University of Derby

Alternative methods for assessing habitat quality in freshwater systems

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Doctor of Philosophy 2020

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ABBREVIATIONS

16S	16S ribosomal RNA
AIC	Akaike Information Criterion
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CL	Chloroplast gene
COI	Cytochrome C Oxidase Subunit 1
Ct	Cycle threshold
CV	Coefficient of Variation
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
eDNA	Environmental DNA
FBA	Freshwater Biological Association
FNU	Formazine Nephelometric Unit
GPS	Global Positioning System
HCMR	Hellenic Center of Marine Research
IUCN	International Union for Conservation of Nature
LOD	Limit of Detection
LOQ	Limit of Quantification
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MT	Mitochondrial gene

NCBI	National Center for Biotechnology Information
ppt	parts per thousand
PSI	Pounds per Square Inch
qPCR	Quantitative PCR
UV	Ultra-Violet
USGS	United States Geological Survey
ZSL	Zoological Society of London

PREFACE

This work, including conceptual design of experiments, data collection and writing within this thesis, has been solely authored by the doctoral candidate with guidance from the supervisory team. Guidance for statistical analysis was provided by Dr Mark Bulling and Dr Alfred Burian.

All ethical considerations had been discussed and approved within the college research ethics committee of the University of Derby.

Where chapters have been published, the doctoral candidate has been the lead author, preparing the manuscript with only guidance from co-authors.

The work contained within this thesis has been disseminated through a number of channels, listed here:

Publications:

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

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., & Sweet, M. (2019). Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. Scientific Reports. doi: 10.1038/s41598-019-50571-9

Mauvisseau, Q., Kalogianni, E., Zimmerman, B., Bulling, M., Brys, R., & Sweet, M. (2020). eDNA based monitoring: advancement in management and conservation of critically endangered killifish species. Environmental DNA. doi: 10.1002/edn3.92

Mauvisseau, Q., Tönges, S., Andriantsoa, R., Lyko, F., & Sweet, M. (2019). Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems. Management of Biological Invasions, 10(3), 461–472. doi: 10.3391/mbi.2019.10.3.04

Mauvisseau, Q., Troth, C., Young, E., Burian, A., & Sweet, M. (2019). The development of an eDNA based detection method for the invasive shrimp *Dikerogammarus haemobaphes*. Management of Biological Invasions, 10(3), 449–460. doi: 10.3391/mbi.2019.10.3.03

Conferences:

June 2018. "Alternative methods for assessing habitat quality in freshwater systems". 3MT. Derby, UK

September 2018. Lecture on Environmental DNA 'eDNA' for CIEEM. Derby, UK

November 2018. "Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches". UK DNA working group. Derby, UK

April 2019. "Alternative methods for assessing habitat quality in freshwater systems".3MT. Derby, UK

June 2019. "Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches". Barcode of Life Conference. Trondheim, Norway

July 2019. "Using environmental DNA detection for improving conservation management plan of critically endangered fish species". FSBI conference. Hull, UK

Posters:

September 2017. "Alternative methods for assessing habitat quality in river systems" Youmares conference. University of Kiel, Germany

December 2017. "Alternative methods for assessing habitat quality in river systems" eDNA working group. University of Manchester, UK

June 2019. "Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches". Barcode of Life Conference. Trondheim, Norway

Awards:

June 2019. Oral presentation, Runner up at the 2019 PGR conference, University of Derby, UK.

Additional publications:

Mauvisseau, Q., Davy-Bowker, J., Bryson, D., Souch, G. R., Burian, A., & Sweet, M. (2019). First detection of a highly invasive freshwater amphipod *Crangonyx floridanus* (Bousfield, 1963) in the United Kingdom. BioInvasions Records, 8(1), 1–7. doi: 10.3391/bir.2019.8.1.01

Davy-Bowker, J., Hammett, M. J., Mauvisseau, Q., & Sweet, M. J. (2018). Rediscovery of the critically endangered 'scarce yellow sally stonefly' *Isogenus nubecula* in United Kingdom after a 22-year period of absence. Zootaxa, 4394(2), 295. doi: 10.11646/zootaxa.4394.2.12

Troth, C., Burian, A., Mauvisseau, Q., Bulling, M., Nightingale, J., Mauvisseau, C., & Sweet, M. (2019). Development and application of eDNA-based tools for the conservation of white-clawed crayfish. BioRxiv, 28.

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M., & Mergeay, J. (2020). Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). Journal of Fish Biology, jfb.14315. doi: 10.1111/jfb.14315

ABSTRACT

“Water, water, everywhere…”. 71% of the earth’s surface is covered by water, freshwater representing 2.5% of it, and only 1% being accessible. Due, largely to a number of anthropogenic activities (pollution, habitats modification) coupled with the impacts of climate change, a dramatic decline in biodiversity is occurring across all earth’s ecosystems. Surprisingly, freshwater ecosystems receive considerably less attention than many other habitats and therefore, effective biodiversity monitoring programs are urgently needed to assess the health and state of the endangered and threatened species in these aquatic systems. Further, current techniques utilised to survey freshwater ecosystems are often considered ineffective, invasive, time consuming and biased. As a result, the implementation of molecular-based detection tools are attractive options as they are often shown to be more sensitive and cost effective. The use of environmental DNA (eDNA) detection is one such molecular tool which is showing promising results, due to its high reliability, sensitivity and non-invasiveness characters. However, recent studies have highlighted potential limitations associated with eDNA-based detection. Such limitations may lead to a decrease in the confidence of this method. The aim of this thesis was to investigate the use of eDNA-based detection across a number of species and a number of systems, all as a proxy of habitat quality. Stringent laboratory practices and validation guidelines were adhered to, allowing for reliable quality assessments of newly designed eDNA assays outlined in this thesis. Moreover, distinct controlled mesocosm experiments allowed the investigation of critical factors, part of the sampling method or analysis processes leading to an optimisation of eDNA collection and decreasing the rates of false negative results. Several comparison between traditional monitoring techniques and the novel assays were also performed aiding in the confidence of these new methods. Interestingly, the results obtained in this thesis shows a similar efficiency between traditional and eDNA-based methods for monitoring invasive species, but a higher efficiency of eDNA detection when detecting rare or low abundant organisms (i.e. those that are endangered or threatened). Furthermore, this thesis reports an extreme example where a species was found at a number of locations within a stretch of a river, yet undetected with the eDNA assay. In this chapter eDNA detection was only possible when I utilised ddPCR rather than qPCR (the more standard technique for assessing eDNA in any given system). Overall, eDNA detection was found to be an effective tool for assessing the presence of invasive and/or endangered species, increasing the

knowledge on their distribution and the impact of future management plans. In this thesis, chapters 2, 3, 4, 5 and 6 are organised as case studies, aiming to highlight benefits and limitations of species-specific detection using eDNA.

ACKNOWLEDGMENT

First, I would like to thank my director of studies Michael Sweet for giving me the opportunity to pursue a PhD and for his support through this whole and challenging process. Many thanks for your help, advice and rapid feedback on the various projects I undertook during these three years. I'm extremely thankful for the freedom you gave me when choosing these research topics and collaborations, and I look forward to keep on working with you in the future.

I would also like to thank my supervisors Andrew Ramsey and especially Rein Brys for your help, support, and numerous and constructive discussions during the various steps of this thesis. Your enthusiasm and help have been exceptional and I'm hopeful that we can keep working together on various topics.

I'm forever grateful to Mark Bulling and Alfred Burian for your patience and support with the statistical analysis and interpretation and especially for your help with my numerous questions when I finally started to understand and enjoy statistics and R software. It was a long learning curve and your commitments were invaluable.

I'm also extremely grateful to Surescreen Scientifics, particularly to Jim Campbell and Troy Whyte for funding this research project. I would like to thank Christopher Troth for his enthusiasm and for his various help during this whole research project.

I would not have pursued a PhD in the eDNA field without Catherine Souty-Grosset and Didier Bouchon. You both gave me the opportunity to discover this field of research during my Master thesis and this is the main reason of my interest to apply molecular detection in monitoring programs.

I am very thankful to the various people who have also helped me across the different stages and projects represented in this thesis: Sabrina Neyrinck, I'm forever grateful for your help and input for ddPCR analysis. Brian Zimmerman and Eleni Kalogianni for your help, support and constructive feedback during the various steps of the *Valencia* project. I would like to thank John Davy-Bowker for his help and expertise in taxonomy and invertebrate kick-sampling, Sina Tönges and Ranja Andriantsoa for their help during the Marmorkrebs project.

I’m thankful for the various support (including the numerous coffee breaks!) of my friends and colleagues Alexandra Karousi, Charitos Zapitis, Lea Barbett, Hannah Lockwood, Martin Stelfox, Briony Norton, Mathieu Sere, Till Roethig, Richard Williams.

I'm thankful to true heroes of the lab: I wouldn't have been able to conduct my project and all these analyses without your work. Many thanks to Angie Ward, Sara Croft, Richard Duff, Graham Souch, Caroline Mills.

Enfin, merci à toute ma famille et amis et plus particulièrement à mes parents et mes sœurs Christophe, Christine, Alix et Flore pour votre aide et pour avoir supporté mes nombreuses digressions et explications sur mes sujets de recherches pendant ces années. Papa, ta passion et ton intérêt pour les animaux ne sont évidemment pas étrangers à mon enthousiasme pour étudier l’écologie aquatique et à ma continuelle recherche d’animaux divers et variés dans les mares, rivières, fosses, jardins et autres depuis mon enfance. Recherche qui finira par inspirer la fameuse phrase « reposes ça où tu l’as trouvé ! ».

Chapter 1: Introduction

1.1 Current state

In 2012, the planet Earth was estimated to be home to approximately 9 million species of plants, animals, protists and fungi (Cardinale et al., 2012). However, it is now generally accepted that we are entering the sixth known mass extinction event, mainly caused by anthropogenic activities, with species extinction rates 1000x higher than natural background rates (Brooks et al., 2006; Ceballos et al., 2015; Ceballos and Ehrlich, 2018). The number of species that is already lost is unknown, but impact of increasing anthropogenic activities including pollution, habitat destruction or over exploitation, coupled with changes to climate are altering nearly every ecosystem on our planet and lead to a dramatic decline of overall biodiversity (Cardinale et al., 2012; Harley, 2011; Hooper et al., 2012). This decline of biodiversity is not only devastating for the health of ecosystems but also dramatically threatens the services they provide, impacting our own well-being (Brooks et al., 2006; Ceballos et al., 2015). As specified by the IUCN Redlist (https://www.iucnredlist.org/), 27% of all assessed species in the world are now threatened with extinction. It is therefore imperative to minimise this species loss with well managed conservation activities and regulated controls on land usage/management (Butchart et al., 2010; Hooper et al., 2012). However, conservation activities, such as habitat management or habitat restoration mainly rely on the knowledge of the current state of the ecosystem (Rosenberg et al., 2000). Such knowledge is obtained by regular monitoring, in which the assessment of species presence, especially bio-indicators, rare or invasive species is particularly important (Rosenberg et al., 2000).

According to the United States Geological Survey, the percentage of the Earth's surface covered in water is estimated around 71%. However, freshwater is representing only 2.5 % of this amount and is mostly situated underground or 'stored' as ice, which means only 1% is 'available' for life to utilize and colonize. In these freshwater systems, biodiversity monitoring is a cornerstone for the evaluation of ecosystem health and status. In Europe, this is further specified by the evaluation of the European Habitats Directive (European Commission, 1992) and Water Framework Directive (European Commission, 2015). In comparison to terrestrial habitats, these aquatic ecosystems (i.e. wetlands or freshwater habitats) are receiving considerably less attention with regards to conservation and management and yet, are suffering from dramatic declines in species

diversity (Davidson, 2014; Reid et al., 2019). One of the main problems is that aquatic environments are difficult to monitor, especially in case of rare, endangered and/or invasive species. Nonetheless, an adequate insights in the presence and composition of aquatic species and communities is needed for a reliable assessment of ecosystem health and status, quick implementation of management strategies and for maximizing conservation actions.

1.2 Conventional monitoring methods

Current conventional methods for biodiversity monitoring in freshwater systems include electrofishing or gillnetting (Hering et al., 2018), snorkelling (Darling and Mahon, 2011) and kick-sampling (Mächler et al., 2019). However, these conventional methods are far from perfect. They often require a large sampling effort (Yatsuyanagi et al., 2019), are labour intensive, often ineffective (especially when monitoring species at low densities), time consuming, expensive and ecologically invasive (Forsström and Vasemägi, 2016; Eiler et al., 2018). Additionally, a high level of taxonomic expertise is generally required to correctly identify observed or sampled organisms (Hering et al., 2018; Ushio et al., 2018). However, misidentification remain possible and therefore inaccurate biodiversity assessment is substantial. For example, the invasive amphipod *Crangonyx floridanus* (Bousfield, 1963) was recently discovered in UK (Mauvisseau et al., 2019b, an incidental findings associated with this PhD). This species is almost identical to its sister species *Crangonyx pseudogracilis* (Bousfield, 1958), which was known to be present in the UK since at least 1936 (Crawford, 1937; Dunn et al., 2017). As either molecular analysis or Light and Scanning Electron-Microscopy technology is necessary to distinguish both species, it is highly likely that this ‘newly discovered’ invasive species was misidentified for a relatively long time. While this example highlights an extreme scenario, it also represents a key limitation of currently utilised methods. Indeed, each of these methods is associated with strong sampling bias and high variability among replicates, which can additionally have a significant impact on the habitats or co-occurring organisms (Eiler et al., 2018). For these reasons, a reliable and efficient alternative method for assessing species presence, and habitat quality in freshwater systems would be extremely valuable. Such alternative methods could be the use of molecular detection, a non-invasive, standardized and cost-effective tool allowing reliable detection of aquatic biodiversity in a range of ecosystems.

1.3 eDNA-based detection as an alternative monitoring method?

In the last ten years, molecular detection has gained traction and has been dubbed environmental DNA or eDNA (Ficetola et al., 2008; Thomsen and Willerslev, 2015) (Figure 1.1.). This molecular method relies on the detection of DNA traces from sources such as pieces of skin, faeces, eggs, sperm, blood or mucus left by living (or dead) organisms in their environment (Thomsen and Willerslev, 2015).

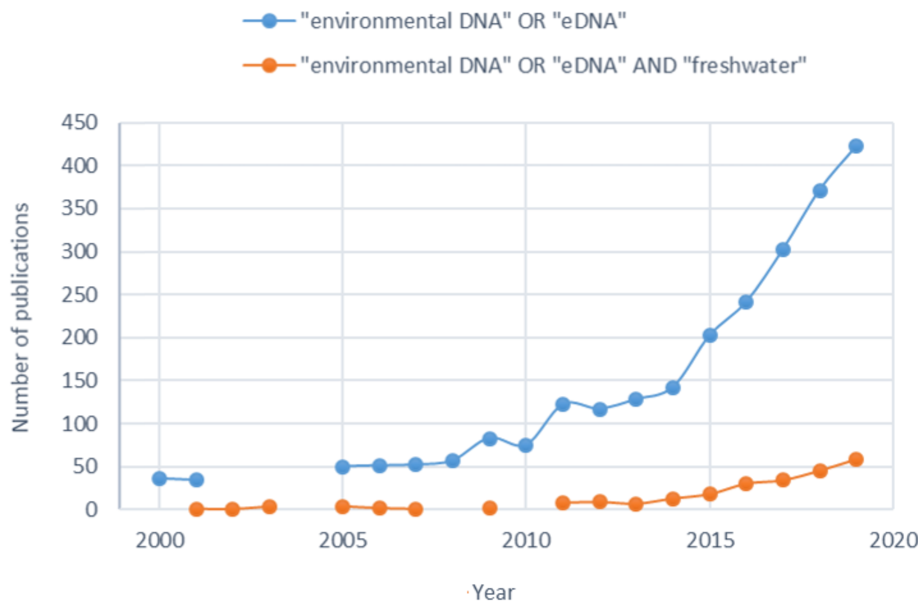

Figure 1.1. Figure highlighting the rise of eDNA research. The number of publication with the keywords "environmental DNA" or "eDNA" and with keywords "environmental DNA" or "eDNA" and "freshwater" was retrieved from Scopus on 13th December 2019. Missing portions represents the absence of publication using the associated keywords search.

In aquatic systems, eDNA can be extracted from water samples and specific species can then be detected through PCR, qPCR or ddPCR (barcoding) (Thomsen and Willerslev, 2015; Tsuji et al., 2019; Wood et al., 2019). Complete communities can also be detected through PCR amplification associated with sequencing (metabarcoding) (Tsuji et al., 2019). Each of these molecular tools have been successfully used for detecting various invasive, endangered, economically important or pathogenic species in both marine and freshwater ecosystems around the world (Dufresnes et al., 2019; Sengupta et al., 2019; Shaw et al., 2019; Yates et al., 2019). Due to the low persistence

of eDNA in the aquatic environments (Collins et al., 2018; Salter, 2018; Li et al., 2019), its detection in water, when avoiding the resuspension of sediment, allows a reliable assessment of current species presence. Moreover, recent studies have highlighted the correlation between eDNA quantification and species biomass/abundance (Ushio et al., 2018; Itakura et al., 2019; Li et al., 2019; Shelton et al., 2019; Yates et al., 2019). In addition, eDNA monitoring has been shown in many cases to be cost effective compared to traditional surveys. For example, as explained in Evans et al., (2017), the assessment of the Brook Trout, *Salvelinus fontinalis* (Mitchill, 1814) is conventionally performed via 100 m triple pass or 300 m single pass electrofishing transect costing 203$US per reach. In this study, the cost of eDNA analysis, including three samples and one blank was estimated to be 75$US. In a prospect of specific-species detection at least, eDNA detection seems to present various advantages compared to conventional monitoring methods.

However, despite the growing interest for this method, many unexplored variables can affect species detection (Deiner and Altermatt, 2014) and a focus is now needed on standardization or method calibration in order to optimize and maximize species detection (Hinlo et al., 2017; Weigand et al., 2019; Zinger et al., 2019). One of the first issues that needs to be addressed is the collection of eDNA from aquatic environments in the first place. For example, while the commercial service for the detection of eDNA from the Great Crested Newt *Triturus cristatus*, (Laurenti, 1768) is based on an ethanol precipitation protocol (Rees et al., 2014), many studies rely on water filtration for collecting eDNA traces (Spens et al., 2017; Majaneva et al., 2018; Sepulveda et al., 2019). Indeed, across many published eDNA studies, the protocols utilised for eDNA capture and extraction vary (Figure 1.2.A.). A recent study aiming to propose a validation scale to assess the readiness of eDNA assays for routine species monitoring highlighted a list of 122 variables allowing to grade a validation from 'incomplete' to 'operational' (Thalinger et al., 2020). This further highlights the rigorous validation needed for meaningful application of eDNA monitoring.

Relatively few eDNA studies follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al., 2009; Mauvisseau et al., 2019a), which were designed to assure reliable assessment of qPCR efficiency. In addition, studies have also indicated that filtration of large amounts of water (on site) reduces cross contamination, stochasticity and variability of eDNA detection in water samples from the same location (Yamanaka et al., 2016; Li et al., 2018; Majaneva et al., 2018). Furthermore, there appears to also

be variability between both the success of an assay (Lear et al., 2018) and the targeted amplified genes (Figure 1.2.B.). However, there are relatively few studies which have even attempted to compare the efficiency of two different gene regions for detecting and quantifying eDNA traces of any given species.

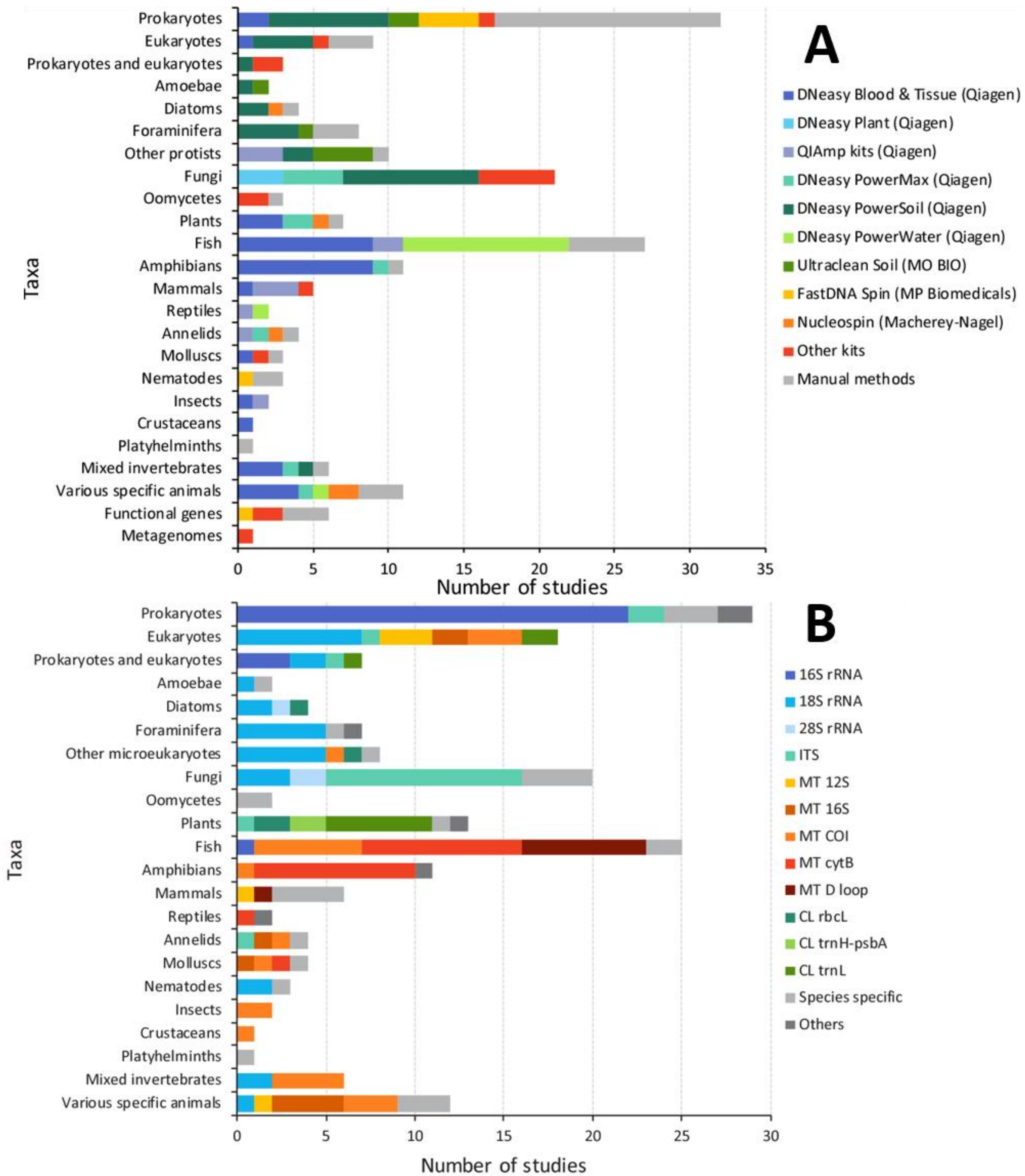

Figure 1.2. A and **B.** Figures extracted from (Lear et al., 2018). Sub-figure **A** is showing the various kits and methods used in studies focussing on the analysis of eDNA originating from different taxa. Sub-figure (**B**) is showing the different gene regions targeted in various studies focused on different taxa, excluding microarray or metagenomic studies (i.e. exclude fish eDNA metabarcoding studies targeting the 12S region). rRNA indicates ribosomal genes, MT indicates mitochondrial genes and CL indicates chloroplast genes.

The impact of the number of natural replicates (i.e. water samples) and technical replicates (i.e. PCR/qPCR/ddPCR replicates) needs to be investigated, as there is little to no standardization across eDNA studies on these key variables. eDNA studies are also prone to the occurrence of inhibition factors (Mauvisseau et al., 2019c; Uchii et al., 2019). This can happen when filtering turbid water and it has previously been shown that the use of a larger pore size filters, as well as dilution or the use of inhibitor removal kits could reduce these effects (Goldberg et al., 2016). However, such actions can reduce the concentration of targeted eDNA and lead to false negative results (Goldberg et al., 2016). For these reasons, the use of alternative technology (ddPCR for example) should be explored as an option. The majority of eDNA barcoding studies currently rely on either conventional PCR or qPCR amplification, despite the potential benefits of ddPCR (Doi et al., 2015a; Mauvisseau et al., 2019c; Wood et al., 2019). Researchers and end users appear to be slow on the uptake of this method for eDNA based studies. This could be due to the high price associated to the whole ddPCR system (around 80 000€, unpublished data from INBO) despite ddPCR being cheaper than qPCR per sample (Mauvisseau et al., 2017). Furthermore, due to the novelty aspect of ddPCR analysis, potential users of the technique could be waiting for additional studies highlighting other potential benefits of this tool.

1.4 Aims of the thesis

The aim of this PhD was to explore the use of eDNA detection as an alternative method for assessing species presence as a proxy for habitat quality in freshwater systems. In order to allow a specific focus on specific parameters, this thesis is split into five different 'case studies'. In these case studies, I aimed to investigate the effect of key parameters, through the detection of endangered or invasive species in a wide variety of freshwater systems.

In the second chapter of the thesis, I investigated the influence of accuracy, repeatability and detection probability on the reliability of species-specific eDNA based approaches. More specifically, I used the critically endangered and well known bioindicator species, the Freshwater Pearl Mussel *Margaritifera margaritifera* (Linnaeus, 1758), as a model organism in a controlled mesocosm experiment. First, I independently validated in laboratory settings following the MIQE Guidelines (Bustin et al., 2009) two previously published assays targeting the COI (Cytochrome C Oxidase Subunit 1) (Carlsson et al., 2017) and 16S (Stoeckle et al., 2015) genes of *M. margaritifera*. After investigating the reliability of these two different assays for detecting and quantifying DNA, I analysed the eDNA samples collected in the controlled mesocosm experiment. This second chapter was the baseline of the work conducted in chapters 3, 4 and 6. In these following chapters, I used the protocol for capturing and extracting eDNA developed and validated by Spens et al., (2017). Here, eDNA is captured by filtering water samples through Sterivex filters™, whereas extraction was done by using a modified version of the Blood and Tissues Kit Qiagen™ protocol. Standardizing the capture and extraction protocol in these chapters was a critical step for allowing a focus on which parameters would influence eDNA detection. Furthermore, this second chapter allowed me to reliably establish an optimal sampling and analytical methodology by assessing the number of natural replicates (i.e. number of eDNA samples) and technical replicates (i.e. number of qPCR replicates) needed for improving the reliability of eDNA measurements. Moreover, an extensive literature review was performed in order to assess the level of compliance (with regards to the MIQE Guidelines) that current eDNA barcoding studies adhered to.

The third chapter focussed on the comparison of eDNA-based detection with the traditionally employed method for fish monitoring (i.e. electrofishing and netting). In this chapter, specific assays were designed for monitoring two critically endangered and one invasive fish species. The distribution of each of these three fish species was assessed through two field surveys combining both traditional and eDNA-based method for monitoring. As in the second chapter, a controlled mesocosm experiment was utilized for determining which optimal pore size of the filters maximized the recovery of eDNA.

