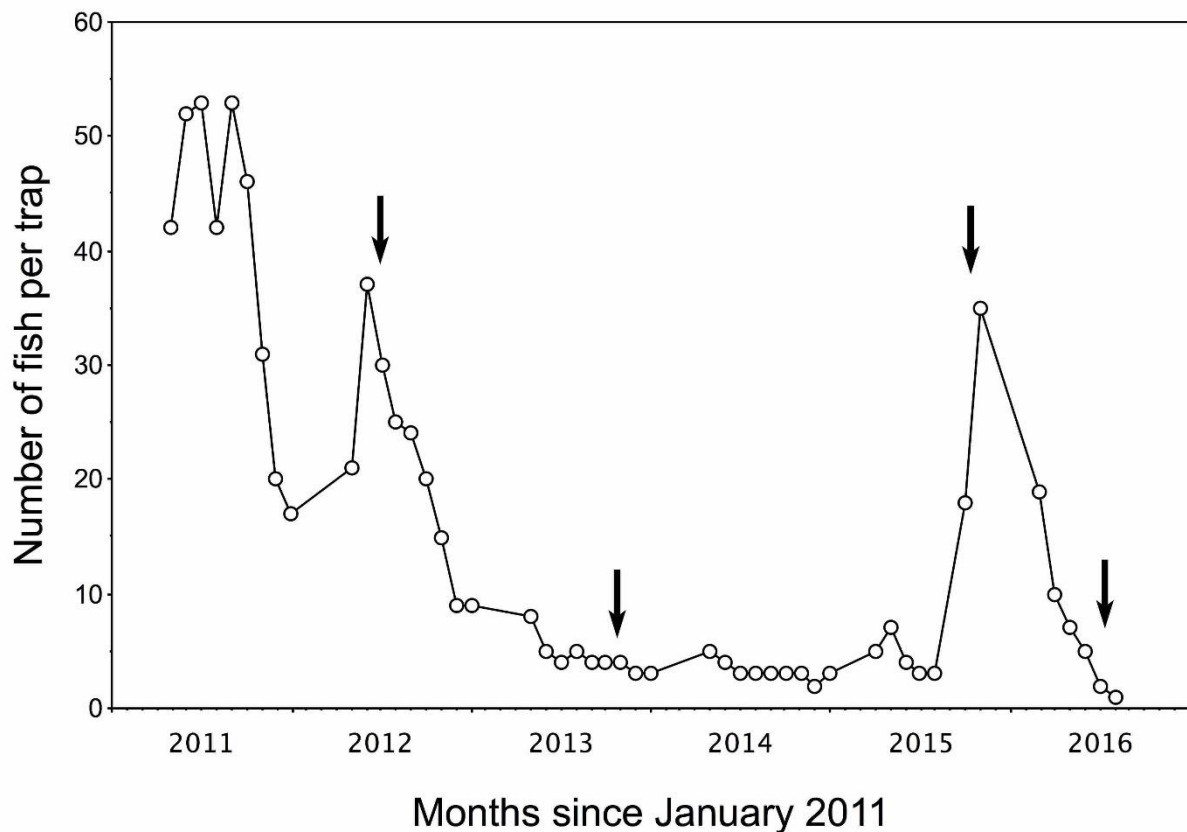
Figure 3.3 Numbers of topmouth gudgeon *Pseudorasbora parva*, calculated on a per trap per month basis, captured by fishery staff between 2011 and 2016 using fish traps (see Methods) placed around the water body's banks on each sampling excursion. The arrows indicate dates of predator biocontrol release, i.e. 400, 200, 400, and 246 Eurasian perch *Perca fluviatilis* (left to right, respectively) of 6–9 cm total length.

In the third sampling step, all sample replicates from the other six angling ponds and from the adjacent small stream proved negative for *P. parva* eDNA. Spiking tests

indicated a small level of inhibition occurring in all six ponds. Following the 1:5 dilution of extracted samples to combat the detected inhibition, no further inhibition was detected. All samples that had previously shown negative for *P. parva* DNA (i.e. previously negative littoral locations and pelagic locations from the infested pond, and all samples from the *a priori* non-infested ponds) also proved to be negative following the 1:5 dilution. These results suggest that the level of inhibition occurring in the samples was not sufficient to mask the presence of DNA during the first analysis.

3.5 Discussion

The current study demonstrates that eDNA surveys are a valuable method for post-evaluation of eradication attempts, with equal, if not greater, power to detect remnant populations of target species than conventional survey methods. Water samples subjected to eDNA analysis confirmed the persistence of a small population of *P. parva* in the infested pond, as indicated from trapping results (Britton et al. 2011b). In the other water bodies, eDNA analysis corroborated trapping results for the other six angling ponds and electrofishing results for the adjacent stream, that indicate it is unlikely the species was present at the time of sampling.

Small-bodied fishes at low population densities can often be difficult to detect, and imperfect detection using conventional methods (electric fishing and trapping) has previously been demonstrated for *P. parva* in 100 m^2 ponds (Britton et al. 2011b). At low population abundances, eDNA surveys may represent the most effective method of confirming the presence of a fish species. For example, eDNA sampling detected the presence of European weather loach *Misgurnus fossilis* in a location where it had not been recorded for 13 years using traditional methods, including fish traps, electrofishing and seine nets (Sigsgaard et al. 2015). In the present study, the spatial heterogeneity of the positive eDNA detections is likely to reflect the heterogeneous distribution of the target species, which has been recorded previously (Li et al. 2010; Davison et al. 2016). The lack of detections from the open water sampling locations is indicative of a distribution favouring shallow vegetated areas in the littoral zone (as previously shown for *P. parva*: Li et al. 2010), or an alternative favoured habitat type that is present in only a few isolated locations around the pond. The trapping of 78 specimens in the vicinity of these sampling points (seven months after the initial water

sample collection), suggests that a small, localised population in this area was the most likely source of the detected eDNA.

Spatial heterogeneity of eDNA is common in lentic water bodies (e.g. Eichmiller et al. 2014), emphasising the need for sufficient water samples to be collected (with adequate spatial coverage) to increase the likelihood of detection of localised species in low abundance. In the present study, only five positive detections resulted from 96 sub-samples of water from 24 locations in the infested lake, demonstrating that the employed sampling strategy was sufficient but that the species could have been missed if fewer samples had been collected. As increased field sample collection will increase financial costs, more work is needed to determine the minimum number of samples required from a water body to provide a high probability of detection.

Detection power could potentially have been improved by modifying the PCR protocol, such as increasing the number of cycles (Rameckers et al. 1997). The sensitivity of detection could arguably be increased by using quantitative real-time PCR (qPCR) protocols, for which higher levels of sensitivity have been reported (Tréguier et al. 2014; Biggs et al. 2015). However, in mesocosm trials, no difference between qPCR and conventional PCR was found in the detection of DNA of target species present at low density (Nathan et al. 2014). A practical consideration is that conventional PCR is financially less costly than qPCR, and therefore more likely to be available to those tasked with the management of invasive species (Davison et al. 2016).

The lack of detection of *P. parva* DNA in the six other lakes on site serves to corroborate the species' absence in angler's catches and conventional surveys undertaken before and after the eDNA survey (fishery owners, pers. comm.). Indeed, no *P. parva* were observed or captured in the adjacent stream during an electrofishing survey carried out a few months after the water samples for eDNA analysis were collected (Environment Agency, pers. comm.). Whilst caution is always needed when declaring a species to be absent on the basis of absence of detection, regardless of the survey method used (Mackenzie 2005; Kéry and Schmidt 2008), the statistically-rigorous sampling protocol used here suggests that it is unlikely that *P. parva* is present in the other nearby, but unconnected, ponds and the stream. PCR-inhibiting compounds in the water are a potential cause of false negatives, but in this case study

the detected inhibition was not sufficient to affect the results. It does demonstrate, however, the importance of incorporating steps in laboratory protocols to assess the extent of inhibition, and if necessary to overcome inhibition by methods such as dilution of samples or addition of bovine serine albumin (Deiner et al. 2015; McKee et al. 2015).

The risk of false positives also needs to be considered when basing management decisions on the results of eDNA surveys. Positive detections should not necessarily be taken as an indication of presence of live organisms, as DNA could enter the water from other sources, e.g. decaying corpses or bird faeces (Merkes et al. 2014; Dunker et al. 2016). Before costly management action is taken, 'ground truthing' (i.e. capture of live individuals) is recommended to corroborate eDNA detection, such as was the case in the present study.

The present study demonstrates the applicability of eDNA surveys to assess the efficacy of eradication attempts in aquatic environments, providing additional support for studies elsewhere in which eDNA analysis was reported to be more sensitive than conventional methods for detecting species present in low abundance. Accurate assessments of the success of eradications is important; the continuation of a monitoring programme after the final individuals have been removed can be costly, whilst conversely the premature declaration of success and resultant cessation of monitoring can be even more costly and potentially nullify previous efforts (Rout *et al.* 2009, 2014). Surveys based on eDNA analysis are therefore an important tool to assist the decision-making process as regards the management of non-native species, both for early detection and rapid response, as well as for the assessment of eradication success. To this end, a nested quantitative PCR protocol is currently being tested in still and running waters for such applications to enhance the sensitivity of the analysis [see Chapter 4]

4. Is it absent or is it present? Detection of a non-native fish to inform conservation management decisions using a new highly-sensitive eDNA protocol

This chapter has been published as:

4.1 Abstract

Environmental managers require a sensitive and reliable means to prove, with the highest level of confidence possible, where non-native fish species exist and where they do not. Therefore, a nested PCR (nPCR) protocol was developed to detect the environmental DNA (eDNA) of a case-study species, topmouth gudgeon *Pseudorasbora parva*, which was recently the subject of a national eradication campaign in the UK. The nPCR protocol was tested in the laboratory and in the field in a series of coordinated surveys (eDNA and conventional sampling with traps) at a commercial angling venue in southern England where an initial eDNA survey, based on conventional PCR (cPCR), found *P. parva* to be present in one of the seven ponds. In the laboratory, the nPCR protocol was on average $100\times$ more sensitive than cPCR, providing a 100% detection rate at DNA concentrations of 3×10^{-8} ng/µL (8 DNA copies per µL). In the field, nPCR and conventional trapping both detected *P. parva* in only one of the seven angling ponds, the same infested pond as in the previous cPCR-based study. Following eradication work on the infested pond, no eDNA of *P. parva* was detected using nPCR in either the formerly infested pond or the adjacent pond, which had been used to quarantine large, commercially valuable fishes. In management applications where the veracity of negative results may be of equal importance as confirmation of positive detections, nPCR protocols provide a useful addition to the analytical toolkit available to inform decision makers responsible for non-native species management.

4.2 Introduction

A major challenge in the conservation and management of aquatic ecosystems to combat biological invasions is the detection of non-native species (NNS), both as an early warning after their initial introduction and as a means of determining where to apply management procedures to eradicate or contain the unwanted NNS (Simberloff et al. 2005). To address this challenge, molecular techniques are being developed to detect species, even when present in very low abundance, from the environmental DNA (eDNA) that these organisms shed into the aquatic environment (Darling and Mahon 2011; Rees et al. 2014b; Davison et al. 2016). These eDNA detection techniques have so far been used primarily to determine distributions (Takahara et al. 2013; Adrian-Kalchhauser and Burkhardt-Holm 2016) or, when next-generation sequencing methods are used, in studies of biodiversity (Taberlet et al. 2012; Hänfling et al. 2016; Keskin et al. 2016; Valentini et al. 2016). Indeed, virtually all applications of eDNA in aquatic environments to date, whether single-species or meta-barcoding based, have aimed to prove species presence. However, conservation management decisions to address biological invasions, such as whether to attempt an eradication, can have immense resource implications and therefore require highly sensitive analytical techniques with which to prove with the highest possible level of confidence where the undesirable NNS is absent, in addition to where it is present.

Another application where eDNA surveys can be particularly valuable is in assessing the efficacy of NNS eradication attempts (Dunker et al. 2016; Davison et al. 2017). A species of particular concern is topmouth gudgeon *Pseudorasbora parva*, a small cyprinid fish that first arrived in Europe as a contaminant of Asian carp consignments to Romania in the 1960s and is now widespread throughout the continent (Gozlan et al. 2010a). In the UK, where it arrived via the same vector in the mid-1980s, *P. parva* has been the subject of a nationwide eradication campaign due to the potential threats the species poses to native fish species (Gozlan et al. 2005; Britton et al. 2007; Great Britain Non-native Species Secretariat 2015). Recent research has demonstrated the value of eDNA techniques, as a complement to conventional sampling/capture methods, for determining *P. parva* distribution within a given area to inform management decisions on eradication procedures (Davison et al. 2017). In that study, at a recreational angling venue in southeast England, conventional PCR-based eDNA

analysis provided evidence, of sufficiently high degree of confidence to form the basis of management decisions, that *P. parva* had survived an attempted eradication in one pond but was not present in six adjacent but unconnected ponds (Davison et al. 2017).

Surveys based on eDNA must consider the risk of errors due to both 'false positives' caused by contamination, and 'false negatives' resulting from failure to capture eDNA in the collected sample, or from limitations of the laboratory tests used (Guillera-Arroita et al. 2017). Conservation management decisions can have serious consequences on the allocation of resources (personnel, consumables, travel) and potential collateral (environmental, socio-economic, ecosystem services) damage, and so must be based on correct information. In terms of economic costs alone, eradication operations for *P. parva* at three UK sites cost between £1.90 and £7.90 per m^2 of water surface (Britton et al. 2008). Monitoring of eradication success, by any survey technique, also provides a challenge for managers; if success is declared prematurely and management operations ceased, the invasive species can re-establish, resulting in continued ecological impacts and increased management costs (Rout et al. 2009). Field and laboratory eDNA protocols of the highest possible accuracy and reliability are necessary in order to provide a high degree of confidence in the survey results so that they can be used to inform management decisions. This is effectively a transition from 'proof of presence' to 'proof of absence', because a positive eDNA detection can normally be substantiated through conventional sampling, albeit with greater effort when extremely rare (infrequent) species are concerned. Whereas, in the case of a negative detection for a species' eDNA, it may be impossible, or practically impossible, to prove that the target species is absent, except where the water body can be drained down in a manner that allows all specimens to be captured (again there remains the possibility of specimens of the target species being missed; e.g. Pot et al. 1984). Therefore, a more accurate and reliable eDNA approach is needed, even if this involves increased financial cost.

To date, single-species eDNA surveys have typically used either conventional PCR (cPCR) or quantitative PCR (qPCR) detection protocols (Goldberg et al. 2016). Although qPCR is generally considered to be more sensitive than cPCR (Wilcox et al. 2013; Tréguier et al. 2014; Biggs et al. 2015), some recent studies have reported little difference in their detection ability, e.g. for fish at low density in a mesocosm

experiment (Nathan et al. 2014), and for invasive freshwater mussels in a river system (De Ventura et al. 2017). A potentially more sensitive approach is nested PCR (nPCR), which consists of two steps: (i) a cPCR, followed by (ii) a qPCR performed on the product of the cPCR. This protocol is expected to increase detection sensitivity but has so far received little used in aquatic eDNA studies, with one exception being for the detection of salmonid fish in rivers (Clusa et al. 2017).

The aim of the present study was to develop an approach, based on conventional and molecular detection methods, to determine the presence or absence of *P. parva* and inform management decisions on where eradication efforts are warranted. The specific objectives of this study were to: 1) directly compare the sensitivity of cPCR and qPCR protocols in laboratory trials; 2) develop a more sensitive eDNA protocol, based on a nPCR approach, and evaluate its sensitivity; 3) undertake coordinated sampling (conventional trapping and eDNA surveys) of ponds at a known *P. parva* site to determine the species presence/absence in each water body prior to an eradication attempt; and 4) undertake coordinated sampling of any infested water body after eradication to check for continued persistence.

4.3 Methods

4.3.1 Overview and study site

The laboratory and field investigations took place in three stages: i) development and laboratory testing of PCR protocols and their eDNA detection sensitivity; ii) a coordinated, pre-eradication survey of the seven water bodies at a commercial fishery in south-east England using conventional and eDNA approaches; and iii) a post-eradication survey of two of these water bodies, the angling pond subjected to eradication measures (henceforth the 'infested pond') and the adjacent 'holding' (quarantine) pond where the rescued (i.e. large, commercially-valuable) fishes were held during and after the eradication work (details given here below).

The commercial fishery (latitude 51°N, longitude 0°E) is the same angling venue where a previous eDNA survey, using cPCR (Davison et al. 2017), demonstrated *P. parva* to be present in only one of the venue's seven human-made angling ponds (areas of 0.5

to 2.4 ha). These ponds are surface-water fed only, i.e. not connected with each other nor with an adjacent stream that flows along the eastern side of the venue (see map in Figure 3.1 in Chapter 3), and any outflows from the ponds discharge into a gravel and reed bed filter that does not retain surface water. An invasive population of *P. parva* was discovered in the infested pond at least as early as April 2004. An eradication attempt was conducted by the fishery owners (intensive trapping combined with introduction of a piscivorous fish species), but the persistence of a low-density population of *P. parva* was confirmed by cPCR of water samples and focused intensive trapping at the locations where DNA of *P. parva* was found (Davison et al. 2017).

4.3.2 Protocol sensitivity testing

Sensitivity tests were conducted using DNA extracted from *P. parva* dorsal muscle tissue (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) collected from a population in southern England; the sequence has been deposited in the open-source database Genbank (www.ncbi.nlm.nih.gov/genbank) with the accession number KR092385 (Davison et al. 2016). Several different approaches to defining limit of detection (LOD) and limit of quantification (LOQ) have been suggested, as reviewed by Hunter et al. (2017). In this study, LOD is defined as the minimum amount of target DNA at which positive detections were recorded in one or more replicates (following the definition used in other eDNA studies, e.g. Takahara et al. 2013; Tréguier et al. 2014; Biggs et al. 2015). LOQ is generally defined as the lowest amount of target DNA which yields an acceptable level of precision and accuracy (Currie 1995; Tréguier et al. 2014). In the present study, LOQ has been specifically defined as detection in 100% of replicates as per Agersnap et al. (2017). Tests to determine LOD and LOQ were applied to two sources of DNA, referred to hereafter in this paper as "total DNA" and "plasmid DNA". "Total DNA" refers to DNA extracted directly from muscle tissue, and therefore comprises both genomic and mitochondrial DNA. "Plasmid DNA" refers to targeted mitochondrial DNA obtained using cloning to create a plasmid solution for use as a standard, enabling calculation of DNA copy numbers. Concentrations of both total and plasmid DNA were measured using a Nanodrop® ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and calculated with the software ND-1000 v3.8.1 (Thermo Scientific).

To obtain the plasmid DNA, a preliminary cPCR using total DNA from *P. parva* was performed to amplify the 350 base-pair target region (Table 4.1). Cloning was performed using a TOPO® TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, U.S.A.) with PCR 4-TOPO® vector including competent cells (*Escherichia coli*), following the manufacturer's recommended protocol. Bacterial colonies were grown on agar plates with ImMedia™ Amp Blue (Invitrogen). Colonies not displaying blue colouration were selected and inoculated in a liquid medium containing 40 mL of LB-Medium (MP Biomedicals, Santa Ana, CA) and 50 µg/ml of Ampicillin. The plasmids were isolated using QIAprep® Spin Miniprep Kit (Qiagen) and tested with a cPCR to verify the success of the incorporation of the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) target gene sequence into the plasmid. Copy numbers for plasmid DNA standards were calculated from DNA concentrations and base-pair lengths using the equation of Godornes et al. (2007).

4.3.3 Conventional PCR (350 bp)

The cPCR used in all field surveys, and in sensitivity testing (referred to hereafter as cPCR 350) used specific primers to amplify *P. parva* DNA, designed to amplify a 350 base-pair sequence of the mtCOI gene (Table 4.1). Specificity of these primers was tested *in silico* against all sequences in the NCBI Genbank database using the NCBI Primer Blast software (www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al. 2012). The primers were also tested experimentally in cPCRs against 0.1 ng genomic DNA extracts from Cyprinidae species which are likely to occur at the study site: common carp *Cyprinus carpio*, common bream *Abramis brama*, roach *Rutilus* and rudd *Scardinius erythrophthalmus*, with none of the triplicate cPCRs showing amplification for any of these species (Davison et al. 2016).

Table 4.1 Primers used for conventional and quantitative PCR of topmouth gudgeon *Pseudorasbora parva.* The cPCR primers targeting a 350 base-pair amplicon (cPCR 350) were designed by Davison et al. (2017). The primer pair used for cPCR targeting a 101 base-pair amplicon (cPCR 101) and for qPCR (with the addition of a FAM probe) was designed for the present study.

	Forward primer (5'-3')	Reverse primer (5'-3')	FAM probe (5'-3')
cPCR 350	CCTCTTCCGGA GTAGAGGCT	TAGGATTGGG TCTCCTCCCC	Not applicable
cPCR 101	GTGTTTCATCAAT TCTAGGCGCAAT	AGCTCATACAAAT AAGGGCGTTTGA	Not applicable
qPCR	GTGTTTCATCAAT TCTAGGCGCAAT	AGCTCATACAAAT AAGGGCGTTTGA	ATATAAAACCTCC AGCTATTTCC

A further pair of cPCR primers, referred to hereafter as cPCR 101, were designed to amplify a target region of 101 base pairs. The purpose of this primer pair was to enable a direct comparison in sensitivity tests with the pair targeting a longer region (cPCR 350), to assess whether length of target region affected sensitivity. This primer pair was used only for comparative sensitivity testing in the laboratory, and was not used in the field surveys.

Conventional PCRs were performed with a total reaction mixture of 20 µL containing 2 µL of DNA samples (total DNA, plasmid or eDNA), 0.5 µM of each specific primer, 10 µL of HotStar Taq® Plus DNA polymerase 2× (Qiagen Fast Cycling PCR Kit) and 2 µL of Coral Load Fast Cycling Dye 10× (Qiagen). De-ionised water was added to obtain the total mixture volume. The cycling conditions were 95°C for 5 min, followed by 35 cycles of 96°C for 5 s, 62°C for 5 s and 68°C for 12 s, with a final extension at 72°C for 1 min. PCR products were visualised after 60 min of electrophoresis migration on 2% agarose gel, stained with SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen). For both laboratory validation trials and eDNA field samples, five cPCR replicates were analysed in each machine run, on three discrete machine runs (i.e. 15 replicates in

total). Each machine run included a negative control (de-ionised water) to check for contamination.

4.3.4 Quantitative and nested PCR

Specific *P. parva* primers and probes were designed for qPCR to amplify a 101 base-pair sequence of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, occurring within the sequence amplified by the cPCR 350 primers (Table 4.1). The primers were successfully tested for specificity *in silico* against all sequences in the Genbank database using NCBI Primer-BLAST software, in which no species likely to be present at UK freshwater sites corresponded to the primer pair to within four base pair mismatches (a level of mismatch within that used for assessing specificity by recent fish eDNA studies, e.g. Harper et al 2019b). The primers were also tested experimentally against genomic DNA of *C. carpio, A. brama, R. rutilus* and *S. erythrophthalmus*, with no amplification observed. Real-time qPCRs were performed using an Applied Biosystems™ Step One™ system (Applied Biosystems, Foster City, CA, USA) using the following thermocycling profile: 2 min at 50°C, 10 min at 95°C, followed by 35 cycles of 15 s denaturation at 95°C, and 60 s annealing-extension at 60°C.

PCRs were performed in a 20 µL reaction mixture containing 2 µL of total DNA, plasmid or eDNA, 1 µL of assay mix (18 µM forward and reverse primers and 5 µM probe) for the targeted species (Applied Biosystems™), 10 µL of TaqMan® Genotyping Master Mix (Applied Biosystems™) and 7 µL of de-ionised water. Samples and standards were analysed in triplicate (i.e. three independent replicates of each sample, with three wells for each replicate as per machine instructions). The standard curve comprised a range of five or six dilutions of a selected standard (plasmid or total DNA), acting as positive samples to confirm reaction efficacy. The dilution series was constructed from the standard on the day of analysis. Finally, the lengths of the qPCR products were checked using 2% agarose gel electrophoresis after addition of DNA Gel Loading Dye (6×) (Invitrogen).

The nPCR protocol consisted of two steps: i) a cPCR, using the cPCR 350 primer pair and the protocol described above; and if the initial cPCR produced a negative result, then ii) a qPCR was performed on 2 µL from each completed cPCR. Five cPCR replicates were performed on each sample. Each cPCR replicate that produced a negative result was then subjected to qPCR in triplicate.

4.3.5 Coordinated pre- and post-eradication surveys

Water samples were collected on 6 and 7 September 2016 from 12 littoral zone locations spread at approximately equal distances from each other around the shores of all ponds, using the same statistically informed sampling protocol developed specifically for these ponds (Davison et al. 2017). Water samples were collected at about 1.5 m distance from the bank using a 183-cm-long sampling pole fitted with a 500 mL polypropylene sampling cup (Camlab Ltd, Cambridge, UK), which, between samples, was disinfected thoroughly with Microsol 3+ sterilising solution (Anachem Ltd, Luton, UK) and washed with de-ionised water. New sampling poles and cups were used for each pond to ensure no contamination risk. The sampling cup was moved in a standardised manner from the bank, to the greatest extent reached by the pole, ensuring no contact with the bottom sediment. At each sampling location, three replicates of 300 mL water, obtained using the sampling cup, were injected through a Sterivex-GP 0.22 µm sterile filter cartridge (EMD Millipore, Billerica, MA, USA) using a 50 mL sterile syringe (Thermo Scientific) that is designed to attach directly onto the cartridge's input opening. Cartridges from each location were sealed in individual plastic bags and immediately frozen (–20°C) for transportation back to the laboratory. On each sampling day, water from a sterilized bottle of de-ionised water from the laboratory was also filtered, handled and transported in the same manner as the pond samples, and analysed in the laboratory to test for contamination.