In the fourth chapter, the efficiency of eDNA detection was assessed and compared to kick-sampling for detection and monitoring of a critically endangered species, the stonefly *Isogenus*

nubecula (Newman 1833). I was a part of the research team who recently rediscovered this species after a period of 22-years of absence (Davy-Bowker et al., 2018). Here, two different amplification methods of eDNA-based detection were compared (i.e. qPCR and ddPCR) to address the issues due to the occurrence of this species in very low abundance at few sites and in a fast flowing river. I also utilised an occupancy modelling approach to investigate the influence that specific environmental factors have on the probability of eDNA detection using ddPCR analyses.

The fifth chapter used a different eDNA fixation method. Ethanol precipitation is the only accredited commercially utilised protocol in the UK for the detection of the Great Crested Newt *T. cristatus* (Rees et al., 2014; Biggs et al., 2015; Harper et al., 2018b). Therefore, I investigated the possibility of using this method for the early detection of an invasive species in the UK. I designed and validated a novel assay to explore the effect of distance between the sampled sites and the consistency of eDNA detection for the demon shrimp *Dikerogammarus haemobaphes* (Eichwald, 1841). This chapter also highlighted the potential limitation of this method, yet still allowed the presence of this species to be mapped across a network of rivers and canals.

In the final research chapter, I developed and validated an assay for monitoring a highly invasive crayfish species *Procambarus virginalis* (Lyko, 2017). *P. virginalis* is different from other crayfish primarily due to its parthenogenetic reproduction capabilities. It is also known to be a carrier of the pathogenic fungus *Aphanomyces astaci* (Schikora, 1906), lethal to European crayfish. As a result, the species has previously been reported as the 'perfect invader' (Jones et al., 2009), and therefore, an early detection tool is critical for limiting its spread.

Finally, I conclude by highlighting the potential benefits and limitations of eDNA-based detection as an alternative method for assessing species presence as a proxy for habitat quality in freshwater systems. Through reporting these results, it is hoped that the findings of this thesis allow a more comprehensive understanding of the potential benefits and limitations associated with eDNA detection for monitoring aquatic species as a proxy for habitat quality.

Chapter 2: Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches

This chapter has been published as: Mauvisseau, Q., Burian, A., Gibson, C., Brys, R., Ramsey, A., Sweet, M., 2019. Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. Scientific Reports 9. https://doi.org/10.1038/s41598-018-37001-y

2.1 Abstract

Environmental DNA (eDNA) barcoding has a high potential to increase the cost-efficiency of species detection and monitoring in aquatic habitats. However, despite vast developments in the field, many published assays often lack detailed validation and there is little, to no commonly (agreed upon) standardization of protocols. Here, the reliability of eDNA detection and quantification was evaluated using published primers and assays targeting the Freshwater Pearl Mussel as a model organism. Limits of detection were first assessed for two different target genes (COI and 16S) following the MIQE guidelines, and then the reliability of quantification was tested in a double-blind mesocosm experiment. Results revealed that different methodological indicators, namely accuracy, repeatability and detection probability affected the reliability of eDNA measurement at the different levels tested. The selection of the optimal analytical method was mainly determined by detection probability. Both the COI and 16S assays were highly specific for the targeted organism and showed similar accuracy and repeatability, whilst the LOD was clearly lower for the COI based approach. In contrast, the reliability of eDNA quantification hinged on repeatability, reflected by the scattering (r^2=0.87) around the relationship between eDNA and mussel density in mesocosms. Finally, a bootstrapping approach, which allowed for the assignment of measures associated with repeatability of samples, revealed that variability between natural replicates (i.e. accuracy) strongly influenced the number of replicates required for a reliable species detection and quantification in the field.

2.2 Introduction

Environmental DNA (eDNA) is a novel molecular technique, which can facilitate via the analysis of water samples (in this context), the detection and monitoring of organisms and communities in aquatic habitats that are difficult to monitor with more traditional methods (Bohmann et al., 2014; Bylemans et al., 2016a, 2016b; Hinlo et al., 2017). The technique is based on the amplification of fragments of DNA originating from skin, hairs, mucus or gametes for example, all of which can be shed by both living and dead organisms alike (Bohmann et al., 2014; Doi et al., 2015a; Spear et al., 2015; Mauvisseau et al., 2017, 2018; Cowart et al., 2018). Assays can be either non-targeted (i.e. a metabarcoding approach) or targeted at specific species (Thomsen et al., 2011). Further, the application of advanced amplification methods such as quantitative Polymerase Chain Reaction (shortened to qPCR, also known as real-time PCR) or digital droplet PCR (ddPCR also known as digital PCR) allows the quantification of target DNA in natural habitats. Accordingly, correlations between species abundance and eDNA detection and quantification has recently been demonstrated for several species (Takahara et al., 2012; Nathan et al., 2014; Lacoursière-Roussel et al., 2015; Eichmiller et al., 2016b; Evans et al., 2016; Yamamoto et al., 2016; Baldigo et al., 2017; Mauvisseau et al., 2017). However, a common limitation of many eDNA based quantification approaches is that only a few cases report rigorous validation steps at a satisfactory level under controlled laboratory conditions (Nathan et al., 2014; Evans et al., 2016). In many examples, validation steps which have been implemented simply depend on correlative comparison with field surveys (Lacoursière-Roussel et al., 2015; Yamamoto et al., 2016; Baldigo et al., 2017). Field surveys, however, have been shown to be often highly variable and underrepresent true species abundance and diversity (Lacoursière-Roussel et al., 2015). Meaning, it could therefore be argued that there is little information on the reliability of eDNA assays with regard to quantifiable data.

The reliability of eDNA based quantification does not only depend on the repeatability and accuracy of quantification, but also on sensitivity, which is linked to the detection probability of any given approach (see Fig. 2.1: definitions used within chapter 2).

In this chapter, the following terms refer from an eDNA perspective:

Reliability: Degree to which the result of all aspects of assay evaluation can be precise and repeatable.

Specificity: Correct amplification of the targeted species and no positive results from closely related species.

Detection probability: Probability that the analysis of a technical or natural replicate that contains DNA of the target species results in a positive detection.

Sensitivity: Synonymous with the Limit of Detection (LOD), which is according to the MIQE guidelines (Bustin et al., 2009) defined as the last dilution step of the standard that results in detection of the targeted DNA with a Ct below 45.

Efficiency: Degree to which the amplification of all DNA copies, in all PCR reactions, can be precise and repeatable.

Repeatability of quantification: In an eDNA context standard curves are based on mean of measurements. Hence, repeatability represents the spread (r^2) of data around regression lines used to standardise quantification.

Accuracy of quantification: Variability of measurements contributing to a data point. Includes both, variability in natural and technical replicates. At low replicate number, low accuracy is likely to decrease repeatability.

Figure 2.1. Box defining the terms related to eDNA assay validation

For instance, the efficiency and reliability of qPCR assays depend on whether they follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines or MIQE for short (Bustin et al., 2009). In particular, validation of any novel assays should at the very least highlight the LOD and the LOQ. Detection of eDNA under natural conditions is typically characterized by large variability, due to limited dispersion capacity of eDNA and strong variation in eDNA release and decay, which can also lead to relative low detection probabilities above the LOD (Rice et al., 2018). A strategy to improve the accuracy of measurement and reduce the effects

of natural variability is to increase the number of replicate samples (Pilliod et al., 2013). Inhibition factors and limitations of the amount of water filtered, can, on the other hand, increase the level of variation seen in any replicate, thereby effecting the assays repeatability (McKee et al., 2014). Considering the analysis of an eDNA sample using six qPCR replicates, the efficiency of eDNA detection and quantification in a targeted approach can be separated in to one of five different categories (Figure 2.2.).

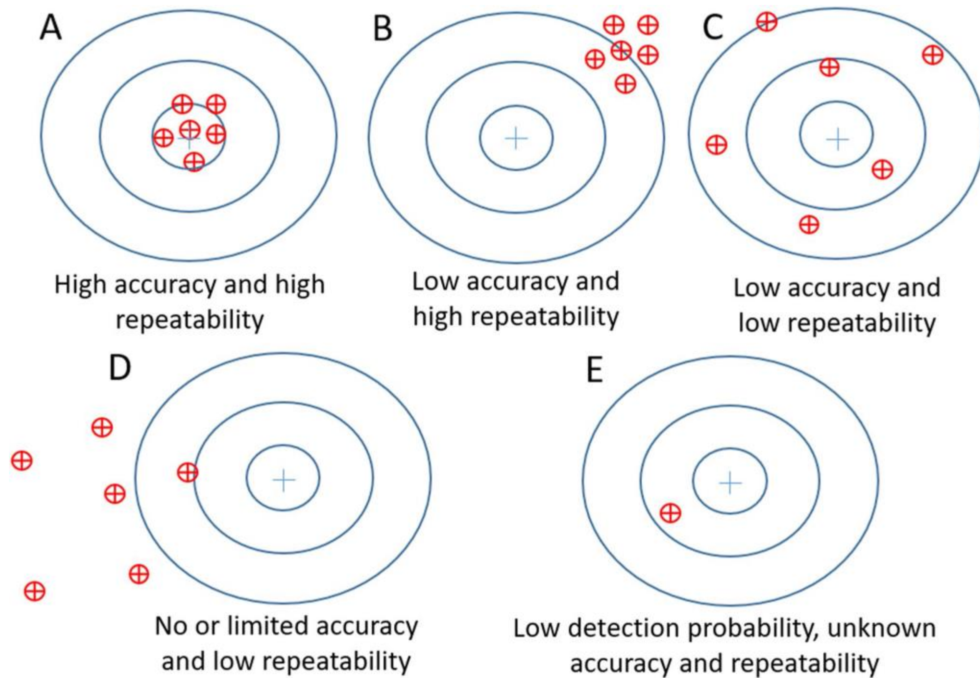

Figure 2.2. Represent the theoretical variations in eDNA reliability: Blue circles represent a “target” whereby the inner centre circle would represent the higher accuracy. The red circles with crosses in the middle represent “replicate” samples (either natural or technical). Scenario in this figure are as follow: eDNA measurements with high accuracy and high repeatability **A**, low accuracy and high repeatability **B**, low accuracy and low repeatability **C** and low or limited accuracy and low repeatability **D**. Panel **E** reflects a case where detection probability is low and hence the accuracy and repeatability of the analysis are unknown.

High accuracy and high repeatability for example (Figure 2.2.A.), will lead to a high efficiency of eDNA detection and quantification. High accuracy with low repeatability (Figure 2.2.B.) and low accuracy with low repeatability (Figure 2.2.C.) would, in contrast, lead to a medium efficiency of eDNA detection and poor efficiency of eDNA quantification. Finally, no or very limited accuracy and low repeatability (Figure 2.2.D.) will lead to both poor eDNA detection and quantification. However, depending on the number of positive technical replicates (i.e. qPCR wells for the same sample), eDNA detection can also be obtained with unknown accuracy and repeatability and lead to a low detection probability (Figure 2.2.E.). Besides detection (presence-absence, or species richness), a challenging question is whether we can relate any given species amplicon abundance (i.e. quantification values of the targeted DNA fragment) to the density of said species in its habitat. Because of the low persistence of eDNA particles in aquatic environments, species detection via eDNA allows a reliable survey of species present at any given location (Dejean et al., 2011; Maruyama et al., 2014). However, despite reportedly being a cheaper and more reliable method for species detection than traditional survey methods (Smart et al., 2015, 2016; Evans et al., 2017), the vast majority of eDNA studies appear to lack detail in the validation of the methods or assays used. For example, as checked in 80 articles (see full list in Appendix 1) focussing on the eDNA detection of species using barcoding techniques which were published between January 2017 and January 2018 and only 10 mentioned the MIQE Guidelines (Bustin et al., 2009) (See MIQE Guidelines in Appendix 2). Clear method standardisation from field sampling to DNA analysis would greatly improve insights on advantage and disadvantages linked to specific eDNA assays and ultimately increase the transparency and end user confidence.

In this chapter, the reliability, detection and quantification limits of different eDNA approaches was systematically assessed using the Freshwater Pearl Mussel, *M. margaritifera* as a target organism. More specifically, the aim was to evaluate accuracy, repeatability and detection probability of two previously designed assays targeting distinct gene regions (COI and 16S) using qPCR (Stoeckle et al., 2015; Carlsson et al., 2017). Therefore, I first tested the reliability of both assays by establishing standard curves and determining LOD and LOQ following MIQE Guidelines (Bustin et al., 2009; Hunter et al., 2017). In a second step, I examined the potential of the approaches to serve as an indicator for species abundance. For this purpose, six stable mesocosms with varying mussel densities were established, sampled, and, compared in a double-

blind procedure eDNA copy numbers and mussel abundance in mesocosms. In this instance, double blind meant that two teams of researchers were involved in the sampling. The first team collected the water (see methods) while the second team filtered the water without knowing its origin. Furthermore, the abundance of mussels associated with each sample/mesocosm was unknown until all laboratory assessment has been completed. Results shows how the number of water samples per mesocosm (i.e. natural replicates) and the number of qPCR replicates (i.e. technical replicates) are linked to the reliability of quantification. This led to recommendations for field sampling protocols.

2.3 Materials and methods

2.3.1 Study species and system

The target species of this chapter was the rare and protected Freshwater Pearl Mussel (*M. margaritifera*), a large (~14cm) bivalve with a maximum life span of over 100 years and a generation time of 30 years (Carlsson et al., 2017; Moorkens et al., 2018). While it was once a dominant and functionally important species, it has since declined across the majority of its former range by upwards of 62%. The species was therefore classified as endangered throughout Europe in 1996 (Moorkens et al., 2018). Application of eDNA approaches on mussel species are in principle characterised by a relative low sensitivity (Carlsson et al., 2017) and hence investigations with *M. margaritifera* represent a suitable yard stick to assess the reliability of eDNA based species quantification.

The experimental part of this chapter was performed at the Freshwater Biological Association (FBA) Ark station in Windermere; a unique facility which has been holding this critically endangered species under controlled conditions for the past 10 years. At the time of the experiment, 167 adult *M. margaritifera* (from six different river populations) were housed in six independently maintained mesocosms. The experimental mesocosms were circular, 1.6 m^3 in size and continuously supplied with water filtered through a 20-micron Hydrotech Drumfilter HDF800-series. The water was obtained directly from Lake Windermere, and no other physical or chemical treatment was utilised. Prior to the experiment, water samples from before and after the facility filtration process were tested in order to ensure the absence of targeted DNA in the water entering

each mesocosm. Additionally, various physio-chemical water parameters were measured to confirm the match with environmental conditions in natural breeding sites. The experimental mesocosms have been designed to reflect the natural environment of the targeted species, by mimicking clean and fast flowing environment and addition of naturally co-occurring fish species. The successful reflection of their habitats has been highlighted by several reproduction events.

2.3.2 Sampling and PCR protocols

Tissue samples (n = 12) from Bivalve species: *Margaritifera margaritifera*, *Margaritifera falcata* (Gould, 1850), *Anodonta anatina* (Linnaeus, 1758), *Anodonta cygnea* (Linnaeus, 1758), *Unio pictorum* (Linnaeus, 1758), *Dreissena rostriformis bugensis* (Andrusov, 1897), *Dreissena polymorpha* (Pallas, 1771), *Corbicula fluminea* (Müller, 1774), *Truncilla truncata* (Rafinesque, 1820), *Quadrula quadrula* (Rafinesque, 1820), *Lampsilis siliquoidea* (Barnes, 1823) and *Cumberlandia monodonta* (Say, 1829) were collected to establish standard curves and the specificity of the approach. Tissue samples were preserved in absolute ethanol and kept at -80°C until extraction (see below). Water samples (for eDNA analysis) were taken on the 1st November 2017. From each mesocosm, three 1L water samples were collected with a sterile polypropylene ladle from the water surface. Samples were collected in a sterile plastic bag (Whirl-Pak® 1242 ml Stand-Up Bag Merck®, Darmstadt, Germany) and filtered with a 50-mL syringe (sterile Luer-Lock™ BD Plastipak™, Ireland) through a sterile 0.45 µm Sterivex™ HV filter (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Millipore®, Germany) (Spens et al., 2017). To avoid contamination, disposable nitrile gloves were used during the sampling process and replaced between each sample. All filters were stored in 50 mL tubes (Falcon™ 50 ml Conical Centrifuge Tube, Fisher Scientific, Ottawa, Canada) at -80°C before extraction.

From both the water and tissue samples, DNA was extracted using the Qiagen DNeasy® Blood and Tissue Kit following manufacturers' guidelines. For the water samples, a slight modification to these were applied following methods outlined in (Spens et al., 2017). Control samples, i.e. water samples without traces of *M. margaritifera* DNA and separate samples consisting of ddH_2O were also extracted as above. Pipettes and tube holders were disinfected and regularly decontaminated under UV treatment. All other lab equipment and surfaces were regularly disinfected using 10% bleach solution and ethanol before the analysis.

PCR amplification was performed on a Gen Amp® PCR System 9700 (Applied Biosystem) by using two sets of pre-designed species-specific primers (Stoeckle et al., 2015; Carlsson et al., 2017). The set designed by (Carlsson et al., 2017) targeted the COI mitochondrial gene while the set designed by (Stoeckle et al., 2015) targeted the DNA sequence of the 16S rRNA subunit (Table 2.1).

	(Stoeckle et al., 2015)		**(Carlsson et al., 2017)**	
Target	16S		COI	
Species	PCR	qPCR	PCR	qPCR
Margaritifera margaritifera	Amplification	Amplification	Amplification	Amplification
Margaritifera falcata	None	None	None	None
Anodonta anatina	None	None	Amplification	None
Unio pictorum	None	None	None	None
Anodonta cygnea	None	None	None	None
Dreissena rostriformis bugensis	None	None	None	None
Dreissena polymorpha	None	None	None	None
Corbicula fluminea	None	None	None	None
Truncilla truncata	None	None	Amplification	None
Quadrula quadrula	None	None	None	None
Lampsilis siliquoidea	None	None	None	None
Cumberlandia monodonta	None	None	Amplification	None

Table 2.1. Results of PCR and qPCR reactions using the primers and probes targeting the COI and 16S gene of *M. margaritifera* on 12 differents mussel species.

PCR reactions were performed in a 25 µL total volume with 12.5 µL of 2x PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10 µM), 9.5 µL of ddH_2O and 1 µL of DNA template. For the COI primers, the PCR protocol followed that outlined in (Carlsson et al., 2017) with slight modifications. Briefly, an initial warming step at 50°C for 2 min and denaturation at 95°C for 10 min, was followed by 35 cycles 95°C for 15s and 60°C for 1 min. For the 16S primer, the PCR protocol followed (Stoeckle et al., 2015), with slight modifications. These included, an initial denaturation at 95°C for 15s, followed by 35 cycles of 95°C for 15s, 60°C for 10s and 72°C for 20s. Products from PCR were visualized on 2% agarose gel stained with GelRed™. qPCR programmes were similar to PCR programmes but were performed with 55 instead of 35 cycles. For this chapter, a complementary probe (6-FAM- TCCAGTTAATCATAGAACTTCATCAAA-

BHQ-1) was designed to work with the 16S primers. This was done with Geneious Pro R10 software http://www.geneious.com; and as in (Kearse et al., 2012). The probe was assessed for specificity against DNA sequences retrieved from tissue samples of the targeted species and from closely related species and other mussel species that potentially can live in the same ecosystem as *M. margaritifera* (See Table 2.1.) along with other sequences retrieved from NCBI (i.e. National Centre for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/). For the qPCR assay targeting the COI, the probe described by (Carlsson et al., 2017) was used. The total amplicon size (including primers) was 83 bp for COI and 172 bp for 16S. Specificity of primers and probes were assessed *in-silico* using Geneious Pro R10 software and *in-vitro* by PCR and qPCR. Primers and probes were tested against tissues of eleven other mussel species (See Tables 2.1. and 2.2.). qPCR assays were performed in a final volume of 25 µl using 12.5 µl of PrecisionPlus qPCR Master Mix with ROX (Primer Design, UK), 1 µl of each primer (10 µM), 1 µl of the corresponding probe (2.5 µM), 6.5 µl of ddH_2O and 3µl of extracted DNA on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems).

Margaritifera margaritifera

Target	Primers	Sequence (5'-3')	Source
COI	Forward	TTGTTGATTCGTGCTGAGTTAGG	(Carlsson et al., 2017)
COI	Reverse	GCATGAGCCGTAACAATAACATTG	(Carlsson et al., 2017)
COI	Probe	6-FAM- CCTGGTTCTTTGCTGGGT -BHQ-1	(Carlsson et al., 2017)
16S	Forward	CAACCCTGGAACCGCTAAAG	(Stoeckle et al., 2015)
16S	Reverse	GGCTGCGCTCATGTGAATTA	(Stoeckle et al., 2015)
16S	Probe	6-FAM- TCCAGTTAATCATAGAACTTCATCAAA-BHQ-1	This chapter

Table 2.2. Primers and probes for the detection of environmental DNA traces released by the Freshwater Pearl mussel *M. margaritifera*

First, calibration curves were established by analysing a 1:10 dilution series of the DNA from tissue samples from *M. margaritifera* (7.8 ng/µl, Nanodrop 2000 Spectrophotometer, Thermofisher Scientific). This dilution series ranged from 10^{-1} to 10^{-7}. Ten technical replicates were ran for each dilution step in order to assess the LOD and LOQ (Bustin et al., 2009; Hunter et

al., 2017). The LOD was defined as the last standard dilution when the targeted DNA was detected and quantified in at least one qPCR replicate with a Ct under 45. The LOQ (and therefore the sensitivity of the assay) was defined as the last standard dilution when the targeted DNA was detected and quantified in at least 90% of replicates of the standard dilution with a Ct under 45. Each PCR and qPCR, with DNA extracted from tissues, was run in duplicate and was replicated at least two times. At least two negative controls were included in each run. Then, the DNA extracts obtained from all water samples from the mesocosm-experiment were analysed in six technical replicates in qPCR with at least four negative controls and two replicates of the dilution series from 10^{-1} to 10^{-4} as positive controls.

2.3.3 Statistical analysis

Standard dilution series obtained for the COI and 16S-based assays were used for determining the LOD and LOQ (Tréguier et al., 2014; Hunter et al., 2017). Linear regressions between dilution factor of tissue samples and the DNA concentration (i.e. means of technical replicates used) were established and r^2 of the regression was evaluated as a measure of repeatability of qualification. Further, the relationship between; (a) detection probability, i.e. the percentage of technical replicates that lead to a positive result, and (b) the coefficient of variation (CV, calculated as standard deviation divided by mean) of technical replicates within a sample to the dilution rate of tissue samples was examined in a regression analysis.

While experimental samples from mesocosms were analysed with both genetic assays, the 16S assay showed a lower detection probability than the COI (see results), therefore, further analysis was only conducted using the COI assay. The relationships between eDNA detection and mussel density in mesocosms was assessed in an ordinary least square regression analysis where r^2 representing the repeatability of quantification. The effect of mussel densities on detection probability and accuracy (i.e. CV within natural replicates) was likewise evaluated in linear regressions. The importance of natural variability, represented by the variability between natural replicates, was analysed using a one-way ANOVA. Regression analyses were tested for non-linearities by establishing separate regression models for non- and log-transformed data and comparing the models fit using the AIC and log-transformed data as necessary. Residuals were analysed, and no pattern or autocorrelation was found. Homogeneity of variance was evaluated

using a Bartlett test prior to ANOVAs and if necessary, measurements were transformed to achieve homoscedasticity. If transformations did not culminate in homogeneity of variances, a pairwise Wilcox test was used instead of ANOVA.

Finally, the effects of the number of technical and natural replicates on the reliability of eDNA measurements were investigated using a boot-strap approach and the results of the mesocosm experiment as a data pool. For a given combination of natural and technical replicates, data from each mesocosm was subsampled 10,000 times and the mean eDNA concentration for each subsample was calculated. Based on these simulations, (i) the mean probability of false negative detection across all mesocosms and (ii) the mean probability to achieve an incorrect result were determined. A "false negative" was thereby defined as a case when DNA was present in a mesocosm (as in all cases in this chapter), but undetected by the assay. An "incorrect result" on the other hand, was defined as a case when the mean eDNA concentration ranged outside the confidence interval of the regression between mussel density and eDNA concentration. This procedure was repeated for all possible combinations of 1-3 natural and 1-18 technical replicates. All statistical analyses and models were performed with R version 3.4.1 (R Core Team (2018)).

2.4 Results

Both sets of primers and probes were found to be specific *in-silico*. Moreover, the primers from (Stoeckle et al., 2015) targeting the 16S gene of *M. margaritifera* were specific against all other mussels tested when using standard PCR (Table 2.1.). The same result was achieved when the probe, (designed in this chapter), was added for use in qPCR (Table 2.2.). Primers from (Carlsson et al., 2017) aimed at targeting the COI gene of *M. margaritifera* amplified the targeted species and DNA extracted from *Anodonta anatina*, *Truncilla truncata*, and *Cumberlandia monodonta* when run with conventional PCR. However, the addition of the probe (designed in the study by Carlsson et al., 2017) increased specificity when utilising qPCR and resulted in the single detection of the target species, *M. margaritifera*.

The analysis of the two calibration curves revealed different LOD and LOQ for the two assays (Figure 2.3.). The COI assay proved to be consistently the more sensitive approach with the LOD and the LOQ falling at 0.78 and 7.8 pg mussel tissue, respectively (Figure 2.3.A.). The 16S assay

resulted in a detection of DNA at 0.78 pg mussel tissue. However only one out of 10 replicates was positive showing Ct of 45.74, which does not fulfil the requirements specified in the MIQE guidelines (Bustin et al., 2009). Hence, the LOD under these rules was 7.8 pg and the LOQ was found to be 78 pg (Figure 2.3.B.).

There was no significant difference (paired t-test, T = 0.3, p = 0.79) between the accuracy of the two assays, or between the natural replicates (ANOVA, p = 0.12). Further, the repeatability (indicated by the r^2 of the calibration curves), was quite similar for the two assays (COI: Adjusted $r^2 = 0.99$; 16S: Adjusted $r^2 = 0.97$). Further, the detection probability of both assays decreased with the dilution rate of tissue samples (16S assay $r^2 = 0.88$ and COI assay $r^2 = 0.85$) (Figure 2.3.C.). Likewise, the accuracy of both assays (which was represented by the CV of technical replicates), decreased with the dilution of sample DNA ($\log(y) = -0.23\log(x) - 4.86$; Adjusted $r^2 = 0.52$; $p = 0.04$). At the LOD, the CV was 0.09 and 0.04 for the COI and 16S assays, respectively (Figure 2.3.D.).

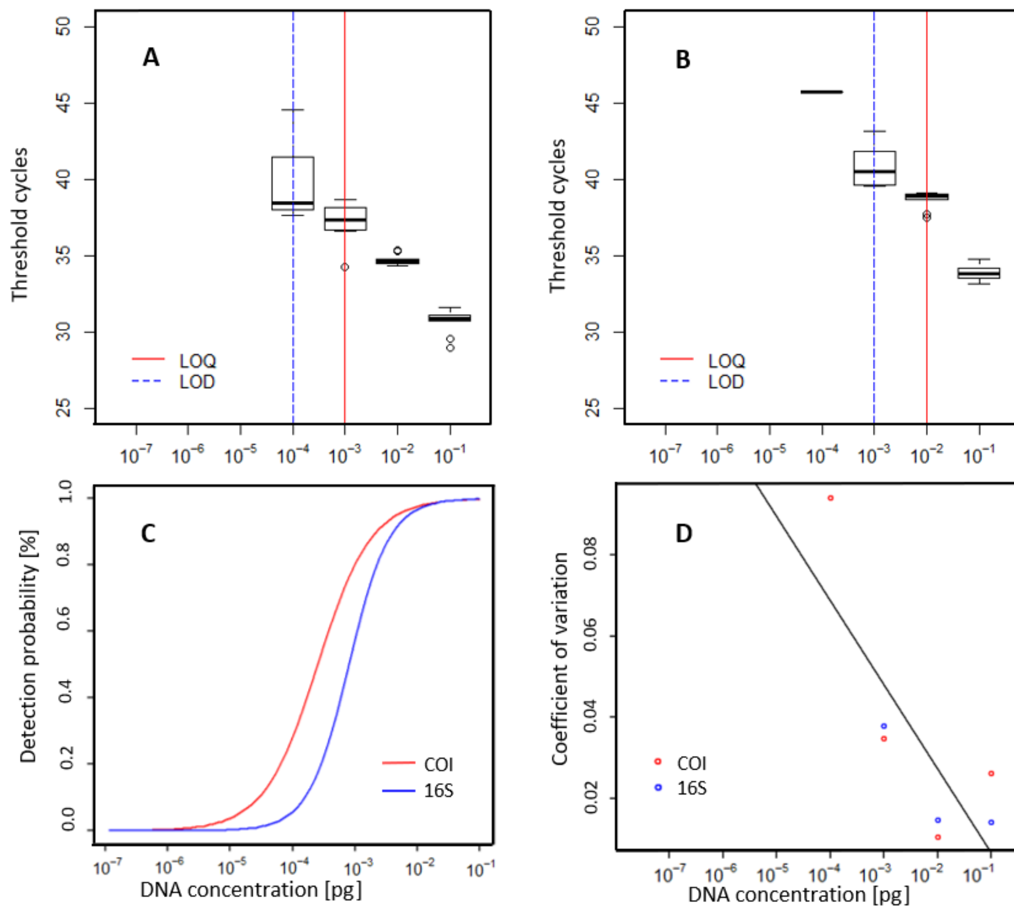

Figure 2.3. Assessment of standard curves used for quantifying *M. margaritifera* DNA as well as for determining the LOD and LOQ with qPCR targeting the COI **A** and the 16S region **B**. Standard curves were obtained with the same 1:10 dilution series from a starting concentration of 7.8 ng with 10 replicates per concentration. The Ct are representing the minimum number of qPCR amplification cycles leading to positive detection. **C**: The relationship between the detection probability and DNA concentration for the COI and 16s assays. **D**: The coefficient of variation of eDNA measurements and its relation to DNA concentration in the standard curve. The black line represents the regression equation for the relationship. Data was pooled from both assays as results did not diverge significantly between methods.

In this mesocosm experiment, environmental conditions were as follows: Temperature was 11.96 ± 0.05 ˚C, pressure 15.1 ± 0.02 PSI, turbidity 0.21 FNU ± 0.13, pH 7.03 ± 0.01, rugged DO 10.06 ± 0.03 mm L^{-1} and conductivity 45.56 ± 0.21 µS cm^{-1}. Flow rates were kept constant within mesocosms and only varied slightly between them (0.75 to 1.03 Ls^{-1}). Water samples from Lake Windermere (before and after the facilities internal filtration process), were all found to be negative for the presence of eDNA from *M. margaritifera* using both assays, showing that the eDNA from the mesocosms is not being recirculated from the lake.

The COI assay resulted in positive DNA signals in 100% of the mesocosms. The 16S assay, on the other hand, detected mussel DNA in only four out of six mesocosms and showed lower detection probability at the level of natural and technical replicates (Figure 2.4.). After this result, it would have been preferable to test the DNA of the six different populations to assess if there were various genetically distinct haplotypes present (i.e. did two of these populations have point mutations in the conserved 16S region where the assay targeted?). However, this was not possible (at the current time) as these animals are part of a breeding program and therefore tissue collection was avoided. That said, this should be considered in future studies. Furthermore, the detection probability for the 16S assay was higher for technical replicates than for natural replicates, a result due to the absence of eDNA detection in several of the mesocosms. For the above reasons, further statistical analyses focus only on the COI assay.

A significant negative correlation was found between, mussel density and the logged Ct of detection in the mesocosm experiment ($y = -1{,}422x + 36{,}842$, $r^2 = 0.88$, $p < 0.01$), hence the Ct

increased logarithmically with decreasing mussel densities in the mesocosms (Figure 2.5.). However, neither detection probability nor the CV of the mesocosms were significantly related to the Ct of detection and mussel densities in the mesocosms ($p > 0.19$). Consequently, five out of six of the mesocosms ranged outside the confidence interval of the relation between detection probability and the number of Ct leading to detection, which was established based on data from the standard curve (Figure 2.6.).

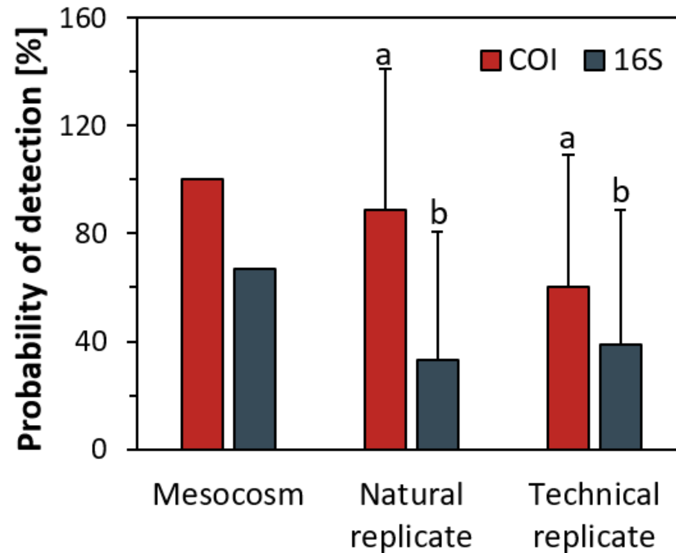

Figure 2.4. Probability of detecting eDNA in mesocosms (n = 6), natural replicates (n = 18) and technical replicates (n = 108) for the COI and the 16s assay. Error bars represent standard deviation and letters a, b relates to significant differences between assay at the natural and technical replicate level assessed by paired t-tests.

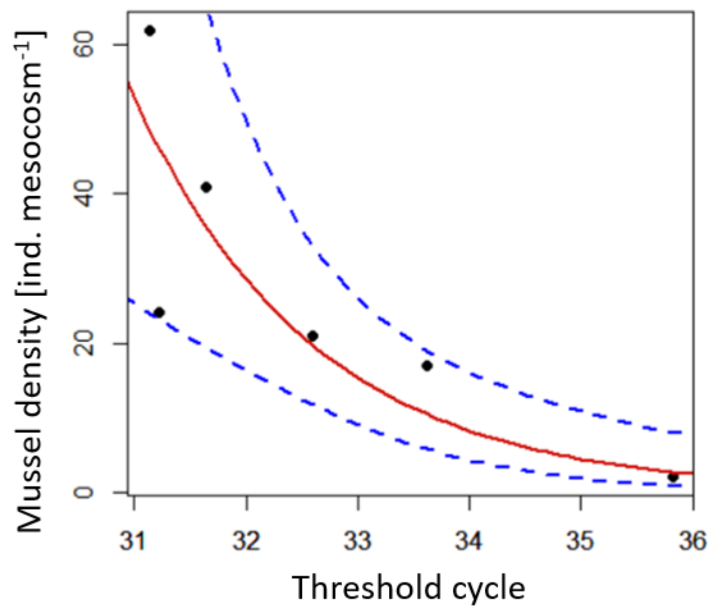

Figure 2.5. Relationship between the number of individuals of *M. margaritifera* present per mesocosm and the Ct of eDNA detection of mesocosm samples (points represent average of natural replicates) generated by the COI assay. Red line represents the regression equation of the relationship, doted blue lines indicate the confidence interval.

It was furthermore interesting to test if natural replicates taken from the same mesocosm showed a significant difference in their Ct of detection. In five out of the six mesocosms, a sufficient number of replicates yielded positive results to allow for this test to be conducted. In two of these five mesocosms, natural replicates showed significantly different results ($p < 0.03$) with regard to their Ct of detection. With mean difference of 1.1 and 1.5 Ct. A bootstrapping approach (to assess the effect of reduced replicate numbers on the reliability of measurements), revealed that a high number of replicates was required to ensure method sensitivity and accurate eDNA quantification (Figure 2.7.). The reduction of natural replicates had thereby a more negative impact on method reliability than the reduction of technical replicates, highlighting the importance of taking multiple water samples at the same field site.

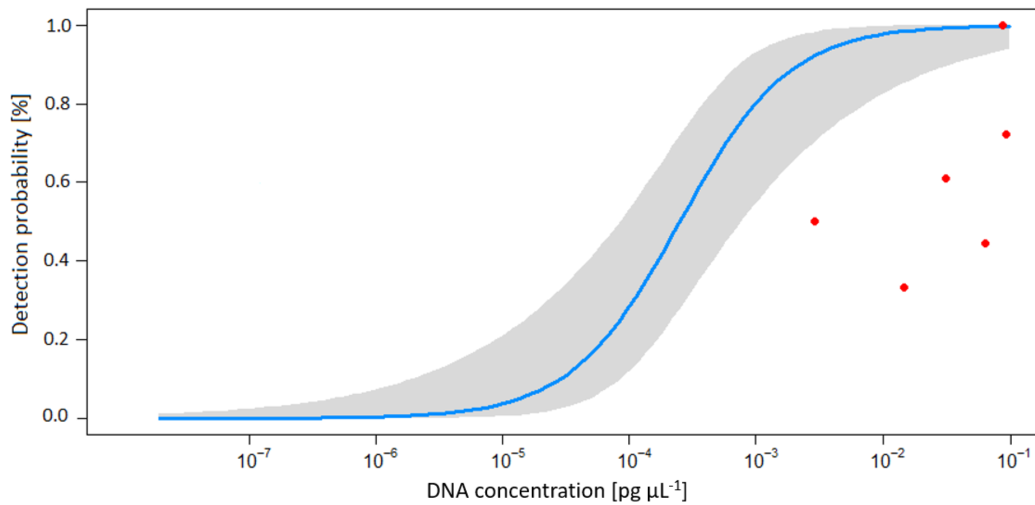

Figure 2.6. Change in detection probability with increasing DNA concentration in samples analysed with the COI assay. The blue curve and the grey-shaded area reflect the regression, established by analysing the standard curve, and its confidence interval. Red dots represent data from the mesocosm experiment. The standard curve for eDNA quantification (Figure 2.3.A.) was used to convert Ct of detection measured in mesocosm samples to DNA concentrations.

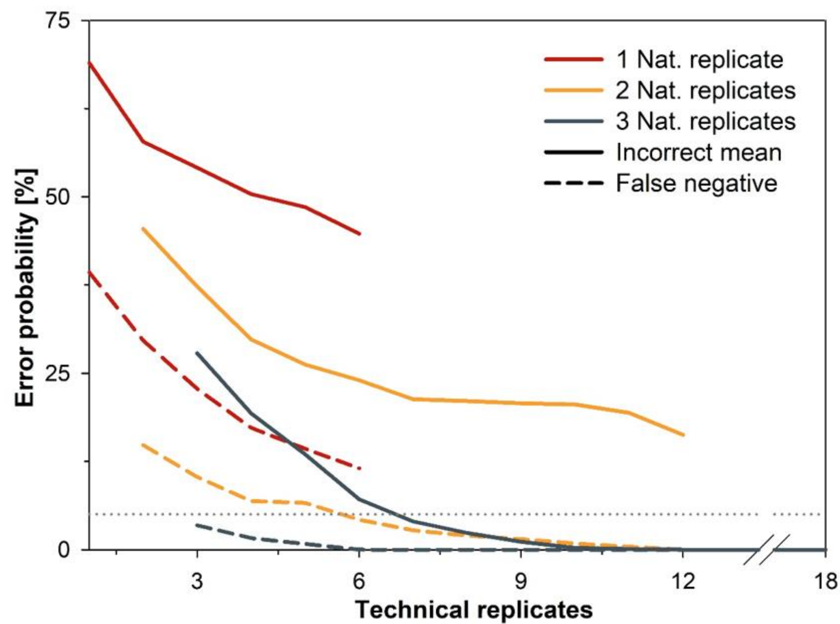

Figure 2.7. Impact of sampling design on the reliability of eDNA measurements. Results were generated by subsampling data from the mesocosm experiment in a bootstrap approach and reveal the change of the probability to attain false negatives or incorrect means, i.e. means that range

outside the confidence interval of the original relationship, with increases in the number of natural and technical replicates. Statistical simulations were based on results of the COI assay.

2.5 Discussion

In this chapter, I assessed the reliability of two different assays targeting the COI and 16S gene, which have been previously designed for the assessment of environmental DNA of the endangered Freshwater Pearl Mussel, *M. margaritifera*. Originally, the COI assay showed non-specificity during PCR, but in combination with a species-specific probe during qPCR, the specificity of this assay substantially increased and only targeted *M. margaritifera* (Carlsson et al., 2017). In contrast, the 16S assay showed specificity during both conventional PCR and qPCR. Applying both assays on the eDNA samples of the mesocosm experiment revealed that the efficiency of COI outperformed the 16S assay in terms of the LOD and LOQ, whereas the Ct appeared to be lower for the same dilution of standard samples. Despite being specific to *M. margaritifera*, detection of eDNA using the 16S assay failed in two out of the six mesocosms, where known mussels were present. These findings reemphasize the call by (Mahon et al., 2013) about the importance of rigorous *ex-situ* tests (under controlled experimental conditions) in order to validate assays before the use of eDNA in the field. Although already published primers and qPCR assays were applied, method validation against MIQE guidelines and an additional test under controlled experimental conditions was necessary to select the most efficient and reliable primers/probe for qPCR quality (Bustin et al., 2009; Hunter et al., 2017). Furthermore, the size of both markers could also explain the difference in the efficiency of eDNA detection. As specified in various studies (Hänfling et al., 2016; Jo et al., 2017), larger fragments of DNA (172 bp for 16S) degrade more rapidly than small fragments (83 bp for COI), and are therefore less abundant in natural environments (Bista et al., 2017; Wei et al., 2018).

A key finding of this chapter was that method validation and obtained sensitivity of primers and qPCR under lab-settings can differ largely from results attained in more natural environmental conditions. High concentration of mussel DNA in the standard dilution led to high detectability and high efficiency of eDNA detection and quantification compared to high dilution standards. As described in (Hunter et al., 2017), qPCR detection had minor variation among replicates of

samples. However, instrumental response shows poor reproducibility at low eDNA concentrations which is typical of eDNA samples (Hunter et al., 2017). Here, the same results were found to be achievable (when exploring the standard curves) (Figure 2.6.) using both assays assessed. Surprisingly, when a high number of mussels were present in a given mesocosm, there was observation of a higher detection probability amongst replicates than in mesocosms with low numbers of mussels. Furthermore, in this chapter, the LOD for eDNA from *M. margaritifera* (10^{-4} ng) was similar to that shown in (Carlsson et al., 2017) i.e. quite high when compared to other animal groups (Tréguier et al., 2014; Buxton et al., 2017). For example, for the invasive crayfish *Procambarus clarkii* (Girard, 1852) and for the endangered newt *T. cristatus*, LODs of 10^{-7} ng and lower were reported (Tréguier et al., 2014; Buxton et al., 2017). A relatively high LOD represents a potential limiting factor for detecting eDNA from mussel species with low abundance in the field. However, operating relatively close to the LOD in this second chapter, allowed to assess the reliability of eDNA detection and quantification under stress-conditions, which are likely frequently encountered during *in-situ* eDNA assessments. There is also a slight possibility that PCR inhibitors were affecting the eDNA detection in this chapter. Although no test for PCR inhibition were performed within the collected samples, inhibition (if present) would be most likely low for three main reasons. The water entering each mesocosms was filtered (through a 20-micron Hydrotech Drumfilter HDF800-series), in the absence of any other physical or chemical treatment. As the eDNA samples were taken within centimetres of the mussels, this is unlikely to play an important factor here. Third and finally, inhibition has also been shown to be driven by compounds produced via various biological processes of phytoplankton and plant matter for example (McKee et al., 2014), the filtered water would have removed the vast majority of these compounds.

Interestingly, a positive relationship between eDNA quantification and the mussels density in mesocosms was illustrated, highlighting similar levels of repeatability as seen in various studies on other organisms (Takahara et al., 2012; Lacoursière-Roussel et al., 2015; Eichmiller et al., 2016b; Mauvisseau et al., 2017). In this second chapter, however, the relationship between the number of mussels present in the mesocosm experiment and the eDNA quantification was non-linear. Therefore, although quantification appears to be possible for *M. margaritifera* using eDNA, further studies still need to be conducted in order to assess the effects of various environmental

variables on species quantification. In fact, one of the key objectives in any eDNA study should be the exploration of factors that increase and decrease the eDNA sheading rate per species and in this instance, this chapter highlighted that density is certainly one to take into account. In this chapter, experimental mesocosms were used, and it is still unknow if these results would be similar in natural environments, as higher flow rate could decrease the eDNA signal of individuals present in low abundance. Furthermore, several ecological aspects (i.e. stage of life, seasonality…) could potentially lead to different results, as well as the use of other detection/quantification tools, such as ddPCR. Exploring the effect of biological and environmental factors including temperature, pH, flow rate and sedimentation, as in (Strickler et al., 2015; Eichmiller et al., 2016a; Buxton et al., 2018) for example, will improve our understanding of the variability of eDNA sheading rates under natural conditions. Furthermore, the method of filtration could also be explored in more detail and may be important in optimising the assay for management and mitigation applications. Here, the utilised enclosed Sterivex filters were highlighted by (Spens et al., 2017) as being desirable. However, these remain costly and the use of cellulose nitrate filters has been recently proposed to be better than (or at least equal to) the Sterivex method (Majaneva et al., 2018). The reduced cost of these filters means they should certainly be considered for use in future studies.

Finally, I assessed how to improve the efficiency of eDNA sampling strategies for *M. margaritifera*. The statistical modelling approach utilised revealed that the collection of three natural replicates per field location is required to ensure a high reliability of eDNA detection and quantification. However, an even higher number of natural replicates should be collected (four to six for example), as this will likely further increase the repeatability and accuracy of species quantification in the field and having more than three allows for the possible failure or poor extraction of DNA from any one given sample. On the other hand, the number of technical replicates could be reduced because the analyses of four technical replicates (per natural replicate), was sufficient to reduce the expected error probability below 1% (Figure 2.7.). Based on these findings, it is also recommended to use standard dilutions on each PCR plate, both as a positive control but also for estimating the LOD of the analysed samples (Hunter et al., 2015, 2017, 2018). Thereby, the MIQE guidelines can be used for assessing the efficiency of any newly developed assay and should be a minimum standard for all eDNA studies moving forward (Bustin et al., 2009; Hunter et al., 2017).

In conclusion, these findings reveal that different methodological aspects influence the reliability of eDNA assays at various levels. Method selection was mostly dependant on detection probability and the LOD, as accuracy and repeatability were similar for both assays assessed in this chapter. However, species quantification mostly relied on repeatability, despite the use of three natural replicates from mesocosms scattered around regression predictions. Finally, method efficiency represented by the minimum effort for obtaining robust results was dependant on accuracy and detection probability of measurements. These factors were proven to be critical because of the observed high variability between natural replicates and the detection probability of ~50% as this is clearly above the LOD.

Chapter 3: eDNA based monitoring: advancement in management and conservation of critically endangered killifish species

This chapter has been published as: Mauvisseau Q, Kalogianni E, Zimmerman B, Bulling M, Brys R, Sweet M. (2020) eDNA-based monitoring: Advancement in management and conservation of critically endangered killifish species. *Environmental DNA*. https://doi.org/10.1002/edn3.92

3.1 Abstract

Ecosystems are currently changing at unprecedented rates due to anthropogenic influences. Application of appropriate management regimes and mitigation measures requires knowledge of ecological community composition and monitoring of any changes that occur. Environmental DNA-based monitoring is becoming increasingly common and offers substantial potential as a non-invasive method associated with highly repeatable and reliable results. In this chapter, river systems in Western Greece that have been strongly impacted by anthropogenic activities and the spread of an alien invasive fish species, the Eastern mosquitofish (*Gambusia holbrooki*) were monitored. This invasive species has been credited as the major cause for the drastic declines of two endemic killifish species (*Valencia letourneuxi* and *Valencia robertae*). Here, the efficacy of an eDNA-based method of detection for all three species was investigated, as an alternative to traditional methods for monitoring. Initially, a mesocosm experiment provided material for the design and validation of the sampling protocol. This was followed by two sampling periods in the field conducted in autumn 2017 and 2018, comparing the novel eDNA assays with the conventional surveying methods in respectively six and 20 systems. eDNA detection consistently outperformed the traditional monitoring methods for both *V. letourneuxi* and *V. robertae* and was comparable for the invasive *G. holbrooki*. This supports the now increasing body of literature, highlighting the benefits of species-specific, targeted eDNA assays for the assessment of threatened and/or invasive species, one which can be utilised by conservation organisations and government bodies alike. However, care should always be taken when designing such tools and strict validation steps should be adhered to, particularly with respect to minimising the probability of false positives and negatives.

3.2 Introduction

Declining biodiversity, driven primarily by increasing anthropogenic activities such as habitat destruction and/or over exploitation, coupled with effects of climate change, is significantly altering nearly every ecosystem on our planet (Harley, 2011; Cardinale et al., 2012; Hooper et al., 2012). The extent of the loss in species is now so great, that many describe this as a sixth mass extinction event (Ceballos et al., 2015). These losses are leading to negative impacts on ecosystem functioning, threatening the services they provide (Brooks et al., 2006; Ceballos et al., 2015). Therefore, there is an urgent need to reduce the rate of species loss through effective conservation management and regulating controls on land and water usage/management (Butchart et al., 2010; Hooper et al., 2012).

Wetlands and freshwater habitats in particular, are now among the most endangered habitats in the world (Davidson, 2014; Reid et al., 2019). As well as impacts of climate change, these habitats are exposed to a number of other substantive threats, including anthropogenic development, pollution, impact caused by species introduction/biological invasions, and more general causes of habitat degradation (Reid et al., 2019). However, there are well documented examples of habitat restoration in such ecosystems that involve relatively little effort and cost (Palmer et al., 2005; Wohl et al., 2005; Hnig et al., 2011; Morandi et al., 2014; Dolédec et al., 2015; Lyon et al., 2019). Effective habitat restoration or habitat management requires knowledge of the current state of an ecosystem, as well as monitoring over time, in which the documentation of species presence is particularly important (Rosenberg et al., 2000). Further, the early detection of invasive species would be beneficial in such instances in order to implement management plans quickly and thereby minimize any effect of such species (Vander Zanden et al., 2010; Xia et al., 2017). That said, it is often problematic to assess rare and invasive species, as in many instances they are in low abundances, which make the majority of traditional methods of surveying freshwater habitats (such as netting and electrofishing) ineffective as they require substantial amounts of time, effort and expertise to yield useful results (Eiler et al., 2018). These requirements lead to constraints on the number and extent of surveys that can be undertaken.

Environmental DNA (eDNA) detection is now widely utilised as an alternative tool for monitoring a number of species (Sepulveda et al., 2019; Thomas et al., 2019; Vilaça et al., 2019; Wacker et

al., 2019). As eDNA is non-invasive in nature (relying on the detection of DNA traces left by living or dead organisms in their environment), species which are present even in low abundance can be efficiently detected with no impact on the sampled habitat or co-occurring species (Thomsen and Willerslev, 2015). Indeed eDNA based methods are already, for example, commercially available for the detection of endangered species in the United Kingdom (Harper et al., 2018b). Utilisation of eDNA based detection methods can also allow for larger-scale surveys to be undertaken, with comparatively lower effort employed compared to traditional methods (Yatsuyanagi et al., 2019).

In this chapter, I assess the use of environmental DNA detection as a rapid and effective tool for monitoring the occurrence of two threatened freshwater killifish species *Valencia letourneuxi* (Sauvage, 1880) and *Valencia robertae* (Freyhof et al., 2014) and of the alien invasive *Gambusia holbrooki* (Girard, 1859) in Greek aquatic systems. Once widely distributed in Western Greece (Barbieri et al., 2000), the distribution ranges of the two native species has now been drastically reduced over the last 40 years. The decline has been linked to anthropogenic habitat modification and competition from the non-native Eastern mosquitofish, *G. holbrooki* (Kalogianni et al., 2010, 2019). *G. holbrooki*, originated from the United States and Mexico is now widespread throughout much of Southern Europe where it was introduced in the early 1920s (Ribeiro and Leunda, 2012; Piria et al., 2018). *G. holbrooki* was initially introduced to control mosquito populations through predation of the larvae and is now the most widespread alien freshwater fish species in Greece (García-Berthou et al., 2005; Economou et al., 2007). Although successful to a degree in its aim, it spread rapidly and was uncontrollable due to its early maturation, viviparity and high reproductive rates. The species was much more adaptable than originally thought and exhibited high behavioural plasticity further ensuring its success even in degraded habitats (Vargas and de Sostoa, 1996). Due to the spread of this invasive species and the other anthropogenic stressors mentioned above, *V. letourneuxi* has been listed amongst the 'world's 100 most threatened species' (Baillie and Butcher, 2012; Freyhof et al., 2014). The range of both *Valencia* species is thought to now be restricted to only 12 systems and these populations are thought to be vulnerable. Indeed, they have already gone extinct from two sites where they were historically known to be clinging on (Kalogianni et al., 2010; unpublished HCMR data). Due to the threatened status of these species, traditional methods for assessing their presence and absence (electrofishing and netting

for example) are far from ideal due to their often destructive nature (Kalogianni et al., 2010; Vogiatzi et al., 2014). As both species are strictly protected by the Bern Convention (Appendix II) and by Presidential Decree (No. 67/1981 of the Greek state Barbieri et al., 2002; Kalogianni et al., 2010; Freyhof et al., 2014), finding a less invasive survey method is urgently needed to ensure effective management and mitigation of these endangered species continues.

Therefore, this chapter aimed to investigate the use of eDNA detection as a reliable alternative tool for monitoring the two Valencia species and *G. holbrooki* across Western Greece. Species-specific assays were developed and validated, and a controlled mesocosm experiment allowed to optimize the sampling protocol. Finally, two independent field surveys (combining both eDNA detection and conventional fish surveying methods) were conducted to assess the reliability of these methods, illustrating a proof of concept for this method to be utilised in future conservation programs.