Conventional trapping in each of the angling ponds consisted of ten, previously unused, rectangular minnow traps of 45 cm length and 25 cm width and height with 3 mm mesh, which were deployed on 7 September 2016 (i.e. same date as eDNA surveying, the use of new traps to avoid potential DNA contamination). Traps were baited using fishmeal pellets (21 mm diameter) and exposed for 12 h, with the numbers of fish captured recorded for *P. parva* only. Only five traps were used in the pond

known to contain *P. parva* due to a periodical check of the traps revealing high numbers of *P. parva* captured. Once *P. parva* presence was confirmed, the traps were retrieved.

Post-eradication surveys (eDNA, trapping) were completed approximately six months after the fishery undertook procedures to eradicate *P. parva*. This consisted of complete drain-down of the infested (i.e. treatment) pond during which the larger and more commercially valuable fish were collected, passed through a salt bath (≈ 30 ppt) and placed into one of the adjacent ponds, henceforth the 'quarantine' pond. On 8 June 2017, three replicate water samples of 300 mL were collected (as described above) at 12 littoral zone locations from the treatment and the quarantine ponds. These samples were collected and analysed in the same manner as described above.

4.3.6 Laboratory processing of the pond-water samples

In the laboratory, DNA was extracted from the cartridges using a PowerWater Sterivex™ DNA Isolation Kit (MoBio, Carlsbad, CA, USA), with a final elution volume of 100 mL. The extracted sample was then diluted 1:5 in deionised water to dilute potential inhibitors (McKee et al. 2015), and a nPCR then performed using the conditions described above on 2 µL of diluted sample. To confirm that negative results in the qPCR were not detection errors ('false negatives') caused by PCR inhibition, five replicate samples from four locations per pond were spiked with 0.01 ng of *P. parva* total DNA and compared against controls of deionised water spiked with the same DNA quantity.

Sample extraction, PCR preparation and post-PCR analyses were each undertaken in separate rooms of a laboratory dedicated to molecular biology, observing strict anti-contamination procedures (no transfer of equipment between rooms; changing of lab coats when moving between rooms; thorough cleaning of all equipment and surfaces before and after use, and treating of equipment under UV light; use of sterile filter tips for pipettes). Increased risk of contamination is an important consideration with nested PCR protocols, due to the increased handling of amplified DNA. This risk was

minimised by placing prepared reagents into well plates in a fume cabinet in a separate room from where the completed cPCR template was added, using different pipettes and gloves.

4.3.7 Statistical analysis

Differences between treatments in the sensitivity testing (plasmid DNA cPCR 350 vs plasmid DNA cPCR 101; plasmid DNA cPCR 350 vs plasmid DNA qPCR; total DNA cPCR 350 vs total DNA qPCR) were tested by Permutational (Univariate) Analysis of Variance (PERANOVA). This was based on a one fixed-factor design consisting of Detection rate at two levels. PERANOVA was carried out in PERMANOVA+ v1.0.8 for PRIMER v6.1.18 (Anderson et al. 2008), using a Euclidean distance, 9999 permutations of the residuals under a full model (Anderson and Robinson 2001), and with statistical effects evaluated at α = 0.05. Notably, the advantage of PERANOVA compared to 'traditional' (fully parametric) ANOVA is that the stringent assumptions of normality and homoscedasticity, which often prove unrealistic when dealing with biological data sets, are 'relaxed' considerably.

4.4 Results

4.4.1 Sensitivity testing

No statistical difference (permutational ANOVA) in sensitivity was observed between cPCR 350 and qPCR, in laboratory trials using plasmid DNA ($F_{1,20}{}^{\#}$ = 0.924, P = 0.415; # = permutational) or total DNA ($F_{1,20}{}^{\#}$ = 0.569, P = 0.480; # = permutational). Nested PCR proved the most sensitive of the three protocols (Figures 4.1 and 4.2; Table 4.2). In trials on plasmid DNA (Fig 1b), the LOQ was 3.34×10^{-8} ng/µL using nPCR, corresponding to 8 DNA copies per µL, compared to 3.34×10^{-6} ng/µL (764 copies per µl) using both cPCR 350 and qPCR. At target DNA concentrations below LOQ, cPCR 350 produced more positive detections than qPCR for a given concentration. The LOD for qPCR was 3.34×10^{-8} ng/µL (76 copies per µL), with no positive detections at 3.34×10^{-9} ng/µL, whereas both cPCR 350 and nPCR produced detections at the lowest DNA concentration tested, 3.34×10^{-10} ng/µL (<1 copy per µL), in 20% and 27% of replicates, respectively.

When total DNA was tested (Figure 4.1), nPCR again proved more sensitive than cPCR 350 or qPCR in terms of LOQ. In all three protocols, a more sensitive LOQ (i.e. more detections at low concentrations) was achieved when using plasmid DNA than when using total DNA.

Table 4.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ) in laboratory sensitivity tests for *Pseudorasbora parva* primers, using conventional (cPCR), quantitative (qPCR) and nested (nPCR) PCR protocols. LOD is defined here as the lowest DNA concentration detected in any replicate (cPCR: 15 replicates; qPCR: 9 wells, forming 3 independent replicates). LOQ is defined as lowest DNA concentration detected in all replicates.

	Plasmid DNA			Total DNA		
	cPCR	qPCR	nPCR	cPCR	qPCR	nPCR
LOD (ng/µL)	3.34×10^{-10}	3.34×10^{-8}	3.34×10^{-10}	9.79×10^{-8}	9.79×10^{-5}	9.79×10^{-8}
LOQ (ng/µL)	3.34×10^{-6}	3.34×10^{-6}	3.34×10^{-8}	9.79×10^{-2}	9.79×10^{-4}	9.79×10^{-6}
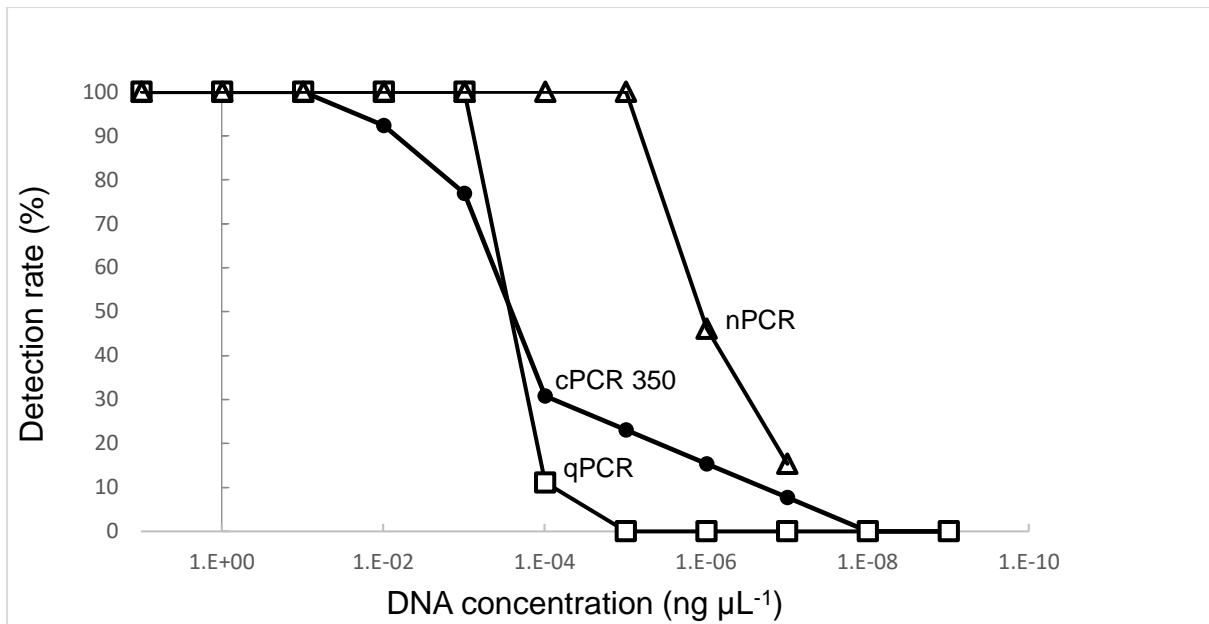
Figure 4.1 Sensitivity test results for conventional PCR (cPCR 350, 15 replicates), quantitative PCR (qPCR, 3 independent replicates) and nested PCR (nPCR, up to 15 replicates if undetected at cPCR stage) detection of topmouth gudgeon *Pseudorasbora parva* in the laboratory using total DNA standards.
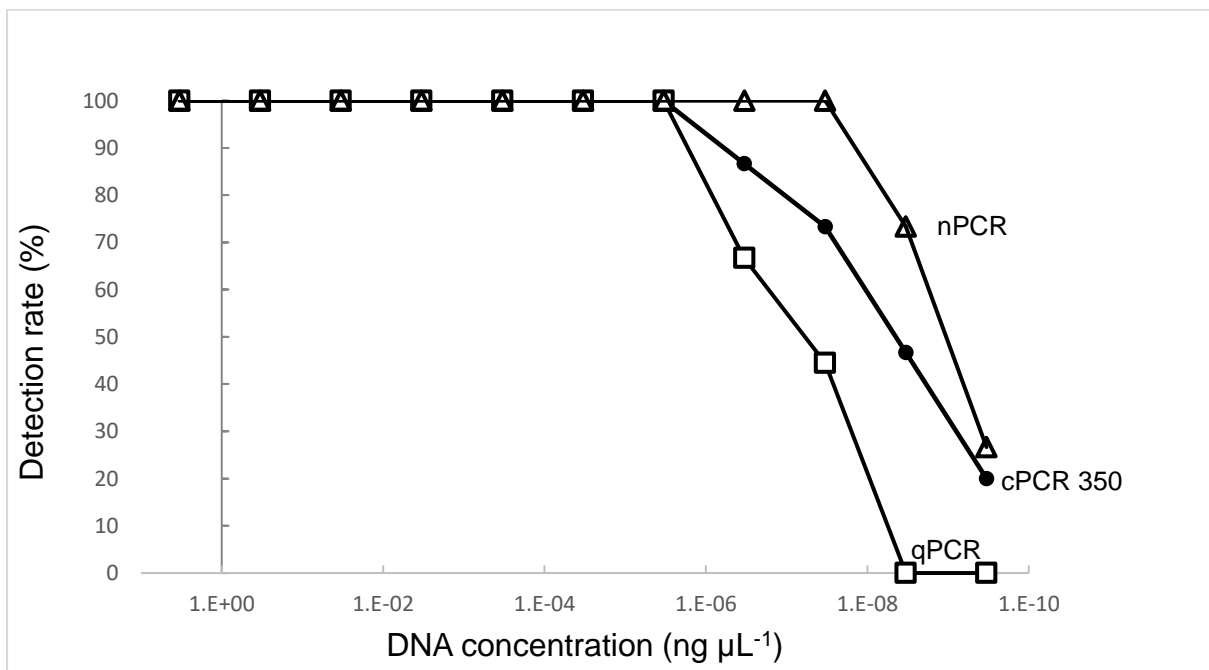
Figure 4.2 Sensitivity test results for conventional PCR (cPCR 350, 15 replicates), quantitative PCR (qPCR, 3 independent replicates) and nested PCR (nPCR, up to 15 replicates if undetected at cPCR stage) detection of topmouth gudgeon *Pseudorasbora parva* in the laboratory using plasmid DNA standards.

Standard Curve
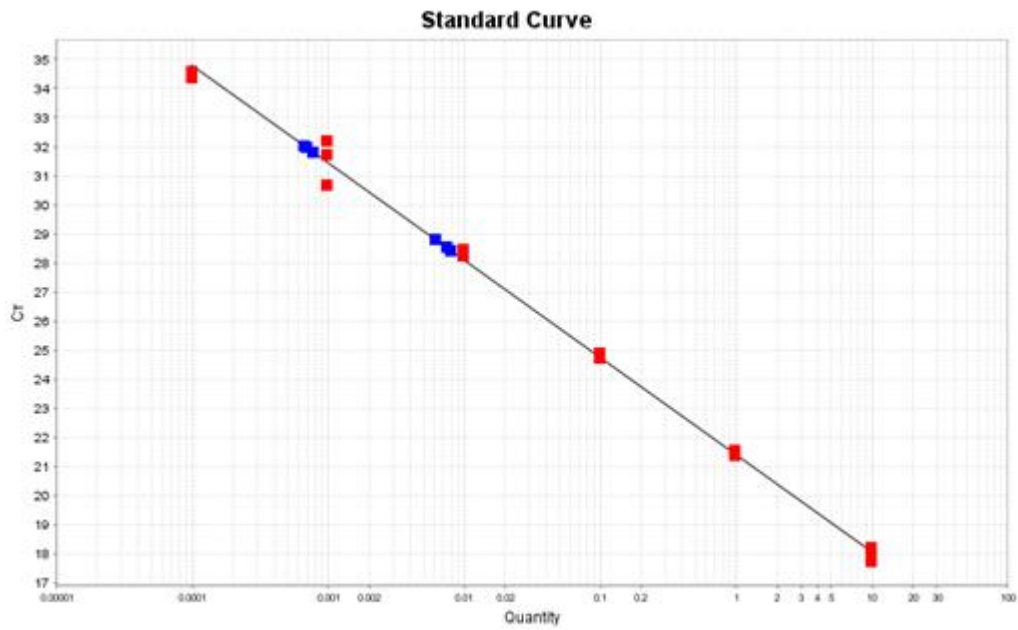
Target: Target 1 Slope: -3.343 Y-Inter: 21.406 R²: 0.996 Eff%: 99.116

Standard Curve
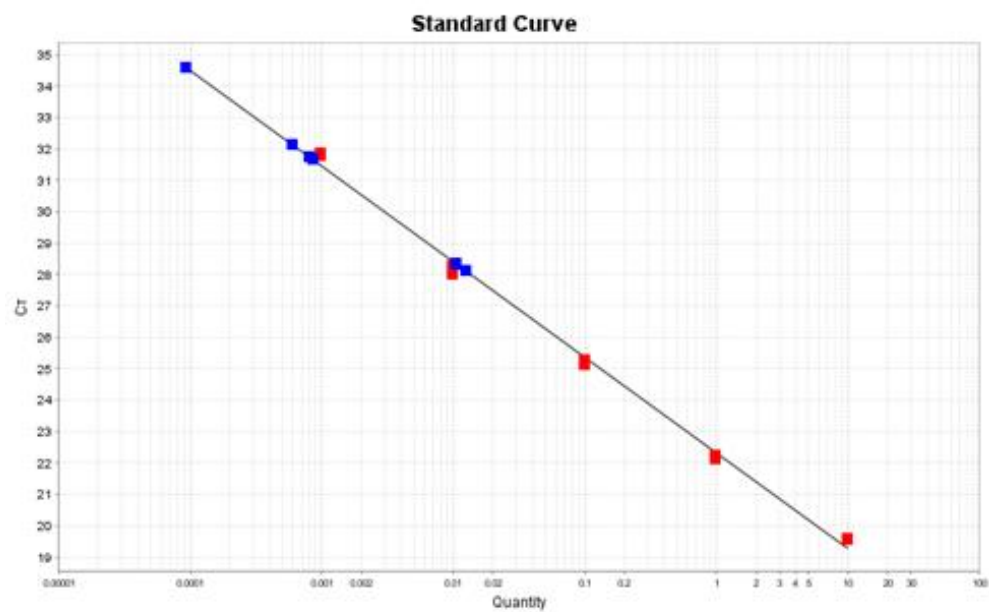
Target: Target 1 Slope: -3.045 Y-Inter: 22.321 R²: 0.996 Eff%: 112.998

Figure 4.3. Quantitative PCR plots (2 independent replicates of each dilution) of sensitivity test using total DNA. Each plot contains one replicate set of dilutions on a separate machine run (blue diamonds), calibrated against a standardised calibration curve (red diamonds). r^2= 0.996 (top plot), 0.996 (bottom plot).
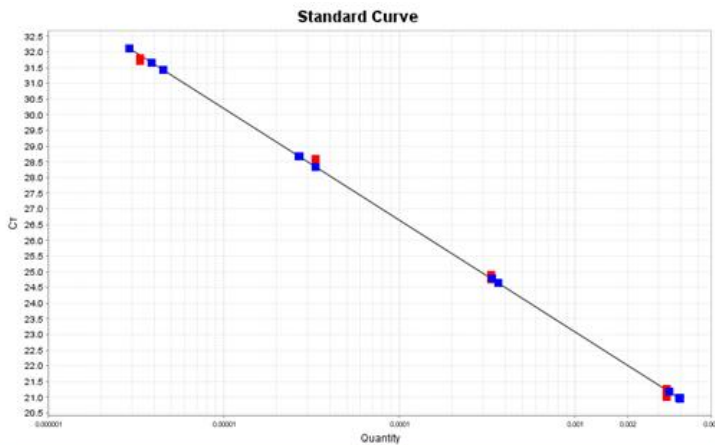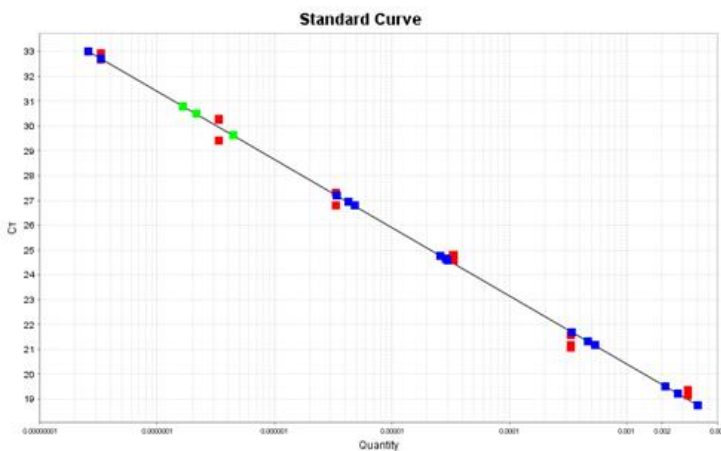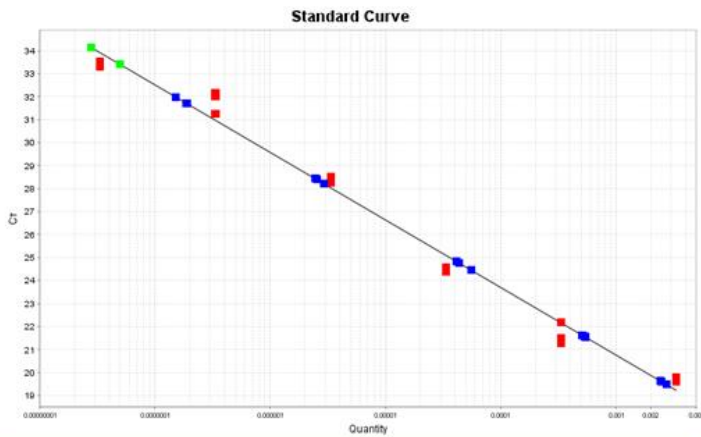
Figure 4.4. Quantitative PCR plots (3 independent replicates of each dilution) of sensitivity test using plasmid DNA. Each plot contains one replicate set of dilutions on a separate machine run (blue diamonds), calibrated against a standardised calibration curve (red diamonds). r^2= 0.999 (top plot), 0.994 (middle plot), 0.985 (bottom plot).
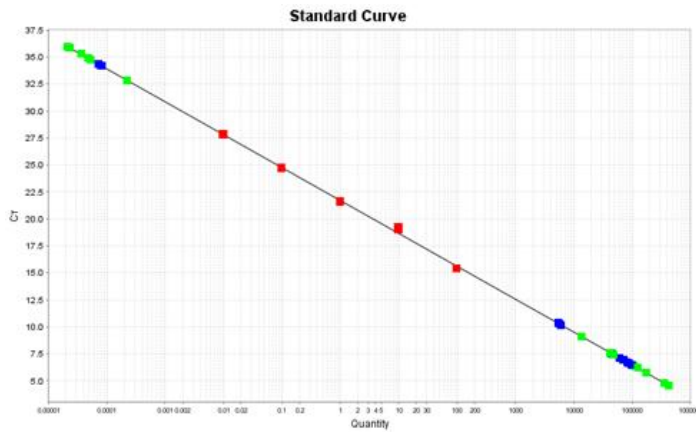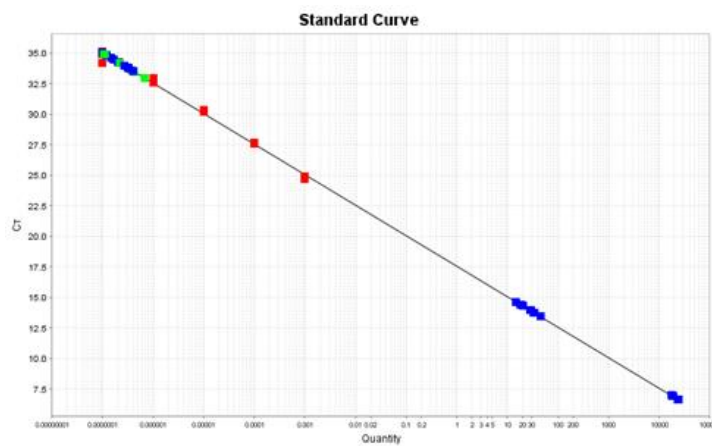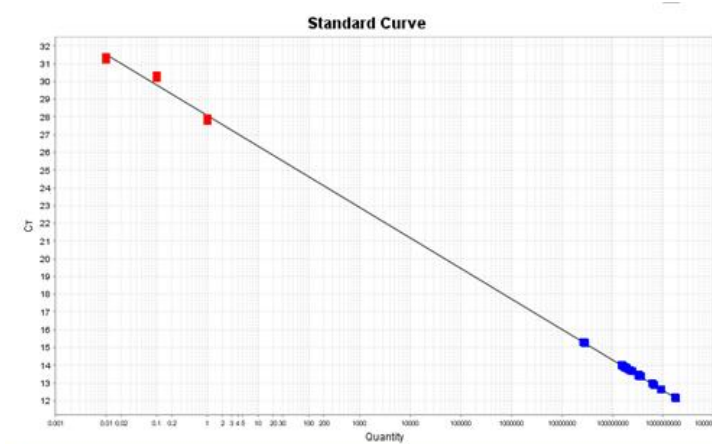
Figure 4.5. Quantitative PCR plots (3 independent replicates of each dilution) of sensitivity test for nPCR, using total DNA. Each plot contains one replicate set of dilutions on a separate machine run (blue diamonds), calibrated against a standardised calibration curve (red diamonds). $r^2 = 0.997$ (top plot), 0.992 (middle plot), 0.948 (bottom plot).
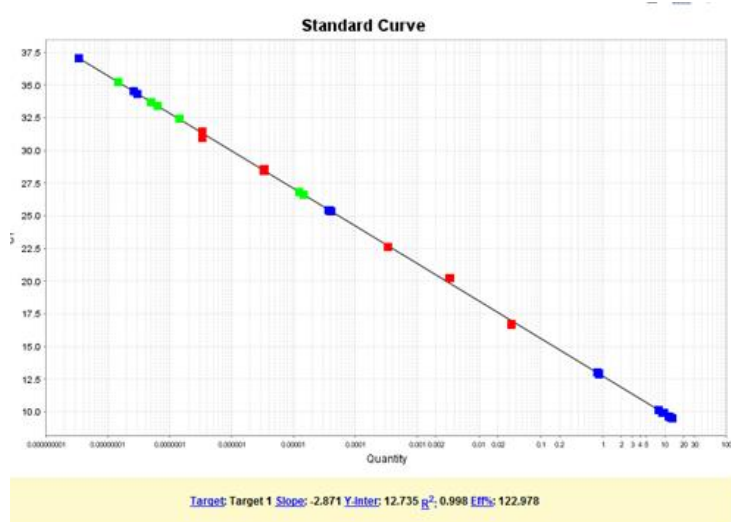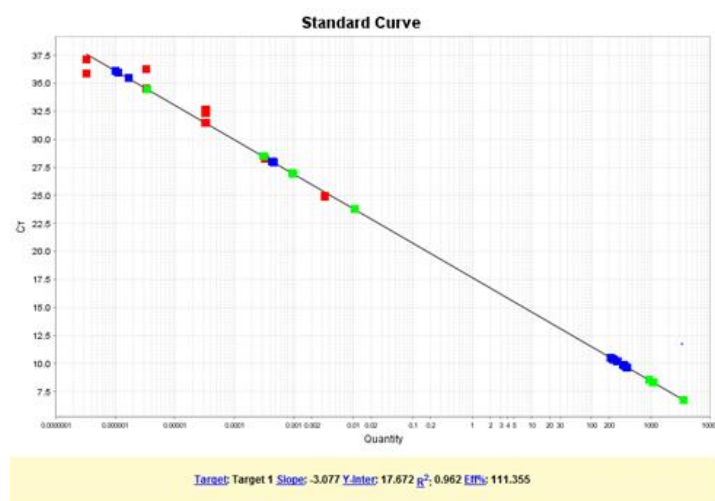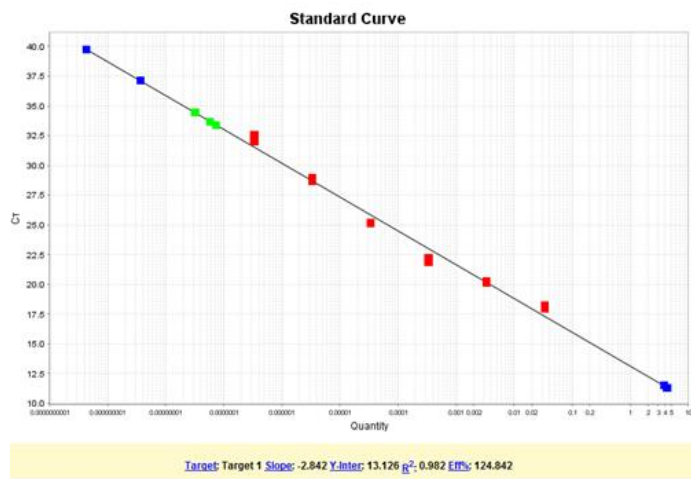
Figure 4.6. Quantitative PCR plots (3 independent replicates of each dilution) of sensitivity test for nPCR, using plasmid DNA. Each plot contains one replicate set of dilutions on a separate machine run (blue diamonds), calibrated against a standardised calibration curve (red diamonds). r^2= 0.982 (top plot), 0.962 (middle plot), 0.998 (bottom plot).
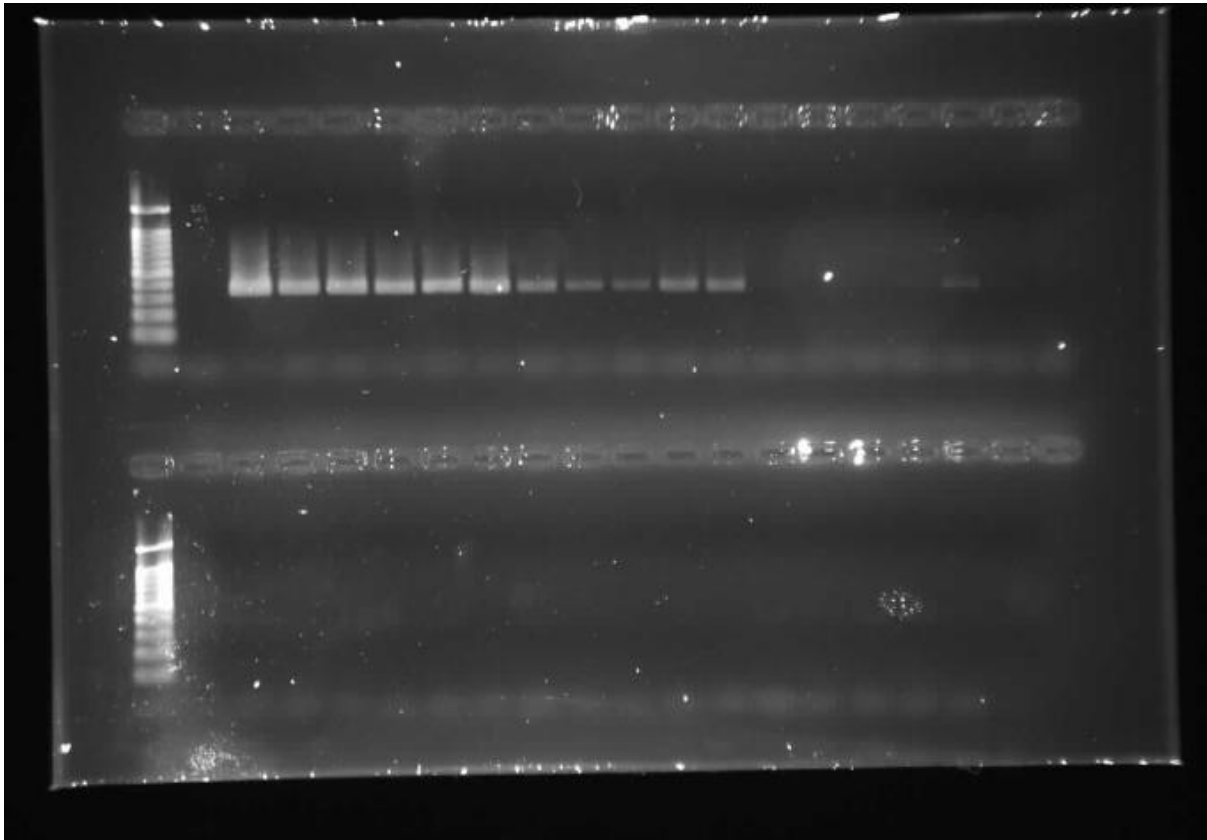
Figure 4.7. Conventional PCR gel photo (five replicates of each dilution) of sensitivity test using total DNA, demonstrating the drop-off in detection.