3.3 Methods

3.3.1 Assay development

Species-specific primers and probes targeting the Cytochrome C Oxidase subunit 1 gene (COI) of *Valencia letourneuxi*, *Valencia robertae* and *Gambusia holbrooki* were designed using the Geneious Pro R10 Software (https://www.geneious.com; Appendix 3; Kearse et al., 2012). These were tested against DNA sequences retrieved from the NCBI database (National Centre for Biotechnology Information; https://www.ncbi.nlm.nih.gov/) from 23 fish species known to be, or likely to be present in the same ecosystems with the targeted organisms (See Appendix 3). The size of the fragments amplified were 113 bp in length for *V. letourneuxi*, 137 bp for *V. robertae*, and 167 bp for *G. holbrooki* (see Table 3.1.). The specificity of each assay was assessed *in-silico* using the previously mentioned DNA sequences (See Appendix 3.A). After *in-silico* validation, the specificity of each assay was tested *in-vitro* with PCR and qPCR using DNA extracted from the following co-occurring species: *Anguilla anguilla* (Linnaeus, 1758), *Economidichthys pygmaeus* (Holly, 1929), *G. holbrooki*, *Gasterosteus aculeatus* (Linnaeus, 1758), *Pelasgus thesproticus* (Stephanidis, 1939), *Pelasgus stymphalicus* (Valenciennes, 1844), *Squalius peloponensis* (Valenciennes, 1844), *V. letourneuxi* and *V. robertae*. DNA was extracted from

tissue samples of these species using the Qiagen DNeasy® Blood and Tissue kit following the manufacturer's instructions. Additional information on the assays development are provided in Appendix 3.B.

3.3.2 eDNA extraction

As in chapter 2, eDNA was extracted from the filters with the Qiagen DNeasy® Blood and Tissue Kit, following the extraction workflow for Sterivex filters outlined in Spens et al. (2017). Extraction of eDNA samples was performed in a separate clean PCR-free room (different than that used for extraction of the tissue samples identified above). This is in order to reduce potential cross-contamination between the samples. Two different types of 'negative controls' were also utilised throughout this study. The 1st consisted of a field control whereby two independent 1 L samples of tap water were filtrated through a Sterivex filter in the field at the time of eDNA sampling. The 2nd consisted in two extraction controls, where water was substituted instead of the samples. All laboratory equipment was regularly disinfected and decontaminated under UV-radiation throughout the whole analysis process. All other laboratory surfaces were disinfected using 10% bleach and ethanol prior to analysis.

Species	**Primers**	**Sequences (5'-3')**	**Target**
Valencia robertae	Forward	ATGGCCTTCCCCCGAATGAA	COI
	Reverse	GCTAAGTTTCCGGCCAGAGG	COI
	Probe	CTTCCTCTGGCGTCGAGGC	COI
Valencia letourneuxi	Forward	TGGGGGTTTTGGCAACTGAC	COI
	Reverse	GGAGGAGAAGAAACGAGGGGGG	COI
	Probe	CATAGCCTTCCCTCGGATAAAC	COI
Gambusia holbrooki	Forward	GTGCCCCAGACATAGCCTTT	COI
	Reverse	TACAGAAGGTCCGGCATGTG	COI
	Probe	AAGATGCGAGGAGGAGGAGA	COI

Table 3.1. Primers and probes designed in this chapter for the detection of eDNA traces from *V. robertae*, *V. letourneuxi* and *G. holbrooki* in freshwater systems.

3.3.3 PCR and qPCR

Primers specificity was assessed using PCR before conducting qPCR. PCR amplifications were performed, each with two technical replicates on a Gen Amp PCR System 9700 (Applied Biosystem) with each set of the three species-specific primers developed in this chapter (Table 3.1). PCR protocols and conditions were the same for the three targeted species. In brief, PCR reactions were conducted in a 25 µL reaction with 12.5 µL of PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10 µM), 9.5 µL of ddH2O and 1 µL of DNA template. Optimal PCR conditions were as follows: thermal cycling at 50 °C for two min and 95 °C for 10 min, followed by 35 cycles at 95 °C for 15s and 62 °C for 1 min. At least one positive (DNA extracted from tissue samples, (*V. letourneuxi* (21 ng/ µL), *V. robertae* (9.2 ng/ µL) and *G. holbrooki* (9.7 ng/ µL)) and one negative (no template) control were included for each PCR. PCR amplification were confirmed by electrophoresis on 2% agarose gel stained with 3 µL of GelRed ™ Nucleic Acid Gel Stain, Biotium. Product sizes from amplified DNA were checked with visual comparison with PCRBio Ladder IV (PCRBIOSYSTEMS).

qPCR reactions were performed on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems) with the assays designed in this chapter (Table 3.1). The specificity of each assay was further confirmed by qPCR using two replicates of DNA extracted from the species mentioned above. qPCR protocols and conditions were the same across all three targeted species. These consisted of a 25 µL final volume, using 12.5 µL of PrecisionPlus qPCR Master Mix with ROX (Primer Design, UK), 1 µL of each primer (10 µM), 1 µL of probe (2.5 µM), 6.5 µL of ddH2O and 3 µL of extracted DNA. Optimal qPCR conditions were as follow: thermal cycling at 50 °C for two min and 95 °C for 10 min followed by 50 cycles of 95 °C for 15s and 62 °C for 1 min.

3.3.4 qPCR analysis

Standard curves were established by analysing a 1:10 dilution series of DNA extracted from tissue samples of *V. letourneuxi* (2.1 ng/ µL, Nanodrop 2000 Spectrophotometer, Thermofisher

Scientific), *V. robertae* (9.2 ng/ µL) and *G. holbrooki* (9.7 ng/ µL) following the MIQE Guidelines (Bustin et al., 2009; Mauvisseau et al., 2019a) (Appendix 2). For the three species, the dilution series ranged from 10^{-1} to 10^{-8} using 10 'technical replicates' (i.e. qPCR replicates) for each dilution step, allowing for the assessment of the Limit of Detection (LOD) and Limit of Quantification (LOQ) (Figure 3.1) (Bustin et al., 2009; Tréguier et al., 2014; Mauvisseau et al., 2019a). As in chapter 2, the LOD was identified as the last dilution of the standard curve in which the targeted DNA is amplified with a cycle threshold (Ct) below 45 (Mauvisseau et al., 2019a, 2019c). The LOQ was identified as the last dilution of the standard curve where the targeted DNA is amplified and quantified in at least 90% of the qPCR replicates with a cycle threshold below 45 (Mauvisseau et al., 2019a, 2019c). When validating the specificity of each assay (with qPCR), at least two positive (tissue samples of the respective species) and two negative controls (filtered sterile water) were included. All eDNA samples were analysed using six technical replicates. Each run analysing eDNA samples also contained two replicates of each six dilution points ranging from 10^{-1} to 10^{-5} (the same as used for establishing the standard curves) as positive control, and six negative controls (no template).

3.3.5 *Ex-situ* testing of the eDNA assays

At the Zoological Society of London (ZSL), UK (https://www.zsl.org/), 128 *V. robertae* specimens were housed over three different mesocosms ('A', 'B' and 'C') as a part of a conservation and breeding programme. This opportunity was used for testing the sampling protocols and assessed the reliability of the developed assay (Appendix 4). Mesocosm 'A' housed 40 juveniles in a 500 L aquarium (equating to a fish biomass of 10 g), 'B' housed a mix of 12 adults and 10 juveniles in 626 L (biomass of 19.8 g) and 'C' housed 66 adults in 723 L (biomass of 101.5 g) (Appendix 4). As part of the breeding programme, eggs were collected on a daily basis and stored in mesocosm 'B' for development and hatching. However, this was not ideal for the purposes of this experiment and the potential implications of this limitation are addressed later in the discussion of this chapter.

In each mesocosm, ten x 1 L water samples were collected from the surface using a sterile polypropylene ladle and a sterile plastic bag (Whirl-Pak® 1242 mL Stand-Up Bag Merck®,

Darmstadt, Germany). As two filter types were compared for assessing their efficiency for eDNA collection, five of these samples were filtrated through a sterile 0.45 µm Sterivex ™ HV filters (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Millipore®, Germany), and the remaining five were filtrated through a sterile 0.22 µm Sterivex ™ GP filters (Sterivex™ filter unit, GP with luer-lock outlet, Merck®, Millipore®, Germany). All filters were then locked using sterile luer lock caps, put into 50 mL Falcon tubes (Falcon™ 50 ml Conical Centrifuge Tube, Fisher Scientific, Ottawa, Canada) and immediately frozen at -20°C. To avoid contamination, disposable nitrile gloves were used during each step of the sampling.

3.3.6 *In-situ* trial 1 (A) for *V. robertae* and *V. letourneuxi*

This component tested the newly designed assays for detecting eDNA from both *V. letourneuxi* and *V. robertae in-situ*. Eight samples from six sites spanning six distinct aquatic systems (stream, wetland, or canal) were sampled over two days (from 26th to 27th September 2017) (Table 3.2, see also Appendix 5). These six aquatic systems are distributed in the two distinct geographical areas where *V. letourneuxi* and *V. robertae* are present (Appendix 5, Fig. S1). More specifically, based on previous biodiversity assessment (Table 3.2), the presence of *V. letourneuxi* was expected at locations '1A' to '4A' and the presence of *V. robertae* was expected at locations '5A' and '6A'. However, all locations were analysed with both Valencia's assays, as a complementary step assessing their reliability. At sites '1A' - '4A', a single sample up to 1 L of water (depending on the turbidity) allowing a full cover of the site, was taken from the water surface in the same manner as that detailed above. Two samples, spaced at least 20 m apart, were collected from sites '5A' and '6A' due to site characteristics (i.e. to allow a better coverage of both riverbanks with dense riparian coverage). The samples were then mixed and filtrated through a sterile 0.45 µm Sterivex ™ HV filter (see results from *ex-situ* methods). The filters were then immediately fixed with 2 mL of absolute ethanol, locked using sterile luer lock caps and stored into a sterile 50mL Falcon tube (Falcon™ 50 ml Conical Centrifuge Tube, Fisher Scientific, Ottawa, Canada) at -20°C until DNA extraction.

After eDNA sampling, conventional methods of fish sampling were also applied at '1A', '3A', '4A' and '6A' to assess for the presence or absence of *V. letourneuxi* and *V. robertae*. At three sites ('1A', '3A' and '4A') sampling was conducted with a D-shaped frame net with a 1.5 m wooden handle (in conjunction with smaller dip nets). Specimens were fished using these nets

depending on site accessibility by two persons during 15 min in a 20 m transect. At '6A' (which was the only site that was wadable), a seine net was used (5 trials) in conjunction with smaller dip nets. Due to the variability of habitats and fishing method used, only relative abundance (% contribution) data are presented for purposes of comparison (See Appendix 5).

Sites	Species targeted	Suspected presence	Fish sampled	eDNA detection	pH	TC°	Locations sampled	Volume (mL)	Date
1A	*V. letourneuxi*	Yes[1]	No	0/6	6.6	16.0	1	1000	27/09/2017
2A	*V. letourneuxi*	Yes[2]	NA	0/6	6.9	15.0	1	1000	27/09/2017
3A	*V. letourneuxi*	Yes[3]	No	0/6	-	-	1	1000	27/09/2017
4A	*V. letourneuxi*	Yes[4]	Yes	1/6	6.8	17.9	1	300	27/09/2017
5A	*V. robertae*	Yes[5]	NA	12/12	6.6	17.7	2	1000	26/09/2017
6A	*V. robertae*	Yes[6]	Yes	9/12	6.6	18.8	2	1000	26/09/2017

Table 3.2. Table depicting the sites sampled in 2017, the fish species targeted at each location, their suspected presence (based on past observation and more recent fish surveys), the fish sampling results (if the targeted fish were captured or not during this survey), eDNA detection results including the number of positive qPCR replicates, pH, water temperature, the number of locations sampled in each aquatic system, the volume filtered at each location and the sampling date. Fish sampling was not performed in sites '2A' and '5A', as these systems are part of an ongoing enhancement/reintroduction conservation programme (see NA: not performed). Only 300 mL of water was filtered at the site '4A' due to high water turbidity. [1]: Last confirmed record 2013; [2]: Translocation action undertaken in 2015 and 2016; [3]: Last confirmed record 2007; [4]: Last confirmed record 2016; [5]: Last confirmed record in 2015; [6]: Last confirmed record in 2016 (based on unpublished HCMR data).

It was not possible to sample for fish at two of the sites ('2A' and '5A'), since these were the "recipient sites" of a then ongoing enhancement/translocation programme (undertaken annually during 2015-2017) and actions leading to potentially disturb populations there were forbidden. Within the frame of this conservation programme, *V. letourneuxi* individuals from '4A' and *V. robertae* individuals from '6A' were collected during this enhancement/translocation programme with a variety of methods (seine net, D net and small nets) and transferred, bottled with water and oxygen, to the "recipient sites", i.e. *V. letourneuxi* individuals to '2A', in order to establish a new

population in this spring-fed stream, and *V. robertae* individuals to '5A' with the intention of enhancing the local population there (Table 3.2, Appendix 5). All field equipment was disinfected with a chlorine solution between locations. To avoid potential cross contamination, eDNA sampling was performed before traditional fishing.

3.3.7 *In-situ* trial 2 (B) for all three fish species

The second component of this case study encompassed all three target species and was mapped against a more invasive conventional survey method (sampling using electrofishing or netting). Here, the first trial run (trial 1 A) was expanded to a proof of concept for conservation practices, whereby twenty aquatic systems were surveyed using both the newly designed eDNA assays and the more traditional fishing methods (i.e. electrofishing or netting). This was conducted over a two week period (from 16th to 28th October 2018). It is important to note that the identifier codes used in the two different sampling events do not correspond to the same sampling site (i.e. sites '1A' and '1B' are two different locations).

All sites were sampled at one location, with eDNA sampling being performed before any fishing action. In trial 2 B, two independent water samples up to 1 L were collected. Variation in the volume of water filtered was dependant on the turbidity. Hereafter these samples were refer to as natural replicates. They were collected from the surface and filtered through a sterile 0.45 µm Sterivex ™ HV filter. These eDNA samples were fixed and stored as described above.

The majority of the sites were sampled using electrofishing, with the exception of four, that were sampled using a D-shaped frame net (Table 3.3, Figure 3.3, see also Appendix 7, Table S2). This was due to high water salinity at these sites. When electrofishing was undertaken at depths < 1.5 m (wadable sites), a single 100 m electrofishing pass was conducted from downstream to upstream. At sites that were > 1.5 m in depth (non-wadable sites), electrofishing was undertaken from the bank. Electrofishing was performed using a Honda GX160 3KW generator (150 m cable, 1.5 m anode pole, 5-10A DC output, voltage range 300–600 V). At the four brackish sites ('7B', '8B', '11B' and '13B', See Appendix 6, Table S2), sampling was conducted with a D-shape net (minimum 8 sweeps and maximum 22 sweeps). Due to the variability of habitats and fishing method used, only relative abundance (% contribution) data were presented for purposes of

comparison, as well as information on historical presence of the target species (Appendix 6, Table S3).

Sites	*V. letourneuxi*		*V. robertae*		*G. holbrooki*		pH	TC°	Volume (mL)	Date
	Fishing	*eDNA*	*Fishing*	*eDNA*	*Fishing*	eDNA				
1B	Yes	0/12	/	-	No	0/12	7.84	16.8	1000	16/10/2018
2B	No	6/12	/	-	No	4/12	7.31	16.7	1000	17/10/2018
3B	No	0/12	/	-	Yes	0/12	7.81	19.5	1000	17/10/2018
4B	NP	0/12	NA	-	NP	0/12	7.11	19.5	750	17/10/2018
5B	No	0/12	/	-	Yes	4/12	7.41	16.7	1000	18/10/2018
6B	No	0/12	/		Yes	12/12	7.54	16.7	1000	18/10/2018
7B	No	3/12	/	-	Yes	1/12	7.77	18.5	1000	19/10/2018
8B	No	0/12	/	-	Yes	0/12	7.76	17.9	1000	19/10/2018
9B	No	9/12	/	-	Yes	12/12	7.58	16.5	1000	19/10/2018
10B	Yes	12/12	/	-	Yes	12/12	7.58	16.7	1000	20/10/2018
11B	No	12/12	/	0/12	Yes	6/12	7.49	16.7	1000	21/10/2018
12B	No	0/12	/	-	No	1/12	7.82	16.8	1000	21/10/2018
13B	/	2/12	Yes	12/12	Yes	12/12	7.22	18.2	1000	22/10/2018
14B	/	0/12	Yes	0/12	Yes	0/12	7.52	16.7	1000	22/10/2018
15B	/	-	No	8/12	Yes	12/12	7.42	17.2	1000	23/10/2018
16B	/	-	No	12/12	No	0/12	7.64	17.0	1000	24/10/2018
17B	/	-	No	0/12	Yes	3/12	7.49	17.6	1000	26/10/2018
18B	/	-	No	12/12	No	7/12	7.43	17.2	1000	28/10/2018
19B	/	-	Yes	6/12	No	0/12	7.45	18.0	1000	28/10/2018
20B	/	-	Yes	6/12	No	0/12	7.52	19.1	1000	28/10/2018

Table 3.3. Table depicting the sampled locations in 2018, the fishing results (i.e. whether the targeted fish were found during fish sampling conducted in 2018, see also Appendix 6), eDNA detection results including the number of positive qPCR replicates for each targeted species, pH and water temperature, the volume of water sampled for each natural replicate and the sampling date. Due to high turbidity and pollution at the sampling location '4B', only 750 mL was filtered for both natural replicates and no fishing was performed (see NA: not performed and / when the site was outside of the known geographical area of the targeted fish). Sites '1B' to '12B' are within the geographical range or *V. letourneuxi* , while sites '14B' to '20B' are in the geographical range of *V. robertae*, with the exception of '17B'. Site '13B' is located at the junction of the ranges of the two Valencia species. Due to the detection of both Valencia species at site '13B', the

neighbouring locations (i.e. '11B' and '14B') were also investigated for both Valencia species using eDNA detection.

A number of these sites were targeted due to previous knowledge of the species' historical home ranges. Specifically, *V. letourneuxi* presence was expected and therefore assessed against 12 sites ('1B' to '12B') and *V. robertae* presence was expected and assessed at seven sites ('13B'-'16B' and '18B' to '20B'). '17B', fell outside the known range of both species, and was therefore used as a negative control (Table 3.3, Figure 3.3, Appendix 6). Sites '11B', '13B' and '14B', were at the border of the ranges of the two Valencia species, and therefore, the presence of both *V. letourneuxi* and *V. robertae* was assessed. Further, *G. holbrooki* presence was assessed across all 20 sites ('1B' to '20B', Table 3.3, Figure 3.3, Appendix 6). It should be noted that *G. holbrooki* was expected to be absent at five of the sampled locations (see Appendix 6 for more detail into why). Presence or absence of *G. holbrooki* was unsure at a further site, due to the absence of historical data ('2B'; Appendix 6).

Finally, at each sampling location, physicochemical water quality parameters, i.e. water temperature (°C), salinity (ppt) and pH were measured using a Portable multiparameter Aquaprobe AP- 200 with a GPS Aquameter (Aquaread AP 2000) (Table 3.3, Appendix 6, Table S2). All field equipment was disinfected with a chlorine solution between locations. To avoid potential cross contamination, eDNA sampling was performed before conducting traditional fishing and water quality measurements.

3.3.8 Statistical analysis

In order to assess the effect of the filters pore size on the Cycle threshold (Ct) for the detection of *V. robertae* in the mesocosm experiment, a one-way ANOVA analyses were performed. For each of the natural replicates, six technical replicates were ran using qPCR analysis. Therefore, where only one natural replicate was sampled, six technical replicates were obtained, whilst where two natural replicates were sampled, twelve technical replicates were obtained (Tables 3.2 and 3.3). Statistical analyses were performed with R version 3.4.1 (R Core Team (2018)).

3.4 Results

All assays designed in this chapter were species-specific to the intended targeted fish species using both PCR and qPCR. The standard curves gave a LOD for *V. letourneuxi*, at 0.02 pg per μl^{-1} of

36.30 ± 0.82 Ct (8/10 qPCR replicates) and the LOQ was indicated at 0.2 pg per μl^{-1} at 34.10 ± 0.88 Ct (10/10 qPCR replicates) (Slope=-3.392, Y-inter= 18.288, R^2= 0.985, Eff= 97.137) (Figure 3.1A). For *V. robertae*, the LOD was 0.92 pg per μl^{-1} at 39.26 ± 0.20 Ct (2/10 qPCR replicates) and the LOQ was 9.2 pg per μl^{-1} at 37.10 ± 0.67 Ct (10/10 qPCR replicates) (Slope=-3.243, Y-inter=30.234 , R^2= 0.841, Eff= 103.416) (Figure 3.1B). For *G. holbrooki*, the LOD was 0.97 pg per μl^{-1} at 41.96 ± 2.65 Ct (5/10 qPCR replicates) and the LOQ was 9.7 pg per μl^{-1} at 36.71 ± 0.74 Ct (10/10 qPCR replicates) (Slope=-3.959, Y-inter=24.922, R^2= 0.951, Eff= 78.889) (Figure 3.1C). All negative controls showed no amplification for any species throughout the experiment.

In the mesocosm experiment, all natural and technical replicates showed a positive amplification of *V. robertae* regardless of fish abundance and biomass. There was no significant difference between the Ct values acquired from either the 0.22 µm or 0.45 µm filters (ANOVA, Df= 1, F-value= 0.138, *p*= 0.71), nor between the Ct values acquired with the different fish biomass (ANOVA, Df= 1, F-value= 0.793, *p*= 0.374). However, it is worthy of note that system 'B' (which housed the eggs) had the lowest mean Ct value (24.14 ± 0.54). This is compared to system 'A' (housing low biomass and a medium number of fish (29.98 ± 0.57)) and system 'C' (the highest biomass and highest number of fish (28.34 ± 0.32)) (Figure 3.2.).

In the first of the *in-situ* trials (conducted on six aquatic systems in Autumn 2017), I was able to confirm the reliability of the designed assays for the two killifish species (Table 3.2., Appendix 5, Table S1). All eDNA samples collected during this trial were tested with both killifish assays as a complementary step for assessing the specificity and reliability of the method. *V. letourneuxi* was detected using both eDNA and fish sampling in one site ('4A'). This matched historical survey data (see Table 3.2). At two sites ('1A' and '3A'), it was not possible to detect *V. letourneuxi* with either eDNA or fish sampling. This was contrary to historical presence but matched more recent data (Table 3.2, Appendix 5, Table S1). Finally, there was no eDNA signal at a site where a translocation action had been previously undertaken ('2A'). *V. robertae* was detected using both eDNA and fish sampling at one site ('6A'). The presence of *V. robertae* was detected at one further site ('5A') only with eDNA. This site is a very small wetland where the fish had been found in the past (Appendix 5, Table S1). There was no fish sampling performed at site '5A' due to an enhancement program being undertaken a year before this sampling event. *V. robertae* was not detected at any of the sites where *V. letourneuxi* was found to be present.

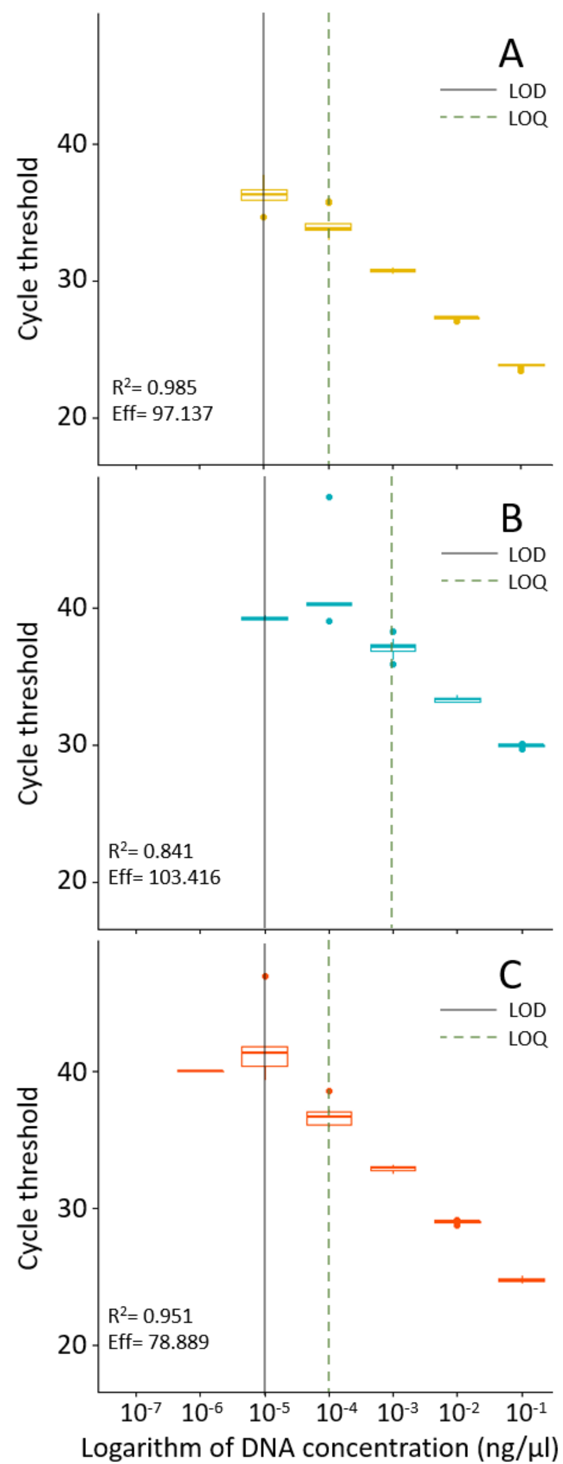

Figure 3.1. All standard curves were established by analysing a 1:10 dilution series of DNA extracted from *V. letourneuxi* (2.12ng/ µL) (A), *V. robertae* (9.2 ng/ µL) (B) and *G. holbrooki* (9.7

ng/ µL) (C). All standard curves ranged from 10^{-1} to 10^{-8} with 10 technical replicates used for each dilution steps in order to assess the LOD and LOQ. The Ct represents the number of qPCR amplification cycles required for a positive amplification of each targeted DNA fragment.

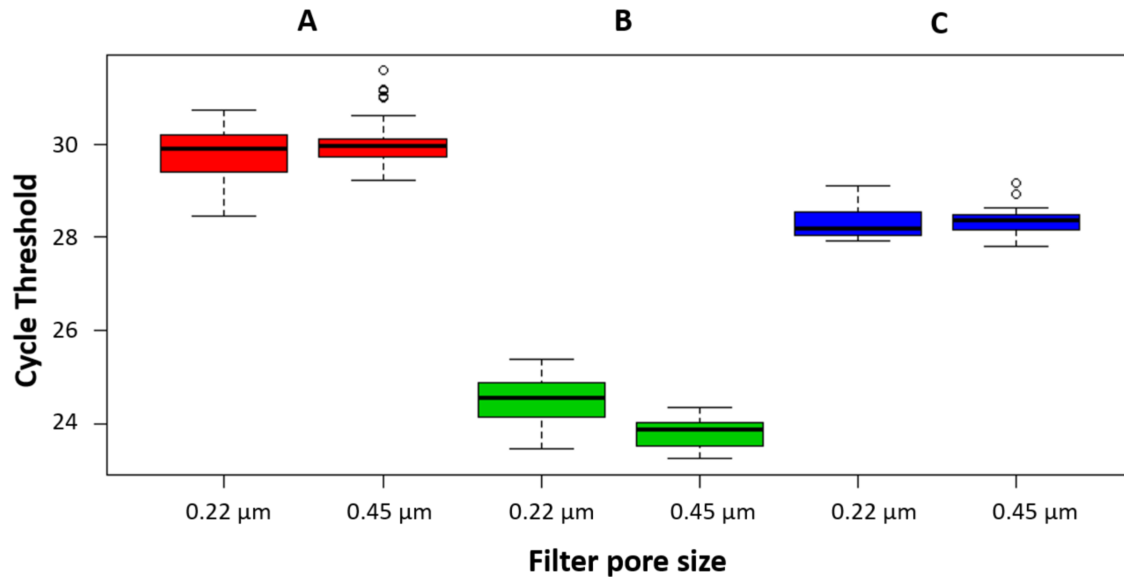

Figure 3.2. Comparison between the Ct values and the filter pore size in the mesocosm experiment. Mesocosm A contained 40 juveniles (biomass of 10 g), mesocosm B contained a mix of 12 adults and 10 juveniles (biomass of 19.8 g) and mesocosm C contained 66 adults (biomass of 101.5 g). All eggs were collected daily from system C and kept in system B for hatching.