Wells in top row (numbered from left):

1= Ladder. 2= Negative control. 3 = Positive control. 4-8 = Five replicates of 9.79×10^{-3} ng/µL DNA (5 positive detections). 9-13 = Five replicates of 9.79×10^{-4} ng/µL DNA (5 positive detections). 14-18 = Five replicates of 9.79×10^{-5} ng/µL DNA (2 positive detections). 19-20 = Two replicates of 9.79×10^{-6} ng/µL DNA (no positive detections).

Wells in bottom row (numbered from left):

 1= Ladder. 2-4 = Three replicates of 9.79×10^{-6} ng/µL DNA (no positive detections). 9-13 = Five replicates of 9.79×10^{-7} ng/µL DNA (no positive detections). 14-18 = Five replicates of 9.79×10^{-8} ng/µL DNA (no positive detections). 19-20 = Five replicates of 9.79×10^{-9} ng/µL DNA (no positive detections).

Although the LOQ showed less sensitivity using cPCR than using qPCR, at concentrations below qPCR LOQ there was a higher probability of detection using

cPCR 350 (e.g. 31% of replicates at 9.79×10^{-5} ng/µL) than using qPCR (11% of replicates at 9.79×10^{-5} ng/µL). As with plasmid DNA, the number of detections using qPCR declined to zero before cPCR and nPCR (qPCR no detections at or below 9.79×10^{-6} ng/µL, nPCR detections in 15% of replicates at 9.79×10^{-8} ng/µL, cPCR 350 detections in 8% of replicates at 9.79×10^{-8} ng/µL).There were no statistically significant differences in sensitivity between cPCR 350 and cPCR 101 ($F_{1,20}$# = 0.569, $P = 0.480$; # = permutational).

4.4.2 Field surveys

In pre-eradication surveys, both eDNA and trapping, *P. parva* was detected in one pond only, the known infested (i.e. treatment) pond, with eDNA detection at the first (cPCR) stage of the nPCR protocol, thus confirming the previous cPCR results of Davison et al. (2017). All water samples from the other six ponds of this angling venue were negative for DNA of *P. parva*, and this included all replicates at both the cPCR and qPCR stages of the nPCR protocol. No inhibition was detected in any sample (following the dilution steps undertaken to reduce inhibition). All positive controls (total DNA controls in the cPCR stage, and plasmid DNA standard curves in the qPCR) demonstrated successful amplification.

In the post-eradication surveys, all water samples and trapping from both the treatment pond and the quarantine pond yielded negative results, including all replicates at both the cPCR stage and the final qPCR stage of the nPCR protocol. No inhibition of eDNA was detected, and all positive controls demonstrated successful amplification.

4.5 Discussion

This nPCR protocol proved to be applicable for evaluating the success of the *P. parva* eradication operation. The greater sensitivity of nPCR in the present study, relative to cPCR or qPCR approaches, confirms the results of two similar studies (Clusa et al. 2017; Jackson et al. 2017). The LOQ of the nPCR was lower than the detection limit of 1×10^{-5} ng/µL reported by Clusa et al. (2017), whereas Agersnap et al. (2017) demonstrated a higher level of sensitivity (LOD and LOQ of one copy per µL of

extracted sample) using qPCR than was recorded in this study. A LOD of less than one copy number per reaction has been reported in other studies using qPCR (Hunter et al. 2017; Serrao et al. 2017).

The increased sensitivity demonstrated by this nPCR protocol is likely to be largely a consequence of the increased number of cycles, with 70 in the two rounds of amplification compared to 35 in the cPCR or qPCR alone. Increased sensitivity could potentially have been obtained from the cPCR or qPCR protocols by increasing the number of cycles to 45–55 as used in some other eDNA studies (e.g. Tréguier et al. 2014; Biggs et al. 2015). However, one potential advantage of the nPCR approach is that it uses refreshed (new) reagents after 35 cycles. Length of DNA amplicon targeted by the primers is another factor that could conceivably affect sensitivity (Deagle et al. 2006), but comparative testing of cPCR primers for two different fragment lengths (101 bp and 350 bp) showed no statistical difference in the present study. Piggott (2016) similarly found no evidence that target amplicon size was a limiting factor in eDNA detectability. Caution also needs to be exercised when extrapolating the results of sensitivity trials conducted on relatively long DNA fragments to eDNA surveys, which may be targeting partially degraded shorter fragments. Further work is needed to show that the increased sensitivity shown by nPCR does result in significantly improved detection ability when analysing the shorter fragments present in environmental samples.

In comparative tests using plasmid DNA, qPCR was not found to be more sensitive than cPCR (Figure 4.2), although it should be noted that there were differences between the two protocols, e.g. different reagents and fragment target lengths. It should also be noted that number of replicates varied between treatments, with three independent replicates (nine wells) in qPCR and fifteen in cPCR, which may have affected results due to the stochasticity inherent in PCR amplification, as more qPCR replicates may have produced more detections. This similarity in sensitivity of the two approaches contradicts some other studies (e.g. Tréguier et al. 2014; Biggs et al. 2015; Piggott 2016) but is consistent with others (e.g. Nathan et al. 2014; De Ventura et al. 2017). Indeed, at concentrations below the 100% detection limit, more detections were obtained using cPCR than with qPCR. In such circumstances, where both

positive and negative results are obtained from a set of replicates, it would be important in a management context to set a threshold limit on the number of positive replicates required before assigning presence/absence status. De Ventura et al. (2017) similarly found cPCR to provide a more robust protocol than qPCR in a direct comparison, producing fewer false negatives at very low target DNA concentrations. This is despite the inherent challenge of interpretation of weak bands, which in cPCR may be on the borderline of visual acuity of the observer. These findings, particularly when the lower financial costs are considered, demonstrate that cPCR remains an effective tool, particularly for routine monitoring and/or survey applications.

Plasmid DNA provided a much lower value for sensitivity (LOQ) than total DNA, using all three PCR protocols. This demonstrates one of the difficulties in comparing protocol sensitivity between different studies, with some reporting values for DNA sensitivity based on total tissue-extracted DNA (e.g. Tréguier et al. 2014; Biggs et al. 2015;) whereas others base these values on plasmid DNA (e.g. Takahara et al. 2012; Jane et al. 2015). The use of plasmid DNA enables the calculation of copy numbers for reporting relative sensitivities of protocols as recommended by Goldberg et al. (2016).

'Proving a negative', i.e. declaring a species to be absent using any survey method, is difficult due to the inherent uncertainty (imperfect detection) that is associated with any form of field sampling approach (Rout et al. 2009; Britton et al. 2011b). Detection rates using conventional methods are well known to vary according to the gear used (e.g. Jackson and Harvey 1997), in the use of citizen science (e.g. Ruiz-Gutierrez et al. 2016), and the spatial scale used (Barry and Elith 2006). Even a moderate variation in the technique used to apply a sampling method, such as how the dip net is handled in the water during point-abundance sampling by electrofishing (Copp and Garner 1995), will affect the likelihood of capturing a species and consequently its 'detection' for purposes of calculating species richness. However, any increase in the sensitivity of a sampling protocol, such as seen here with the nPCR protocol, provides increased confidence in a negative detection result. For example, the nPCR protocol presented here was up to 100× more sensitive at detecting plasmid DNA than the cPCR protocol used previously to survey these same water bodies (Davison et al. 2017). The increased sensitivity did not, in this instance, result in detections that would not have been achieved by cPCR or conventional trapping, and further field trials on water

bodies containing smaller populations of the target species would be beneficial to demonstrate the merit of the technique. The extent to which this increased sensitivity translates into increased detections in environmental samples (i.e. to assess the level of sensitivity that is required from the laboratory protocol to detect eDNA from a small fish population in a water body of a given size) requires further study. False negatives can derive from several stages in sampling design, in both field and laboratory (Darling and Mahon 2011). In field applications, it is conceivable that modifications to sampling strategy, such as sampling location (within a water body) or quantity of water filtered, may have as great an effect on the overall result (positive/negative) as improvements to the sensitivity of the laboratory protocol.

The results of the eDNA survey of the seven water bodies matched the results of the conventional sampling, with both methods detecting *P. parva* in the known infested pond, and neither method detecting the species in the six remaining ponds. Experimental trials (in 100 m^2 mesocosms) have demonstrated that trapping is not completely effective at detecting *P. parva* at low densities (Britton et al. 2011b). Baited traps (deployed for 1 h) showed 100% detection at densities >0.5 m^{-2}, but imperfect detection at densities of 0.02 and 0.1 m^{-2} (Britton et al. 2011b). Surveys of other fish species using eDNA methods have frequently proved more effective than conventional methods at detecting species (e.g. Takahara et al. 2013; Janosik and Johnston 2015; Sigsgaard et al. 2015). Further study would be needed to determine the efficiency of eDNA sampling, relative to conventional sampling, at varying population densities, as such comparisons will be species specific (Hinlo et al. 2017a) and are also likely to be site specific.

Following draw-down of the infested pond by the fishery owners, the negative eDNA detection for *P. parva* in the infested and quarantine ponds indicated that eradication of the infested pond had been successful and that it was highly unlikely that any *P. parva* were transferred into the quarantine holding pond. The six-month delay between eradication and water sampling would have provided sufficient time for: (i) any surviving *P. parva* to introduce more eDNA into the ponds (and possibly even reproduce, as spawning in southern England occurs between April and June; Beyer 2008), and (ii) any remaining DNA from dead/removed *P. parva* to degrade to non-detectable levels (Dejean et al. 2011). The present study, therefore, clearly

demonstrates the potential of eDNA surveying as a tool to identify which water bodies require eradication efforts and to assess the success of those eradication attempts (Dunker et al. 2016; Davison et al. 2017).

Environmental DNA surveys are often seen as a less-expensive substitute for conventional methods (Biggs et al. 2015; Sigsgaard et al. 2015), although this does not hold true for all sampling programmes under all circumstances (Smart et al. 2016). For example, to detect brook trout *Salvelinus fontinalis* in streams, eDNA analysis imposed 67% less cost than triple-pass electrofishing and required lower sampling effort (Evans et al. 2017). However, for simple presence/absence detection in that study, eDNA was more expensive than electrofishing, when fishing operations were halted upon first detection of the target species. Conventional fish surveying approaches can also provide information on population structure, which eDNA surveys cannot, and therefore eDNA sampling may in some applications represent a complement, rather than an alternative, to conventional sampling.

The work required to confirm definitive absence of an invasive fish species in a pond would be both costly and potentially environmentally destructive. The enhanced sensitivity of the nPCR protocol provides increased confidence that the negative results obtained were not 'false negatives', but this comes at increased financial cost. In the present study, the extra analysis involved with the two steps of the nPCR protocol increased the combined costs of consumables and laboratory analysis by 1.6×. Despite these considerations, nPCR protocols are recommended for any eDNA survey in which a high level of confidence is required in the declaration of a negative result, e.g. where the presence or absence of a species will form the basis of decisions for management action.

5. Assessing non-native fish distributions in river basins using environmental DNA

This chapter has been submitted as a paper:

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5.1 Abstract

Environmental DNA (eDNA) is increasingly used to inform decisions and strategies for non-native fish management, initially in still waters but also in water courses, by detecting the presence and plotting the distribution of newly-arrived and established non-native species that are in too low abundance for efficient detection using conventional surveillance methods. A recently-developed nested PCR protocol was used to assess the current distributions of three non-native fish species at a catchment level for two river basins in southern England (River Test, Hampshire; River Ouse, Sussex) known previously to contain two small-bodied invasive non-native fish species (topmouth gudgeon *Pseudorasbora parva*, sunbleak *Leucaspius delineatus*) as well as a non-native species predicted to become invasive under future climate conditions (pumpkinseed *Lepomis gibbosus*). In these two river catchments, water samples were collected at locations from headwater streams to the estuary. Pumpkinseed and sunbleak were both detected downstream of an angling venue in the Sussex Ouse catchment known to contain those species, with upstream expansion of sunbleak suggested by eDNA detected at a few upstream locations. Neither sunbleak nor topmouth gudgeon were detected from eDNA in water samples from various locations in the River Test catchment, where both species were first introduced to the U.K., suggesting that neither species has persistent populations in that river catchment.

5.2 Introduction

Accurate mapping of biological invasions is essential to inform management decisions on strategies for eradication, control or containment, so as to avoid or minimise

impacts (Simberloff et al. 2005). In aquatic environments, molecular techniques based on environmental DNA (eDNA) shed by organisms into the water have successfully detected a range of taxa (e.g. Darling and Mahon 2011; Rees et al. 2014b; Davison et al. 2016), and in particular fish species at low densities in still waters (e.g. Takahara et al. 2013; Lacoursière-Roussel et al. 2016; Davison et al. 2017) and in lotic systems (e.g. Minamoto et al. 2012; Keskin, 2014). These methods have sometimes outperformed traditional survey methods; for example, a river-basin wide eDNA-based study detected Chinook salmon *Oncorhynchus tshawytscha* at six locations outside of its previously known range (Laramie et al. 2015).

Non-native fishes introduced to still waters in a river's flood plain can be particularly problematic when they gain access to tributary streams and adjacent floodplain water bodies, which serve as dispersal pathways to invade other suitable habitats (e.g. Fobert et al. 2013). Monitoring methods for freshwater fishes in water courses have generally been successful in capturing larger-bodied, more abundant species, with small-bodied fishes in low density potentially avoiding detection (e.g. Bohlin et al. 1989; Rogers et al. 2003). Environmental DNA is potentially detectable when the target species occurs at very low densities, providing a means of mapping the distributions of these species at a catchment level, thus providing the necessary information upon which to develop control and containment measures.

The aim of the present study was to assess the distribution of three non-native fish species in two river catchments in southern England in a synchronic manner (*sensu* Amoros et al. 1987), which resembles the approaches developed by government agencies to monitor fish stocks annually (e.g. Adjers et al. 2006) or every three years such as for the River Great Ouse, England (e.g. Coles et al. 1985). The three non-native fish species are the: 1) North American freshwater sunfish, pumpkinseed *Lepomis gibbosus*, which was introduced to England as an ornamental fish (the equivalent of the koi carp) in either the late 1800s or early 1900s (Copp et al. 2002), has existed in (or in the vicinity of) the River Ouse (Sussex) since at least as early as the 1910s (Wheeler and Maitland 1973) and was found to be relatively widespread but in low-to-moderate abundance in the Sussex Ouse in 2001 (Klaar et al. 2004; Copp et al. 2010a); 2) sunbleak *Leucaspius delineatus*, a small-bodied cyprinid native to continental Europe, and 3) topmouth gudgeon *Pseudorasbora parva*, a small-bodied

invasive cyprinid native to parts of Asia that is of particular concern due to its threat as a host of the pathogen *Sphaerothecum destruens* (Gozlan et al. 2005).

Both *P. parva* and *Leucaspius delineatus* were accidentally introduced to an ornamental fish farm (now defunct) at Crampmoor on Tadburn Lake Stream, a tributary of the River Test, Hampshire, as a contaminant of golden orfe *Leuciscus idus* consignments from continental Europe (Farr-Cox et al. 1996; Pinder and Gozlan 2003). The known UK distribution of *Leucaspius delineatus* remained limited to the southern English counties of Hampshire and Somerset until an established population was discovered in a commercial fishery on a tributary of the Sussex Ouse (Zięba et al. 2010), whereas *P. parva* became much more widely distributed in the UK (Pinder and Gozlan, 2003) as a contaminant of fish movements (Copp et al. 2010b).

The specific objectives of the present study were to: 1) design quantitative PCR (qPCR) primers for detection of *Lepomis gibbosus* and *Leucaspius delineatus* for use in a recently-developed nested PCR protocol (Davison et al. 2019); 2) survey lotic sites for the eDNA of *Lepomis gibbosus* and *Leucaspius delineatus* in the catchment of the Sussex Ouse, with spatially intensified sampling close to the discharge into the stream of a known still-water site for both species; 3) survey sites in the catchment of the River Test for eDNA of *P. parva* and *Leucaspius delineatus*, with spatially intensified sampling in the vicinity of the discharge of the fish farm into which those two species are known to have been imported as contaminants; and 4) assess whether use of a nested PCR protocol can result in increased field detections when compared to conventional PCR alone.

5.3 Methods

5.3.1 Study sites

The two study catchments were the River Ouse (Sussex) and River Test (Hampshire). The Sussex Ouse has a length of 62 km, discharging into the English Channel at Newhaven (https://en.wikipedia.org/wiki/River_Ouse,_Sussex). Two of the Ouse's second-order tributaries, Sheffield Stream and Batts Bridge Stream (Figure 6.1), were sampled more intensively due to the known presence of *Lepomis gibbosus* and

Leucaspius delineatus in upstream water bodies of one or both of these two tributaries. In the Sheffield Stream catchment, *Lepomis gibbosus* and *Leucaspius delineatus* are established in ponds of a commercial angling venue that exchanges water with the stream near Danehill, East Sussex (Zięba et al. 2010; Copp et al. 2017; Bašić et al. 2018). The angling venue and its water exchanges with Sheffield Stream are described in Fobert et al. (2013). In the Batts Bridge Stream catchment, *Lepomis gibbosus* is known to have been established in Boringwheel Lake (Villeneuve et al. 2005; Fobert et al. 2013), a 400-year old, 2.6 ha former mill pond now used as a commercial fishery for rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* (Figure 5.1). The stream passes through this former mill reservoir, continuing along the stream bed after overtopping a gated weir. Both tributaries, which are of variable width (1.0–4.3 m) and depth (0.05–1.5 m), are described in Fobert et al. (2013).

The River Test is a chalk river of high conservation interest (Natural England 1997), dropping 90 m in elevation to discharge into the English Channel via Southampton Water (https://en.wikipedia.org/wiki/River_Test). Water sampling sites focused on the receiving water course of a former (now-disused) fish farm at Crampmoor, Hampshire (Figure 6.2), the introduction site of *P. parva* and *Leucaspius delineatus* to England (Pinder and Gozlan 2003). Crampmoor Stream runs adjacent to, and received escapee fish from, the fish farm via an unscreened discharge pipe, eventually joining Tadburn Lake Stream (width 0.7–2.0 m), which itself subsequently joins the River Test at Romsey (Figure 6.2). A population of *P. parva*, but not *Leucaspius delineatus*, was found to persist in the fish ponds and in the stream (Beyer et al. 2007) until action to eradicate *P. parva* at this site took place in 2014–2015 as part of a national programme (Environment Agency 2014; Great Britain Non-native Species Secretariat 2015). Following this eradication, the pathogen *S. destruens* was detected in water samples collected downstream of the fish farm (Sana et al. 2018). An established population of *Leucaspius delineatus* is known to be present in the nearby Stoneham Lakes complex (Beyer et al. 2010), but its persistence in the Test downstream of Crampmoor remains unknown.

5.3.2 Sample collection and preparation

Water sampling was conducted on 14–15 June 2016 (Sussex Ouse) and 16 June 2016 (Hampshire Test). Water samples were collected at about 1.5 m distance from one bank, or from the mid-point of the stream at sites where the stream was <3 m wide. A 500 mL polypropylene sampling cup, attached to a polypropylene sampling pole of 183 cm length (Camlab Ltd, Cambridge, UK), was used to collect the water. Care was taken to avoid any contact with the bottom sediment. At each sampling location, three replicates of 300 mL water, obtained using the sampling cup, were injected through a Sterivex-GP 0.22 µm sterile filter cartridge (EMD Millipore, Billerica, MA, USA) using a 50 mL sterile syringe (Thermo Scientific) attached to the cartridge's input opening. Cartridges from each location were sealed in individual plastic bags and immediately frozen (–20°C) for transportation back to the laboratory. Contamination between sites was avoided by disinfecting the pole and cup thoroughly using Microsol 3+ sterilising solution (Anachem Ltd, Luton, UK) and then rinsing with de-ionised water. On each sampling day, water from a sterilized bottle of de-ionised water from the laboratory was also filtered, handled and transported in the same manner as the stream samples, and analysed in the laboratory to test for contamination.

In the laboratory, DNA was extracted from the cartridges using a PowerWater Sterivex™ DNA Isolation Kit (MoBio, Carlsbad, CA, USA), producing a final elution volume of 100 mL. The extracted sample was then diluted 1:5 in deionised water to dilute potential inhibitors (McKee et al. 2015).

5.3.3. Primer design and nested PCR protocol

Water samples were subjected to a nested PCR (nPCR) protocol that involves two steps: Step 1 consists of a conventional PCR (cPCR, which in the event of non-detection is followed by Step 2, which involves a quantitative PCR (qPCR) on the product of Step 1 (Davison et al. 2019). The nPCR protocol proved 100× more sensitive in laboratory sensitivity tests on DNA derived from *P. parva* tissue (Davison et al. 2019). To complement the existing qPCR primer for *P. parva*, qPCR primers were designed for *Lepomis gibbosus* and *Leucaspius delineatus* (Table 5.1) to amplify a section of the mitochondrial cytochrome *c* oxidase I (COI) gene. Conventional PCR

primers for all three species were designed and tested for sensitivity and specificity for a previous study (Davison et al. 2016), as were the qPCR primers for *P. parva* (Davison et al. 2019). The qPCR primers for *Lepomis gibbosus* and *Leucaspius delineatus* were designed for the present study, and tested for sensitivity and specificity in a similar manner.

Table 5.1: Primers used for conventional and quantitative PCR of topmouth gudgeon *Pseudorasbora parva*, pumpkinseed *Lepomis gibbosus* and sunbleak *Leucaspius delineatus.* The cPCR primers were designed by Davison et al. (2017). The qPCR primers for topmouth gudgeon were designed by Davison et al. (2019), and for the other two species as part of the present study.

Species, PCR type, (amplicon size in bp)	Forward primer (5'-3')	Reverse primer (5'-3')	FAM probe (5'-3')
P. parva cPCR (350)	CCTCTTCCGGA GTAGAGGCT	TAGGATTGGG TCTCCTCCCC	Not applicable
P. parva qPCR (101)	GTGTTTCATCAAT TCTAGGCGCAAT	AGCTCATACAAAT AAGGGCGTTTGA	ATATAAAACCTCC AGCTATTTCC
L. gibbosus cPCR (350)	CTAATAATTGGCG CCCCCGA	CGGACCAGACA AACAGTGGT	Not applicable
L. gibbosus qPCR (83)	GCTGGCACGGGCTGAA CCGGCAACCTAGC	GAGAAAATAGTGA GATCAACGGATGCT	CCGGCAACCTAGC CCACGCC
L. delineatus cPCR (251)	TTCGAGCCGAAC TAAGCCAR	GGCCTCAACCC CAGAAGAAG	Not applicable
L. delineatus qPCR (93)	CCCACGCCTTCGT AATAATTTTCTT	CGGGCGCACCAA TCATTAG	CGGGTTTGGAAAC TGACTCGT

DNA extracted from dorsal muscle tissue (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) of fish collected from populations in southern England was used as a positive control in cPCRs and for calibration of qPCRs; these sequences have been deposited in the open-source database Genbank (www.ncbi.nlm.nih.gov/genbank) with the accession numbers KR092382 (*Lepomis gibbosus*), KR092383 (*Leucaspius delineatus*) and KR092385 (*Pseudorasbora parva*) (Davison et al. 2016). Concentrations of tissue-extracted DNA used for construction of qPCR calibration curves were measured using a Nanodrop® ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and calculated with the software ND-1000 v3.8.1 (Thermo Scientific). Specificity of all primers was tested *in silico* against sequences in the NCBI Genbank database using the NCBI Primer Blast software (www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al. 2012), and tested experimentally against 0.1 ng genomic DNA extracts from fish species likely to co-occur at the study sites: common carp *Cyprinus carpio*, common bream *Abramis brama*, roach *Rutilus rutilus* and rudd *Scardinius erythrophthalmus*, with none of the triplicate PCRs showing amplification for any of these species (Davison et al. 2017).

Conventional PCRs were performed on 6 µL of eDNA sample, with the reaction mixture containing 0.5 µM of each specific primer, 10 µL of HotStar Taq® Plus DNA polymerase 2× (Qiagen Fast Cycling PCR Kit) and 2 µL of Coral Load Fast Cycling Dye 10× (Qiagen), made up to 20 µL with the addition of de-ionised water. The PCR cycling conditions consisted of an initial denaturation step of 95°C for 5 min, followed by 32 cycles of 96°C for 5 s, 62°C for 5 s and 68°C for 12 s, with a final extension step at 72°C for 1 min. PCR products were visualised after 60 min of electrophoresis migration on 2% agarose gel, stained with SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen). Five cPCR replicates were analysed for each sample location. Each machine run included a negative control (de-ionised water) to check for contamination.

Environmental DNA samples that provided a negative result at the cPCR stage were subjected to further analysis (the second stage of the nested PCR protocol), with qPCR performed on the products from three of the five cPCR replicates. Real-time qPCRs were performed on an Applied Biosystems Step One™ system (Applied Biosystems, Foster City, CA, USA), in a 20 µL reaction mixture containing 2 µL of

DNA sample, 1 μL of assay mix (18 μM forward and reverse primers and 5 μM probe) for the targeted species (Applied Biosystems), 10 μL of TaqMan® Genotyping Master Mix (Applied Biosystems) and 7 μL of de-ionised water. The thermocycling profile used was 2 min at 50°C, 10 min at 95°C, followed by 35 cycles of 15 s denaturation at 95°C, and 60 s annealing-extension at 60°C. The standard curve, applied on each qPCR run, comprised a range of five 10-fold dilutions of tissue-derived DNA. Each qPCR run incorporated a negative control (deionised water).

Sample extraction, PCR preparation and post-PCR analysis were performed in separate rooms of a dedicated molecular biology laboratory, observing strict anti-contamination procedures (no transfer of equipment between rooms; changing of lab coats when moving between rooms; thorough cleaning of all equipment and surfaces before and after use, and treating of equipment under UV light; use of sterile filter tips for pipettes). An important consideration when using nested PCR protocols is the increased risk of sample contamination, due to increased handling of amplified DNA. This risk was minimised by preparing reagents in a separate room to that in which the completed cPCR template was added, using different pipettes and gloves.

5.4 Results

Positive eDNA detections were obtained in the Sussex Ouse catchment for both *Leucaspius delineatus* and *Lepomis gibbosus* from locations in the Sheffield Stream, but not from Batts Stream (Figure 5.1). Both species were recorded at the two sites immediately downstream of the commercial angling venue, which has lakes known to contain both species (Zięba et al. 2010; Fobert et al. 2013). Additionally, *Leucaspius delineatus* DNA was recorded at three sites upstream of the angling venue, and two sites further downstream (one on the Sussex Ouse immediately downstream of its confluence with Sheffield Stream). At both locations with positive detections for eDNA of pumpkinseed, all five replicates of the cPCR provided a positive result. The eDNA signal for *Leucaspius delineatus* was strongest at the location immediately downstream of the angling venue, with all five cPCR replicates proving positive. At the remaining six locations with *Leucaspius delineatus* detections, only 1–2 cPCR replicates proved positive, with comparatively faint bands, but in each case proceeding with the nPCR on three of the negative replicates produced a positive result.
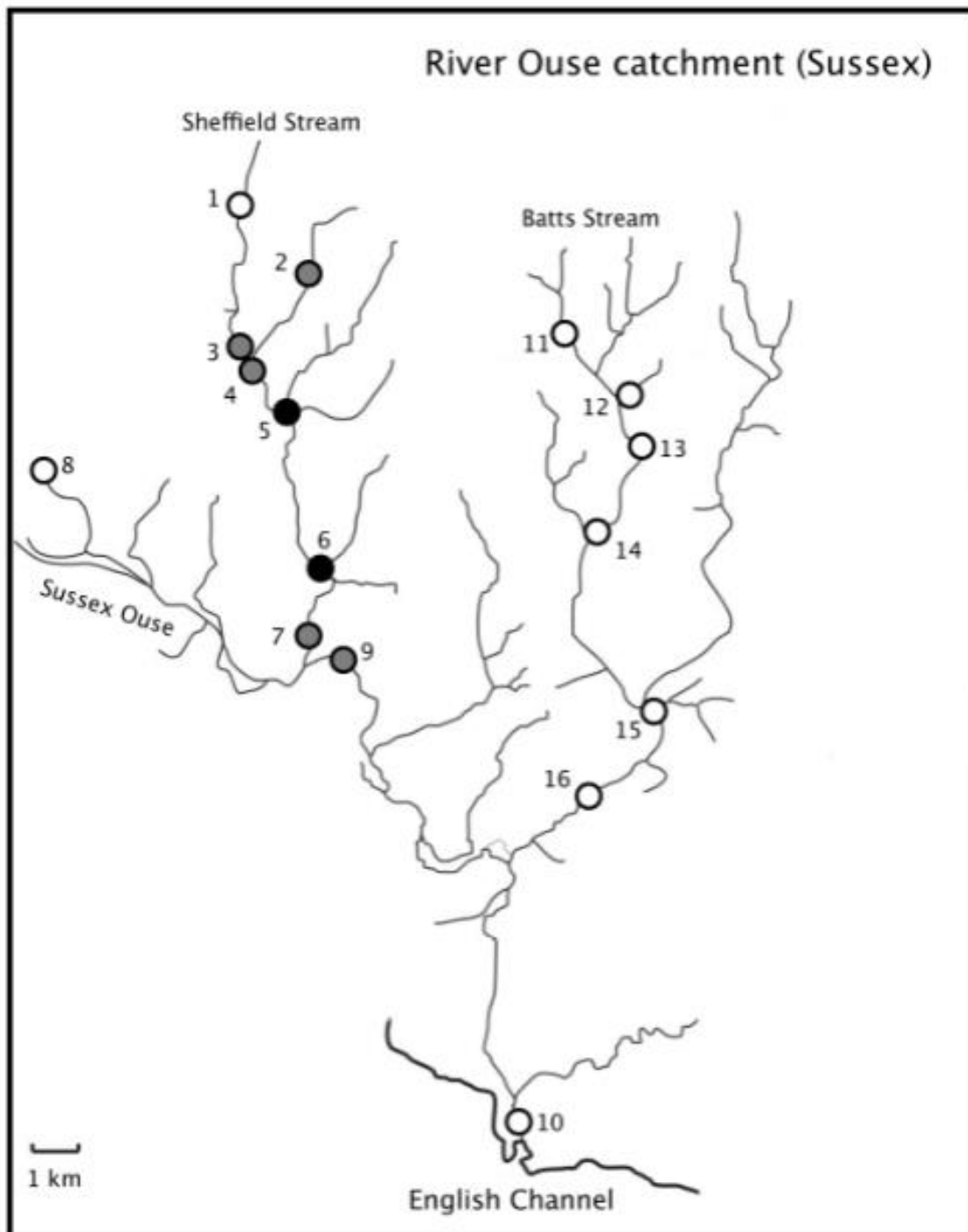
Figure 5.1 Map of water collection sites (WGS84 coordinates) in River Ouse catchment (Sussex) for eDNA analysis of pumpkinseed *Lepomis gibbosus* (all sites) and sunbleak *Leucaspius delineatus* (sites 1–10). Black-filled circles = sites where both species were detected. Grey-filled circles = sites where only *L. delineatus* was detected. Open circles = sites with no detections. 1 = Sheffield Stream at Collingford (51.028885, 0.007233); 2 = Tanyards Farm Stream (51.027380, 0.013906);

Figure 5.1 continued: 3 = Sheffield Stream above Tanyards Fishery (51.023912, 0.009229); 4 = Sheffield Stream at upper end of Tanyards Fishery (51.023486, 0.009990); 5 = Sheffield Stream below Tanyards Fishery (51.018459, 0.014657); 6 = Sheffield Stream at Eastbridge (51.007024, 0.017481); 7 = Sheffield Stream at lower end of Sheffield Park (51.993436, 0.019276); 8 = River Ouse at Ardingly (51.028445, −0.0894816); 9 = River Ouse at Fleching Mill (50.987522, 0.027771); 10 = River Ouse estuary at Piddinghoe (50.810306, 0.033544); 11 = Batts Stream at Batts Bridge (50.991580, 0.068360); 12 = Batts Stream at Cackle Street (51.016889, 0.075606); 13 = Batts Stream at Old Forge Lane (51.013028, 0.078568); 14 = Batts Stream at Hole Farm (51.020853, 0.072426); 15 = Confluence of Batts Stream and Shortbridge Stream at Powder Mill (50.991621, 0.068564); 16 = Shortbridge Stream (50.992533, 0.094205).

On the River Test catchment, no positive eDNA detections were obtained for either *Leucaspius delineatus* or *P. parva*, including on the stretch of Tadburn Lake Stream running adjacent to, and receiving overflow discharge from, the ponds on the site formerly known as Crampmoor Fish Farm (Figure 5.2).
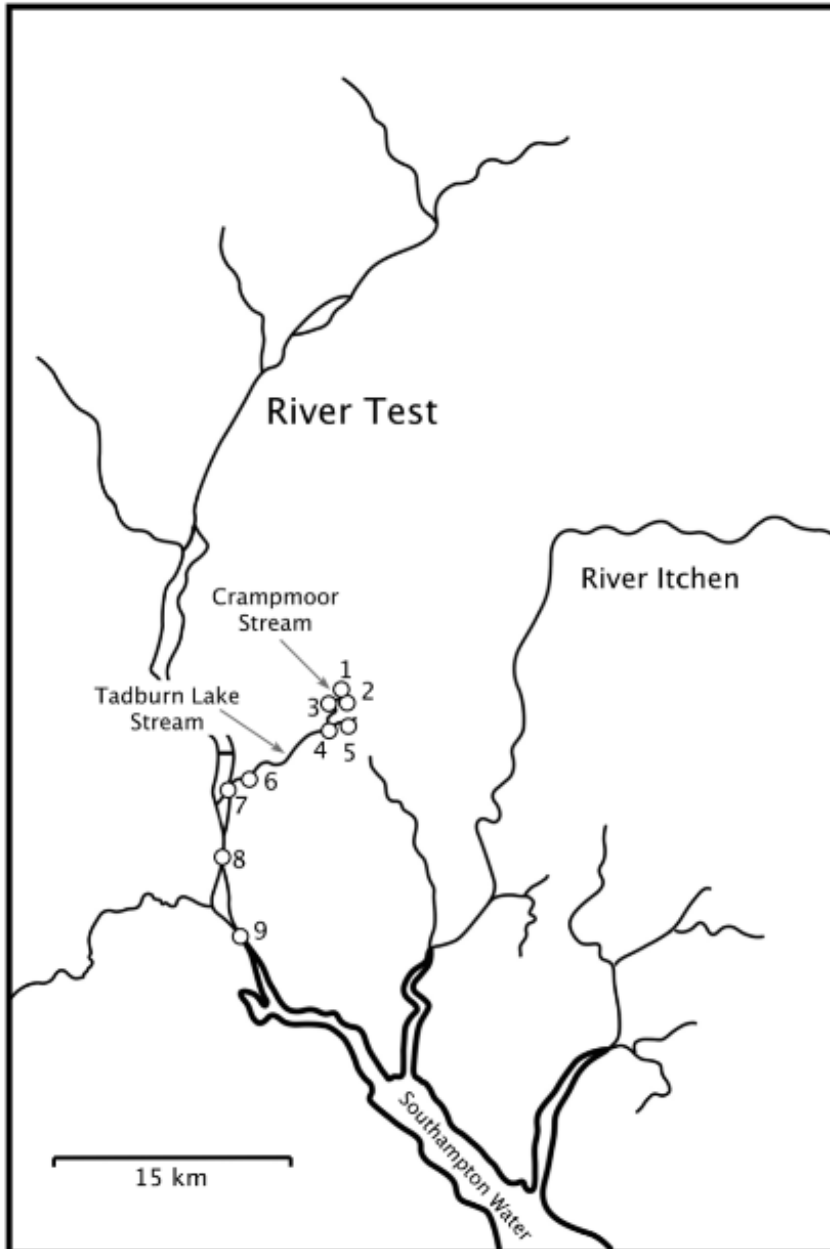
Figure 5.2 Map of water sampling sites (WGS84 coordinates) on the River Test catchment, Hampshire. Open circles indicate sites with no detection of topmouth gudgeon *Pseudorasbora parva* or sunbleak *Leucaspius delineatus*. 1 = Crampmoor Fishery stream, above ponds (51.001136, −1.44476); 2 = Crampmoor Fishery stream, adjacent to ponds (50.999826, −1.447869); 3: Crampmoor Fishery stream, below ponds (50.999556, −1.451109); 4: Crampmoor Stream, at confluence with Tadburn (50.996450, −1.454585); 5: Tadburn Lake (50.996234, −1.454135); 6: Tadburn, in Romsey (50.986037, −1.498767); 7: River Test, in Romsey, above confluence with Tadburn (50.984605, −1.504131); 8: River Test, at Lee Farm Longbridge (50.959004, −1.496252); 9: River Test, at Salmon's Leap (50.926528, −1.486266).

5.5 Discussion

The eDNA surveys provided a relatively rapid assessment of the target species distributions within the two river catchments (Figure 5.1 and 5.2), requiring one day of water sampling on each catchment, and 24–39 h of laboratory analysis per species per catchment. Detections of *Lepomis gibbosus* eDNA in Sheffield Stream (Sussex Ouse catchment) were consistent with previous surveys (e.g. Klaar et al. 2004; S. Stakėnas and G. H. Copp, unpublished data), which were substantiated by subsequent tagging and drift-net sampling that demonstrated a much lower *Lepomis gibbosus* escapee rate into that stream (via outflow) than in the adjacent Batts Stream catchment from Boringwheel Lake (Fobert et al. 2013). Despite the demonstrated capacity of *Lepomis gibbosus* to make seasonal up- and down-stream movements in Sussex Ouse tributaries (Stakėnas et al. 2013), no *Lepomis gibbosus* eDNA was detected upstream of the angling venue on Sheffield Stream, where the species is well established in several of the angling ponds (Villeneuve et al. 2005; Fobert et al. 2013; Copp et al. 2017). Detections of *Leucaspius delineatus* were more widely dispersed along Sheffield Stream (Figure 5.1), including a few locations upstream of the established populations at the angling venue. This suggests an expansion of the species up the catchment, including the possibly of an established population at the in-stream pond just above site 2 (Figure 5.1).

The lack of *Lepomis gibbosus* eDNA detections anywhere in Batts Bridge Stream catchment would appear to confirm a previous electrofishing survey which documented no *Lepomis gibbosus* captures after 2007 (Jackson et al. 2016), despite *Lepomis gibbosus* having been present in the stream and connected still waters for at least a decade previously (Klaar et al. 2004; Villeneuve et al. 2005). The most important of the connected still waters has been Boringwheel Lake, which discharges into Batts Stream immediately upstream of site 12 (Figure 5.1) and therefore has drip-fed *Lepomis gibbosus* into the stream, leading to the establishment of at least one new population in a garden pond due to an extreme flood event (Fobert et al. 2013).

The lack of *P. parva* and *Leucaspius delineatus* detections in water samples from sites in the Crampmoor and Tadburn Lake streams suggest either that these species are no longer present in that part of the River Test catchment, or that they occur at a

density below the detection limit of this survey. In view of the presence of *Leucaspius delineatus* in some stillwater fisheries that discharge into tributaries of the River Test, such cases of negative detection suggest a need for further, more intensive surveying. Imperfect detection rates by eDNA or conventional methods (e.g. Britton et al. 2011b) are particularly important when the results form the basis of management decisions. When analysing eDNA survey results, lack of detection (i.e. 'false negatives') could result from a number of elements of the laboratory or field protocol that could affect the sensitivity of the technique. Stochastic variability in detection should be expected when seeking to detect low DNA concentrations in streams (Wilcox et al. 2016), and a higher level of field sampling (larger water volume filtered, or increased spatial or temporal repetition) might have produced positive results at more locations in this study.

The use of nPCR did not detect any species at any locations where they were not detected at the initial cPCR stage, although *Leucaspius delineatus* was detected when using nPCR on cPCR replicates that produced negative detections. This suggests that the increased sensitivity of this technique (as demonstrated in the laboratory; Davison et al. 2019) could in some cases result in improved field detectability. The enhanced sensitivity of the nested PCR protocol provides increased confidence that species that are present in the streams are not being missed as 'false negatives', but at increased financial cost and risk of laboratory contamination. Such nPCR protocols may prove particularly useful in running water, where eDNA signals from upstream populations are likely to be highly diluted.

The potential of eDNA surveying for surveying fish in running water, clearly demonstrated by this and other studies, needs to be assessed in comparison to other survey methods. Advantages include the ability to undertake surveys when factors such as weather or water depth would preclude the use of electrofishing, or reduce its effectiveness. In terms of financial cost, eDNA surveys are often regarded as less expensive than conventional methods (Biggs et al. 2015; Sigsgaard et al. 2015; Evans et al. 2017). Whilst eDNA surveying can provide a wider geographic coverage more rapidly than would be possible using conventional methods, the likelihood that positive eDNA detections may result from animals occurring some distance upstream of the sampling site (potentially up to 12.3 km; Deiner and Altermatt 2014) means that

accurate population mapping may require a combination of eDNA and other methods. Further attempts to map species presence in catchments using eDNA would benefit from gaining a more complete knowledge of the dynamics of eDNA in running water systems.

6. How much sampling effort is needed to detect a small lentic fish population using eDNA?

This chapter is in advanced stages of production for submission as a paper:

Davison, P.I., Vilizzi, L., Wesley, K., Britton, J.R. and Copp, G.H., in prep. How much sampling effort is needed to detect a small lentic fish population using eDNA? [title, author order and journal to be confirmed]

6.1 Abstract

Environmental DNA approaches have proved to be effective, sometimes out-performing traditional methods of fish surveying, and have also been suggested as saving economic costs, a key consideration for environmental management agencies. The detection of fish at very low population density, and in situations where the veracity of negative results is important, requires a statistically-rigorous sampling programme, tailored to the species and water body in question to account for spatial heterogeneity of DNA, and a highly sensitive laboratory protocol with sufficient replicates to account for stochastic variation in PCRs. These considerations potentially increase the economic cost. To assess the relative cost-effectiveness of statistically-rigorous eDNA surveys in comparison to conventional methods (trapping and electrofishing), field trials were conducted in six ponds in Hertfordshire, southern England, which contained fish populations typical of angling ponds. Fish of two species known not to occur in the ponds and selected as 'surrogate' non-natives, barbel *Barbus barbus* (a native species representative of benthic-feeding fishes) and rainbow trout *Oncorhynchus mykiss* (a pelagic-shoaling species) were stocked into the six ponds at a population density of 100 fish of each species per hectare. Water sampling for eDNA, conducted 6–7 days following fish stocking, detected *B. barbus* in three of the ponds and *O. mykiss* in all six ponds. Neither conventional method detected *O. mykiss* in any pond, and imperfect detection rates for *B. barbus* at this low fish density were also demonstrated by both trapping (successful at two ponds) and electrofishing (also successful at two ponds). Economically, the most cost-effective method for detecting *B. barbus* varied between ponds, with eDNA sampling (£615–£1600, dependent on pond size), trapping (£900,

for 2 days of a fishery consultant's time) and electrofishing (£450, for one day of a consultant's time) each proving the most cost-effective method on ponds where other methods failed.

6.2 Introduction

Numerous studies have demonstrated the effectiveness of environmental DNA (eDNA) surveying for detecting a wide range of aquatic taxa (Rees et al. 2014b). In addition to frequently proving more effective than conventional methods at detecting elusive target species (e.g. Takahara et al. 2013; Hinlo et al. 2018; Nakagawa et al. 2018), several authors have suggested that such eDNA surveys can be conducted at a lower financial cost than conventional fish surveys (Biggs et al. 2015; Sigsgaard et al. 2015; Nakagawa et al. 2018). This is a key consideration when making the step from scientific trials to applied surveys to address management questions.

Key applications of eDNA surveying include the management of non-native fish, for which the technique can be used to provide an early warning of newly invading species (Blackman et al. 2018), to map established populations (Adrian-Kalchhauser and Burkhardt-Holm 2016) and to assess eradication attempts (Dunker et al. 2016; Davison et al. 2017, 2019). These applications may require attempted location of very low populations, close to the limit of detection of any available survey methods, as a successful invasion can be initiated by a very low number of founding individuals (Kalinowski et al. 2010), or an eradication attempt may have reduced the population to low levels (Davison et al. 2017). Detection of low populations of a target fish species, whether using either eDNA or conventional survey methods, will require a higher sampling effort, at increased financial cost. In the case of eDNA surveying, sensitivity of detection could be increased by using more sensitive laboratory techniques (e.g. nested PCR; Davison et al. 2018) or increasing the number of field or laboratory replicates, with both options resulting in increased laboratory time and consumable costs. Such factors could at least partially negate the perceived financial benefits of eDNA surveying.

False negatives, i.e. the failure of eDNA or other survey methods to detect a population, can have serious deleterious consequences on the success and financial costs of invasive species management operations. For example, a false declaration of a species' absence could lead to a premature end to a surveillance and control programme, enabling the organism to expand its population and range (Britton et al. 2011b; García-Díaz et al. 2017; Furlan et al. 2019). Consequently, field and laboratory protocols of the highest possible accuracy and reliability are necessary. Imperfect detection is a consideration with all survey methods (molecular and conventional).

The aim of the present study was to directly compare the effectiveness and cost-efficiency of conventional and molecular detection methods for detecting fish present at low density in a water body. The specific objectives of this study were to: 1) to undertake co-ordinated sampling (eDNA surveys, conventional trapping, and electrofishing surveys) on six ponds to compare the effort required to detect fish present at a known low density; and 2) to use the eDNA survey to inform sampling strategy, by determining how many samples are required to have a high probability of detecting the target species.