In the second *in-situ* trial (October 2018) it was possible to detect *V. letourneuxi* at two sites using fish sampling, and six with eDNA (only site '10B' was positive using both methods, Table 3.3, Figure 3.3A, see also Appendix 6, Table S2 and Table S3). For *V. robertae*, its presence was detected at a further four sites using fish sampling, and six with eDNA. Here, three of the seven sites where this fish was detected were positive for both methods (Table 3.3, Figure 3.3B). The non-native and invasive species *G. holbrooki* was detected at fifteen sites; twelve using fish sampling, and twelve with eDNA, nine of these were positive across both methodologies (Table 3.3, Figure 3.3A and B).

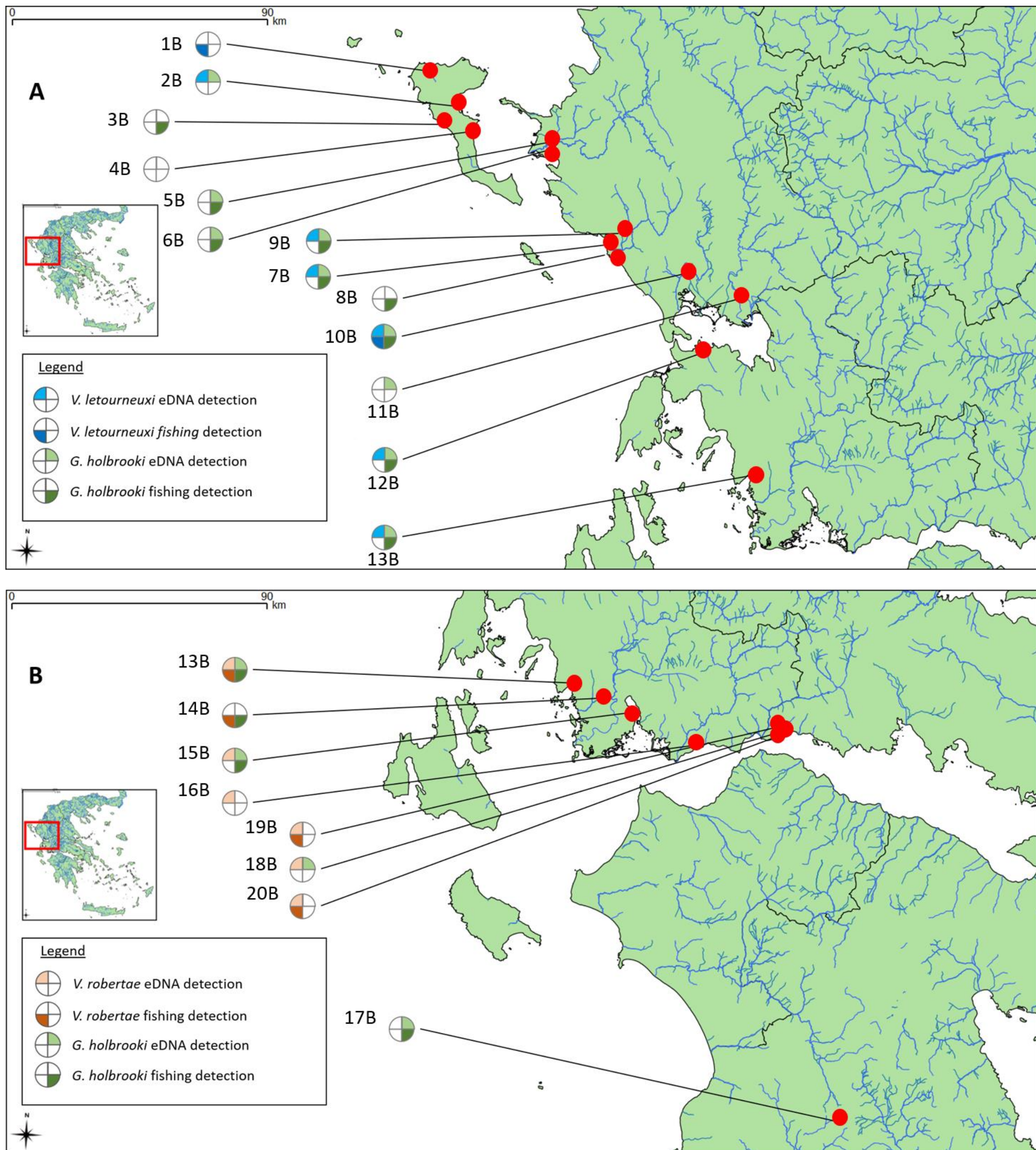

Figure 3.3. Map showing the freshwater network and locations sampled in Western Greece during the survey conducted in autumn 2018. Sub-figure **A** show the eDNA and electrofishing results of *V. letourneuxi* and *G. holbrooki*. Sub-figure **B** show the eDNA and electrofishing results of *V. robertae* and *G. holbrooki*. Red points represent the sampled locations, blue lines represent the

main rivers. Blue pie charts indicate detection of *V. letourneuxi*, green pie charts represent the detection of *G. holbrooki* and orange pie charts represent the detection of *V. robertae*.

3.5 Discussion

In this chapter, species-specific assays were developed, laboratory validated, and then tested in the field. Two threatened killifishes and one non-native invasive species, known to be spreading across Europe at a rapid rate were targeted (Grapputo et al., 2006; Freyhof et al., 2014). After an *in-vitro* validation step, following the protocol outlined in Appendix 3 (Tréguier et al., 2014), all assays were shown to be species-specific and gave high reliability in both *in-situ* and *ex-situ* trials. Interestingly, a few recent studies have indicated that the fragment size that eDNA-based assays target, can influence detection rates (Bylemans et al., 2018). Although the target fragments of the assays designed in this chapter ranged between 113 and 167 bp, no variation in the reliability of the assays was observed, with all performing well and accurately. However, it is important to note that the sensitivity of the assays varied substantially. The standard curves of all three indicated a similar Limit of Detection, all be it relatively high compared to some other species specific assays such as those reported by Klymus et al., (2019). There were slight differences regarding the Limit of Quantification, which was lower for *V. robertae* for example. Further, the assays for *V. letourneuxi* and *G. holbrooki* had an $R^2 > 0.95$, whilst *V. robertae* $R^2 < 0.95$. It should also be noted that the efficiency of the assay targeting *G. holbrooki* fell outside the standard range ($80 < \text{Efficiency} < 120$). Therefore, although all assays worked well in both the laboratory and in the field, optimization of the *V. letourneuxi* assay is the only one complete, whilst the assays for *V. robertae* and *G. holbrooki* could be optimized further.

The laboratory validation was very detailed for one of the targeted species: *V. robertae*. This was due to close collaborations with a current conservation programme led jointly by the ZSL and the HCMR. For this species, a controlled *ex-situ* mesocosms was utilised and allowed an assessment of the effect eDNA sampling had on the reliability of the results. Surprisingly, there was no variation in the detection rate when sampling with either of the two filter sizes tested. Meaning that, at least in this instance, either filter could be utilised without compromising assays effectiveness. However, the larger pore size of the two was chosen, as in the field, filters can get clogged by sediment, affecting the amount of water that can be filtered (Goldberg et al., 2016). It is well known that filtering large volumes of water is optimum (for any type of eDNA sampling

applied), and improves detection probability (Mächler et al., 2015; Hunter et al., 2019a; Sepulveda et al., 2019). In this same system, it was not possible to test the effect of biomass on the eDNA assay. However, there was no indication of biomass or fish abundance affecting detection rate for *V. robertae*. This is in contrast to some studies which have indicated that quantification of eDNA (especially with regard to fish) may be used to estimate biomass (Evans et al., 2016; Mauvisseau et al., 2019a). This could be due to the experimental set up utilised in the controlled experiment (i.e. various fish sizes, stage of life).

Interestingly, the mesocosm which additionally held the eggs prior to hatching, did result in higher Ct values of eDNA amplification compared to the two other mesocosms. This suggests that the eggs may have increased the amount of eDNA in the system, alternatively the result may also be explained by an increase in the sloughing rates of the fishes tissue and/or mucus directly (Klymus et al., 2015). Regardless of the reason, this result implies that the best sampling time in the field for eDNA from this species is likely to be during the spawning period, starting early in summer until autumn (Kalogianni et al., 2010). Finally, the lack of detection of *V. letourneuxi* (with both eDNA and traditional sampling) at sites '1A' and '3A' during the first trial is not surprising as both sites have been highly degraded in recent years and no records of the species have been noted since 2013 and 2007 respectively (HCMR unpublished data).

Regarding the conservation actions currently being undertaken, results from the first field trial (in 2017) indicate a positive eDNA signal for the site '5A', where an enhancement action was undertaken for *V. robertae* in 2015-2016. This conforms with the last confirmed physical detection in late 2015 at this pristine habitat. However, there was unfortunately no positive signal at site '2A', where a translocation action occurred for *V. letourneuxi* in 2015-2016. It was hoped that the utilisation of a more sensitive technique (compared to the traditional more invasive fish sampling methods previously used) would have indicated the species presence, even if it was at very low abundances (Thomsen et al., 2011; Mächler et al., 2014; Sigsgaard et al., 2015). However, as this was not the case, the translocation may not have been successful. That said, it should be noted that during this first field test, only one natural replicate was taken for eDNA analysis and at two or more would have been preferable as found in chapter 2 (Mauvisseau et al., 2019a), in order to reduce the possibility of a false negative result.

Indeed, two natural replicates were taken at each of the sites in the second *in-situ* trial (conducted in 2018), thereby increasing confidence in these results. During this survey, it was demonstrated again that eDNA approach outperformed conventional survey techniques (Table 3.3.). Using novel assay designed in this chapter, it was possible to highlight six sites where *V. letourneuxi* was found and six sites for *V. robertae*. These locations fell within the historical range of the target species but had steep banks, abundant vegetation cover and/or deep waters, making traditional surveying difficult (Kalogianni et al., 2010, 2019). Therefore, eDNA-based method offers a non-invasive and safer alternative for monitoring these species, especially at locations which present difficulties of applying more traditional sampling methodologies. Interestingly, there were two locations, one for *V. letourneuxi* and one for *V. robertae* (site '1B' and '14B'), where a negative signal was obtained in the eDNA sample, but the fish were found via the traditional survey sampling. These two confirmed 'false negatives' for the newly developed eDNA assay in this chapter, therefore, indicate a level of limitation. Such discrepancies could be explained by a number of factors, such as (i) hydrology, (ii) the low abundance of the target species (iii) inhibition and/or (iv) issues with the sampling of the eDNA sample, such as insufficient number of natural replicates or insufficient volume of water collected. More specifically, site '14B' is a large riverine habitat with high discharge and therefore eDNA is likely to be considerably diluted and may be rapidly removed from the site of origin. As highlighted in Pont et al., 2018, eDNA concentration and detectability can be impacted by both dilution and river transport, leading to false negative detection. It should also be noted that eDNA transport could also lead to false positive results up to more than 100km in large river (Pont et al., 2018). Site '1B', in contrast, is a much smaller system but with abundant vegetation cover, and both inhibition and/or insufficient number of natural replicates, combined with an insufficient volume of water collected, could explain this false negative result. Reducing the chance of false negatives should be a priority in an eDNA-based survey and therefore increasing the number of natural replicates – previously shown in chapter 2, as well as increasing the filtering capacity should be considered in combination with the utilisation of an internal positive control aimed at assessing levels of inhibition (Goldberg et al., 2016; Mauvisseau et al., 2019a, 2019c; Sepulveda et al., 2019). Interestingly, the third assay (targeting the non-native and highly invasive eastern mosquito fish - *G. holbrooki*) showed no variation between eDNA sampling and traditional fish sampling. *G. holbrooki* is known to be present in considerably higher densities than the two critically endangered *Valencia* species (Kalogianni et al., 2010). Such high

densities may therefore be playing a yet undetermined role on the reliability or sensitivity of this assay. Alternatively, the result may have been driven more from variation with regard to the accessibility of the sites, inhibition and/or the number of natural replicates taken at any given time. Future studies should therefore focus on these aspects in order to more fully understand the complex interaction between biotic and abiotic factors affecting the sensitivity of these targeted assays and their performance against the more traditional survey methods. That said, the mesocosm experiment conducted in this chapter showed no apparent effect of biomass on the reliability of detection. This contraction in results may be explained by the very different characteristics of the two studied systems (i.e. small closed artificial system and larger open natural and dynamic systems with high levels of water flow).

There were also some instances of false negatives throughout this chapter. Regardless of the cause, such result highlights an important issue which needs to be dealt with. That said, this is the same for both eDNA detection and traditional methods and is acknowledged as a norm in most survey techniques. In an attempt, to try and ascertain the impact of such false negatives on the end result (and therefore the management/conservation plan which would be implemented), occupancy modelling has shown some promise (Mackenzie et al., 2002; Mackenzie and Royle, 2005; Dorazio and Erickson, 2018). Further, such models can help to assess the influence that specific environmental factors have on the probability of detection or account for the imperfect detection of eDNA (Schmidt et al., 2013; Hunter et al., 2015; Schmelzle and Kinziger, 2015; Lahoz-Monfort et al., 2016; Dorazio and Erickson, 2018; Hunter et al., 2019b; Sutter and Kinziger, 2019). An interesting approach would be to capitalize on multiple datasets from different survey methods. Combined with sufficient replication (not available in this chapter), this would allow to identify false positive/negative detection and increase the reliability of eDNA-based assessments. However, in this chapter, (due to the limited number of habitats hosting the target species and thus the relatively low number of locations surveyed) occupancy modelling may not have helped in this regard. The uniformity of environmental characteristics of the sampled locations, combined with a relatively low number of natural replicates would have led to incorrect estimation from an occupancy model. Further, the killifishes preferred habitats were intentionally targeted in this study (spring-fed wetlands with clear waters and rich surface vegetation) and focused on sites which had known historical presence of the species (Kalogianni et al., 2010, 2019). This meant

that the environment surveyed was largely uniform across the various samples. Further, and as stated earlier, the number of habitats hosting the targeted species in this study was limited due to the endangered nature of the two Valencia species. Further, the temporal effect of eDNA sampling on either the killifish or *G. holbrooki* are not yet fully understood and further studies should assess if there is an optimal time for sampling or more importantly when results are less reliable (a time not to survey). Finally, as with everything the number of replicates is likely to play an important role. If money is no option, a large number of replicates will obviously give the best and therefore the more reliable result (Erickson et al., 2019). However, two to three natural replicates with four technical replicates has proven to be more than sufficient in a number of other species specific targeted assays (Mauvisseau et al., 2019a).In conclusion, these field efforts confirmed the usefulness of eDNA monitoring for the detection of both threatened killifishes and a non-native and highly invasive species, regarded as responsible for the decline of many native, and often threatened freshwater species across Europe (Grapputo et al., 2006; Freyhof et al., 2014). One major benefit of this new tools for surveying these species is the non-invasive nature of the technique. Electrofishing in particular should be reconsidered as a method as it can be potentially harmful to the fish (both target and non-target alike) (Snyder, 2003; Miranda and Kidwell, 2010). That said, despite the very promising initial results of all three eDNA assays more intensive field tests are recommended to be undertaken in the future. Such field trials should, where possible, include a more balanced distribution of sites with known presence or absence of the target species (Farrington et al., 2015; Guan et al., 2019). That said, when dealing with critically endangered species this is obviously not always possible and so such a balance was only possible for the invasive species in this context.

Regardless, the advent of novel eDNA approaches aimed at targeting specific species of conservation priority and/or non-native invasive species that have deleterious effects on native wildlife (as demonstrated here), is going to be a logical step forward in environmental monitoring, due to the non-invasive nature of the method. Combined with citizen science, they could pave the way to larger scale conservation programmes, along with improving management decisions associated with already existing programmes, as demonstrated in this chapter. Although, new assays require a high level of validation to ensure reliability and confidence in the results, the advent of novel eDNA approaches offers optimistic perspectives as complementary tools for assessing species distribution.

Chapter 4: Improving detection capabilities of a critically endangered freshwater invertebrate with environmental DNA using digital droplet PCR

This chapter has been published as: Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., Sweet, M., 2019. Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. Scientific Reports. https://doi.org/10.1038/s41598-019-50571-9

4.1 Abstract

Isogenus nubecula is a critically endangered *Plecoptera* species. Considered extinct in the UK, the species was recently rediscovered in one location of the river Dee in Wales after 22 years of absence. As many species belonging to the *Perlodidae*, this species can be a bio-indicator, utilised for assessing water quality and health status of a given freshwater system. However, conventional monitoring of invertebrates via kick-sampling for example, is invasive and expensive (time consuming). Further, such methods require a high level of taxonomic expertise. Here, the traditional kick-sampling method was compared with the use of eDNA detection using qPCR and ddPCR-analyses. In spring 2018, twelve locations on the river Dee were sampled for eDNA analyses. *I. nubecula* was detected using kick-sampling in five of these locations, three locations using both eDNA detection and kick-sampling and one location using eDNA detection alone - resulting in a total of six known and distinct populations of this critically endangered species. Interestingly, despite the eDNA assay being validated *in-vitro* and *in-silico*, and results indicating high sensitivity, qPCR analysis of the eDNA samples proved to be ineffective. In contrast, ddPCR analyses resulted in a clear detection of *I. nubecula* at four locations suggesting that inhibition most likely explains the big discrepancy between the obtained qPCR and ddPCR results. It is therefore important to explore inhibition effects on any new eDNA assay. Following the results of this chapter, ddPCR may well be the best option for the detection of aquatic organisms which are either rare or likely to shed low levels of eDNA into their environment.

4.2 Introduction

Monitoring biodiversity in freshwater systems is a cornerstone of the evaluation of the European Habitats Directive, the European Water Framework Directive and the general evaluation of ecosystem health and status (European Commission, 1992, 2000, 2015). The assessment of freshwater biodiversity relies on biological monitoring methods, in which, the use of biodiversity indicators is an essential component of its evaluation. Various aquatic macroinvertebrates, such as mayflies, stoneflies and caddisflies (*Ephemeroptera*, *Plecoptera* and *Trichoptera*) are commonly used as bio-indicator organisms for water quality and ecosystem assessments (Hering et al., 2004; Sweeney et al., 2011; Morinière et al., 2017). This is down to their rapid reaction to anthropogenic change such as levels of pollution, climate change, fracking, mining, and the construction of hydroelectric stations for example (Burton et al., 2014; Álvarez-Troncoso et al., 2015; Dedieu et al., 2015; Morinière et al., 2017).

Traditional monitoring of macroinvertebrates via kick-sampling and/or capture-recapture methods, is, however, costly (i.e. time consuming), labour intensive and, above all, known to be limited in effective detection of populations below a certain threshold (Forsström and Vasemägi, 2016; Morinière et al., 2017). Further, such methods are ecologically invasive i.e. they increase the risk of injury to the target (and non-target) organism. The morphological identification of these bio-indicators is also often challenging, especially at the immature life stages (Haase et al., 2010; Pfrender et al., 2010; Zhou et al., 2011; Morinière et al., 2017), so a high level of taxonomic expertise is therefore usually required to avoid any possible misidentification and therefore misrepresentation (Ushio et al., 2018; Mauvisseau et al., 2019b).

The use of molecular approaches for biomonitoring, for example the detection of environmental DNA (eDNA), may overcome a number of these issues (Baird and Sweeney, 2011). Moreover, the use of eDNA increases efficiency, reliability and allows for a more rapid species identification and ultimately detection (Morinière et al., 2017), whilst minimising any associated impacts on the species and the environment. All aquatic organisms shed DNA traces in their environment (Thomsen and Willerslev, 2015), and it is now possible to detect a specific species (barcoding) or assess an entire community (metabarcoding) by sampling an aquatic system and amplifying the existing DNA traces using PCR (Polymerase Chain Reaction) based techniques (Thomsen and

Willerslev, 2015). Since the implementation of eDNA techniques in environmental studies last decade, it has been proven to be successful for the monitoring of invasive (Klymus et al., 2015; Adrian-Kalchhauser and Burkhardt-Holm, 2016; Dougherty et al., 2016; Mauvisseau et al., 2018, 2019d), endangered (Rees et al., 2017; Harper et al., 2018a) and/or economically important species from a wide range of taxa (Mauvisseau et al., 2017; Atkinson et al., 2018; Shaw et al., 2019). However, few studies have used eDNA for monitoring rare or indicator macroinvertebrate species (Mächler et al., 2014; Fernández et al., 2018; Wei et al., 2018).

A typical example of such a bioindicator *Plecoptera* is the Scarce Yellow Sally stonefly, *I. nubecula* (*Perlodidae*, *Plecoptera*) (Newman 1833). This critically endangered species has been reported as extinct or undetected in numerous countries from which it was originally present (Davy-Bowker, 2003; Davy-Bowker et al., 2018). Also in the UK, it was considered as extinct until recently, when *I. nubecula* specimens were rediscovered after a 22-year period of absence at a location in the river Dee, North Wales, UK (Davy-Bowker et al., 2018). Moreover, a total of 14 individuals were recorded on that spot during two kick-sampling surveys. The aim of this chapter was to design a novel single species eDNA based primer/probe assay for the detection of *I. nubecula* and to compare the efficiency of qPCR and ddPCR versus traditional kick-sampling.

4.3 Methods

4.3.1 Primers and probe design

As in chapter 3, a species-specific set of primers and probe, targeting the COI gene of *I. nubecula* was designed using the Geneious Pro R10 Software (https://www.geneious.com; Kearse et al., 2012). This assay amplifies a 124 bp fragment using the forward primer (5' – CCAGAAGCCTTGTAGAAAAC – 3'), the reverse primer (5' – ACCCCGGCTAGATGAAGAGA – 3') and a probe (6-FAM – CCCCACTCTCTGCTGGAATT – BHQ-1). Specificity of the assay was assessed *in-silico* by comparing against sequences from 21 genetically similar invertebrate species which had all been previously submitted to the NCBI https://www.ncbi.nlm.nih.gov/) see Appendix 7 for full list. The specificity of the assay was tested *in-vitro* using PCR and qPCR, with DNA extracted from the nine invertebrate species (closely related or likely to be present in the same ecosystem). These included; *I. nubecula*, *Leuctra*

hippopus (Kempny, 1899), *Perlodes mortoni* (Klapálek, 1906), *Nemoura lacustris* (Pictet, 1865), *Leuctra geniculata* (Stephens, 1836), *Nemoura erratica* (Claassen, 1936), *Taeniopteryx nebulosa* (Linnaeus, 1758), *Diura bicaudata* (Linnaeus, 1758) and *L. fusca* (Linnaeus, 1758).

4.3.2 eDNA samples

12 locations from the River Dee, were sampled for eDNA between 9th March 2018 and 1st of April 2018 (Figure 4.1. and Table 4.1.). These locations were chosen following previous knowledge of historical observations in 1981 and 1982 (Davy-Bowker et al., 2018). At each location, three independent (i.e. A, B and C) 1L water samples (referred to here after as natural replicates) were collected using a 40mL sterile polypropylene ladle and placed into a sterile plastic bag (Whirl-Pak® 1242 ml Stand-Up Bag Merck®, Darmstadt, Germany) following methods and findings from chapter 2 (Mauvisseau et al., 2019a). Sub-samples were regularly collected from surface water downstream to upstream (to avoid disturbing sediments), across the width or the bank of the river, depending on the access and weather conditions following the method outlined in (Mauvisseau et al., 2019e). Each independent 1L water sample was then filtered with a sterile 50 mL syringe (sterile Luer-Lock™ BD Plastipak™, Ireland) through a sterile 0.45 µm Sterivex™ HV filter (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Millipore®, Germany). Sterivex filters were immediately placed in a freezer bag and stored at - 80°C until further analysis in the laboratory. At each location, new sterile equipment and disposable nitrile gloves were used during the sampling process to avoid contamination. A 'positive' eDNA sample was collected by creating an isolated mesocosm onsite, which consisted of river water from site W4 and 11 specimens of *I. nubecula* stored for 1 hour. Two negative control samples were additionally filtered in the field with sterile ddH_2O in parallel with the natural samples, to control for potential cross-contamination during the workflow.

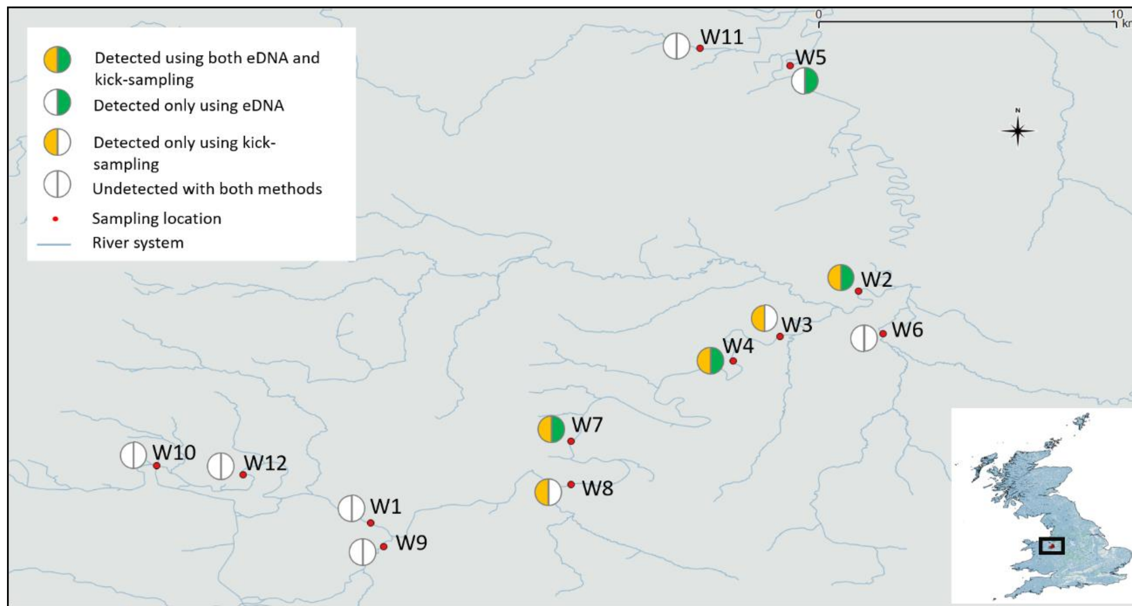

Figure 4.1. Map showing the 12 locations of the river Dee sampled with both kick-sampling and eDNA survey for monitoring *I. nubecula* in Wales, United Kingdom. Red dots are showing the sampled locations, half green circle the locations positive with eDNA detection using ddPCR, the half orange circle the locations were *I. nubecula* was found using kick-sampling. Locations W5 and W6 were not surveyed using kick-sampling method.