6.3 Methods

6.3.1 Study sites and species

Six ponds in Hertfordshire, southern England were selected as study sites, of size 0.2 to 3.5 ha (Table 6.1). Five of these were artificial ponds situated in close proximity on a commercial fishery (centred on 51.842N, 0.136E), and contained a representative range of fish species typical of many such sites in southern England (Table 6.1). The other pond, Hanbury Manor Pond 17 (51.828N, 0.036W), was an ornamental pond on a golf course, and contained only common carp *Cyprinus carpio* and tench *Tinca tinca*.

Table 6.1 Description and location (WGS084 latitude, longitude) of ponds selected as study sites, including number of fish stocked to give an equal density of 100 fish per ha, and other fish species present: northern pike *Esox lucius*, Eurasian perch *Perca fluviatilis*, European catfish *Silurus glanis*, common carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella*, crucian carp *Carassius carassius*, goldfish *Carassius auratus*, orfe *Leuciscus idus*, roach *Rutilus rutilus*, rudd *Scardinius erythrophthalmus*, silver bream *Blicca bjoerkna*, common bream *Abramis brama*, chub *Squalius cephalus*, tench *Tinca tinca*, gudgeon *Gobio gobio*.

Pond name	Location	Size (ha)	No. of rainbow trout and barbel stocked	Other fish species present
Hurricane	51.844722, 0.128611	0.22	22	perch, carp, rudd, silver bream, tench
Spitfire	51.835833, 0.140278	0.43	43	perch, European catfish, carp, grass carp, roach, rudd, tench, gudgeon
Hanbury Manor Pond 17	51.828056, 0.036389	0.47	47	carp, tench
Lysander	51.837222, 0.135556	0.66	66	perch, carp, crucian carp, goldfish, orfe, roach, rudd, common bream, chub, tench
Wellington	51.842222, 0.136111	2.45	245	pike, perch, carp, roach, gudgeon
Lancaster	51.845000, 0.126111	3.08	308	pike, carp, roach, rudd, common bream, gudgeon

Due to legal and ethical limitations on stocking of invasive fish species into UK water bodies, two non-invasive species were selected to act as a proxy for invasive fish. These species were chosen to represent fishes expected to show different behaviours and distributions within the lake. Barbel *Barbus barbus*, native to water courses in parts of eastern England, was chosen to represent a benthic-living cyprinid, with a broadly similar ecology to topmouth gudgeon *Pseudorasbora parva*, a current species of concern in the UK (Environment Agency 2014). Rainbow trout *Oncorhynchus mykiss,* non-native to the UK but legally permitted to be stocked in certain still waters, was selected to represent a more pelagic shoaling species. Neither of these species has been found capable of reproducing in still waters in the UK, so any specimens surviving at the study site after the experiment would pose no long-term ecological risk.

The fish assemblages of each pond were unlikely to contain specimens of either *B. barbus* or *O. mykiss*) because the long-term lease holder of angling rights has never captured or introduced either of these species in these human-made, private access ponds. Nonetheless, water samples were collected from each lake on 15 June 2018, prior to fish stocking, to confirm for the absence of eDNA of the two target species.

6.3.2 Primer design and testing

For both species, new primer pairs were designed for this study (Table 6.2). Primers were designed to amplify segments of the mitochondrial cytochrome *c* oxidase I (COI) gene, for which many sequences were available in the publicly-accessible Genbank database (https://www.ncbi.nlm.nih.gov/genbank/) for both species (*B. barbus,* n = 49; *O. mykiss*, n = 241; with no significant intra-specific variation). The NCBI Primer-BLAST software (Ye et al. 2012) was used for primer design.

Table 6.2 Primers designed for conventional PCR of rainbow trout *Oncorhynchus mykiss* and barbel *Barbus barbus*.

Species	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon target length (base pairs)
O. mykiss	ACAGCCCATGCCTTCGTTAT	ACTGAGAGATGGCTGGAGGT	361
B.barbus	CCGAATTAAGCCAACCCGGA	CCTGCCAAGTGGAGTGAGAA	346

Sensitivity tests were conducted on DNA from dorsal muscle tissue of *B. barbus* and *O. mykiss* specimens supplied by the UK Environment Agency, extracted using a QIAGen Blood and Tissue Extraction Kit (QIAGen, Hilden, Germany) to the manufacturer's protocol. The concentration of this extracted DNA was measured using a Nanodrop ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and calculated with the software ND-1000 v3.8.1. The DNA was diluted with deionised water to produce a standard series featuring 10-fold dilutions (10^0 to 10^{-9} ng/µL), with three replicate series run per species.

These sensitivity tests determined that the designed primers could reliably detect DNA at concentrations of 10^{-6} ng/µL (*O. mykiss*: 7.74×10^{-6} ng/µL; *B. barbus* 2.68×10^{-6} ng/µL), with all three replicates showing detection at this concentration (i.e. limit of quantitation, LOQ, defined here as detection in all replicates, as per Agersnap et al. (2017)). The limit of detection (LOD, here defined as the minimum amount of target DNA at which positive detections were recorded in one or more replicates, as per Tréguier et al. (2014)) for *O. mykiss* was 7.74×10^{-7} ng/µL, with this concentration being detectable in two of the three replicate series. For *B. barbus*, the LOD was unchanged from the LOQ. These LODs/LOQs are a similar order of magnitude to

those observed in the primers previously designed and tested for *Pseudorasbora. parva* (Davison et al. 2019 [Chapter 4 of this thesis]).

Specificity tests on the primers, conducted *in silico* using the NCBI Primer-BLAST tool, showed no species likely to occur in UK freshwater environments with fewer than three base pair matches to either set of designed primers. This level of 'acceptable mismatch' is the same as that used in recent papers on freshwater fish eDNA (e.g. Harper et al. 2019b).

Additionally, the *B. barbus* primers were tested in the laboratory against tissue-extracted DNA from four species of the same family (Cyprinidae) known to occur in some of the study sites: *Cyprinus carpio*, *Abramis brama*, *Rutilus rutilus* and *Scardinius erythrophthalmus*. No amplification of DNA of these species was observed. Additional laboratory sensitivity tests were not conducted for the *O. mykiss* primers as no other species in the same order (Salmoniformes) were deemed likely to occur at the study sites.

6.3.3 Fish stocking and field sampling

Simulating usual fishery practices, both fish species were acquired from commercial sources and maintained in holding tanks for up to one week, prior to being stocked into the six ponds on 27 June 2018. Both species were stocked into the six water bodies at a stocking density of 100 fish per ha. This low stocking level was chosen to represent a population that has been shown to be near the threshold level for fish trapping, e.g. for *P. parva* (Britton et al. 2011b), but was intended to be large enough for a high likelihood of survival of at least some of the newly introduced fish in the period between stocking and sampling, a concern as they could have been at increased risk of predation due to their naivety in new surroundings. Numbers stocked into each pond to attain the required density (22-308 fish of each species) are shown in Table 6.1. Mean total lengths of stocked fish were: *B. barbus* = 108 mm; *O. mykiss* = 115 mm. In each lake, fish were released at a single point, by operatives otherwise unconnected with the sampling design and with no knowledge of water sampling locations.

The number of trapping and water sampling locations in each pond was determined by the surface area, with four bankside locations for every 0.5 ha, as informed by previous experiments (Davison et al. 2017). As the smaller water bodies in this study were < 0.5 ha, water samples were collected from a minimum of four locations around those ponds. Sampling locations were situated at equal distances around the pond perimeter.

The eDNA sampling was conducted on 3 July 2018 (Hurricane, Spitfire, Lysander and Hanbury Manor ponds) and on 4 July 2018 (Wellington and Lancaster ponds), 6–7 days after fish stocking. Water samples were collected using a 500 ml polypropylene sampling cup on a 183 cm sampling pole (Camlab Ltd, Cambridge, UK), which between samples was disinfected with Microsol 3+ sterilising solution (Anachem Ltd, Luton, UK) and rinsed with de-ionised water. New sampling poles and cups were used for each pond, to ensure no contamination risk. The sample was collected by moving the sampling cup in a standardised manner from the bank, to the greatest extent reached by the pole (approximately 1.5 m from the shore), ensuring no contact with the bottom sediment. At each sampling location, three replicates of 1L water were obtained using the sampling cup and filtered through multiple Sterivex-GP 0.22 µm sterile filter cartridges (EMD Millipore, Billerica, MA, USA) using a sterile 50 ml syringe (Thermo Scientific) attached directly onto the cartridge's input opening. To ensure filtration of a constant volume (1L) in each replicate, multiple filters were used as required to account for varying rates of filter clogging (due to variation in turbidity) between ponds. Three cartridges were used to filter each 1L replicate in all ponds except for Wellington, where four cartridges were necessary.

Cartridges from each sampling location were sealed in individual plastic bags and immediately frozen (–20°C) for transportation back to the laboratory. For each pond, one sterilised bottle of de-ionised water from the laboratory was transported and stored in the same manner as the pond samples, and filtered immediately following the samples from that pond on the same equipment, to test for field contamination or contamination during the filtering process.

6.3.4 Conventional trapping and electrofishing

After water samples had been collected, crab nets of 60 cm length and 30 cm diameter, with 12 mm mesh (catalogue number 6200500, Lineaeffe, Bologna, Italy) were deployed for 24 hours at each sampling point. Date of deployment in the four smaller ponds was 3 July 2018; in Wellington pond 4 July 2018; and in Lancaster pond 5 July 2018. The traps were baited using fishmeal pellets. Upon recovery of the traps, all fish captured were recorded and measured (TL to nearest mm).

Immediately after recovery of the traps from each pond, electrofishing operations were conducted from a fibreglass boat powered by an electric motor by two professional operatives, using a generator-powered DC electrofishing unit. A transect route around the perimeter of the pond was followed at a constant speed (covering the entire pond in 15–45 mins). All fish of the target species were counted.

6.3.4 Laboratory processing of the pond-water samples

In the laboratory, DNA was extracted from the cartridges using a PowerWater Sterivex^{TM} DNA Isolation Kit (MoBio, Carlsbad, CA, USA), following the manufacturer's protocol, with a final elution volume of 100 mL. The multiple cartridges used at each location to filter 1 L of water were pooled at this stage. The extracted sample was then diluted 1:5 in deionised water to dilute potential inhibitors ((McKee, Spear, and Pierson, 2015), and a conventional PCR then performed.

Conventional PCRs were performed on 6 µl of DNA sample. The reaction mixture contained, 0.5 µM of each specific primer, 10 µl of HotStar Taq® Plus DNA polymerase 2× (Qiagen Fast Cycling PCR Kit) and 2 µl of Coral Load Fast Cycling Dye 10× (Qiagen), with de-ionised water added to obtain the total mixture volume of 20 µl. The cycling conditions were 95°C for 5 min, followed by 35 cycles of 96°C for 5 s, 62°C for 5 s and 68°C for 12 s, with a final extension at 72°C for 1 min. PCR products were visualised after 60 min of electrophoresis migration on 2% agarose gel, stained with SYBR^{TM} Gold Nucleic Acid Gel Stain (Invitrogen). Five cPCR replicates were analysed for each sample location.

To test for inhibition, samples from four locations in each pond were spiked with 0.01 ng of tissue-derived DNA of each species. These spiked samples were compared against controls of deionised water containing the same DNA quantity.

Contamination risk was reduced by undertaking the four stages of the process (extraction from tissue, extraction of environmental samples, PCR preparation, and post-PCR sample handling) in separate rooms of a molecular biology laboratory, observing strict anti-contamination procedures (no transfer of equipment between rooms; changing of lab coats when moving between rooms; thorough cleaning of all equipment and surfaces before and after use, and regular treating of equipment under UV light; use of sterile filter tips for pipettes).

6.4. Results

6.4.1 Detection results

There was partial success in *B. barbus* detection, i.e. in five of the six ponds (Table 6.3), with *B. barbus* detected in three ponds using eDNA sampling, i.e. at 50% of sampling locations in the two smaller water bodies (Spitfire and Lysander) and 17% of sampling locations in the largest pond (Lancaster). Partial success was also encountered in detecting *B. barbus* by conventional sampling methods, with specimens caught in traps in two ponds, and caught by electrofishing in one of those ponds and in another pond. In one pond (Wellington), *B. barbus* were not detected by any method. No positive detections were encountered from any of the negative control blanks.

Oncorhynchus mykiss eDNA was recorded in all six ponds (Table 6.4), and was widely distributed around the ponds (positive detections at 75–100% of sampling locations in the four ponds <1 ha, and 41–70% of sampling locations in the two larger ponds). Conversely, no specimens of this species were detected using trapping or electrofishing. No positive detections were encountered on any of the negative control 'field blanks'.

Table 6.3 Detections of barbel *Barbus barbus* by eDNA, trapping and electrofishing survey methods. Five PCR replicates were performed at each site.

Pond	No. of eDNA sampling and trapping sites	Sites with positive DNA detections (number of +ve PCR replicates at each site)	No. of fish trapped / No. of traps with positive detections	Electrofishing detections (No. of fish captured)
Hurricane	4	0	0/0	1
Spitfire	4	2 (5, 5)	0/0	0
Hanbury Manor Pond 17	4	0	2/2	0
Lysander	6	3 (5, 5, 4)	3/2	6
Wellington	20	0	0/0	0
Lancaster	24	4 (5, 5, 5, 5)	0/0	0

Table 6.4 Detections of rainbow trout *Oncorhynchus mykiss* by eDNA, trapping and electrofishing survey methods. Five PCR replicates were performed at each site.

Pond	No. of eDNA sampling and trapping sites	Sites with positive eDNA detections (number of +ve PCR replicates)	No. of fish trapped / No. of traps with positive detections	Electrofishing detections (No. of fish captured)
Hurricane	4	4 (5, 5, 5, 5)	0/0	0
Spitfire	4	3 (5, 5, 3)	0/0	0
Hanbury Manor Pond 17	4	4 (5, 5, 4, 4)	0/0	0
Lysander	6	6 (5 at all sites)	0/0	0
Wellington	20	14 (5 at all sites)	0/0	0
Lancaster	24	10 (5 at 9 sites, 3 at 1 site)	0/0	0

6.4.2 Analysis of economic costs

The economic costs of detecting each species by each of the methods (eDNA, trapping and electrofishing) were calculated for each pond. The method used and assumptions made to calculate these costs were:

1) *Cost of eDNA sampling*: Total cost comprises: i) time spent by a Cefas operative of intermediate pay grade to prepare equipment and to collect water samples in the field, following a sampling regime of four sample sites per 0.5 ha of pond area; ii) time spent by Cefas operative to analyse water samples, ceasing analysis after first detection of the species (assuming a sample analysis sequence by which samples collected at opposite ends of the pond are analysed first, in batches of four sample locations, and then intermediate gaps filled in sequentially so as to maximise chances of detection of spatially heterogenous DNA); iii) consumable costs necessary for continuing analysis until point of first detection, using the sample analysis sequence described above;

2) *Cost of trapping* (commercial cost): Total cost is based on the daily rate of £450 charged by a commercial fisheries consultant. As commercial agencies typically charge by a daily rate rather than an hourly rate, this represents the 'effective cost' of such work. For 24-hour trap deployment, two days are required (one for trap deployment, one for trap recovery)

3) *Cost of electrofishing* (commercial cost): Total cost is based on the daily rate charged by a commercial fisheries consultant. As commercial agencies typically charge by a daily rate rather than an hourly rate, this does not take time of first detection into account; and

4) *Combined cost of electrofishing and trapping* (commercial cost: Total cost for a commercial fisheries consultant to undertake combined trapping and electrofishing. This requires two days (one day for trap deployment, and one day for trap recovery and electrofishing operations).

Table 6.5 Economic costs of sampling for barbel *Barbus barbus* and rainbow trout *Oncorhynchus mykiss* by three methods (eDNA sampling, trapping and electrofishing) during June 2018. Costs are presented without parentheses (a return on resources invested) for those cases where positive detections were recorded by that method in that pond and within parentheses in cases where the species was not detected (i.e. losses). Costs calculated based on £120/hour are explained in footnotes for the conventional sampling methods, and incorporate equipment preparation time and time on site until time of first detection.

Species	Pond	eDNA sampling cost (£)	Trapping commercial cost (£)	Electrofishing commercial cost (£)	Combined trapping and electrofishing cost (£)
B.barbus	Hurricane	(616)	(900)[1]	450[1]	900[9]
	Spitfire	616	(900)[1]	(450)[2]	(900)[10]
	Hanbury Manor 17	(616)	900[1]	(450)[2]	900[11]
	Lysander	672	900[2]	450[4]	900[12]
	Wellington	(2991)	(900)[3]	(450)[5]	(900)[13]
	Lancaster	1597	(900)[3]	(450)[6]	(900)[14]
O. mykiss	Hurricane	616	(900)[1]	(450)[4]	(900)[15]
	Spitfire	616	(900)[1]	(450)[2]	(900)[10]
	Hanbury Manor 17	616	(900)[1]	(450)[2]	(900)[10]
	Lysander	672	(900)[2]	(450)[8]	(900)[16]
	Wellington	995	(900)[3]	(450)[5]	(900)[13]
	Lancaster	1094	(900)[3]	(450)[6]	(900)[14]

Costs per hour: [1] £80; [2] £90; [3] £195; [4] £85; [5] £100; [6] £110; [7] £85; [8] £95; [9] £160; [10] 170; [11] £130; [!2] £140; [13] £295; [14] 305; [15] £165; [16] £185.

Economically, the most cost-effective method for detecting *B. barbus* varied between ponds, with eDNA sampling (£615–£1600, dependent on pond size), trapping (£900, for 2 days of a fishery consultant's time) and electrofishing (£450, for one day of a consultant's time) each proving the most cost-effective method on ponds where other methods failed. The general trend was for eDNA sampling costs to become relatively greater as size of pond increased; this is because an increased number of samples needed to be collected, whereas the costs of a fishery consultant remains the same regardless of pond size.

The among-pond variations in eDNA sampling costs (Table 6.5) are due to: the different number of samples that required processing to point when a positive result was obtained; the time taken for sampling (greater on larger water bodies); and the number of filter cartridges necessary for sampling larger water bodies.

6.5 Discussion

The two species, stocked at the same densities, demonstrated very different detection rates by the three methods. The eDNA surveying for *O. mykiss* was successful in all six ponds, contrasting with no catches by trapping or electrofishing, demonstrated that eDNA surveying provided the best detection method for this pelagic-dwelling species. The results for *B. barbus* were less consistent, and consequently less easy to interpret. In some ponds, *B. barbus* were detected in the eDNA analysis of water samples but not by conventional methods, whereas in other ponds the opposite was the case, and in one pond (Wellington) there were no detections by any method. Such inter-species variability in detectability in eDNA surveying has implications for both the planning of single-species targeted surveys, and the interpretation of metabarcoding studies of fish communities.

There are several possible explanations for the greater success in detecting *O. mykiss* eDNA. As a species exhibiting more pelagic behaviour, their movements (and shedding of DNA into the water) might cover a greater area of the pond, thereby

dispersing eDNA to more sampling locations. This could also be aided by *O. mykiss* occupying higher positions in the water column than *B. barbus*, which would be expected to provide more opportunity for dispersal of eDNA on currents around the water body than might be expected for DNA shed by fish nearer the pond bottom. The patchy distribution of eDNA in ponds, with large variation on fine spatial scales, is an important consideration for sampling strategy. Cage experiments have shown a strong decrease in eDNA detection probability with distance from the cage, with most species of fish and amphibians undetectable from a few metres away (Harper et al. 2019a). The probability of eDNA detection of caged northern pike *Esox lucius* declined from 89% at 1 m distance to 57% at 10 m and 27% at 40 m (Dunker et al. 2016). The weather conditions during the week between fish stocking and water sampling were warm and still, with little wind and consequently little water mixing – in large still water bodies it may be advisable to conduct sampling on days following windy weather when trying to detect small populations.

Little information is available on the likely home range of juvenile *B. barbus* in lentic environments, but it is possible that the small number of stocked fish remained in localised locations in the pond, which may have been a considerable distance from any eDNA sampling site on the pond's perimeter. For example, fish inhabiting the centre of the largest pond (Lancaster) would have been 70 m from the nearest sampling point, in contrast to the smallest pond (Hurricane) in which any fish would have been within 30 m of a sampling point. Diel movements by young *B. barbus* are known to occur in river systems, appearing in denser shoals near the bank during darkness (Copp 2004; Copp et al. 2005a), with daytime distances from the bank ranging from 4 to 8 m (Bischoff and Scholten 1996). And given the propensity of young *B. barbus* to disperse during daylight hours (Bischoff and Scholten 1996), there may have been a reduced likelihood of encountering the species' eDNA during the daytime than at night.

In addition to the possibility of fish behaviour influencing the patchy distribution of eDNA in the ponds, the inter-species differences may have been influenced by different shedding rates. The quantity of DNA shed by an individual organism depends

on many factors, including developmental state (Maruyama et al. 2014), size (Klymus et al. 2015), and stress (Pilliod et al. 2014), and even differences in diet have been shown to produce 10-fold changes in DNA shedding rate in Asian carp *Hypophthalmichthys sp.* (Klymus et al. 2015), so equal biomasses of two fish species might be expected to shed different quantities of DNA into the environment.

Some of the ponds used in the present study were inhabited by predatory fish, and all were accessible to piscivorous birds. Whereas *B. barbus* were captured immediately following eDNA sampling in three of the ponds (and an individual was also caught in Lancaster pond by an angler on 1 August 2018, approximately five weeks after the study, providing confirmation of their continued persistence), no *O. mykiss* were observed in any pond following stocking. It is therefore possible that there were no individuals alive at time of eDNA sampling. The persistence time for eDNA in ponds is known to be variable and to depend on a suite of environmental factors (Bista et al. 2017), but as DNA fragments have been reported as persisting for 17–21 days after the organisms have been removed (Dejean et al. 2011), the *O. mykiss* DNA signal detected could have originated from fish predated soon after stocking.

The costs of eDNA sampling are determined in part by the number of sample replicates required, both in the field and in the laboratory, to provide a positive or negative result to the required degree of accuracy. A higher number of PCR replicates (minimum of 12; Harper et al. 2019) are now considered necessary to detect low abundance DNA in environmental samples than was typically used for earlier studies, increasing costs. The cost of analysis also depends on the methods used, as the conventional PCRs used in this study are less expensive than qPCRs (in terms of consumables and initial purchase of equipment).

The high economic costs for sampling that failed to provide positive detections in the present study may be considered as losses, and these would be expected to increase with increased fishing effort to achieve a detection as smaller populations are targeted (Furlan et al. 2019). The ultimate cost of sampling is influenced by various factors

associated with the duration of effort to achieve either a detection or a level of certainty (of absence) of the species that is acceptable to environmental managers upon which to make a management decision (Davison et al. 2019). Determining when to stop allocating resources to the search for a species and to declare the species as absent is a critical decision for environmental managers, which will vary depending on context.

The imperfect detection rates obtained by eDNA sampling for *B. barbus* (detections from only three ponds) suggest that 100 fish per hectare could be around the threshold population size for reliable detection of a small benthic fish. The number of locations in each pond recording a positive eDNA detection was also lower than for *O. mykiss* (16.6% to 50% for *B. barbus* compared to 41% to 100% for *O. mykiss*). Further work is needed to elucidate the threshold lowest number of fish that could be detected from their eDNA in a water body of a given area, and to determine the amount of sampling necessary to enable a 95% probability of detection. Water sampling for eDNA should be considered as part of the toolkit available to environmental managers, and for some species under some conditions will be the most effective method of detection available.

7. Discussion

7.1 Key findings of this thesis

The projects undertaken as part of this thesis, presented as chapters in roughly chronological order, represent a series of steps in method development from initial method development to its deployment in a range of environments (lentic and lotic systems) to answer applied questions in non-native fish management.

The key findings of this body of work are:

- Environmental DNA can represent an effective method of detecting freshwater fishes, including species present at low densities. This was demonstrated by the detection of a low population of topmouth gudgeon *Pseudorasbora parva* at a fishing lake in Kent, where an eradication attempt reduced fish density to a presumed low level (Chapter 3), and corresponds with many other studies which have achieved a greater detection rate with eDNA surveying than with other methods (e.g. Takahara et al. 2013; Hinlo et al. 2018; Nakagawa et al. 2018).

- The ability to detect these low populations means that the technique lends itself to assessment of eradication programmes, in which populations will be present at a low level. Analysis for eDNA was used to prove failure (Chapter 3) and demonstrate highly probable success (Chapter 4) of eradication attempts for *P. parva*. The utility of eDNA sampling as a method of assessing fish eradication success has been reported by Dunker et al. 2016 (northern pike *Esox lucius*), Furlan et al. 2019 (common carp *Cyprinus carpio*) and Robinson et al. 2019 (*P. parva*), in addition to Davison et al. 2017 and Davison et al. 2019 (*P. parva*, this study).

- The field trial (Chapter 6) demonstrated the difficulty in "proving a negative", with failure to detect *Barbus barbus* in three ponds at a density of 100 fish per hectare. This suggests that this density could be close to the eDNA detection threshold for this species (and also the detection threshold of the conventional

methods deployed). Such failure to detect a low density population is probably to be expected at times given the low rates of DNA dispersal reported in still water (Harper et al. 2019a; Li et al. 2019b), and the patchiness of DNA observed in the 1.4 ha infested pond in Chapter 3. Further work is needed to elucidate the sampling effort required to detect these low fish populations, with the caveat that any such study is likely to be very species-and site- specific.

- In comparison trials against conventional and quantitative PCR, the nested PCR protocol developed in Chapter 4 proved considerably more sensitive in the detection of low quantities of tissue-derived eDNA. Although used to test field samples in Chapters 4 and 5, the nested PCR did not provide any new detection locations beyond those provided by the first conventional PCR stage. However, as the target species were likely missing from many of those sampling sites (particularly the *P. parva* eradication sites), further work is needed to determine whether the increased sensitivity will translate into increased field detections.

- The investigation into sensitivity of conventional and quantitative PCR protocols (Chapter 4) suggested that conventional PCR could be equally as sensitive, despite the concerns over reliability of visualisation. This contradicts some studies which have found quantitative PCR to be more sensitive (e.g. Tréguier et al. 2014; Biggs et al. 2015; Piggott 2016), but it may be that it depends on the specific primers in question. Robinson et al. (2019) developed novel qPCR primers for *P. parva* which achieved a higher detection rate than the conventional PCR primers developed in this thesis (Chapter 2; published as Davison et al. 2017), demonstrating that primers used may have important effects on attribution of positive or negative status.

- The application of eDNA sampling to the mapping of a river catchment (Chapter 5) provided a rapid 3-day assessment of distribution which broadly matched the expected distribution based on extensive previous fieldwork input. This corroborates the findings of other studies which have shown the cost-

effectiveness of eDNA sampling at a catchment level (e.g. Nakagawa et al. 2018; Pont et al. 2018).

Methodological advances are likely to continue to increase the sensitivity of eDNA techniques, and in turn their applicability to non-native species management applications. Indeed, the recommended methods have evolved over the duration of this PhD. For example, water filtration (as used throughout this PhD) has been shown to be a more effective eDNA capture method than the precipitation protocols used in a number of early papers (e.g. Ficetola et al. 2008; Dejean et al. 2012), and is now almost universally recognised as a more effective method (P. Davison, personal discussions at DNAquaNet Working Group). The course of this PhD saw a shift from collecting water bottles to filter in the laboratory using a vacuum pump (Chapters 1-2) to filtering the water in the field using a Sterivex filter cartridge (Chapters 3-5), with consequent advantages in ease of collection and storage, and reduced contamination ability. This switch in filtering methods is in line with many recent studies, with the filter cartridges offering advantages in terms of contamination avoidance and ease of transport from the field, in addition to giving a high DNA yield (Spens et al. 2017).

As methods developed over the course of this PhD, one of the main inconsistencies in methodology evident between the Chapters is the analysis of different water volumes. Comparatively small volumes, of 80 mL from ponds at Crampmoor Fishery and just 30 mL from the experimental ponds (both Chapter 2), were sampled in the initial trials. The positive results demonstrated that such small water volumes can be effective, but more consistent positives (e.g. in the pond at Crampmoor Fishery with low density) may have been obtained if larger water volumes had been sampled. Similarly, the 100 mL subsamples filtered in the initial survey for *Pseudorasbora parva* in the fishing lakes (Chapter 3) gave patchy positive results only at one end of the infested lake – more consistent positives across that water body *may* have been achieved from larger volumes. From Chapter 4, larger volumes were filtered: three replicates of 300 ml in both Chapter 4 (fishing lakes) and Chapter 5 (rivers/streams). To maximise the possibility of detection of known low density fish in lakes in Chapter 6, three replicates of 1L was sampled. Published studies use a wide range of water

volumes (15 mL to over 6 L; Hunter et al. 2019), but mesocosm studies have shown that increased water volume increases the likelihood of capturing 'rare' DNA in a sample (e.g. probability of detection increased 4.4 times when increasing water volume by 4 times; Hunter et al. 2019). One of the key limitations on volume that can be filtered is the clogging of the filters by debris; this can be overcome by increasing pore size or by combining filters at the extraction stage, apparently without loss of DNA detection probability (Harper et al. 2019; Hunter et al. 2019).

To combat potential effects of inhibition in environmental samples, which can be particularly prevalent in ponds (Jane et al. 2015), in Chapters 3 and 5, extracted samples were diluted in water following recommendations of several authors (e.g. Biggs et al. 2015; McKee et al. 2015). This method is now not recommended, as it may further reduce the detection probability of 'rare' DNA in a sample, and Harper et al. (2019) review other methods such as different extraction protocols or the addition of bovine-serum albumen to PCR reactions. It is unknown whether detection probability would have been significantly affected in this study.

7.2 Development of the research field during this PhD, and future research questions

The field of environmental DNA research has grown considerably over the seven-year course of this PhD study. At the start of this PhD (March 2013), <25 papers on aquatic macrobial DNA had been published, although at that early stage these did include the first papers relating to detection of non-native freshwater fishes (Takahara et al. 2012, 2013). By 2021, many new papers on the subject are appearing each month, and indeed a peer-reviewed journal, *Environmental DNA*, devoted entirely to the topic was initiated in 2019 (Bernatchez 2019).

As of 2021, although eDNA surveying is increasingly being used in small-scale research projects, there are relatively few cases in which eDNA surveying has been

incorporated into routine monitoring programmes. This is particularly the case for non-native species, where considerable method validation that is needed before a method can be used in an applied context, particularly where the results have legal or resource implications. Non-native species, especially if newly arrived or undergoing eradication, may occur at low density and be difficult to detect when compared to other eDNA applications (for example, they may evade detection by metabarcoding community analyses, unlike the commoner species in the community) – but the consequences of a false negative detection are potentially greater. For example, failure to detect a species may lead to delays in management, enabling it to increase and leading to far greater management costs when it is discovered in the future, while premature declaration of an eradication (due to a false lack of evidence of the species persistence) can also be financially costly (Rout et al. 2009). Similarly, a false positive detection that leads to an unnecessary eradication operation could have serious unintended consequences for the environment (due to effects on non-target organisms), to landowners and livelihoods (e.g. due to temporary closure of fisheries), and to financial resources of agencies. Financial costs can be considerable, with eradication operations at three sites for *Pseudorasbora parva* costed at between £1.90 and £7.90 per m^2 of water surface (Britton et al. 2008).

The prime example in the UK where barriers to eDNA-based monitoring have been overcome is surveying for the great crested newt *Triturus cristatus*, a species of conservation importance which needs to be surveyed for before development is permitted. Following extensive method validation (Biggs et al. 2015; Buxton et al. 2017; Rees et al. 2017; Harper et al. 2018), eDNA surveying is now admissible as legal proof of the presence or absence of *T. cristatus* (Natural England 2015), and an eDNA analysis service is offered by a number of consultancies.

In a UK context, the seven-year duration of this PhD has seen considerable progress being built between academic researchers and monitoring agencies to bridge the gap between research studies and routine monitoring applications. This is continuing to grow under the auspices of, for example, the UK DNA Working Group and its associated specialist Technical Groups. Discussion at these forums often focusses on

the need for greater validation of the methods under a wider range of conditions; the need for increased clarification on the sampling effort required, such that it can be realistically incorporated into limited monitoring budgets; and the need for increased communication of method potential and limitations to end-user agencies (P. Davison, personal experience). One example of an effort to bridge this communication gap is the production of a clear scale by which to grade the degree of validation tests carried out on a PCR assay (by the DNAquaNet group), which enables stakeholders to evaluate published papers. In spite of the challenges, the next 5-10 years are likely to see increased uptake of eDNA as a survey method. There is considerable discussion currently about whether molecular monitoring could be used to effectively monitor new metrics for biodiversity or ecosystem function which could provide more powerful information than those metrics currently measured – for example when looking at disturbance gradients in benthic marine ecosystems.

With the global increase in non-native species introductions showing no sign of abating (Seebens et al. 2020), their management is likely to become increasingly important to avoid conservation, social and economic impacts. There are also increasing regulatory imperatives to manage non-native species appropriately in the UK and Europe, with assessments of their status required to monitor compliance with the Water Framework Directive (Boon et al. 2020), Marine Strategy Framework Directive (Long 2011), and the European Union Invasive Alien Species regulations (Beninde et al. 2015) which have been transposed into UK law. The UK Government's 25 Year Environment Plan (HM Government 2018) draws attention to the threats caused by non-native plants and animals, stating that "by adopting a policy of early and effective intervention, we can save time and money, and spare the environment from greater impacts from breaches in bio-security".

The emphasis is on detecting any new invaders as early as possible, and eDNA surveying is one element of the toolkit that may be appropriate for such monitoring. Monitoring can be focussed on sites at high risk of new regional introductions, such as ports and marinas in the case of marine non-native species, or fish farms and aquaculture facilities in the case of freshwater fish. If eradication of a population is the

desired outcome, before it successfully establishes, then rapid management action is required. For some species with significant impacts, UK rapid response plans have been formulated prior to their arrival in the country, with a good current terrestrial example being the Asian hornet *Vespa velutina*, whose isolated nests have swiftly been destroyed since the first records in 2016, preventing it from establishing in the country so far.

A hierarchy of measures are available to be deployed against aquatic invasive species, including freshwater fishes. For those species that have not yet arrived, horizon scanning is a useful exercise to identify potential new arrivals (Roy et al. 2014), which can then be risk screened or risk assessed to determine those likely to cause problems, enabling the prioritisation of resources for management (Mandrak and Cudmore 2015). The most cost-effective management methods will stop the species from arriving in the first place, by targeting the pathway of introduction via legislation or by education (e.g. information campaigns targeted at people using the aquatic environment, or the 'Be Plant Aware' programme informing gardeners about the risks posed by invasive plants).

Once an invasive animal or plant has established, the options could include attempted eradication (locally from one water body, or nationally), or control and containment. Both options are potentially costly, both in terms of resources and in collateral damage to native species, and the advantages and disadvantages need to be assessed using a risk-based decision framework (Britton et al., 2011). Freshwater fish eradications can be successful, typically using piscicides (or drainage of smaller water bodies), with many of the 77 eradication attempts reviewed by Rytwinski et al. (2019) reporting success. Invasive fish eradications should be prioritised at sites from which they can spread into other water courses, thereby acting to contain the population. It should be remembered that if a non-native species appears environmentally 'benign' at one location, it may be in a lag phase before a future population increase (Crooks and Soule 1999); it may also be a species which will benefit from future climatic conditions (Britton et al. 2010a).

It is generally agreed that targeted detections of a species of interest are currently best undertaken using species-specific PCR assays rather than metabarcoding, as differences in species detection reads obtained by metabarcoding are little understood (Bylemans et al. 2019; Wood et al. 2019). Future increases in metabarcoding sensitivity might make that approach as powerful for single-species detection in future (Taberlet et al. 2012b). Currently, surveillance programmes for newly-arrived non-native species, for example in ports or estuaries, would benefit from a combination of both methods, with metabarcoding of samples to search for a suite of potential arriving species (Sard et al. 2019) supported by single-species targeting of key problem species (P. Davison, personal discussions with experts at marine eDNA workshops).

The PCR protocol varies between studies, with quantitative PCR (qPCR) increasingly preferred over conventional PCR (cPCR). There is a widespread perception that qPCR has greater sensitivity (P. Davison, personal experience), and several studies have indeed found this to be the case (Tréguier et al. 2014; Biggs et al. 2015; Piggott 2016), despite their similar sensitivity demonstrated in Chapter 4 of this report (and published as Davison et al. 2019). In this PhD, cPCR was used for the majority of the studies (Chapter 2-6), with the addition of a nested qPCR stage in Chapters 4 and 5. The results of the comparative trials (Chapter 4), combined with lower financial cost (important in the context of developing monitoring protocols for agency use) provided justification for use of cPCR in the field trials in Chapter 6.

This thesis focusses entirely on presence/absence detection, but a key research question still under investigation is whether fish abundance can be accurately determined from eDNA signal, measured either as the number of reads of a species in metabarcoding studies, or the fluorescence signal obtained by qPCR analysis. Correlations have been found between fish abundance and eDNA signal. Lacoursiére-Roussel et al. (2016), comparing 12 lakes, found a significant positive correlation between relative fish abundance (measured as catch-per-unit-effort in gillnet surveys) and eDNA concentration. In contrast to other studies, they found a less pronounced

relationship with biomass (Lacoursiére-Roussel et al. 2016). A promising future development for assessment of populations using eDNA, which will be made possible with increased sensitivity of the technique, is likely to be detection of other genes which can be used for population genetics analysis (Adams et al. 2019).

A key knowledge gap is a clear understanding of the 'ecology of eDNA' (Barnes and Turner 2016) in terms of its dispersal and degradation in the aquatic medium. When laboratory procedures have been optimised, this knowledge gap might account for some 'false negatives' (as occurred in Chapter 6 of this thesis). Very few published studies report definite 'false-negatives', i.e. a lack of detections where they would definitely be expected; one such recent example concerns a failure to detect killer whales *Orcinus orca* from water samples collected in close proximity (10 -20 m) to observed animals (Pinfield et al. 2019). More analysis of the failures, as well as the successes, of eDNA detection are needed to advance the field. Environmental managers would benefit from clearer communication of both the potential benefits and limitations of the technique, and more field trials are needed to determine key elements of sampling strategy (such as the sampling effort required to attain a high probability of detection in different water bodies).

An environmental manager deciding whether to employ eDNA surveys or traditional methods, for example to assess whether a particular fish species is present in a complex of lakes, should base their decision on resources available and the relative need for proof of presence or absence. The cost of molecular methods in the laboratory needs to be weighed up against the costs of sample collection (whether by netting, trapping, electric fishing, or water sampling) for an acceptable probability of getting the correct positive or negative decision for each water body. This will depend on the species – with those with high levels of catchability and at high abundance likely to be best detected using conventional methods, but those elusive species at low abundance likely to be detected most effectively using eDNA surveys. It may be that a combined approach will be required; for example, a rapid eDNA survey to determine presence/absence in each water body, followed by conventional surveys to provide abundance information on those water bodies that generated positive detections.

Before starting on a survey in situations where a positive detection will have consequences (e.g. financial deployment of resources, implications for landowners, or environmental impacts of management actions), environmental managers should be aware of the need to set a threshold for what constitutes a positive eDNA detection (Jerde 2019). Is one detection in a single replicate enough to initiate action, or will further proof of captured specimens be necessary?

Based on current knowledge, the advice to environmental managers who have decided that eDNA surveys will be the best method to detect non-native freshwater fishes would be:

1) Use single-species targeted PCRs in cases where detection of a certain species is of key importance. Whereas if knowledge of the fish community is more important, or if conducting surveillance for a large number of potential non-native species, metabarcoding will be a more cost-effective method.

2) Either conventional or quantitative PCRs can be used, as sensitivity can be similar (see Chapter 4). A literature search should be conducted to discover if primers have been developed for the species in question and tested, preferably on field samples as well as in the laboratory. If not, then primers (for either cPCR or qPCR) will need to be their designed and tested, adding to the financial cost. Guidelines for designing and evaluating primers, such as the MIQE guidelines for qPCR (Minimum Information for Publication of Quantitative PCR Experiments; Bustin et al. 2009), should be followed to ensure robust and repeatable results.

3) Design the field sampling programme carefully with respect to optimising for the species (seasonal considerations, preferred habitat within the water bodies) and site (likely levels of water mixing). Despite several field studies (Chapter 6 of this thesis; Dunker et al. 2016; Harper et al. 2019a) which have shown that eDNA is patchily distributed in ponds and that detection probability declines within metres, it is still difficult to define the minimum amount of sampling required for a given species in a given water body. Advances in knowledge of eDNA 'ecology' (Barnes and Turner 2016) and occupancy modelling analyses (Neto et al. 2020) will increasingly be able to inform strategy. The general rule,

however, is that for species in low abundance in still waters, a high number of field replicates may need to be collected, increasing financial costs.

4) Use a protocol which filters a large volume of water (at least 1L), as opposed to precipitation-based methods, to increase probability of detecting species in low abundance (Harper et al. 2019b). Filtration in the field using cartridges (Spens et al. 2017) may be the most effective method, in comparison to transport of water to the laboratory for filtration there. Transport and storage should be considered carefully to avoid DNA degradation, e.g. from repeated freezing and thawing of samples.

5) Use a high number of PCR replicates (further increasing costs) to detect DNA in small quantities, due to stochasticity during amplification (Harper et al. 2018). Twelve qPCR replicates may be the minimum required (as per Harper et al. 2018) to ensure a high probability of detection.

6) Ensure that all appropriate controls are in place to ensure robustness of reported results. These include: rigorous contamination avoidance procedures, both in the field and in the laboratory; negative control samples collected in the field to test for potential contamination; positive controls such as addition of non-target DNA sequences to test that DNA is not lost at any stage of the storage, filtering or extraction process; positive and negative controls during PCR amplification. Unexpected positive results, or positive results which will have management action consequences, should be sequenced as a safeguard against unforeseen problems with primer specificity.

The potential for eDNA surveying to enhance our knowledge of the distribution of aquatic organisms is now beyond doubt. Future advances in knowledge and capabilities are likely to ensure its status as an important tool for monitoring non-native species and wider biodiversity, in many cases providing the best survey tool available.
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8. References

Adams, C.I., Hoekstra, L.A., Muell, M.R. and Janzen, F.J., 2019. A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA under field conditions. *Diversity*, 11 (4), 50.

Adams, C.I., Knapp, M., Gemmell, N.J., Jeunen, G.J., Bunce, M., Lamare, M.D. and Taylor, H.R., 2019. Beyond biodiversity: can environmental DNA (eDNA) cut it as a population genetics tool? *Genes*, 10 (3), 192.

Adjers, K, Appelberg, M., Eschbaum, R., Lappalainen, A., Minde, A., Repecka, R. and Thoresson, G., 2006. Trends in coastal fish stocks of the Baltic Sea. *Boreal Environment Research*, 11 (1), 13–25.

Adrian-Kalchhauser, I. and Burkhardt-Holm, P., 2016. An eDNA assay to monitor a globally invasive fish species from flowing freshwater. *PLoS One*, 11 (1), e0147558.

Agersnap, S., Larsen, W.B., Knudsen, S.W., Strand, D., Thomsen, P.F., Hesselsøe, M., Mortensen, P.B., Vrålstad, T. and Møller, P.R., 2017. Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. *PLoS One*, 12 (6), e0179261.

Allen, Y., Kirby, D.S., Copp, G.H. and Brazier, M., 2006. Toxicity of rotenone to topmouth gudgeon *Pseudorasbora parva* for eradication of this non-native species from a tarn in Cumbria, England. *Fisheries Management and Ecology*, 13 (5), 337–340.

Amberg, J.J., Merkes, C.M., Stott, W., Rees, C.B. and Erickson, R.A., 2019. Environmental DNA as a tool to help inform zebra mussel, *Dreissena polymorpha*, management in inland lakes. *Management of Biological Invasions*, 10 (1), 96–110.

Amoros, C., Rostan, J.C., Pautou, G. and Bravard, J.P., 1987. The reversible process concept applied to the environmental management of large river systems. *Environmental Management*, 11 (5), 607–617.

Anderson, L.W.J., 2005. California's reaction to *Caulerpa taxifolia*: a model for invasive species rapid response. *Biological Invasions*, 7 (6), 1003–1016.

Anderson, M.J., Gorley, R.N. and Clarke, K.R., 2008. *PERMANOVA+ for PRIMER: guide to software and statistical methods.* Plymouth: PRIMER-E.

Anderson, M.J. and Robinson, J., 2001. Permutation tests for linear models. *Australian and New Zealand Journal of Statistics*, 43, 75–88.

Andújar, C., Arribas, P., Yu, D.W., Vogler, A.P. and Emerson, B.C., 2018. Why the COI barcode should be the community DNA metabarcode for the metazoa. *Molecular Ecology*, 27, 3968-3975.

Antognazza, C.M., Britton, R.J., Read, D.S., Goodall, T., Mantzouratou, A., De Santis, V., Davies, P., Aprahamian, M., Franklin, E., Hardouin, E.A. and Andreou, D., 2020. Application of eDNA metabarcoding in a fragmented lowland river: Spatial and methodological comparison of fish species composition. *Environmental DNA*, 3 (2), 458-471.

Baker, C.S., Steel, D., Nieukirk, S. and Klinck, H., 2018. Environmental DNA (eDNA) from the wake of the whales: droplet digital PCR for detection and species identification. *Frontiers in Marine Science*, 5, 133.

Bakker, J., Wangensteen, O.S., Chapman, D.D., Boussarie, G., Buddo, D., Guttridge, T.L., Hertler, H., Mouillot, D., Vigliola, L. and Mariani, S., 2017. Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact. *Scientific Reports*, 7 (1), p.16886.

Barnes, M.A. and Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, 17 (1), pp.1-17.

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L. and Lodge, D.M., 2014. Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science and Technology*, 48 (3), 1819–1827.

Barry, S. and Elith, J., 2006. Error and uncertainty in habitat models. *Journal of Applied Ecology*, 43, 413–423.

Bašić, T, Copp, G.H., Edmonds-Brown, V.R., Keskin, E., Davison, P.I. and Britton, J.R., 2018. Limited trophic consequences of non-native sunbleak *Leucaspius delineatus* for native pond fishes in experimental ponds. *Biological Invasions*, 21 (1), 261–275.

Bayley, P.B. and Peterson, J.T., 2001. An approach to estimate probability of presence and richness of fish species. *Transactions of the American Fisheries Society,* 130 (4), 620–633.

Beninde, J., Fischer, M.L., Hochkirch, A. and Zink, A., 2015. Ambitious advances of the European Union in the legislation of invasive alien species. *Conservation Letters*, 8 (3), 199-205.

Bernatchez, L., 2019. Welcome to Environmental DNA! *Environmental DNA*, 1, 3–4.

Beyer, K., 2008. *Ecological implications of introducing* Leucaspius delineatus *(Heckel, 1843) and* Pseudorasbora parva *(Temminck and Schlegel, 1842) into inland waters in England.* Thesis (PhD). University of Hull.

Beyer, K., Copp, G.H. and Gozlan, R.E., 2007. Microhabitat use and interspecific associations of introduced topmouth gudgeon *Pseudorasbora parva* and native fishes in a small stream. *Journal of Fish Biology*, 71 (Suppl. D), 224–238.

Beyer, K., Gozlan, R.E. and Copp, G.H., 2010. Social network properties within a fish assemblage invaded by non-native sunbleak *Leucaspius delineatus*. *Ecological Modelling*, 221 (17), 2118–2122.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R.A., Foster, J., Wilkinson, J.W., Arnell, A., Brotherton, P. and Williams, P., 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*, 183, 19–28.

Billman, H.G., St-Hilaire, S., Kruse, C.G., Peterson, T.S. and Peterson, C.R., 2011. Toxicity of the piscicide rotenone to Columbia spotted frog and boreal toad tadpoles. *Transactions of the American Fisheries Society*, 140 (4), 919–927.

Blackman, R.C., Hänfling, B. and Lawson-Handley, L., 2018. The use of environmental DNA as an early warning tool in the detection of new freshwater invasive non-native species. *CAB Reviews*, 13 (010), 1-15.

Blanchet, S., 2012. The use of molecular tools in invasion biology: an emphasis on freshwater ecosystems. *Fisheries Management and Ecology*, 19 (2), 120–132.

Bohlin, T., Hamrin, S., Heggberget, T.G., Rasmussen, G. and Saltveit, S.J., 1989. Electrofishing – theory and practice with special emphasis on salmonids. *Hydrobiologia*, 173 (1), 9–43.

Bohmann, K., Evans, A., Gilbert, M.T.P., Carvalho, G.R., Creer, S., Knapp, M., Yu, D.W. and de Bruyn, M., 2014. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology and Evolution*, 29 (6), 358–367.

Boon, P. J., Clarke, S. A., & Copp, G. H. (2020). Alien species and the EU Water Framework Directive: a comparative assessment of European approaches. *Biological Invasions*, 22 (4), 1497-1512.

Boussarie, G., Bakker, J., Wangensteen, O.S., Mariani, S., Bonnin, L., Juhel, J.B., Kiszka, J.J., Kulbicki, M., Manel, S., Robbins, W.D. and Vigliola, L., 2018.

Environmental DNA illuminates the dark diversity of sharks. *Science Advances*, *4* (5), eaap9661.

Britton, J.R. and Shepherd, J.S., 2005. Biometric data to facilitate the diet reconstruction of piscivorous fauna. *Folia Zoologica*, 54 (1–2), 193–200.

Britton, J.R., Davies, G.D., Brazier, M. and Pinder, A.C., 2007. A case study on the population ecology of a topmouth gudgeon (*Pseudorasbora parva*) population in the UK and the implications for native fish communities. *Aquatic Conservation: Marine and Freshwater Ecosystems,* 17 (7), 749–759.

Britton, J.R., Brazier, M., Davies, G.D. and Chare, S.I., 2008. Case studies on eradicating the Asiatic cyprinid *Pseudorasbora parva* from fishing lakes in England to prevent their riverine dispersal. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 18 (6), 867–876.

Britton, J.R., Cucherousset, J., Davies, G.D., Godard, M.J. and Copp, G.H., 2010a. Non-native fishes and climate change: predicting species responses to warming temperatures in a temperate region. *Freshwater Biology*, 55 (5), 1130–1141.

Britton, J.R., Davies, G.D. and Brazier, M., 2010b. Towards the successful control of the invasive *Pseudorasbora parva* in the UK. *Biological Invasions*, 12 (1), 125–131.

Britton, J.R., Copp, G.H., Brazier, M. and Davies, G.D., 2011a. A modular assessment tool for managing introduced fishes according to risks of species and their populations, and impacts of management actions. *Biological Invasions*, 13 (12), 2847–2860.