Sample ID	*I. nubecula*	eDNA (ddPCR)	Time (s)	Volume (ml)	Date	pH	O_2	Latitude	Longitude
W1	0	No	60	350	31/03/2018	7.48	12.5	52.952759	-3.0232733
W2	3	Yes	60	200	01/04/2018	7.53	11.9	53.024980	-2.8760059
W3	30	No	60	700	09/03/2018	6.69	11.9	53.010679	-2.8998019
W4	16	Yes	120	1000	09/03/2018	6.52	11.8	53.003120	-2.9138314
W5	ns	Yes	45	750	15/03/2018	7.83	11.4	53.095257	-2.8967275
W6	ns	No	60	300	01/04/2018	7.82	12.5	53.011702	-2.8686273
W7	1	Yes	90	750	14/03/2018	7.67	11.6	52.978139	-2.9627502
W8	1	No	90	750	14/03/2018	7.8	10.7	52.964635	-2.9628967
W9	0	No	90	750	11/03/2018	6.75	11.8	52.945402	-3.0194684
W10	0	No	60	300	31/03/2018	7.74	13	52.970460	-3.0879607
W11	0	No	45	500	11/03/2018	6.63	11.6	53.100487	-2.9239146
W12	0	No	90	750	15/03/2018	7.69	10.9	52.967603	-3.0619060

Table 4.1. Table depicting the kick-sampling results for *I. nubecula* (i.e. how many specimens found at each site), the eDNA results using ddPCR analysis (i.e. if one natural replicate was

positive to *I. nubecula* DNA), the amount of time spent performing kick-sampling and eDNA sampling, the amount of water filtrated for all natural replicate at each site, the sampling date, pH, dissolved oxygen and GPS coordinate. The sites inaccessible for conducting a kick-sampling were marked "ns".

4.3.3 DNA extraction

DNA extraction from both the eDNA samples and the tissue samples (utilised for validating the assay) was done using the Qiagen DNeasy® Blood and Tissue Kit as in chapters 2 and 3. The manufacturer's instructions were followed for performing DNA extraction from tissue samples. Sterivex filters were extracted following the methods outlined in (Spens et al., 2017). All laboratory equipment was disinfected and decontaminated using UV treatment prior to conducting any laboratory work. Laboratory equipment and surfaces were regularly disinfected using 10% bleach and absolute ethanol before conducting analyses.

4.3.4 PCR

PCR amplifications were performed on a Gen Amp PCR System 9700 (Applied Biosystem) using the primers described above. PCR reactions were performed in a 25 µL reaction, with 12.5 µL of PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10 µM), 9.5 µL of ddH2O and 1 µL of template DNA. Optimal PCR conditions were performed under thermal cycling 50 °C for 2 min and 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60°C for 1 min. For each PCR (with DNA from tissue samples), at least one positive and one negative control were included. PCR within this chapter were performed in Derby University.

4.3.5 qPCR

qPCR amplifications were performed on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems) in final volumes of 25 µL, using 12.5 µL of PrecisionPlus qPCR Master Mix with ROX (Primer Design, UK), 1 µL of each primer (10 µM), 1 µL of probe (2.5 µM), 6.5 µL of ddH2O and 3 µL of extracted DNA. qPCR conditions were as follow: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. For each qPCR with DNA

from tissue samples, at least two positive and two negative controls were included. A standard curve was established by analysing a 1:10 dilution series of DNA extracted from *I. nubecula* (68.2 ng/ µL, Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific) following the MIQE Guidelines (Bustin et al., 2009) (Appendix 2). qPCR analyses conducted within this chapter were performed in Derby University.

4.3.6 ddPCR

ddPCR was conducted using the Bio-Rad QX200 ddPCR system in a 20 µL total volume. Each reaction contained 10 µL Bio-Rad ddPCR supermix for probes (no dUTP), 750 nM of each primer, 375 nM probe, 3 µL DEPC water, and 4 µl template DNA. Twenty microlitres of the PCR mix was pipetted into the sample chambers of a Droplet Generator DG8 Cartridge (Bio-Rad, cat no. 1864008), and 70 µL of the Droplet Generation Oil for Probes (Bio-Rad, cat no. 186-4005) was added to the appropriate wells. The cartridges were covered with DG8 Gaskets (Bio-Rad, cat no. 1863009) and placed in a QX200 Droplet Generator (Bio-Rad) to generate the droplets. After droplet generation, the droplets (40 µL) were carefully transferred to a ddPCR 96-well plate (Bio-Rad, cat no. 12001925). The PCR plate was sealed with pierceable foil (Bio-Rad, cat no. 181-4040). PCRs were performed using a C1000 TouchTM Thermal Cycler with a 96-well Deep Reaction Module (Bio-Rad). PCR conditions were 10 min at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C and extension at 60°C for 1 min, with ramp rate of 2°C s^{-1}, followed by 10 min at 98°C and a hold at 12°C. Droplets were then read on a QX200 droplet reader (Bio-Rad). All droplets were checked for fluorescence and the Bio-Rad's QuantaSoft software version 1.7.4.0917 was used to quantify the number of *I. nubecula* copies per µL. Thresholds for positive signals were determined according to QuantaSoft software instructions. All droplets beyond the fluorescence threshold (3500) were counted as positive events, and those below it as negative events. All eDNA samples were analysed in duplicate (one replicate undiluted and one replicate diluted 1:2). One positive control (i.e. DNA extracted from *I. nubecula* at a concentration of 1 ng/ µL diluted 1:100 (Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific)), one No Template Control (i.e., IDTE pH 5.0) and the two negative field controls were additionally included. The LOD using the ddPCR was assessed following the method outlined in (Baker et al., 2018). A serial dilution of a DNA extracted from *I. nubecula* was conducted. The starting point

was an initial 1: 100 dilution of extracted genomic DNA from *I. nubecula* at 1 ng/ µL, followed by a serial 1:5 dilution. The serial dilution included ten replicate of each dilution. ddPCR analyses conducted within this chapter were performed in INBO (Belgium).

4.3.7 Estimation of the LOD and LOQ

To become estimates of the LOD and LOQ for the primer/probe assay used on both the qPCR and ddPCR machines, a dilutions range from 10^{-1} to 10^{-9} was set-up with 10 technical replicates used for each of the dilution steps. Following (Bustin et al., 2009), the LOD was defined as the lowest concentration in which 95% of positive samples were detected. The LOQ was defined as the last standard dilution in which the targeted DNA was detected and quantified in at least 90% of positive samples, as previously defined in chapters 2 and 3 (Mauvisseau et al., 2019a, 2019d). All eDNA samples were then analysed with six technical replicates as in chapters 2 and 3 (Mauvisseau et al., 2019a, 2019d) on a qPCR plate, with six negative controls and a positive control dilution series from 10^{-1} to 10^{-6} in duplicate.

4.3.8 Kick-sampling

Kick-sampling was performed using the standard of the FBA (UK), i.e. using a kick-sampling net with a 1 mm mesh (see detailed protocol: https://www.fba.org.uk/practical-guidance-sampling-and-collecting). Sampling duration was recorded at each site and varied depending on access, depth, river flow, or weather conditions (Table 4.1.). Perlodidae specimens found during kick-sampling were either preserved in 99% ethanol or kept alive as a part or a separate rearing experiment. Specimens were identified in the laboratory by two independent taxonomy experts (John Davy-Bowker & Michael Hammett) using a low-power binocular microscope with cold light source and using an identification key (Hynes, 1963, 1977).

4.3.9 Statistical analysis

A site occupancy modelling approach (Mackenzie et al., 2002; MacKenzie et al., 2006; Royle and Dorazio, 2008) was utilised to assess the effect of environmental covariates on the presence of eDNA of *I. nubecula* and to estimate the detection probability. This hierarchical modelling

framework has the advantage of accounting for the risk of false negative results when estimating the probability of detection. This analysis was run with the ddPCR data (Appendix 8). Covariates tested included: (i) turbidity (likely to inhibit the PCR reaction, with the volume of filtered water being used as a proxy), (ii) pH, (iii) dissolved oxygen concentration, (iv) amount of time including eDNA sampling and kick-sampling spent at each location as indicator of the field conditions and (v) human accessibility as a binary indicator (possible to perform kick-sampling/absence of kick-sampling survey) (Appendix 9). Analyses were performed using the 'eDNAoccupancy' package (Dorazio and Erickson, 2018; Hunter et al., 2019b) in the R statistical programming environment (R Core Team 2018). Model selection and interpretation followed procedures given in (Dorazio and Erickson, 2018; Hunter et al., 2019b). The model was fitted using the 'occModel' function from the described package. MCMC chains ran for 11,000 iterations, with 10,000 retained for obtaining parameter estimates and credible intervals.

4.4 Results

4.4.1 Specificity and validation of eDNA assay using PCR, qPCR and ddPCR

The primers and probe designed in this chapter were species-specific *in-silico* and *in-vitro* with both conventional PCR and qPCR. The negative controls or samples with DNA from non-target species did not amplify with either method. For qPCR, I analysed the standard curve and compiled the LOD and LOQ as per the MIQE guidelines and chapters 2 and 3 (Bustin et al., 2009; Mauvisseau et al., 2019a). The LOD was 6.82×10^{-6} ng DNA μL^{-1} at 39.29 ± 2.00 Ct and the LOQ was 6.82×10^{-4} ng DNA μL^{-1} at 34.48 ± 0.95 Ct (Slope= -3.86, Y inter= 19.52, R^2= 0.97, Eff%= 81.63) (Figure 4.2.). Using ddPCR, five replicates from the dilution which equated to 0.08 pg of DNA yielded a positive detection (mean 0.05 copy per μL^{-1}) and only one replicate of the next dilution (i.e. 0.016 pg) yielded to a positive detection of *I. nubecula* (0.08 copy per μL^{-1}). All replicates from further dilution and negative controls were negative. However, as specified in (Baker et al., 2018) (at the lower end of detection), the lower 95% confidence can limit overlap with potential artefact in the negative control. For this reason, 0.08 pg of DNA was considered to be the lowest amount able to be detected using ddPCR and only samples > 0.5 copy per μL^{-1} were considered (as in Baker et al., 2018) to meet the threshold for a positive detection.

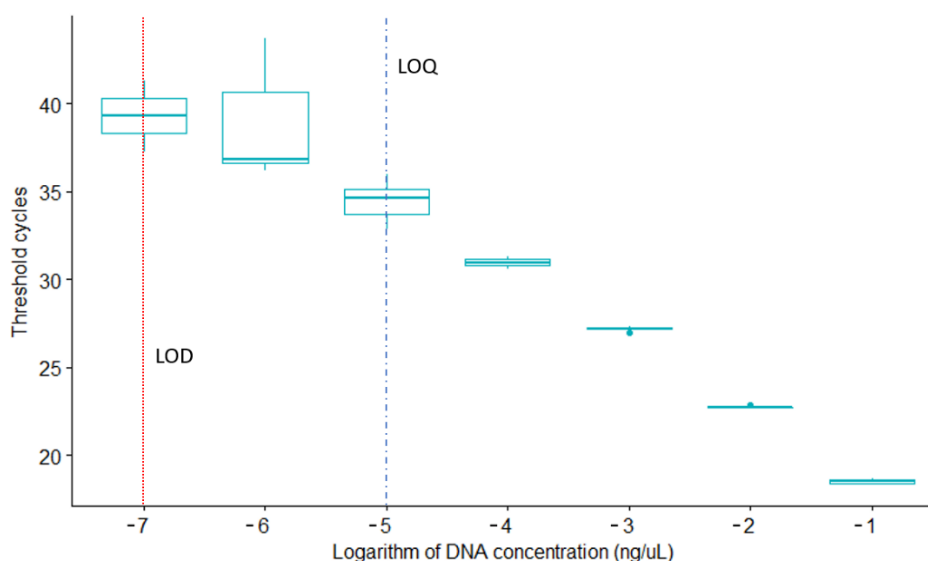

Figure 4.2. Standard curve assessing the LOD and LOQ for the qPCR assays detecting the DNA traces of *I. nubecula*. Both limits were calculated from a 1:10 serial dilution with 10 replicates per concentration. The LOD was 6.82 10^{-6} ng DNA μL^{-1} at 39.29 ± 2.00 Ct and the LOQ was 6.82 10^{-4} ng DNA μL^{-1} at 34,48 ± 0,95 Ct (Slope= -3.86, Y inter= 19.52, R^2= 0.97, Eff%= 81.63).

4.4.2 Kick sampling assessment

I. nubecula was found at 5 sites along the River Dee, whereas the species could not been found at five other sites (Figure 4.1., Table 4.1.). Abundance ranged from just one individual at two sites, at W7 and W8, up to a highest density of 30 individuals at W3. Two of the sites surveyed for eDNA were not assessed via kick sampling due to dangerous access and weather conditions (Table 4.1.).

4.4.3 Comparison of qPCR versus ddPCR analyses

Despite the success of the assay *in-silico* and *in-vitro*, no amplification could be obtained via qPCR on any of the eDNA samples (Table 4.2.). Even the 'positive control eDNA sample' which consisted of 11 *I. nubecula* individuals housed in a 1 litre mesocosm for a period of one hour before filtering (see methods). During each run, the positive dilution range indicated the assay ran without any issue (Slope= -3.65 / -4.05, Y inter= 19.22 / 26.46, R^2= 0.98 / 0.99, Eff%= 76.46 / 88.03). In

contrast, the ddPCR analysis revealed a positive detection of *I. nubecula* at four sampling locations (Figure 4.1., Table 4.2.). Concentration ranged from 0.6 to 0.14 copy per μL^{-1} in the eDNA samples. The 'positive eDNA' sample generated a DNA concentration of 5.4 copies per μL^{-1} (undiluted) and 8.2 copies per μL^{-1} (diluted).

	qPCR			**ddPCR**					
Sample ID	undiluted			undiluted			diluted		
	NR A	NR B	NR C	NR A	NR B	NR C	NR A	NR B	NR C
W1	-	-	-	-	-	-	-	-	-
W2	-	-	-	-	0.8	0.7	-	-	-
W3	-	-	-	-	-	-	-	-	-
W4	-	-	-	-	-	0.7	0.7	-	0.14
W5	-	-	-	0.7	0.7	0.14	-	-	0.6
W6	-	-	-	-	-	-	-	-	-
W7	-	-	-	-	-	-	-	0.7	-
W8	-	-	-	-	-	-	-	-	-
W9	-	-	-	-	-	-	-	-	-
W10	-	-	-	-	-	-	-	-	-
W11	-	-	-	-	-	-	-	-	-
W12	-	-	-	-	-	-	-	-	-
'positive control'		-			5.4			8.2	

Table 4.2. Table depicting the eDNA detection results using qPCR and ddPCR techniques on diluted and undiluted (1:2) natural replicates (NR) sampled at each field location. '-' depicts the absence of eDNA detection using qPCR and/or ddPCR. Quantification values of ddPCR results are displayed in copy per μL^{-1}. Natural replicates were analysed using six technical replicates with qPCR and without replicates using ddPCR. All samples revealed a negative result for *I. nubecula* eDNA using qPCR. DNA from the targeted specie was amplified in samples from four field locations and in the 'positive control'.

The site occupancy modelling approach did not reveal any significant effect of the environmental variables on the presence of eDNA or on the probability of detection (Tables 4.3. and 4.4.). Probabilities of *I. nubecula* occurrence were relatively low and ranging from 0.45 to 0.53 (Table 4.4.). Probabilities of eDNA detection at each sampling sites ranged from 0.59 at site W5 where

all ‘natural replicates’ where found to be positive using ddPCR to 0.27 at site W10, a site with high turbidity where no stonefly were found.

Bayesian estimates of model parameters				
	Mean	50%	2.5%	97.5%
β Intercept	0.135	0.086	-1.107	1.610
β Accessibility	-0.232	-0.189	-1.678	1.113
α Intercept	0.970	0.933	-0.265	2.506
α Volume	0.151	0.166	-1.026	1.178
α pH	0.156	0.134	-1.118	1.671
δ Intercept	-0.136	-0.136	-0.847	0.619
δ Volume	0.275	0.292	-0.486	1.054
δ O_2	-0.037	-0.087	-2.037	2.102
δ Time	-0.149	-0.153	-0.845	0.575
Monte Carlo SE of Bayesian estimates				
	Mean	50%	2.5%	97.5%
β Intercept	0.0345	0.0418	0.0420	0.0316
β Accessibility	0.0305	0.0372	0.0305	0.0474
α Intercept	0.0391	0.0434	0.0516	0.0332
α Volume	0.0220	0.0258	0.0439	0.0302
α pH	0.0306	0.0342	0.0407	0.0462
δ Intercept	0.0166	0.0189	0.0204	0.0255
δ Volume	0.0156	0.0182	0.0225	0.0178
δ O_2	0.0667	0.0704	0.0840	0.0819
δ Time	0.0188	0.0199	0.0207	0.0249

Table 4.3. Table depicting the of the Bayesian estimates for effects of covariates on the probability of occurrence at a site (ψ). (α) and (δ) parameters are covariates for the conditional probability of eDNA presence in a sample (θ) and for its detection (p). (β) parameters are covariates of the estimated occupancy (ψ). Means represent estimated parameter values and last two columns represent the boundaries of the 95% credible intervals.

Site	ψ	θ	p
W1	0.45	0.79	0.33
W2	0.45	0.76	0.31
W3	0.45	0.77	0.52
W4	0.45	0.75	0.48
W5	0.53	0.87	0.59
W6	0.53	0.81	0.32
W7	0.45	0.86	0.46
W8	0.45	0.87	0.52
W9	0.45	0.77	0.47
W10	0.45	0.80	0.27
W11	0.45	0.75	0.49
W12	0.45	0.86	0.50

Table 4.4. Table depicting the Bayesian estimates for the probabilities of occurrence (ψ), the conditional probabilities of eDNA presence in a sample (θ) and eDNA detection (p) of *I. nubecula* at each sampling site of the river Dee and its tributaries.

4.5 Discussion

In this chapter, I compared the use of kick-sampling and eDNA detection for monitoring a critically endangered bioindicator macroinvertebrate. While eDNA detection approach using qPCR showed high sensitivity (Figure 4.2.), with no false positive results during the validation process and assessment of the MIQE guidelines (Appendix 2), however, it was not possible to amplify DNA traces of *I. nubecula* in any of the eDNA samples. This is surprising as one should expect positive detection at least in the five locations where the species was found via kick-sampling, and especially in the ’positive eDNA’ sample. These observations thus clearly pose doubts on the concept of eDNA using the qPCR methodology. Potential explanations for these false negative observations might be (i) an incorrect sampling protocol, (ii) the presence of PCR inhibitors in the DNA extracts, or (iii) a very limited shedding rate of the targeted species (Goldberg et al., 2016). As previously shown in chapter 2, the sampling design of any eDNA based study can affect the reliability of detection (Mauvisseau et al., 2019a). In this case, however, this was accounted for by taking, for example, three natural replicates at each site and incorporating six technical PCR replicates per sample, following the findings of chapter 2.

Most likely, inhibition of the qPCR assay is the most responsible aspect for the false negative detections, is it has also been found to be the case in other studies (McKee et al., 2015; Hunter et al., 2019a). One can assess for inhibition via the use of internal positive controls, such as spiked synthetic DNA or different from the targeted species (Goldberg et al., 2016). Limited detection or complete failure of such internal controls may then clearly show the occurrence of inhibition factors. If there is inhibition, two methods can be used to overcome this issue. The first method is to dilute the DNA extracted from the field sample (Goldberg et al., 2016), whilst the second is the use of an inhibitor removal kit (McKee et al., 2014; Goldberg et al., 2016). However, both methods have been shown to reduce the yield of target DNA in the extracted sample (Goldberg et al., 2016). In this chapter, qPCR showed no results from the eDNA samples and it was hypothesised that inhibition may be an important driver for the false negative observations in this assay. I did not use an inhibitor removal kit in order to avoid reducing the amount of DNA extracted from the field samples. Instead, the samples were ran on a ddPCR with two different dilutions. ddPCR has been shown to outperform qPCR in some other studies (Doi et al., 2015a, 2015b; Uthicke et al., 2018; Hunter et al., 2019b) by simply detecting and quantifying lower amounts of DNA and being less sensitive to inhibition. Findings of this chapter also support these observations as it was possible to detect the presence of *I. nubecula* eDNA at four distinct locations. Three of them matched with the positive results from the kick-sampling survey. Interestingly, the analysis of the 'positive eDNA sample' showed an increase from 5.4 copies per µL (undiluted) to 8.2 copies per µL (diluted), indicating that inhibition was still affecting the ddPCR (although not strong enough to block amplification in this instance). This finding indicates that there are substantial inhibiting factors affecting the primer/probe assay used and may explain the false negative results following qPCR analyses.

The very low *I. nubecula* eDNA concentrations in the samples also indicate that this species is characterized by very low shedding rates. Moreover, in all locations, the eDNA concentration ranged from only 0.6 to 0.14 copies per µL and up to 8.2 copies per µL in the 'positive eDNA' sample. As this chapter is the first study to use ddPCR for detecting low populations of endangered invertebrates in fast flowing rivers, it is not possible to compare these results with previously published studies. Besides the fact that invertebrates are generally found to shed only limited amounts of eDNA in the water, potential other explanatory variables could be the high flow rate

of the river and low temperature during sampling. Sampling was undertaken at the end of winter/beginning of spring, when environmental conditions such as high flow rates or flood events could have decreased and diluted the quantity of DNA traces. However, this was unavoidable for this species as *I. nubecula* emerges from March onwards (Hynes, 1977; Davy-Bowker, 2003) and so for this species sampling time could not be altered.

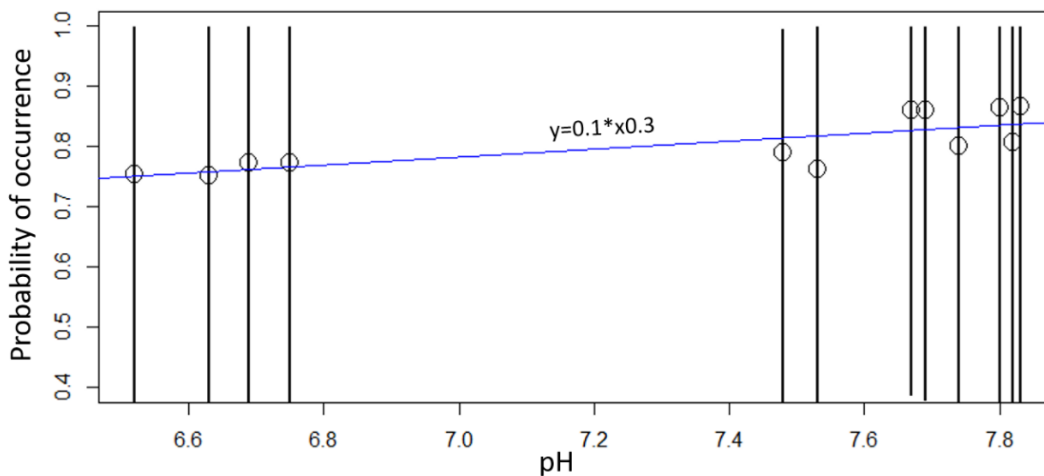

Figure 4.3. Estimated probability of occurrence of *I. nubecula* eDNA with the pH of each sampling sites. Dots are representing each sampling locations, the black lines are representing the estimates of posterior medians with 95% credible intervals and the blue line the regression analysis.

Finally, when sampling for any eDNA study, it is useful to have an understanding of the ecology of the species under study, such as the species habits and preferred habitat in which it occurs. However, again, as *I. nubecula* was recently rediscovered in Wales, there is very little information on this species (Davy-Bowker et al., 2018). The site occupancy modelling approach was also not able to identify any specific variable which would have a significant effect on the probability of detection of this species (Figure 4.3., Tables 4.3. and 4.4.), which is quite logic as all the sites were located in the same study system. A recent study by (Hunter et al., 2019b) on Burmese pythons similarly acknowledge that occupancy modelling approach analyses have certain limitations, mainly driven by the number of locations sampled and restricted range of environmental values collected. In addition, the species in question appears to be rare, and its distribution may be subject to high degrees of stochasticity with regard to population dynamics. Thereby resulting in the effects of the underlying environmental drivers of its distribution being harder to detect. Further

work will therefore be necessary in order to increase the understanding of the ecology of *I. nubecula* if we want to optimize the sampling protocol and conservation plans for this species. Notably, site occupancy modelling is most flexible using the Bayesian statistical framework, and this allows the combination of prior information along with information gained from new sampling data to produce a more informed post experimental understanding, allowing the combination of previous data with current data to produce more robust results (Royle and Dorazio, 2008; Kéry and Schaub, 2012).

In conclusion, even if the highest standards of validation are undertaken in the design and implementation of an eDNA based PCR or qPCR assay (Mächler et al., 2014; Fernández et al., 2018; Wei et al., 2018), false negative results can appear by inhibition factors (Goldberg et al., 2016), low shedding rates from the target species (Klymus et al., 2015; Vörös et al., 2017) or low population sizes (Dougherty et al., 2016). This chapter represent an extreme scenario, in which none of the eDNA samples showed any amplification via qPCR despite the fact that populations of *I. nubecula* were present. However, positive detection (using ddPCR) were obtained at most of the locations where the species was found via the physical survey effort. Less than ten studies have (at the time of writing) utilised this technology for eDNA assays (Doi et al., 2015b, 2015a; Hunter et al., 2017; Baker et al., 2018; Hamaguchi et al., 2018; Lafferty et al., 2018; Uthicke et al., 2018; Hunter et al., 2019b), but this is likely to increase significantly due to the apparent benefits observed in this chapter for example. Caution should therefore be taken with any negative results derived from assays reliant solely on qPCR for the reasons given above.

Chapter 5: The development of an eDNA based detection method for the invasive shrimp *Dikerogammarus haemobaphes*

This chapter has been published as: Mauvisseau, Q., Troth, C., Young, E., Burian, A., Sweet, M., 2019. The development of an eDNA based detection method for the invasive shrimp *Dikerogammarus haemobaphes*. MBI 10, 449–460. https://doi.org/10.3391/mbi.2019.10.3.03

5.1 Abstract

Dikerogammarus haemobaphes is a freshwater gammarid crustacean native to the Ponto-Caspian region. However, the species is rapidly spreading throughout Western Europe and is classed as a highly invasive species. In this chapter, I present a novel eDNA assay aimed at detecting *D. haemobaphes* and demonstrate its suitability with validation steps conducted *in-silico* (computer simulations), *ex-situ* (test of specificity using closely related species) and *in-situ* (within the field). A survey of freshwater systems in the West-Midlands, United Kingdom, highlighted that *D. haemobaphes* was present in 26 out of the 39 sites assessed. Furthermore, it was found that an increase of the distance between two locations increased the probability to attain different eDNA detection results. In conclusion, eDNA detection for *D. haemobaphes* is a promising tool for assessing and mapping the presence/distribution of this invasive amphipod.

5.2 Introduction

Amphipods are a very successful group of invertebrates and many species can impact on the benthic communities and ecosystems of fresh and brackish water systems (van der Velde et al., 2009). Upwards of 1870 species (and sub-species) have been described to date - all of which have been shown to inhabit fresh or inland waters around the world (Väinölä et al., 2008). In some instances, abundances of certain species have been recorded to exceed 5,000 individuals per square metre (Kotta et al., 2013). Their rapid proliferation rates translate to a high potential to function as effective invaders and disrupt natural communities of ecosystems where they are non-native (van der Velde et al., 2009). One example of just such an invader is *Dikerogammarus haemobaphes* (Eichwald, 1841), also referred to as the “demon shrimp”. *D. haemobaphes* originates from the Ponto-Caspian region, however the species has been documented to progressively move across

much of Central and Western Europe over recent years (Bacela et al., 2009). In the United Kingdom, it was first recorded in 2012 in the River Severn (Constable and Birkby, 2016; Aldridge, 2018) and has since spread rapidly through many canal and river networks across the country (Constable and Birkby, 2016). The invasion of this species can lead to significant threats to native species, such as *Gammarus pulex* (Linnaeus, 1758), by direct predation, intensifying resource competition and functioning as vector for new diseases and parasites (Constable and Birkby, 2016).

The far-reaching ecological effects of this invasive species (Gallardo and Aldridge, 2013) highlight the importance to develop an effective early detection and monitoring system to improve the conservation plans for endangered and functionally important native species. However, existing methods used for detecting amphipods (i.e. kick-sampling, capture-recapture) - especially for those occurring at low population densities - are labour-intensive, often ineffective (Forsström and Vasemägi, 2016), time consuming, expensive, ecologically invasive (as it may cause injuries to targeted and non-targeted organisms) (Eiler et al., 2018) and require (in many cases) a high level of taxonomic expertise (Ushio et al., 2018). If such injuries are not a concern for invasive species, it is critical to avoid any potential threats to endangered species or their habitats. New detection techniques facilitating area-wide surveys are therefore urgently required.

All aquatic species leave traces of their DNA within their environment. These DNA fragments may originate from eggs, mucus, faeces or shedding of the epidermis (Thomsen and Willerslev, 2015) and are referred to as environmental DNA (eDNA) (Ficetola et al., 2008). By sampling an aquatic system and amplifying existing eDNA, it is now possible to determine the presence of a given species via means of targeted barcoded qPCR or metagenomics (Dejean et al., 2011; Thomsen and Willerslev, 2015). eDNA methodology has been used successfully over the last 10 years for various target organisms (Hunter et al., 2017; Mauvisseau et al., 2017; Parrondo et al., 2018). eDNA based methods represent a non-invasive tool for assessing species distribution i.e. such methods do not require catching, disturbing or even killing the target organisms. Further, the use of eDNA has been shown to be a highly repeatable and relatively cost-effective method as it requires a lower sampling effort than more traditional survey methods (see Smart et al., 2016; Evans et al., 2017; Bálint et al., 2018; Parrondo et al., 2018).

The aim of this chapter therefore was to further advance eDNA approaches for surveying aquatic amphipods and develop a targeted barcoded eDNA method for *D. haemobaphes*. A new assay was developed and validated, and its specificity tested *in-vitro* and *in-silico* on numerous closely related species and on species, which are likely to share the same habitat as the target organism. The reliability of this assay, i.e. the LOD and LOQ, was assessed following the method outlined in Tréguier et al., (2014) and followed in the chapters 2, 3 and 4. To ensure assay design was optimum, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were utilised (Bustin et al., 2009) (See Appendix 2). Further, the new assay was tested on 39 sites across the West-Midlands, United Kingdom, in order to validate the assay in the field and assess the distribution of *D. haemobaphes* (Table 5.1. and Appendix 10). Based on these results, I present the efficiency of the developed assay and propose recommendations for field sampling protocols.

5.3 Methods

5.3.1 Primers and probe design

Species specific primers, targeting the Cytochrome C Oxidase subunit 1 (i.e. COI) mitochondrial gene of *D. haemobaphes* were designed using the Geneious Pro R10 Software https://www.geneious.com (Kearse et al., 2012) as in chapters 3 and 4. A probe (6-FAM - TTCTTAATATGCGCGCCCCAGGC - BHQ-1) was designed to complement both the forward primer (EY-COI-DhF 5' - GGAGCTTCCTCTATTCTTGGCGCAATT - 3'), and the reverse primer (EY-COI-DhR 5' - GGCCGTGATAAAGACAGACCAGACAAA - 3') in order to increase specificity of the reaction. This resulted in a 117 bp fragment of DNA from the COI region when amplified. Sequences from 23 species (which are either taxonomically similar to *D. haemobaphes*, or likely to be present within the same habitats) were utilised during the development and assessment of the assay sensitivity (Appendix 11).

Locations	eDNA detection	Number of positive replicates	Mean Ct	Collection date	Type of site	Latitude	Longitude
1	Yes	1/6	36,13276291	01/12/2016	Canal	52,7212	-1,7995
2	No	-	-	01/12/2016	Canal	52,7219	-1,7879
3	Yes	1/6	36,32998657	29/11/2016	Canal	52,6121	-1,7025
4	Yes	4/6	36,40121746	29/09/2016	Canal	52,7597	-2,0982
5	Yes	1/6	35,16476822	29/09/2016	Pond	52,7568	-2,0961
6	Yes	1/6	37,29187012	27/10/2016	Canal	52,6616	-1,9336
7	No	-	-	27/10/2016	Canal	52,6594	-1,9301
8	No	-	-	27/10/2016	Canal	52,6641	-1,9397
9	No	-	-	27/10/2016	Canal	52,6566	-1,9263
10	Yes	1/6	36,51880264	27/10/2016	Canal	52,6379	-1,9706
11	Yes	2/6	36,52320671	23/11/2016	Canal	52,5453	-2,0090
12	Yes	3/6	36,98597972	16/11/2016	Canal	52,5240	-2,0484
13	Yes	1/6	35,91026306	16/11/2016	Canal	52,5154	-2,0494
14	Yes	3/6	36,56238302	16/11/2016	Canal	52,5161	-2,0240
15	No	-	-	13/11/2016	Canal	52,5006	-2,1004
16	Inconclusive	3/6	38,43717448	13/11/2016	Canal	52,4872	-2,1160
17	Yes	6/6	37,4743983	13/11/2016	Canal	52,4750	-2,1268
18	Yes	1/6	36,24039078	13/11/2016	Canal	52,4846	-2,0925
19	Yes	3/6	36,27352524	11/11/2016	Canal	52,4891	-2,0733
20	Yes	3/6	36,4181811	13/11/2016	Canal	52,4822	-2,0606
21	Inconclusive	2/6	40,08729935	11/11/2016	Canal	52,4730	-2,0558
22	No	-	-	11/11/2016	Canal	52,4704	-2,0522
23	No	-	-	11/11/2016	Canal	52,4595	-2,0397
24	No	-	-	12/12/2016	Canal	52,4890	-1,4584
25	No	-	-	12/12/2016	Canal	52,5910	-1,3951
26	No	-	-	12/12/2016	Canal	52,6577	-1,4455
27	Yes	2/6	36,7260437	12/12/2016	Canal	52,6800	-1,4947
28	Yes	1/6	37,28199768	01/12/2016	Canal	52,7262	-1,7862
29	Yes	1/6	37,62047958	29/09/2016	Canal	52,7542	-2,0964
30	Yes	3/6	36,47432709	29/09/2016	Canal	52,7378	-2,0942
31	No	-	-	11/10/2016	Pond	52,5274	-2,1582
32	No	-	-	06/10/2016	Reservoir	52,4949	-2,1279
33	Yes	1/6	37,29760361	27/09/2016	Canal	52,4770	-2,1548

Table 5.1. Table depicting eDNA detection results, the number of positive qPCR replicates, the mean Ct of positive replicates, the collection date, the type of site and the GPS coordinates of each sampled locations.

The assay was then tested against extracted DNA from the following species to further ensure specificity; *G. pulex*, the killer shrimp *Dikerogammarus villosus* (Sowinsky, 1894), *Gammarus fossarum* (Koch, 1836), *Sigara fossarum* (Leach, 1817), *Cloeon dipterum* (Linnaeus, 1761), the spinycheek crayfish *Faxonius limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1952), the noble crayfish *Astacus astacus* (Linnaeus, 1758), the narrow-clawed *Pontastacus leptodactylus* (Eschscholtz, 1823), the Louisiana crayfish *P. clarkii* and the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858). DNA was also extracted from four individual *D. haemobaphes*.

5.3.2 eDNA samples

33 locations spanning canals, rivers and reservoirs were sampled across the West Midlands in the United Kingdom (Figure 5.1.). Sampling was conducted between the 29th September 2016 and the 12th December 2016 (Table 5.1.). At each location, a 1L water sample was collected with a sterile polypropylene ladle (see Figure 5.2.). To acquire the 1L water sample, 25 'sub-samples' of 40 mL of water were collected and placed into a sterile plastic bag (Whirl-Pak® 1242 ml Stand-Up Bag Merck®, Darmstadt, Germany) for homogenisation. Out of the 1L sample, 6 X 15 mL were transferred into 6 sterile falcon tubes (Falcon™ 50mL Conical Centrifuge Tube, Fisher Scientific, Ottawa, Canada) containing 1.5 mL 3M sodium acetate and 33.5 mL of absolute ethanol using sterile disposable plastic pipettes. All samples were then stored at -20 °C before further analysis. Furthermore, at 6 additional locations, kick-sampling was undertaken (in addition to the eDNA sampling protocol mentioned above - Appendix 10). However, results from these six locations were only used for confirming the specificity of the eDNA assay to traditional sampling methods and not utilised in the spatial analysis which was conducted on the 33 original sites (highlighted above).

5.3.3 DNA extraction

Laboratory equipment was disinfected using 10% bleach solution and ethanol and decontaminated under UV lights (Mauvisseau et al., 2017; Parrondo et al., 2018). DNA extraction was performed in a clean, PCR free room. The Qiagen DNeasy® Blood and Tissue Extraction Kit was used for eDNA extraction following manufacturers' guidelines. eDNA from the water samples were

extracted following the methods outlined in (Tréguier et al., 2014). DNA pellets from all 6 falcon tubes were pooled together and hence a total water volume of 90 mL per location was analysed. The final DNA elution volume was 100 µL.

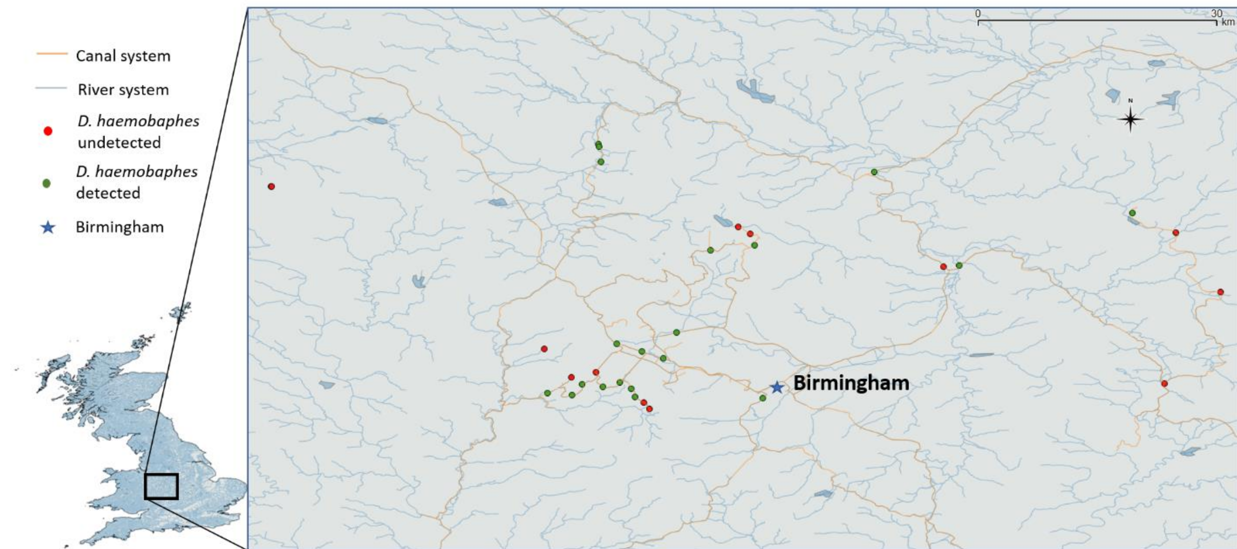

Figure 5.1. Map of the canal and rivers system showing the sampling locations (n = 33) screened for the presence of *D. haemobaphes* in the United Kingdom. DNA of the targeted species was detected in the green locations. Red locations showed the places found to be negative to eDNA detection.

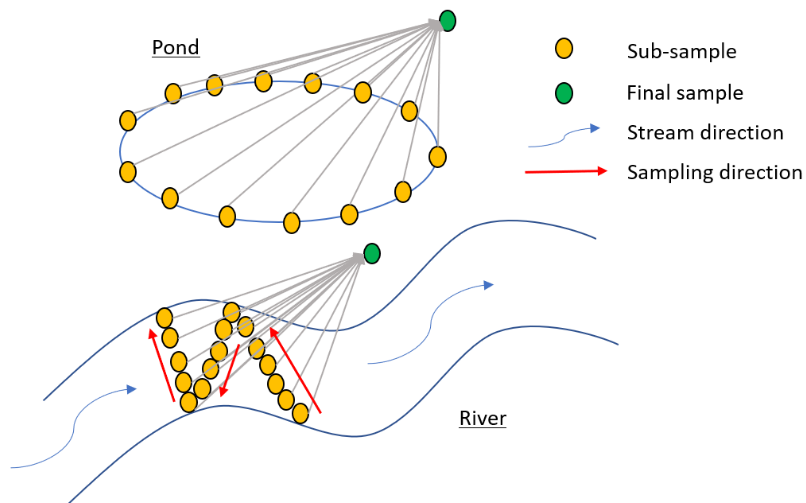

Figure 5.2. Representation of the eDNA sampling protocol in respective freshwater systems. For each location, 25 sub-samples of 40 mL were taken to obtain a representative 1 L final sample of the location. In small river system or canal, sub-samples are taken from across the river/canal and sampled from downstream to upstream. This ensures disturbed sediment washes downstream from the collection point at any given time.

5.3.4 PCR and qPCR amplification

PCR amplifications were performed on a Gen Amp PCR System 9700 (Applied Biosystem). A 25 µL reaction was run for each sample, consisting of 12.5 µL of PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10 µM), 9.5 µL of ddH2O and 1 µL of template DNA. qPCR amplifications were performed on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems) in the same final volume of 25 µL. In contrast to standard PCR, the mixture for qPCR consisted of; 12.5 µL of TaqMan™ Environmental Master Mix 2.0 (ThermoFisher Scientific, UK), 1 µL of each primer (10 µM), 1 µL of probe (2.5 µM), 6.5 µL of ddH2O and 3 µL of extracted DNA. Both PCR and qPCR were performed under the following protocol. Initial denaturation at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 64 °C for 1 min.

A standard curve was established by analysing a 1:10 dilution series of DNA extracted from *D. haemobaphes* (164.1 ng/ µL, Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific) following the MIQE Guidelines (Bustin et al., 2009). Dilutions ranged from 10^{-1} to 10^{-9} with 10 technical replicates used for each of the dilution steps. Following the methods of chapter 2, 3 and 4, the LOD was defined as the last dilution in the standard curve at which eDNA is detected with a Ct below 45. The LOQ was also assessed. LOQ was defined as the last dilution in the standard curve at which eDNA is detected and quantified in at least 90% of the qPCR replicates with a Ct below 45 (Bustin et al., 2009; Hunter et al., 2017). All eDNA samples were then analysed with six technical replicates (Cowart et al., 2018) on a qPCR plate including six negative controls and a positive control dilution series from 10^{-1} to 10^{-5} in duplicate.

5.3.5 Statistical analysis

As in chapters 2, 3 and 4, a standard dilution was undertook for the assay in order to determine the LOD and the LOQ (Bustin et al., 2009; Tréguier et al., 2014; Hunter et al., 2017). Then, the likelihood of obtaining similar eDNA detection results in locations geographically close to each other was also explored utilising the GPS coordinates from the original 33 sampled locations. For this, a matrix distance was calculated containing pairwise distances (in meters) between all data points. Further, a second matrix was established, containing the information on the constancy of eDNA detection between two locations. eDNA based detection was considered as consistent if the target species was either present or absent in both locations. Utilising a logit-regression analysis, it was then assessed whether the probability of obtaining consistent results decreased with the increase in distance between sampling locations. Regression analyses were performed with logged and non-logged data and the most parsimonious model was chosen based on the AIC. Residuals of the regression were checked for autocorrelation, Cook's distance and systematic trends of residuals. All statistical analyses were performed with R version 3.4.1 (R Core Team 2018).

5.4 Results

The assay designed in this chapter was found to be species-specific to *D. haemobaphes*. Both PCR and qPCR did not result in any positive detection of non-target species in all *in-vitro* tests (i.e. none of the PCR and qPCR controls showed any amplification in this chapter). The LOD and the LOQ of the assay determined by an analysis of the standard curve (Figure 5.3.) (Slope= -3.577, Y inter= 18.037, R^2= 0.937, Eff%= 90.341) was assessed and revealed a LOD of 1.641 pg DNA μl^{-1} at 38.236 ± 0.915 Ct and a LOQ of 16.41 pg DNA μl^{-1} at 34.90 ± 0.690 Ct.

The efficiency of the developed assay was confirmed using both kick-sampling and eDNA analysis (Appendix 10). It was possible to detect *D. haemobaphes* using both methods in 4 locations, despite having an inconclusive result in another location due to a Ct value over the LOD. One location was positive only with eDNA methodology. The blank control (see Appendix 10) and all negative control technical replicates showed no amplification with PCR or qPCR during the whole experiment.

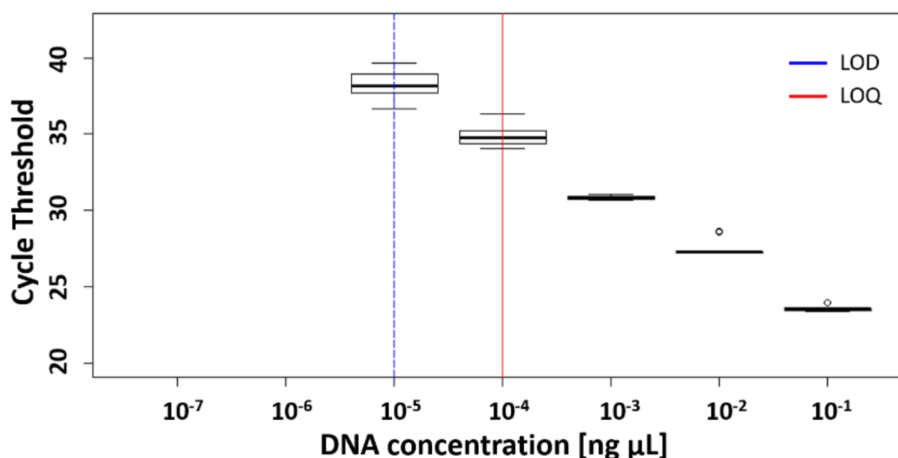

Figure 5.3. Standard curve used for determining the LOQ and LOD relating Ct of the qPCR targeting the COI region of *D. haemobaphes* to DNA dilution steps.

eDNA of *D. haemobaphes* was detected in 21 of the 33 tested locations (Table 5.1.). Mean Ct values from the positive field samples ranged from 35.2 to 40.1. Notably, two sites showed a high mean Ct value, which ranged well above the established LOD. However, both of these sites contained technical replicates with Ct values below the LOD. As the mean Ct values from all positive locations were above the established LOQ, only the presence and absence data were utilised for further assessments.

The analysis of matrices containing the physical distance of sampling locations and the consistency of eDNA measurements revealed that an increase of the distance between two locations increased the probability to attain different eDNA detection results ($p < 0.023$). Interestingly sampling locations within close proximity to each other showed a relatively low eDNA based detection consistency of 68% (Figure 5.4.). Two versions of the regression model containing either logged or non-logged physical distance values were evaluated. While both versions resulted in a significant influence of physical distances, the linear model (non-logged data) was more parsimonious (lower AIC). The relationship showed a large scattering around predictions indicating that there is likely to be a number of factors not included in the analysis influencing the consistency of *D. haemobaphes* detection.

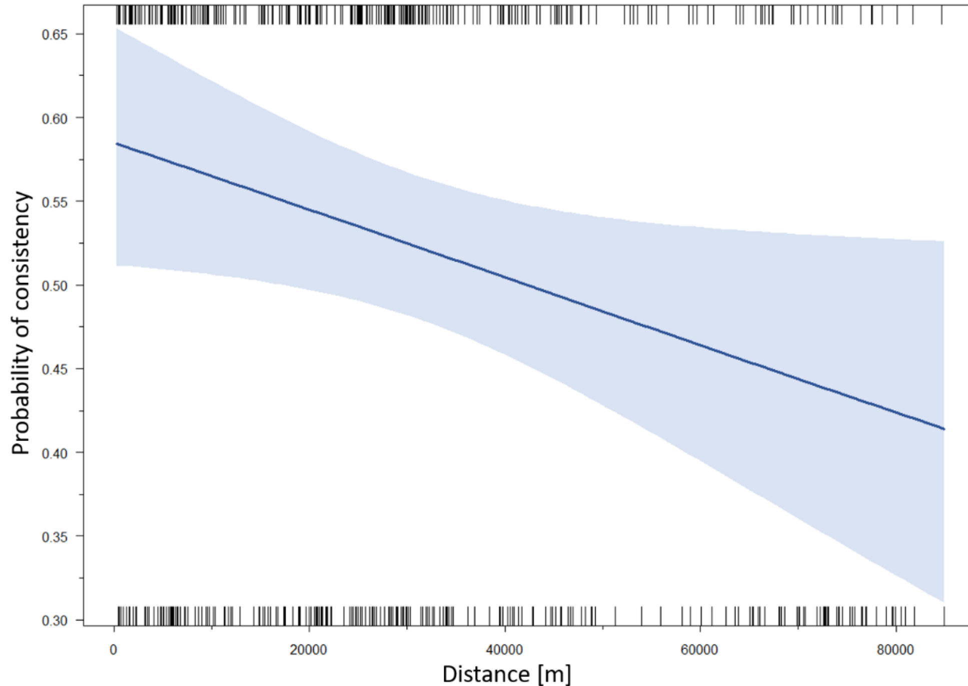

Figure 5.4. Relationship between the probability of obtaining the same eDNA detection result in two different sampling locations (i.e. probability of consistency) and the distance between sites. Distances between sites are stated in meters. The blue line and the blue shaded area reflect the regression equation and its confidence interval, respectively. Black ticks on the upper and lower edge of the graph represent data points.

5.5 Discussion

Here, I introduce a novel eDNA assay, which can be used to assess the presence of *D. haemobaphes*. The approach was shown to be highly sensitive and no false positives were identified, either via *ex-situ* or *in-situ* validation tests. Further, I was able to demonstrate that the assay can successfully detect *D. haemobaphes* in various habitats including lotic and lentic systems such as ponds, canals and faster flowing rivers. Design of a novel eDNA based methods mean that, in contrast to traditional tools such as kick-sampling for example, the environment does not need to be disturbed when any survey is undertaken. Further, the use of eDNA eliminates the need for high level taxonomic expertise. However, despite the specificity of the approach and the reliability of detection, the field assessment highlighted several possible ways for improving the sampling protocols.

First, the amount of eDNA detected was generally low and ranged above the established LOQ in all sites (Table 5.1., Figure 5.3.). This indicates that the field sampling protocol for *D. haemobaphes* was in contrast to protocols developed for other aquatic invertebrates (Yusishen et al., 2018) and therefore not suitable for the accurate quantification of DNA traces, as a proxy for population densities. There are a number of possible explanations for this. For example, population densities may be low for this species and/or *D. haemobaphes* may only shed a reduced amount of DNA into its environment (Buxton et al., 2017). A corroborating reason, however, may be the choice of sampling method utilised in this chapter (Piggott, 2016). Here I opted to use the established ethanol precipitation method introduced by (Tréguier et al., 2014), which is commercially available for the detection of the endangered Great Crested Newt *T. cristatus* (Harper et al., 2018b). This method results in the extraction of eDNA from only 90 mL of water. In contrast, the use of filters is becoming a more widespread and practiced method for eDNA surveys and more often results in the filtration of upwards of 250 mL of water (Rees et al., 2014). Indeed, other studies focusing on macroinvertebrates have utilised filtration successfully to detect eDNA, as in chapter 4, but yet, there is no consensus on the optimal filter type (Niemiller et al., 2017; Harper et al., 2018a). Further studies will therefore benefit from a detailed assessment of sampling design when utilising eDNA of any given species. Moreover, these analysis revealed that an increase of the distance between two locations increased the probability of obtaining different eDNA results between two locations. This is an expected result because samples taken from the same canal-section or from the same river reach are more likely to have a similar habitat suitability than sections far apart from each other. However, sampling locations within close proximity to each other showed a relatively low eDNA detection consistency of 68%. One factor contributing to this finding might be a large habitat heterogeneity. Indeed, tributaries to channels can be in close proximity but may very well have a different species composition than the main channel of the river potentially explaining different result in sampling locations separated by only a few hundred meters. Anthropogenic interventions (such as dams) and-or variation in habitat quality (such as levels of pollution), can also affect the presence and/or dispersion of any given species (invasive or not) and would therefore be picked up as variation in the consistency in the eDNA assays of close environmental replicates.

However, it is important to acknowledge that also the sampling protocol utilised here may have caused relatively large inconsistency in eDNA results of adjacent sampling sites. For example, sampling a small amount of water (90 mL) could increase the stochasticity of eDNA detection (Foote et al., 2012; Wilcox et al., 2016). Protocols of eDNA capture and extraction often varies between studies and for different target species (Deiner et al., 2015). Therefore, the sampling methodology could influence the reliability in the eDNA detection of any given organism and/or any given eDNA assay. The filtration of large amounts of water is one possible approach to reduce the variability of eDNA detection in samples from the same location (Adrian-Kalchhauser and Burkhardt-Holm, 2016) and this has been recommended by several studies for detecting eukaryotes in freshwater ecosystems (Deiner et al., 2015; Hinlo et al., 2017). Alternatively, using multiple field replicates for each sampling locations as highlighted in chapter 2, could also allow for the reduction in the variability of eDNA detection and improve the detectability of the target species even at low abundance. A detailed assessment of the effect of sampling method choice/water volume utilised on the consistency of eDNA detection would be an interesting next step to further improve the efficiency and reliability of eDNA based surveys.

In conclusion, this chapter illustrates a novel and reliable method to assess the presence of *D. haemobaphes* populations. As this proposed assay is non-invasive and can be utilized in a citizen science type program, it can be easily brought into existing biodiversity management plans – especially those tackling the spread of invasive species.

Chapter 6: Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems

This chapter has been published as: Mauvisseau, Q., Tönges, S., Andriantsoa, R., Lyko, F., Sweet, M., 2019. Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems. MBI 10, 461–472. https://doi.org/10.3391/mbi.2019.10.3.04

6.1 Abstract

Procambarus virginalis, also known as the Marmorkrebs is a highly invasive crayfish species characterized by parthenogenetic reproduction. As conservation management plans rely on the accuracy of the presence and distribution information of invasive species, a reliable method is needed for detecting such species in aquatic systems. In this chapter, I developed and validated a qPCR-based assay for monitoring *P. virginalis* at low abundance, by detecting their eDNA traces left in freshwater systems. I was able to implement this new assay *in-situ* at two separate lakes in Germany, where the crayfish were known to be present. Furthermore, the pathogenic fungus *Aphanomyces astaci* was not detected in the locations where the Marmorkrebs were detected. In conclusion, the use of eDNA is therefore a reliable tool for the early detection of this "perfect invader".