Britton, J.R., Pegg, J. and Gozlan, R.E., 2011b. Quantifying imperfect detection in an invasive pest fish and the implications for conservation management. *Biological Conservation*, 144 (9), 2177–2181.

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M. and Mergeay, J., 2020. Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). *Journal of Fish Biology*, 98 (2), 399-414.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. and Vandesompele, J., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, 55, 611-622.

Buxton, A.S., Groombridge, J.J., Zakaria, N.B. and Griffiths, R.A., 2017. Seasonal variation in environmental DNA in relation to population size and environmental factors. *Scientific Reports*, 7, 46294.

Bylemans, J., Furlan, E.M., Pearce, L., Daly, T. and Gleeson, D.M., 2016. Improving the containment of a freshwater invader using environmental DNA (eDNA) based monitoring. *Biological Invasions*, 18 (10), 3081–3089.

Bylemans, J., Gleeson, D.M., Duncan, R.P., Hardy, C.M. and Furlan, E.M., 2019. A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environmental DNA*, 1, 402–414.

Cardás, J.B., Deconinck, D., Márquez, I., Torre, P.P., Garcia-Vazquez, E. and Machado-Schiaffino, G., 2020. New eDNA based tool applied to the specific detection and monitoring of the endangered European eel. *Biological Conservation*, *250*, 108750.

Casey, J.W., Bowser, P.R., Getchell, R.G. and Bain, M.B., 2012. Sensitivity of detecting environmental DNA. *Conservation Letters*, 5 (3), 240.

Clusa, L., Ardura, A., Fernández, S., Roca, A.A. and García-Vázquez, E., 2017. An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of salmonids in eDNA from water samples. *PeerJ* [online], 5, e3045.

Coles, T.F., Wortley, J.S. and Noble, P., 1985. Survey methodology for fish population assessment within Anglian water. *Journal of Fish Biology*, 27, 175–186.

Collins, R.A., Armstrong, K.F., Meier, R., Yi, Y., Brown, S.D.J., Cruickshank, R.H., Keeling, S. and Johnston, C., 2012. Barcoding and border biosecurity: identifying cyprinid fishes in the aquarium trade. *PLoS One* [online], 7 (1), e28381.

Collins, R.A, Armstrong, K.F., Holyoake, A.J. and Keeling, S., 2013. Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. *Biological Invasions*, 15 (6), 1209-1215.

Collins, R.A., Wangensteen, O.S., O'Gorman, E.J., Mariani, S., Sims, D.W. and Genner, M.J., 2018. Persistence of environmental DNA in marine systems. *Communications Biology*, 1(1), 185.

Copp, G.H., 2010. Patterns of diel activity and species richness in young and small fishes of European streams: a review of 20 years of point abundance sampling by electrofishing. *Fish and Fisheries*, 11 (4), 439–460.

Copp, G.H. and Garner, P., 1995. Evaluating microhabitat use of fish larvae and juveniles with Point Abundance Sampling. *Folia Zoologica*, 44, 145–158.

Copp, G.H., Fox, M.G. and Kováč, V., 2002. Growth, morphology and life history traits of a coolwater European population of pumpkinseed *Lepomis gibbosus. Archiv für Hydrobiologie*, 155, 585–614.

Copp, G.H., Bianco, P.G., Bogutskaya, N.G., Erős, T., Falka, I., Ferreira, M.T., Fox, M.G., Freyhof, J., Gozlan, R.E., Grabowska, J., Kováč, V., Moreno-Amich, R., Naseka, A.M., Peňáz, M., Povž, M., Przybylski, M., Robillard, M., Russell, I.C., Stakėnas, S., Šumer, S., Vila-Gispert, A. and Wiesner, C., 2005. To be, or not to be, a non-native freshwater fish? *Journal of Applied Ichthyology*, 21 (4), 242-262.

Copp, G.H., Wesley, K.J., Verreycken, H. and Russell, I.C., 2007. When an 'invasive' fish species fails to invade! Example of the topmouth gudgeon *Pseudorasbora parva. Aquatic Invasions* [online], 2 (2), 107–112.

Copp, G.H., Stakėnas, S. and Cucherousset, J., 2010a. Aliens vs. the natives: interactions between introduced *Lepomis gibbosus* and indigenous *Salmo trutta* in small streams of southern England. *In* Gido, K. B. and D. Jackson, eds. *Community Ecology of Stream Fishes: Concepts, Approaches and Techniques.* Bethesda, MD: American Fisheries Society, 347–370.

Copp, G.H., Vilizzi, L. and Gozlan, R.E., 2010b. Fish movements: the introduction pathway for topmouth gudgeon *Pseudorasbora parva* and other non-native fishes in the UK. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 20, 269–273.

Copp, G.H., Britton, J.R., Guo, Z., Edmonds-Brown, V.R., Pegg, J., Vilizzi, L. and Davison, P.I., 2017. Trophic consequences of non-native pumpkinseed *Lepomis gibbosus* for native pond fishes. *Biological Invasions*, 19 (1), 25–41.

Cowart, D.A., Renshaw, M.A., Gantz, C.A., Umek, J., Chandra, S., Egan, S.P., Lodge, D.M. and Larson, E.R., 2018. Development and field validation of an environmental DNA (eDNA) assay for invasive clams of the genus *Corbicula. Management of Biological Invasions*, 9 (1), 27-37.

Crane, L.C., Goldstein, J.S., Thomas, D.W., Rexroth, K.S. and Watts, A.W., 2021. Effects of life stage on eDNA detection of the invasive European green crab (*Carcinus maenas*) in estuarine systems. *Ecological Indicators*, *124*, 107412.

Crooks, J.A., Soulé, M.E. and Sandlund, O.T., 1999. *Lag times in population explosions of invasive species: causes and implications.* In Sandlund, O.T., Schei, P.J. and Viken, Å (eds) *Invasive species and biodiversity management*, pp. 103-125. Dordrecht/Boston/London: Kluwer Academic Publishers.

Currie, L.A., 1995. Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995). *Pure and Applied Chemistry*, 67 (10), 1699–1723.

Curtis, A.N., Tiemann, J.S., Douglass, S.A., Davis, M.A. and Larson, E.R., 2020. High stream flows dilute environmental DNA (eDNA) concentrations and reduce detectability. *Diversity and Distribution* [online]*,* doi: 10.1111/ddi.13196.

Darling, J.A., 2019. How to learn to stop worrying and love environmental DNA monitoring. *Aquatic Ecosystem Health & Management*, 22 (4), 440-451.

Darling, J.A. and Mahon, A.R., 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research*, 111 (7), 978-988.

Davies, G.D. and Britton, J.R., 2015. Assessing the efficacy and ecology of biocontrol and biomanipulation for managing invasive pest fish. *Journal of Applied Ecology*, 52 (5), 1264–1273.

Davison, P.I., Créach, V., Liang, W-J., Andreou, D., Britton, J.R. and Copp, G.H., 2016. Laboratory and field validation of a simple method for detecting four species of non-native freshwater fish using environmental DNA. *Journal of Fish Biology*, 89 (3), 1782–1793.

Davison, P.I., Copp, G.H., Créach, V., Vilizzi, L. and Britton, J.R., 2017. Applications of environmental DNA analysis to inform invasive fish eradication operations. *The Science of Nature*, 104 (3-4), 35.

Davison, P.I., Falcou-Préfol, M., Créach, V., Davies, G.D., Vilizzi, L. and Copp, G.H., 2019. Is it absent or is it present? A new highly-sensitive eDNA protocol to detect non-native fishes to inform management decisions. *Biological Invasions*, 21 (8), 2549–2560.

Deagle, B.E., Eveson, J.P. and Jarman, S.N., 2006. Quantification of damage in DNA recovered from highly degraded samples–a case study on DNA in faeces. *Frontiers in Zoology*, 3 (1), 11.

Defra, 2008. *The invasive non-native species framework strategy for Great Britain* [online]. London: Department for Environment, Food and Rural Affairs.

Defra, 2011. *Biodiversity 2020: A strategy for England's wildlife and ecosystem services* [online]. London: Department for Environment, Food and Rural Affairs.

Deiner, K. and Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. *PLoS One* [online], 9 (2), e88786.

Deiner, K., Walser, J.C., Mächler, E. and Altermatt, F., 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*, 183, 53–63.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P. and Miaud, C., 2011. Persistence of environmental DNA in freshwater ecosystems. *PLoS One* [online], 6 (8), e23398.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E. and 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 (4), 953–959.

De Ventura, L., Kopp, K., Seppälä, K. and Jokela, J., 2017. Tracing the quagga mussel invasion along the Rhine river system using eDNA markers: early detection and surveillance of invasive zebra and quagga mussels. *Management of Biological Invasions*, 8 (1), 101–112.

Di Muri, C., Lawson Handley, L., Bean, C.W., Li, J., Peirson, G., Sellers, G.S., Walsh, K., Watson, H.V., Winfield, I.J. and Hänfling, B., 2020. Read counts from environmental DNA (eDNA) metabarcoding reflect fish abundance and biomass in drained ponds. *Metabarcoding and Metagenomics*, 4, 97-112.

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K. and Yamanaka, H., 2015. 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 (9), 5601-5608.

Dougherty, M.M., Larson, E.R., Renshaw, M.A., Gantz, C.A., Egan, S.P., Erickson, D.M. and Lodge, D.M., 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *Journal of Applied Ecology*, 53 (3), 722–732.

Drake, J.M. and Lodge, D.M., 2004. Global hot spots of biological invasions: evaluating options for ballast-water management. *Proceedings of the Royal Society London B*, 271 (1545), 575–580.

Dunker, K.J., Sepulveda, A.J., Massengill, R.L., Olsen, J.B., Russ, O.L., Wenburg, J.K. and Anotovich, A., 2016. Potential of environmental DNA to evaluate northern pike (*Esox lucius*) eradication attempts: an experimental test and case study. *PLoS One* [online], 11 (9), e0162277.

Dunn, N., Priestley, V., Herraiz, A., Arnold, R. and Savolainen, V., 2017. Behavior and season affect crayfish detection and density inference using environmental DNA. *Ecology and Evolution*, 7 (19), 7777-7785.

Egan, S.P., Barnes, M.A., Hwang, C.-T., Mahon, A.R., Feder, J.L., Ruggiero, S.T., Tanner, C.E. and Lodge, D.M., 2013. Rapid invasive species detection by combining environmental DNA with Light Transmission Spectroscopy. *Conservation Letters*, 6 (6), 402-409.

Eichmiller, J.J., Bajer, P.G. and Sorenson, P.W., 2014. The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS One* [online], 9 (11), e112611.

Eichmiller, J.J., Best, S.E. and Sorensen, P.W., 2016. Effects of temperature and trophic state on degradation of environmental DNA in lake water. *Environmental Science and Technology*, 50 (4), 1859-1867.

Environment Agency, 2014. *Topmouth gudgeon eradication programme. UK Environment Agency Progress update: July 2014*. [online] Bristol: Environment Agency. Available from www.nonnativespecies.org/downloadDocument.cfm?id=1133 [Accessed: 15 November 2019]

Epanchin-Niell, R.S., 2017. Economics of invasive species policy and management. *Biological Invasions*, 19 (11), 3333-3354.

European Union, 2014. *Regulation (EU) no 1143/2014 of the European parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species.* Official Journal of the European Union 4.11.2014, L317, 35–55.

Evans, N.T., Shirey, P.D., Wieringa, J.G., Mahon, A.R. and Lamberti, G.F.A., 2017. Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries*, 42 (2), 90–99.

Farr-Cox, F., Leonard, S. and Wheeler, A., 1996. The status of the recently introduced fish *Leucaspius delineatus* (Cyprinidae) in Great Britain. *Fisheries Management and Ecology*, 3 (3), 193–199.

Ficetola, G.F., Miaud, C., Pompanon, F. and Taberlet, P., 2008. Species detection using environmental DNA from water samples. *Biology Letters*, 4 (4), 423–425.

Finlayson, B., Somer, W.L. and Vinson, M.R., 2010. Rotenone toxicity to rainbow trout and several mountain stream insects. *North American Journal of Fisheries Management*, 30 (1), 102–111.

Finnoff, D., Shogren, J.F., Leung, B. and Lodge, D., 2007. Take a risk: preferring prevention over control of biological invaders. *Ecological Economics*, 62 (2), 216–222.

Fobert, E., Fox, M.G., Ridgway, M. and Copp, G.H., 2011. Heated competition: how climate change will affect non-native pumpkinseed *Lepomis gibbosus* and native perch *Perca fluviatilis* interactions in the UK. *Journal of Fish Biology*, 79 (6), 1592−1607.

Fobert, E., Zięba, G., Vilizzi, L., Godard, M.J., Fox, M.G., Stakėnas, S. and Copp, G.H., 2013. Predicting non-native fish dispersal under conditions of climate change: case study in England of dispersal and establishment of pumpkinseed *Lepomis gibbosus* in a floodplain pond. *Ecology of Freshwater Fish*, 22 (1), 106–116.

Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A., Salling, A.B., Galatius, A., Orlando, L. and Gilbert, M.T.P., 2012. Investigating the potential use of environmental DNA (e-DNA) for genetic monitoring of marine mammals. *PLoS One* [online], 7 (8), e41781.

Frézal, L. and Leblois, R., 2008. Four years of DNA barcoding: current advances and prospects. *Infection, Genetics and Evolution*, 8 (5), 727–736.

Furlan, E.M., Gleeson, D., Wisniewski, C., Yick, J. and Duncan, R.P., 2019. eDNA surveys to detect species at very low densities: A case study of European carp eradication in Tasmania, Australia. *Journal of Applied Ecology*, 56 (11), 2505-2517.

Gallardo, B., Clavero, M., Sánchez, M.I. and Vilà, M., 2016. Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biology*, 22 (1), 151-163.

García-Díaz, P., Ramsey, D.S., Woolnough, A.P., Franch, M., Llorente, G.A., Montori, A., Buenetxea, X., Larrinaga, A.R., Lasceve, M., Álvarez, A. and Traverso, J.M., 2017. Challenges in confirming eradication success of invasive red-eared sliders. *Biological Invasions*, 19 (9), 2739-2750.

GBNNSS, 2015. *The Great Britain Invasive Non-native Species Strategy August 2015* [online]. York: The Great Britain Non-native Species Secretariat.

Geerts, A.N., Boets, P., van den Heede, S. and Goethals, P., 2018. A search for standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): a lab and field evaluation. *Ecological Indicators*, 84, 564–572.

Genovesi, P., Carboneras, C., Vila, M. and Walton, P., 2015. EU adopts innovative legislation on invasive species: a step towards a global response to biological invasions? *Biological Invasions*, 17 (5), 1307–1311.

Gingera, T.D., Bajno, R., Docker, M.F. and Reist, J.D., 2017. Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. *Management of Biological Invasions*, 8 (3), 287-300.

Godornes, C., Leader, B.T., Molini, B.J., Centurion-Lara, A. and Lukehart, S.A., 2007. quantitation of rabbit cytokine mRNA by real-time RT-PCR. *Cytokine*, 38 (1), 1–7.

Goldberg, C.S., Pilliod, D.S., Arkle, R.S. and Waits, L.P., 2011. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS One* [online], 6 (7), e22746.

Goldberg, C.S., Sepulveda, A., Ray, A., Baumgardt, J. and Waits, L.P., 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science*, 32 (3), 792–800.

Gozlan, R.E., Pinder, A.C. and Shelley, J., 2002. Occurrence of the Asiatic cyprinid *Pseudorasbora parva* in England. *Journal of Fish Biology*, 61 (1), 298–300.

Gozlan, R.E., St-Hilaire, S., Feist, S.W., Martin, P. and Kent, M.L., 2005. Biodiversity: disease threat to European fish. *Nature*, 435 (7045), 1046.

Gozlan, R.E., Andreou, D., Asaeda, T., Beyer, K., Bouhadad, R., Burnard, D., Caiola, N., Cakic, P., Djikanovic, V., Esmaeili, H.R., Falka, I., Golicher, D., Harka, A., Jeney, G., Kováč, V., Musil, J., Nocita, A., Povž, M., Poulet, N., Virbickas, T., Wolter, C., Tarkan, A.S., Tricarico, E., Trichkova, T., Verreycken, H., Witkowski, A., Zhang, C.G., Zweimueller, I. and Britton, J.R., 2010a. Pan-continental invasion of *Pseudorasbora parva*: towards a better understanding of freshwater fish invasions. *Fish and Fisheries*, 11 (4), 315–340.

Gozlan, R.E., Britton, J.R., Cowx, I. and Copp, G.H., 2010b. Current knowledge on non-native freshwater fish introductions. *Journal of Fish Biology*, 76 (4), 751–786.

Guillera-Arroita, G., Lahoz-Monfort, J.J., Van Rooyen, A.R., Weeks, A.R. and Tingley, R., 2017. Dealing with false-positive and false-negative errors about species occurrence at multiple levels. *Methods in Ecology and Evolution*, 8 (9), 1081–1091.

Gustavson, M.S., Collins, P.C., Finarelli, J.A., Egan, D., Conchúir, R.Ó., Wightman, G.D., King, J.J., Gauthier, D.T., Whelan, K., Carlsson, J.E.L. and Carlsson, J., 2015. An eDNA assay for Irish *Petromyzon marinus* and *Salmo trutta* and field validation in running water. *Journal of Fish Biology*, 87 (5), 1254–1262.

Hänfling, B., Handley, L.L., Read, D.S., Hahn, C., Li, J., Nichols, P., Blackman, R.C., Oliver, A. and Winfield, I.J., 2016. Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*, 25 (13), 3101–3119.

Harper, L.R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H.C., Gough, K.C., Lewis, E., Adams, I.P., Brotherton, P., Phillips, S. and 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 (12), 6330–6341.

Harper, L.R., Buxton, A.S., Rees, H.C., Bruce, K., Brys, R., Halfmaerten, D., Read, D.S., Watson, H.V., Sayer, C.D., Jones, E.P. and Priestley, V., 2019a. Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia*, 826 (1), 25-41.

Harper, L.R., Griffiths, N.P., Lawson Handley, L., Sayer, C.D., Read, D.S., Harper, K.J., Blackman, R.C., Li, J. and Hänfling, B., 2019b. Development and application of environmental DNA surveillance for the threatened crucian carp (*Carassius carassius*). *Freshwater Biology*, 64 (1), 93–107.

Harrison, J.B., Sunday, J.M. and Rogers, S.M., 2019. Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B*, 286 (1915), 20191409.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and deWaard, J.R., 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society London B*, 270 (1512), 313–320.

Hinlo, R., Furlan, E., Suitor, L. and Gleeson, D., 2017a. Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. *Management of Biological Invasions*, 8 (1), 89–100.

Hinlo, R., Gleeson, D., Lintermans, M. and Furlan, E., 2017b. Methods to maximise recovery of environmental DNA from water samples. *PloS One*, 12 (6), e0179251.

Hinlo, R., Lintermans, M., Gleeson, D., Broadhurst, B. and Furlan, E., 2018. Performance of eDNA assays to detect and quantify an elusive benthic fish in upland streams. *Biological Invasions*, 20 (11), 3079-3093.

HM Government, 2018. *A green future: our 25 year plan to improve the environment.* [online]. London: HM Government.

Hubert, N, Hanner, R, Holm, E, Mandrak, N.E., Taylor, E, Burridge, M., Watkinson, D., Dumont, P., Curry, A., Bentzen, P., Zhang, J., April, J. and Bernatchez, L., 2008. Identifying Canadian freshwater fishes through DNA barcodes. *PLoS One* [online], 3 (6): e2490.

Hulme, P.E., 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. *Journal of Applied Ecology*, 46 (1), 10–18.

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J.A., Smith, B.J., Hunter, C.T., Reed, R.N. and Hart, K.M., 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS One* [online], 10 (4): e121655.

Hunter, M.E., Dorazio, R.M., Butterfield, J.S.S., Meigs-Friend, G., Nico, L.G. and Ferrante, J.A., 2017. Detection limits of quantitative and digital PCR assays and their influence in presence-absence surveys of environmental DNA. *Molecular Ecology Resources*, 17 (2), 221–229.

Hunter, M.E., Ferrante, J.A., Meigs-Friend, G. and Ulmer, A., 2019. Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. *Scientific Reports*, 9 (1), 1-9.

Huveneers, C., Apps, K., Becceri-Garcia, E.E., Bruce, B., Butcher, P.A., Carlisle, A., Chapple, T., Christiansen, H., Cliff, G., Curtis, T. and Daly-Engel, T., 2018. Future research directions on the 'elusive' white shark. *Frontiers in Marine Science*, 5, 455.

Jackson, D.A. and Harvey, H.H., 1997. Qualitative and quantitative sampling of lake fish communities. *Canadian Journal of Fisheries and Aquatic Sciences*, 54 (12), 2807–2813.

Jackson, M., Myrholm, C., Shaw, C. and Ramsfield, T., 2017. Using nested PCR to improve detection of earthworm eDNA in Canada. *Soil Biology and Biochemistry*, 113, 215–218.

Jackson, M.C., Britton, J.R., Cucherousset, J., Guo, Z., Stakėnas, S., Gozlan, R.E., Godard, M.G., Roussel, J.M. and Copp, G.H., 2016. Do non-native pumpkinseed *Lepomis gibbosus* affect the growth, diet and trophic niche breadth of native brown trout *Salmo trutta*? *Hydrobiologia*, 772 (1), 63–75.

Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.L., Letcher, B.H. and Whiteley, A.R., 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, 15 (1), 216-227.

Janosik, A. M. and Johnston, C. E., 2015. Environmental DNA as an effective tool for detection of imperilled fishes. *Environmental Biology of Fishes*, 98 (8), 1889–1893.

Jerde, C.L., 2019. Can we manage fisheries with the inherent uncertainty from eDNA? *Journal of Fish Biology*, 98 (2), 341-353.

Jerde, C.L. and Mahon, A.R., 2015. Improving confidence in environmental DNA species detection. *Molecular Ecology Resources*, 15 (3), 461–463.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. and Lodge, D.M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, 4 (2), 150–157.

Jerde, C.L., Miller, D.J., Mahon, A.R., Chadderton, W.L. and Lodge, D.M., 2012. Response to Casey et al.'s sensitivity of detecting environmental DNA comment. *Conservation Letters*, 5 (3), 241–242.

Jerde, C.L., Chadderton, W.L., Mahon, A.R., Renshaw, M.A., Corush, J., Budny, M.L., Mysorekar, S. and Lodge, D.M., 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences*, 70 (4), 522–526.

Kalinowski, S.T., Muhlfeld, C.C., Guy, C.S. and Cox, B., 2010. Founding population size of an aquatic invasive species. *Conservation Genetics*, 11 (5), 2049-2053.

Kelly, R.P., Port, J.A., Yamahara, K.M. and Crowder, L.B., 2014. Using environmental DNA to census marine fishes in a large mesocosm. *PLoS One* [online], 9 (1), e86175.

Kéry, M. and Schmidt, B., 2008. Imperfect detection and its consequences for monitoring for conservation. *Community Ecology*, 9 (2), 207–216.

Keskin, E., 2014. Detection of invasive freshwater fish species using environmental DNA survey. *Biochemical Systematics and Ecology*, 56, 68–74.

Keskin, E., Unal, E.M. and Atar, H.H., 2016. Detection of rare and invasive freshwater fish species using eDNA pyrosequencing: Lake Iznik ichthyofauna revised. *Biochemical Systematics and Ecology*, 67, 29–36.

Klara, M., Copp, G.H. and Horsfield, 2004. Autumnal habitat use of non-native pumpkinseed *Lepomis gibbosus* and associations with native fish species in small English streams. *Folia Zoologica*, 53 (2), 189–202.

Klymus, K. E., Richter, C. A., Chapman, D. C. and Paukert, C., 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation*, 183, 77–84.

Lacoursière-Roussel, A., Côté, G., Leclerc, V. and Bernatchez, L., 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *Journal of Applied Ecology*, 53 (4), 1148–1157.

Lance, R.F. and Guan, X., 2020. Variation in inhibitor effects on qPCR assays and implications for eDNA surveys. *Canadian Journal of Fisheries and Aquatic Sciences*, 77 (1), 23-33.

Laramie, M.S., Pilliod, D.S. and Goldberg, C.S., 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation*, 183, 29–37.

Lever, C., 1977. *The naturalised animals of the British Isles.* London: Hutchinson and Co.

Lewin, H.A., Robinson, G.E., Kress, W.J., Baker, W.J., Coddington, J., Crandall, K.A., Durbin, R., Edwards, S.V., Forest, F., Gilbert, M.T.P. and Goldstein, M.M., 2018. Earth Biogenome Project: sequencing life for the future of life. *Proceedings of the National Academy of Sciences*, 115 (17), 4325–4333.

Li, W., Zhang, T. and Li, Z., 2010. Spatial distribution and abundance of small fishes in Xiaosihai Lake, a shallow lake along the Changjiang (Yangtze) River, China. *Chinese Journal of Oceanology and Limnology*, 28 (3), 470–477.

Li, J., Hatton-Ellis, T.W., Lawson Handley, L.J., Kimbell, H.S., Benucci, M., Peirson, G. and Hänfling, B., 2019a. Ground-truthing of a fish-based environmental DNA metabarcoding method for assessing the quality of lakes. *Journal of Applied Ecology*, 56 (5), 1232-1244.

Li, J., Lawson Handley, L.J., Harper, L.R., Brys, R., Watson, H.V., Di Muri, C., Zhang, X. and Hänfling, B., 2019b. Limited dispersion and quick degradation of environmental DNA in fish ponds inferred by metabarcoding. *Environmental DNA*, 1 (3), 238-250.

Lodge, D.M., Stein, R.A., Brown, K.M., Covich, A.P., Brönmark, C., Garvey, J.E. and Klosiewski, S.P., 1998. Predicting impact of freshwater exotic species on native biodiversity: challenges in spatial scaling. *Australian Journal of Ecology*, 23 (1), 53–67.

Lodge, D.M., Turner, C.R., Jerde, C.L., Barnes, M.A., Chadderton, L., Egan, S.P., Feder, J.L., Mahon, A.R. and Pfrender, M.E., 2012. Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Molecular Ecology*, 21 (11), 2555–2558.

Long, R., 2011. The Marine Strategy Framework Directive: a new European approach to the regulation of the marine environment, marine natural resources and marine ecological services. *Journal of Energy & Natural Resources Law*, 29 (1), 1-44.

Mackenzie, D., 2005. Was it there? Dealing with imperfect detection for species presence/absence data. *Australia and New Zealand Journal of Statistics*, 47 (1), 65–74.

Mahon, A.R., Barnes, M.A., Li, F., Egan, S.P., Tanner, C.E., Ruggiero, S.T., Feder, J.L. and Lodge, D.M., 2013a. DNA-based species detection capabilities using laser transmission spectroscopy. *Journal of The Royal Society Interface*, 10 (78), 20120637.

Mahon, A.R., Jerde, C.L., Galaska, M., Bergner, J.L., Chadderton, W.L., Lodge, D.M., Hunter, M.E. and Nico, L.G., 2013b. Validation of e-DNA surveillance sensitivity for detection of Asian carps in controlled and field experiments. *PLoS One* [online], 8 (3), e58316.

Mandrak, N.E. and Cudmore, B., 2015. Risk assessment: cornerstone of an aquatic invasive species program. *Aquatic Ecosystem Health & Management*, 18(3), 312-320.

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M. and Minamoto, T., 2014. The release rate of environmental DNA from juvenile and adult fish. *PLoS One* [online], 9 (12), e114639.

Matter, A.N., Falke, J.A., López, J.A. and Savereide, J.W., 2018. A rapid-assessment method to estimate the distribution of juvenile chinook salmon in tributary habitats using eDNA and occupancy estimation. *North American Journal of Fisheries Management*, 38 (1), 223-236.

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C. and Sweet, M., 2019. Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. *Scientific Reports*, 9 (1), 1-9.

McKee, A.M., Spear, S.F. and Pierson, T.W., 2015. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision and inhibition of environmental DNA samples. *Biological Conservation*, 183, 70–76.

McNeely, J.A., Mooney, H.A., Neville, L.E., Schei, P.J. and Waage, J.K., 2001. *A global strategy on invasive alien species*. Gland, Switzerland and Cambridge, UK: International Union for Conservation of Nature.

Mehta, S.V., Haight, R.G., Homans, F.R., Polasky, S. and Venette, R.C., 2007. Optimal detection and control strategies for invasive species management. *Ecological Economics*, 61 (2-3), 237–245.

Merkes, C.M., McCalla, S.G., Jensen, N.R., Gaikowski, M.P. and Amberg, J.J., 2014. Persistence of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA data. *PLoS One* [online], 9 (11), e113346.

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M.N. and Kawabata, Z., 2012. Surveillance of fish species composition using environmental DNA. *Limnology*, 13 (2), 193–197.

Minamoto, T., Naka, T., Moji, K. and Maruyama, A., 2016. Techniques for the practical collection of environmental DNA: filter selection, preservation and extraction. *Limnology*, 17 (1), 23–32.

Miserez, R., Pilloud, T., Cheng, X., Nicolet, J., Griot, C. and Frey, J., 1997. Development of a sensitive nested PCR method for the specific detection of *Mycoplasma mycoides* subsp. *mycoides* SC. *Molecular and Cellular Probes*, 11 (2), 103–111.

Moyer, G.R., Díaz-Ferguson, E., Hill, J.E., and Shea, C., 2014. Assessing environmental DNA detection in controlled lentic systems. *PLoS One* [online], 9 (7), e103767.

Muha, T.P., Robinson, C.V., Garcia de Leaniz, C. and Consuegra, S., 2019. An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume. *PloS One*, 14 (7), e0219218.

Nagler, M., Insam, H., Pietramellara, G. and Ascher-Jenull, J., 2018. Extracellular DNA in natural environments: features, relevance and applications. *Applied Microbiology and Biotechnology*, 102 (15), 6343-6356.

Nakagawa, H., Yamamoto, S., Sato, Y., Sado, T., Minamoto, T. and Miya, M., 2018. Comparing local-and regional-scale estimations of the diversity of stream fish using eDNA metabarcoding and conventional observation methods. *Freshwater Biology*, 63 (6), 569-580.

Nathan, L.M., Simmons, M., Wegleitner, B.J., Jerde, C.L. and Mahon, A. R., 2014. Quantifying environmental DNA signals for aquatic invasive species across multiple detection platforms. *Environmental Science and Technology*, 48 (21), 12800–12806.

National Center for Biotechnology Information, 2013. *GenBank overview* [online]. Bethesda, Maryland, USA: National Center for Biotechnology Information. Available from: http://www.ncbi.nlm.nih.gov/genbank/ [Accessed 15 November 2019].

Natural England, 1997. *River Test SSSI citation* [online]. Peterborough: Natural England. Available from: https://designatedsites.naturalengland.org.uk/PDFsForWeb/ Citation/2000170.pdf [Accessed 15 November 2019].

Natural England, 2015. *Great crested newts, surveys and mitigation for development projects* [online]. Peterborough: Natural England. Available from: https://www.gov.uk/guidance/great-crested-newts-surveys-and-mitigation-for-development-projects [Accessed 15 November 2019].

Neto, J.G.D.S., Sutton, W.B., Spear, S.F., Freake, M.J., Kéry, M. and Schmidt, B.R., 2020. Integrating species distribution and occupancy modeling to study hellbender (*Cryptobranchus alleganiensis*) occurrence based on eDNA surveys. *Biological Conservation*, 251, 108787.

Olson, Z.H., Briggler, J.T. and Williams, R.N., 2012. An e-DNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water. *Wildlife Research*, 39 (7), 629–636.

Packer, L., Gibbs, J., Sheffield, C., and Hanner, R., 2009. DNA barcoding and the mediocrity of morphology. *Molecular Ecology Resources*, 9 (s1), 42–50.

Padgett-Stewart, T.M., Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K. and Schwartz, M.K., 2016. An eDNA assay for river otter detection: a tool for surveying a semi-aquatic mammal. *Conservation Genetics Resources*, 8 (1), 5–7.

Piggott, M., 2016. Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and Evolution*, 6 (9), 2739–2750.

Pilliod, D.S., Goldberg, C.S., Arkle, R.S. and Waits, L.P., 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquaculture Sciences*, 70 (8), 1123–1130.

Pilliod, D.S., Goldberg, C.S., Arkle, R.S. and Waits, L.P., 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, 14 (1), 109-116.

Pinder, A. C. and Gozlan, R.E., 2003. Sunbleak and topmouth gudgeon — two new additions to Britain's freshwater fishes. *British Wildlife*, 15 (2), 77–83.

Pinfield, R., Dillane, E., Runge, A.K.W., Evans, A., Mirimin, L., Niemann, J., Reed, T.E., Reid, D.G., Rogan, E., Samarra, F.I. and Sigsgaard, E.E., 2019. False-negative detections from environmental DNA collected in the presence of large numbers of killer whales (*Orcinus orca*). *Environmental DNA*, 1 (4), 316-328.

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H. and Dejean, T., 2018. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific Reports*, 8 (1), 10361.

Pot, W., Noakes, D.L.G., Ferguson, M.M., and Coker, G., 1984. Quantitative sampling of fishes in a simple system: failure of conventional methods. *Hydrobiologia*, 114 (3), 249–254.

Pyšek, P., Hulme, P.E., Simberloff, D., Bacher, S., Blackburn, T.M., Carlton, J.T., Dawson, W., Essl, F., Foxcroft, L.C., Genovesi, P., Jeschke, J.M., Kühn, I., Liebhold, A.M., Mandrak, N.E., Meyerson, L.A., Pauchard, A., Pergl, J., Roy, H.E., Seebens, H., van Kleunen, M., Vilà, M., Wingfield, M.J and Richardson, D.M., 2020. Scientists' warning on invasive alien species. *Biological Reviews*, 95, 1511-1534.

Rameckers, J., Hummel, S. and Herrmann, B., 1997. How many cycles does a PCR need? Determinations of cycle numbers depending on the number of targets and the reaction efficiency factor. *Naturwissenschaften*, 84 (6), 259–262.

Rees, H.C., Bishop, K., Middleditch, D.J., Patmore, J.R., Maddison, B.C. and Gough, K.C., 2014a. The application of eDNA for monitoring of the great crested newt in the UK. *Ecology and Evolution*, 4 (21), 4023–4032.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R. and Gough, K., 2014b. The detection of aquatic animal species using environmental DNA– a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, 51 (5), 1450–1459.

Rees, H.C., Baker, C.A., Gardner, D.S., Maddison, B.C. and Gough, K.C., 2017. The detection of great crested newts year round via environmental DNA analysis. *BMC Research Notes,* 10 (1), 327.

Robinson, C.V., Garcia de Leaniz, C., Rolla, M. and Consuegra, S., 2019. Monitoring the eradication of the highly invasive topmouth gudgeon (*Pseudorasbora parva*) using a novel eDNA assay. *Environmental DNA*, 1 (1), 74-85.

Rogers, M.W., Hansen, M.J. and Beard Jr, T.D., 2003. Catchability of walleyes to fyke netting and electrofishing in northern Wisconsin lakes. *North American Journal of Fisheries Management*, 23 (4), 1193–1206.

Rout, T.M., Thompson, C.J. and McCarthy, M.A., 2009. Robust decisions for declaring eradication of invasive species. *Journal of Applied Ecology*, 46 (4), 782–786.

Rout, T.M., Kirkwood, R., Sutherland, D.R., Murphy, S. and McCarthy, M.A., 2014. When to declare successful eradication of an invasive predator? *Animal Conservation*, 17 (2), 125–132.

Roy, H.E., Peyton, J., Aldridge, D.C., Bantock, T., Blackburn, T.M., Britton, R., Clark, P., Cook, E., Dehnen-Schmutz, K., Dines, T., Dobson, M., Edwards, F., Harrower, C., Harvey, M.C., Minchen, D., Noble, D.G., Parrott, D., Pocock, M.J.O., Preston, C.D., Roy, S., Salisbury, A., Schonrogge, K., Sewell, J., Shaw, R.H., Stebbing, P., Stewart, A.J.A. and Walker, K.J., 2014. Horizon scanning for invasive alien species with the potential to threaten biodiversity in Great Britain. *Global Change Biology*, 20 (12), 3859-3871.

Ruiz-Gutierrez, V., Hooten, M.B. and Grant, E.H.C., 2016. Uncertainty in biological monitoring: a framework for data collection and analysis to account for multiple sources of sampling bias. *Methods in Ecology and Evolution*, 7 (8), 900–909.

Ruppert, K.M., Kline, R.J. and Rahman, M.S., 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, 17 (1), e00547.

Rytwinski, T., Taylor, J.J., Donaldson, L.A., Britton, J.R., Browne, D.R., Gresswell, R.E., Lintermans, M., Prior, K.A., Pellatt, M.G., Vis, C. and Cooke, S.J., 2019. The effectiveness of non-native fish removal techniques in freshwater ecosystems: a systematic review. *Environmental Reviews*, 27 (1), 71-94.

Sales, N.G., McKenzie, M.B., Drake, J., Harper, L.R., Browett, S.S., Coscia, I., Wangensteen, O.S., Baillie, C., Bryce, E., Dawson, D.A., Ochu, E., Hänfling, B., Handley, L.L., Mariani, S., Lambin, X., Sutherland, C. and McDevitt, A.D., 2020. Fishing for mammals: Landscape-level monitoring of terrestrial and semi-aquatic communities using eDNA from riverine systems. *Journal of Applied Ecology*, 57 (4), 707-716.

Sales, N.G., Wangensteen, O.S., Carvalho, D.C., Deiner, K., Præbel, K., Coscia, I., McDevitt, A.D. and Mariani, S., 2021. Space-time dynamics in monitoring neotropical fish communities using eDNA metabarcoding. *Science of the Total Environment*, 754, 142096.

Sana, S., Williams, C., Hardouin, E.A., Blake, A., Davison, P., Pegg, J., Paley, R., Zhang, T. and Andreou, D., 2018. Phylogenetic and environmental DNA insights into emerging aquatic parasites: implications for risk management. *International Journal of Parasitology*, 48 (6), 473–481.

Sard, N.M., Herbst, S.J., Nathan, L., Uhrig, G., Kanefsky, J., Robinson, J.D. and Scribner, K.T., 2019. Comparison of fish detections, community diversity, and relative abundance using environmental DNA metabarcoding and traditional gears. *Environmental DNA*, 1 (4), 368-384.

Schenekar, T., Schletterer, M., Lecaudey, L.A. and Weiss, S.J., 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*, (7), 1004-1013.

Schneider, J., Valentini, A., Dejean, T., Montarsi, F., Taberlet, P., Glaizot, O. and Fumagalli, L., 2016. Detection of invasive mosquito vectors using environmental DNA (eDNA) from water samples. *PLoS One* [online], 11 (9), e0162493.

Seebens, H., Bacher, S., Blackburn, T.M., Capinha, C., Dawson, W., Dullinger, S., Genovesi, P., Hulme, P.E., van Kleunen, M., Kühn, I., Jeschke, J.M., Lenzner, B., Liebhold, A.M., Pattison, Z., Pergl, J., Pyšek, P., Winter, M. and Essl, F., 2020. Projecting the continental accumulation of alien species through to 2050. *Global Change Biology*, 27 (5), 970-982.

Serrao, N.R., Reid, S.M. and Wilson, C.C., 2017. Establishing detection thresholds for environmental DNA using receiver operator characteristics. *Conservation Genetics Resources*, 10 (3), 555–562.

Sevilla, R.G., Diez, A., Norén, M., Mouchel, O., Jérôme, M., Verrez-Bagnis, V., van Pelt, H., Favre-Krey, L., Krey, G., The FishTrace Consortium, and Bautista, J.M., 2007. Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome *b* and nuclear rhodopsin genes. *Molecular Ecology Notes*, 7 (5), 730–734.

Shogren, A.J., Tank, J.L., Andruszkiewicz, E., Olds, B., Mahon, A.R., Jerde, C.L. and Bolster, D., 2017. Controls on eDNA movement in streams: Transport, retention, and resuspension. *Scientific Reports*, 7, 1-11.

Shokralla, S., Spall, J.L., Gibson, J.F. and Hajibabaei, M., 2012. Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology*, 21 (8), 1794–1805.

Sigsgaard, E.E., Carl, H. and Møller, P.R., 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, 183, 46–52.

Simberloff, D., 2020. Maintenance management and eradication of established aquatic invaders. *Hydrobiologia* [online], doi: 10.1007/s10750-020-04352-5.

Simberloff, D., Parker, I.M. and Windle, P.N., 2005. Introduced species policy, management, and future research needs. *Frontiers in Ecology and the Environment*, 3 (1), 12–20.

Simon, C., Wiezer, A., Strittmatter, A.W. and Daniel, R., 2009. Phylogenetic diversity and metabolic potential revealed in a glacier ice metagenome. *Applied and Environmental Microbiology*, 75 (23), 7519–7526.

Simpson, T.J., Dias, P.J., Snow, M., Muñoz, J. and Berry, T., 2017. Real-time PCR detection of *Didemnum perlucidum* (Monniot, 1983) and *Didemnum vexillum* (Kott, 2002) in an applied routine marine biosecurity context. *Molecular Ecology Resources*, 17 (3), 443–453.

Smart, A.S., Weeks, A.R., Van Rooyen, A.R., Moore, A., McCarthy, M.A. and Tingley, R., 2016. Assessing the cost-efficiency of environmental DNA sampling. *Methods in Ecology and Evolution*, 7 (11), 1291–1298.

Spear, S.F., Groves, J.D., Williams, L.A. and Waits, L.P., 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation*, 183, 38–45.

Spens, J., Evans, A.R., Halfmaerton, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S., Sigsgaard, E.E. and Hellström, M., 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*, 8 (5), 635–645.

Stakėnas, S., Vilizzi, L. and Copp, G.H., 2013. Habitat use, home range, movements and interactions of introduced *Lepomis gibbosus* and native *Salmo trutta* in a small stream of Southern England. *Ecology of Freshwater Fish*, 22 (2), 202–215.

Stat, M., John, J., DiBattista, J.D., Newman, S.J., Bunce, M. and Harvey, E.S., 2019. Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology*, 33 (1), 196-205.

Stribling, J.B., Pavlik, K.L., Holdsworth, S.M. and Leppo, E.W., 2008. Data quality, performance, and uncertainty in taxonomic identification for biological

assessments. *Journal of the North American Benthological Society*, 27, 906–919.

Strickler, K.M., Fremier, A.K. and Goldberg, C.S., 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, 183, 85–92.

Sunardi, A.T. and Manatunge, J., 2005. Foraging of a small planktivore (*Pseudorasbora parva*: Cyprinidae) and its behavioural flexibility in an artificial stream. *Hydrobiologia*, 549 (1), 155–166.

Taberlet, P., Coissac, E., Hajibabaei, M. and Reiseberg, L.H., 2012a. Environmental DNA. *Molecular Ecology*, 21 (8), 1789–1793.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. and Willerslev, E., 2012b. Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, 21 (8), 2045–2050.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. and Kawabata, Z., 2012. Estimation of fish biomass using environmental DNA. *PLoS One* [online], 7 (4): e35868.

Takahara, T., Minamoto, T. and Doi, H., 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS One* [online], 8 (2): e56584.

Takahara, T., Minamoto, T. and Doi, H., 2015. Effects of sample processing on the detection rate of environmental DNA from the common carp (*Cyprinus carpio*). *Biological Conservation*, 183, 64–69.

Takahashi, M.K., Meyer, M.J., Mcphee, C., Gaston, J.R., Venesky, M.D. and Case, B.F., 2018. Seasonal and diel signature of eastern hellbender environmental DNA. *The Journal of Wildlife Management*, 82 (1), 217–225.

Thomsen, P.F. and Willerslev, E., 2015. Environmental DNA – an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, 183, 4–18.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Møller, P.R., Rasmussen, M. and Willerslev, E., 2012a. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One* [online], 7 (8): e41732.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L. and Willerslev, E., 2012b. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, 21 (11), 2565–2573.

Torsvik, V. and Øvreås, L., 2002. Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology*, 5 (3), 240–245.

Trebitz, A.S., Hoffman, J.C., Darling, J.A., Pilgrim, E.M., Kelly, J.R., Brown, E.A., Chadderton, W.L., Egan, S.P., Grey, E.K., Hashsham, S.A., Klymus, K.E., Mahon, A.R., Ram, J.L., Schulz, M.T., Stepien, C.A. and Schardt, J.C., 2017. Early detection monitoring for aquatic non-indigenous species: Optimizing surveillance, incorporating advanced technologies, and identifying research needs. *Journal of Environmental Management*, 202, 299-310.

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M.A. and 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 (4), 871–879.

Turner, C.R., Barnes, M.A., Xu, C.C.Y., Jones, S.E., Jerde, C.L. and Lodge, D.M., 2014. Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, 5 (7), 676–684.

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y. and Takeshita, M., 2017. Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, 17 (6), e63-e75.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F. and Gaboriaud, C., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, 25 (4), 929–942.

Vander Zanden, M.J., Hansen, G.J.A., Higgins, S.N., and Kornis, M.S., 2010. A pound of prevention, plus a pound of cure: early detection and eradication of invasive species in the Laurentian Great Lakes. *Journal of Great Lakes Research*, 36 (1), 199-205.

Verhelst, P., Boets, P., Van Thuyne, G., Verreycken, H., Goethals, P.L. and Mouton, A.M., 2016. The distribution of an invasive fish species is highly affected by the presence of native fish species: evidence based on species distribution modelling. *Biological Invasions*, 18 (2), 427–444.

Verreycken, H., Van Thuyne, G., and Belpaire, C., 2011. Length–weight relationships of 40 freshwater fish species from two decades of monitoring in Flanders (Belgium). *Journal of Applied Ichthyology*, 27 (6), 1416–1421.

Villeneuve, F., Copp, G.H., Fox, M.G. and Stakėnas, S., 2005. Interpopulation variation in growth and life-history traits of the introduced sunfish, pumpkinseed *Lepomis gibbosus*, in southern England*. Journal of Applied Ichthyology*, 21 (4), 275−281.

Wang, S., Yan, Z., Hänfling, B., Zheng, X., Wang, P., Fan, J. and Li, J., 2021. Methodology of fish eDNA and its applications in ecology and environment. *Science of the Total Environment*, 755 (2), 142622.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. and Hebert, P.D.N., 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 360 (1462), 1847−1857.

Weigand, H., Beermann, A.J., Čiampor, F., Costa, F.O., Csabai, Z., Duarte, S., Geiger, M.F., Grabowski, M., Rimet, F., Rulik, B.,Strand, M., Szucsich, N., Weigand, A.M., Willassen, E., Wyler, S.A., Bouchez, A., Borja, A., Čiamporová-Zaťovičová, Z., Ferreira, S., Dijkstra, K.B., Eisendle, U., Freyhof, J., Gadawski, P., Graf, W., Haegerbaeumer, A., van der Hoorn, B.B., Keresztes, B.J.L., Keskin, E., Leese, F., Macher, J.N., Mamos, T., Paz, G., Pešić, V., Pfannkuchen, D.M., Pfannkuchen, M.A., Price, B.W., Rinkevich, B., Teixeira, M.A.L., Várbíró, G. and Ekrem, T., 2019. DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *Science of the Total Environment*, 678, 499-524.

Wheeler, A. C. and Maitland, P.S., 1973. The scarcer freshwater fishes of the British Isles I. Introduced species. *Journal of Fish Biology*, 5 (1), 49−68.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R. and Schwartz, M.K., 2013. Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS One* [online], 8 (3): e59520.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Sepulveda, A.J., Shepard, B.B., Jane, S.F., Whiteley, A.R., Lowe, W.H. and Schwartz, M.K., 2016. Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char *Salvelinus fontinalis. Biological Conservation*, 194, 209−216.

Williams, F., Eschen, R., Harris, A., Djeddour, D., Pratt, C., Shaw, R.S., Varia, S., Lamontagne-Godwin, J., Thomas, S.E. and Murphy, S.T., 2010. *The economic cost of invasive non-native species on Great Britain* [online]. Wallingford, UK: CABI.

Willoughby, J.R., Wijayawardena, B.K., Sundaram, M., Swihart, R.K. and DeWoody, J.A., 2016. The importance of including imperfect detection models in eDNA experimental design. *Molecular Ecology Resources*, 16 (4), 837-844.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, 63 (10), 3741–3751.

Wimbush, J., Frischer, M.E., Zarzynski, J.W. and Nierzwicki-Bauer, S.A., 2009. Eradication of colonizing populations of zebra mussels (*Dreissena polymorpha*) by early detection and SCUBA removal: Lake George, NY. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 19 (6), 703–713.

Wineland, S.M., Welch, S.M., Pauley, T.K., Apodaca, J.J., Olszack, M., Mosher, J.J., Holmes, J.N. and Waldron, J.L., 2019. Using environmental DNA and occupancy modelling to identify drivers of eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) extirpation. *Freshwater Biology*, 64 (1), 208–221.

Wood, S.A., Pochon, X., Laroche, O., von Ammon, U., Adamson, J. and 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 (6), 1407-1419.

Yates, M.C., Fraser, D.J. and Derry, A.M., 2019. Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environmental DNA*, 1, 5–13.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, 13 (1), 134.

Zaiko, A., Martinez, J.L., Schmidt-Petersen, J., Ribicic, D., Samuiloviene, A. and Garcia-Vasquez, E., 2015. Metabarcoding approach for the ballast water surveillance – an advantageous solution or an awkward challenge? *Marine Pollution Bulletin*, 92 (1−2), 25−34.

Zaiko, A., Schimanski, K., Pochon, X., Hopkins, G.A., Goldstien, S., Floerl, O. and Wood, S.A., 2016. Metabarcoding improves detection of eukaryotes from early biofouling communities: implications for pest monitoring and pathway management. *Biofouling*, 32 (6), 671-684.

Zhang, S., Zhao, J. and Yao, M., 2020. A comprehensive and comparative evaluation of primers for metabarcoding eDNA from fish. *Methods in Ecology and Evolution*, 11 (12), 1609-1625.

Zięba, G., Copp, G.H., Davies, G.D., Stebbing, P., Wesley, K.J. and Britton, J.R., 2010a. Recent releases and dispersal of non-native fishes in England and Wales, with emphasis on sunbleak *Leucaspius delineatus* (Heckel, 1843). *Aquatic Invasions* [online], 5 (2), 155−161.

Zięba, G., Fox, M.G. and Copp, G.H., 2010b. The effect of elevated temperature on spawning frequency and spawning behaviour of introduced pumpkinseed *Lepomis gibbosus* in Europe. *Journal of Fish Biology*, 77 (8), 1850–1855.