6.2 Introduction

Non-indigenous crayfish species are starting to outnumber indigenous crayfish species throughout much of Europe (Holdich et al., 2009). Only five indigenous species were originally found to exist in various freshwater systems across Europe and now eleven non-indigenous species are spreading at alarming rates across this eco-region (Holdich et al., 2009; Kouba et al., 2014). Three of these species are from North America, *P. clarkii*, *Faxonius limosus* (Rafinesque, 1817) and *Pacifastacus leniusculus* (Dana, 1852) and have been classified as the most problematic of the invasive crayfish (Holdich et al., 2009). Several other species, also from North America include; *Faxonius immunis* (Hagen, 1870), *Faxonius juvenilis* (Hagen, 1870), *Faxonius virilis* (Hagen, 1870), *Procambarus*

virginalis (Lyko, 2017), *Procambarus alleni* (Faxon, 1884) and *Procambarus acutus* (Girard, 1852), introduced after 1980 and therefore have a considerably more restricted range (Holdich et al., 2009). This is the same for the two Australian species; *Cherax destructor* (Clark, 1936) and *Cherax quadricarinatus* (von Martens, 1868). In addition to these already established species, there is the substantial risk of further introductions, especially through the aquarium trade where many American or Australian species are still readily available (Holdich et al., 2009). There are a number of well documented examples, whereby certain non-indigenous crayfish have been discarded as they outgrow their tanks (such was the case for *Cherax* or *Faxonius* species) or reproduce excessively (such as *P. virginalis*) (Holdich et al., 2009). Due to these introductions (or at least in part), there has been a global decline of indigenous crayfish (Holdich et al., 2009). This is due largely to increased and direct competition for habitat space and resources, along with many (of these American invasive species) carrying the crayfish plague, *Aphanomyces astaci* (Schikora, 1906), a lethal pathogen affecting native species (Bramard et al., 2006; Schrimpf et al., 2013; Keller et al., 2014; Lipták et al., 2016; Ludányi et al., 2016). As a result of these combined threats, conservation programs and "ark" sanctuary sites are being established in various countries with the goal of protecting the local crayfish biodiversity (Holdich et al., 2009; Reynolds and Souty-Grosset, 2012). Native crayfish are keystone species in freshwater systems and are also useful as bioindicators of pollution (Reynolds and Souty-Grosset, 2012). They have also been proposed as umbrella species, from which the protection is expected to benefit to a large range of co-occurring species, for ecosystem conservation (Reynolds et al., 2013).

Although, many of these invasive species currently have restricted ranges, at least one has the potential to be of major concern (Keller et al., 2014). *P. virginalis*, also known as the Marmorkrebs has been labelled as a "perfect invader" due to its recent speciation and, more specifically, its parthenogenetic reproduction mode (Jones et al., 2009; Vogt et al., 2015; Gutekunst et al., 2018). Distribution via the pet trade and anthropogenic releases have led to an increasing spread of the species in various countries, inside and outside Europe (Chucholl et al., 2012; Vojkovská et al., 2014; Lipták et al., 2016; Ludányi et al., 2016; Pârvulescu et al., 2017; Gutekunst et al., 2018). The species is also remarkably tolerant to changes in habitat parameters and can adapt to temperatures as low as 2 to 3 °C (Veselý et al., 2015; Lipták et al., 2016; Ludányi et al., 2016;

Andriantsoa et al., 2019). Furthermore, *P. virginalis* has also been cited as carrying *A. astaci* (or crayfish plague) (Keller et al., 2014; Lipták et al., 2016).

As European countries have a mandate to prevent the deliberate introduction of exotic species under the Habitats Directive (European Commission, 1992, 2000, 2014; Souty-Grosset et al., 2004), the ability to screen for this particular invasive species would be useful for management of aquatic ecosystems. However, early detection of aquatic organisms, especially when they occur at low densities, has been challenging and often ineffective using currently established methods (Forsström and Vasemägi, 2016). For marbled crayfish, this is further compounded by the nocturnal or crepuscular activity patterns.

In recent years, interest has increased for methods which detect DNA traces within any given environment (known as environmental DNA or eDNA) (Hinlo et al., 2017). This non-invasive method (which targets DNA from skin, blood, mucus or gametes for example) allows a reliable and cost-effective tool for monitoring many different organisms within a wide variety of aquatic habitats, especially when populations are low in abundance (Forsström and Vasemägi, 2016; Eiler et al., 2018). Indeed, the method has already been used to detect many of the current indigenous and native crayfish species mentioned earlier (Tréguier et al., 2014; Figiel and Bohn, 2015; Dougherty et al., 2016; Ikeda et al., 2016; Agersnap et al., 2017; Cai et al., 2017; Dunn et al., 2017; Larson et al., 2017; Cowart et al., 2018; Geerts et al., 2018; Harper et al., 2018; Mauvisseau et al., 2018; Riascos et al., 2018; Rice et al., 2018; Robinson et al., 2018).

The aim of this chapter is to design and validate a qPCR (i.e. quantitative Polymerase Chain Reaction) assay for the detection of the newly described, yet highly invasive, crayfish species *P. virginalis*. The assay was tested *in-vitro* and *in-silico* against various indigenous and non-indigenous crayfish species known to occur throughout Europe. Further, I assessed the reliability of the developed assay i.e. the LOD and LOQ by following the MIQE Guidelines and methods from chapters 2, 3, 4 and 5 (Bustin et al., 2009). 15 locations were then sampled in Germany (which included sites with known presence of *P. virginalis*) (Figure 6.1.). Finally, at all locations shown to contain *P. virginalis* (either via eDNA sampling and/or direct searching), the presence of *A. astaci* was investigated (Appendix 12).

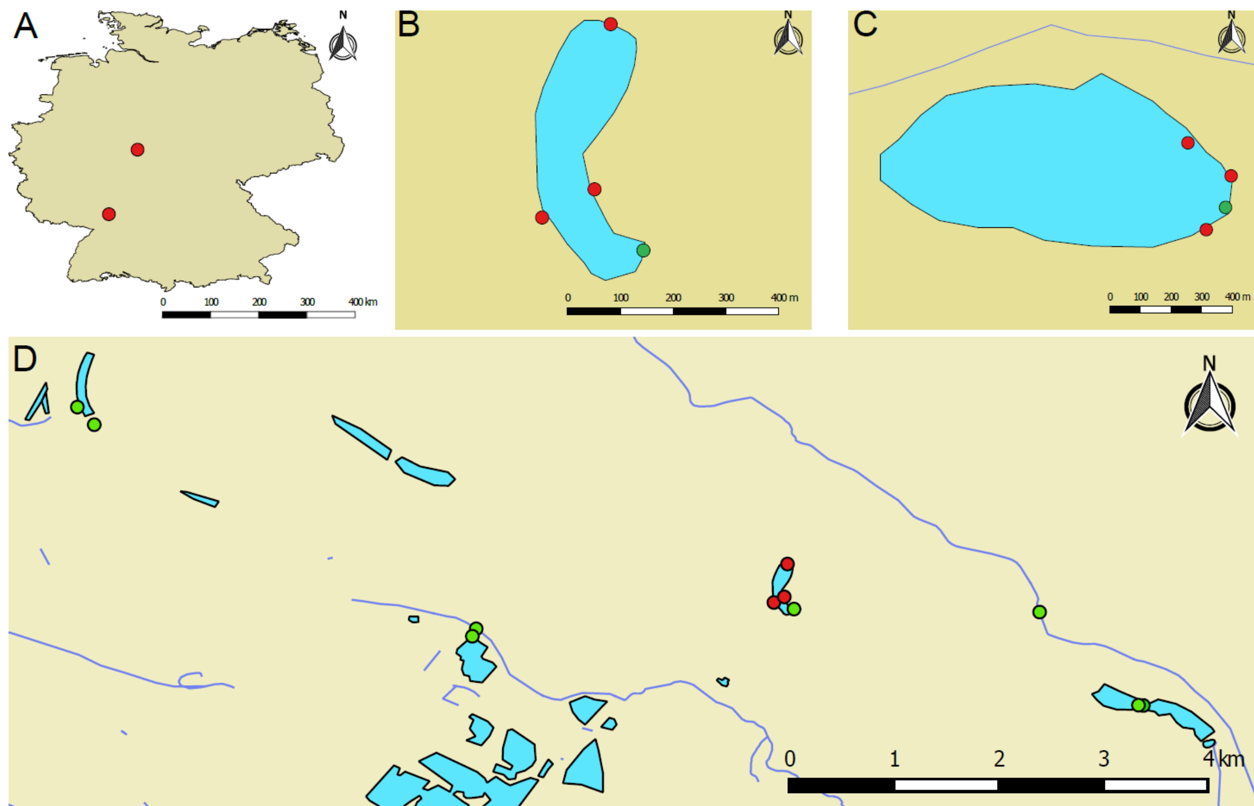

Figure 6.1. Detection of *P. virginalis* eDNA in established marbled crayfish populations in Germany. (**A**) Location of Reilinger See (lower left red circle) and Singliser See (upper right red circle) in Germany. (**B**) Map of Reilinger See with locations of the four sampling sites. Sampling sites with *P. virginalis* eDNA detection are indicated by red circles. (**C**) Map of Singliser See with locations of the four sampling sites. (**D**) Map of Reilinger See and surrounding water bodies with locations of eleven sampling sites.

6.3 Methods

6.3.1 Assay development

Primers and a probe targeting the Cytochrome C oxidase subunit 1 (i.e. COI) mitochondrial gene of *P. virginalis* were designed using the Geneious Pro R10 Software https://www.geneious.com (Kearse et al., 2012) following the method outlined in (Tréguier et al., 2014). In addition to the target species, DNA was extracted from one individual of each of the following crayfish species (present or likely to be present in European freshwater systems): *P. clarkii*, *F. limosus*, *P. leniusculus*, *Astacus astacus* (Linnaeus, 1758), *A. leptodactylus* (Eschscholtz, 1823) and

Austropotamobius pallipes (from a UK population) (Lereboullet, 1858). DNA from each individual was sequenced using Eurofins Genomics (Wolverhampton, UK), as identification of controls using primers and methods described in (Folmer et al., 1994). When designing the assay, COI sequences from *P. virginalis* (GenBank Accession No. KJ690261.1), *P. clarkii* (JN000901.1, JF438002.1), *F. limosus* (JF437991.1, KT959387.1, KT959445.1), *P. leniusculus* (KU603472.1, JF437998.1, JF437996.1), *A. astacus* (JN254661.1, JN254666.1, JN254672.1), *A. leptodactylus* (KU571456.1, KU571460.1, KU571463.1), *A. pallipes* (AB443446.1, AB443448.1) and *P. alleni* (HQ171452.1, HQ171450.1) were obtained. Specificity of the set of primers and probe was assessed *in-silico* using the BLASTn and Primer-BLAST tools from the NCBI https://www.ncbi.nlm.nih.gov/. *In-silico* validation was performed before *in-vitro* tests.

The forward primer Pv-COI-F 5' - GTATAGTTGAGAGGGGAGTA - 3', reverse primer Pv-COI-R 5' - CCATAGTTATACCAGCTGCC - 3' and probe 6-FAM - AGGTATTTTTTCCTTGCA - BHQ-1 were developed to amplify a 189 bp fragment. Primers and the probe were tested via both conventional PCR and qPCR with DNA extracted from the crayfish species mentioned above.

6.3.2 eDNA samples

15 locations including rivers, lakes and one pond were sampled between the 10th May 2018 and the 15th June 2018 in Baden-Württemberg, south-west Germany and in Hessen, central Germany (Figure 6.1.). Eight of the sampled locations were previously known for the presence of *P. virginalis* (Appendix 12) (Dümpelmann and Bonacker, 2012; Lyko, 2017). At each location, two independent 1 L water samples (hereafter referred as "natural replicates") were collected using a sterile polypropylene ladle and placed into a sterile plastic bag (Whirl-Pak® 1242 ml Stand-Up Bag Merck®, Darmstadt, Germany). Samples from rivers consisted of combining water subsamples, regularly sampled from across the width of the rivers, by moving downstream to upstream, in order to avoid disturbing the sediments as shown in chapter 5 (Mauvisseau et al., 2019e). Samples from the lakes and pond consisted of combining surface-water subsamples, sampled across a ten-meter-wide strip, approximately 1 meter away from the bank. Subsamples across the entirety of the two lakes were not possible, as the size, anthropogenic activities, and vegetation cover did not always allow for complete access to all lake side locations. Samples from each location were then filtered with a 50 mL syringe (sterile Luer-Lock™ BD Plastipak™,

Ireland) through a sterile 0.45 µm Sterivex™ HV filter (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Millipore®, Germany). Sterivex filters were then immediately fixed with 2 mL of absolute ethanol and stored at room temperature until the end of the fieldtrip as in chapter 3 (Spens et al., 2017). All filters were then stored at -80 °C in the laboratory before further analysis. Sterile equipment and disposable nitrile gloves were used during the sampling process and replaced at each location to avoid contamination.

6.3.3 DNA extraction

DNA was extracted from both tissue samples and Sterivex filters using the Qiagen DNeasy® Blood and Tissue Kit. DNA was extracted from tissue samples following manufacturers' guidelines. As in chapters 2, 3, and 4, eDNA was extracted from Sterivex filters following the methods described in (Spens et al., 2017). Three control samples consisting of ddH_2O were extracted as above with the Sterivex filters for assessing the absence of cross-contamination. Pipettes and tube holders were disinfected and decontaminated under UV treatment throughout the whole process. All other laboratory equipment and surfaces were regularly disinfected using 10% bleach solution and ethanol before the analysis.

6.3.4 PCR and qPCR protocols

6.3.4.1 Procambarus virginalis

PCR amplifications were performed on a Gen Amp PCR System 9700 (Applied Biosystem) with the set of species-specific primers described above. PCR reactions were performed in a 25 µL reaction, with 12.5 µL of PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10 µM), 9.5 µL of ddH2O and 1 µL of template DNA. Optimal PCR conditions were performed under thermal cycling 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 62 °C for 1 min. For each PCR (with DNA from tissue samples), at least one positive and one negative control were included.

qPCR reactions were performed in final volumes of 25 µL, using 12.5 µL of PrecisionPlus qPCR Master Mix with ROX (Primer Design, UK), 1 µL of each primer (10 µM), 1 µL of probe (2.5 µM), 6.5 µL of ddH2O and 3 µL of extracted DNA on an ABI StepOnePlus™ Real-Time PCR

(Applied Biosystems). Optimal qPCR conditions were performed under thermal cycling 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 56 °C for 1 min.

6.3.4.2 Aphanomyces astaci

For samples which showed the presence of *P. virginalis*, the presence or absence of *A. astaci* was also assessed. Detection of *A. astaci* was performed using the method (including the primers and probe), developed by (Vrålstad et al., 2009; Strand et al., 2011). In brief, qPCR reactions were performed using the forward primer AphAstITS-39F (5'-AAGGCTTGTGCTGGGATGTT-3'), reverse primer AphAstITS-97R (5'- CTTCTTGCGAAACCTTCTGCTA-3') and a MGB probe AphAstITS-60T (5'-6-FAM-TTCGGGACGACCCMGBNFQ-3') in a final volume of 25 µl using 12.5 µl of TaqMan™ Environmental Master Mix 2.0 (ThermoFisher Scientific, UK), 1 µl of each primer (10 µM), 1 µl of the corresponding probe (2.5 µM), 4.5 µl of ddH2O and 5 µl of extracted DNA on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems). qPCR conditions were as follows; warm up at 50 °C for 5 min and denaturation at 95 °C for 8 min, followed by 50 cycles 95 °C for 15 s, 58 °C for 1 min. Negative controls were also collected and run as above.

6.3.5 qPCR analysis

A standard curve was first established by analysing a 1:10 dilution series of DNA extracted from *P. virginalis* (55.2 ng/ µL, Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific) following the MIQE Guidelines and methods from chapters 2, 3, 4 and 5 (Appendix 2) (Bustin et al., 2009). A second standard curve was performed for the analysis targeting *A. astaci*. DNA for this was acquired from the reference isolate of *Aphanomyces astaci* 8866_2 (Department of Environmental and Biological Science, University of Eastern Finland) (13.1 ng/µL, Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific). Again, this was conducted using a 1:10 dilution series similar to that for *P. virginalis* (Bustin et al., 2009). For both targets, the dilution ranged from 10^{-1} to 10^{-9} with 10 "technical replicates" (i.e. qPCR replicates) used for each of the dilution steps in order to assess the LOD and LOQ (Figure 6.2. and 6.3.) (Bustin et al., 2009; Hunter et al., 2017). When running each assay (for *A. astaci* and *P. virginalis*) two positive and two negative controls were also included.

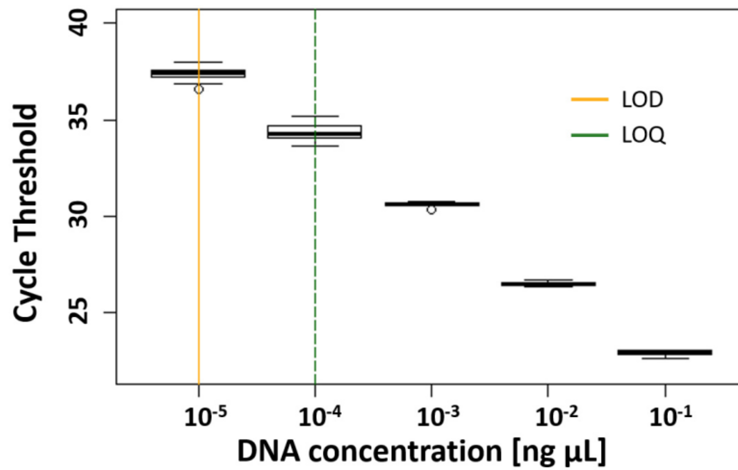

Figure 6.2. Standard curve established by the analysis of a 1:10 dilution series of DNA extracted from *P. virginalis* tissue (55.2 ng/ µL).

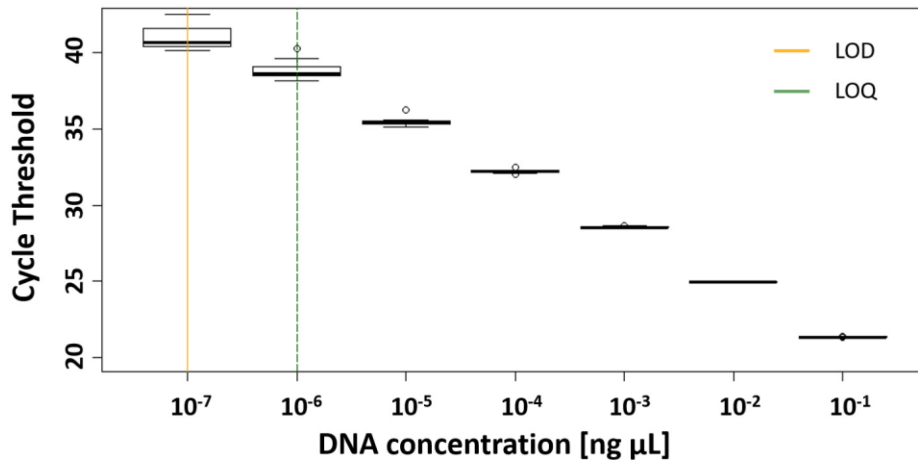

Figure 6.3. Standard curve established by the analysis of a 1:10 dilution series of DNA extracted from a pure *A. astaci* culture isolate (13.1 ng/ µL).

For each of the eDNA samples (i.e. the Sterivex filters) six "technical replicates" were ran, at the same time two further replicates of the dilution series (see above - ranging from 10^{-1} to 10^{-5} for *P. virginalis* and from 10^{-2} to 10^{-6} for *A. astaci*), and six negative control (i.e. blanks) were also run. The negative controls consisted of water free of both *A. astaci* and *P. virginalis* DNA which was collected at the same time as the eDNA samples and in the same way.

6.4 Results

Primers and probes designed in this chapter were found to be species-specific to *P. virginalis* using PCR and qPCR against DNA from the six other crayfish species mentioned above in the assay development section. All negative controls were found to be negative for *P. virginalis* DNA. The standard dilution obtained for the set of primers/probe targeting the COI gene was used for determining the LOD and the LOQ (Bustin et al., 2009; Tréguier et al., 2014; Hunter et al., 2017) (Figure 6.2.). The LOD was identified as the last dilution of the standard curve in which the DNA from the targeted gene is amplified with a Ct below 45 (Bustin et al., 2009; Mauvisseau et al., 2019e). The LOQ was identified as the last dilution of the standard curve in which the DNA from the targeted gene is detected, amplified and quantified in at least 90% of the qPCR replicates with a Ct below 45 as in chapters 2, 3, 4 and 5 (Mauvisseau et al., 2019a, 2019e). After the standard curve analysis (Slope = -3.68, Y inter = 19.27, R^2 = 0.99, Eff% = 86.82), the LOD was found to be 0.552 pg per μl^{-1} at 37.36 ± 0.40 Ct and the LOQ was indicated at 5.52 pg per μl^{-1} at 34.30 ± 0.44 Ct (Figure 6.2.).

In order to detect *P. virginalis* eDNA, water samples were obtained from two lakes in Germany with known stable populations (Figure 6.1.A.) and from rivers, lakes and a one pond surrounding one of the positive sites (Reilinger See, Figure 6.1.B.) with unknown status about the presence of this invasive crayfish. At both Reilinger See, and Singliser See, *P. virginalis* eDNA was detected in three of the four sampled locations (Figure 6.1.B. and 6.1.C. respectively). The mean Ct values ranged from 34.86 ± 1.9 to 29.86 ± 0.12 (Slope = −3.68 / −4.29 (range), Y inter = 17.57 / 18.95 (range), R^2 = 0.98 / 0.99 (range), Eff% = 70.10 / 86.92 (range)) (Appendix 12). These results show that this eDNA assay can detect *P. virginalis* in the majority of samples from established populations.

A qPCR analysis targeting *A. astaci* was conducted on all the natural replicates from the same six locations as those tested for *P. virginalis*. After the standard curve analysis targeting *A. astaci* (Slope = −3.34, Y inter = 11.76, R^2 = 0.99, Eff% = 99.33), the LOD was found to be 1.31 x 10-3 pg per μl^{-1} at 41.09 ± 1.02 Ct and the LOQ was indicated at 1.31 x 10-2 pg per μl^{-1} at 38.83 ± 0.61 Ct (Figure 6.3.). All the natural replicates of the locations positive to *P. virginalis* DNA were found to be negative to the presence of *A. astaci*. All negative controls were found to be negative for *A.*

astaci. These results show that the pathogen is not present at the sites surveyed and at the time of sampling, despite the invasion of *P. virginalis*.

6.5 Discussion

This chapter is the first study to highlight the use of a bespoke eDNA assay for the detection of a highly invasive and parthenogenetic crayfish species (*P. virginalis*) which is spreading throughout Europe and other areas of the globe (including Madagascar) (Gutekunst et al., 2018). In addition to validating the assay *ex-situ* (under controlled laboratory settings) the *in-situ* feasibility was also tested (at eight locations in two lakes in Germany - the epicentre for the invasion of this newly identified species, and seven other locations surrounding Reilinger See (Figure 6.1.B.)). Interestingly, when sub-sampling the same lake (i.e. sampling from multiple sites within the lake), I was only able to detect an eDNA signal from three of the four sites. It was not possible to detect any DNA traces from *P. virginalis* in the seven other sampled locations (lakes, rivers and pond). Therefore, although these results illustrate the efficiency of the assay, it also identifies the need for taking multiple "environmental replicates" from any given location. In large freshwater systems (a pond or lake for example), sub-sampling across the entire banks circumference, then merging and homogenising these sub-samples would allow a more reliable analysis of the entire habitat/ecosystem. However, this is not always practical, and, in this chapter, the lakes were too large or had areas which were inaccessible for such a sample strategy to be undertaken. If only one site at any given location had been sampled, the negative eDNA read would have indicated no *P. virginalis* populations in either of the two lakes sampled (despite knowing to the contrary) i.e. this would have been a false negative. Reasons why such a result may have occurred are likely related to the behaviour of the organism in question. Many crayfish species are known to have patchy distribution (Kershner and Lodge, 1995) and even when populations are high, the eDNA detection rate may not increase in correlation (Rice et al., 2018). Further, the flow or movement of eDNA may not be even across the system. Indeed, although in this chapter, the mean Ct and the number of samples indicating positive eDNA detection varied, I was unable to correlate this with numbers/density of *P. virginalis*. Further work should therefore focus on assessing if this eDNA assay can be used for quantifying *P. virginalis* populations.

P. virginalis has also been shown to be a vector of the pathogenic agent *Aphanomyces astaci* (Lipták et al., 2016). As this pathogen results in the dramatic decline of native species including *A. pallipes*, early detection of the pathogen and the vectors would therefore be invaluable. Interestingly, although it was possible to detect *P. virginalis* in six locations, none of these showed a positive signal for *A. astaci*. This is encouraging and if populations spread from these two main locations it may be the case that *A. astaci* does not spread with them. However, it should be noted that I only sampled for the presence or absence of *A. astaci* at one time point and a more detailed seasonal study should be completed before it can be assessed without any doubt that these populations are pathogen free.

In conclusion, the newly developed eDNA assay presented in this chapter has been shown to be species-specific to *P. virginalis* and can be used *in-situ* to test for unidentified populations of *P. virginalis* across Europe. Such surveying may highlight areas where active management such as physical removal can be concentrated to minimise the spread of this potentially dangerous species. Preliminary data suggests a quantitative approach may be possible with further assessment of known populations in any given environment. Furthermore, the appearance of a false negative highlights the need of multiple 'natural replicate' samples when undertaking eDNA research – particularly for this assay but most likely for all assays developed to date. Finally, I did not detect the presence of crayfish plague in these populations. Although this is a promising finding, it is important to highlight that seasonality could play a yet unknown role in the detection of *A. astaci* and further work should be undertaken to assess if this is the case.

Chapter 7: General discussion of the thesis findings and conclusion

7.1 Introduction

Implementation of molecular-based detection methods for monitoring freshwater ecosystems have been on the rise in the last ten years. However, despite the new method being associated with high reliability and efficiency, care must always be undertaken while validating eDNA-based detection methods in order to keep a high level of confidence in the results. In this thesis, I developed and validated assays for several endangered or invasive freshwater organisms and investigated potential limitations or improvements of molecular-based monitoring. The first part of this thesis focusses on critically endangered species. Chapter 2 aimed to improve the reliability of sampling protocol and chapters 3 and 4 aimed to compare eDNA-based detection with traditional monitoring methods. Finally, chapters 5 and 6 both concentrated on monitoring invasive species through eDNA detection. General advantages, as well as limitations or potential improvement of eDNA methods are further discussed below.

7.2 Chapter 2

In the second chapter of this thesis I independently validated, by following the MIQE Guidelines, two previously published assays targeting the 16S and COI genes of a critically endangered species (Bustin et al., 2009; Stoeckle et al., 2015; Carlsson et al., 2017). This allowed me to identify and evaluate the impacts of the following variables (i) accuracy, (ii) reliability and (iii) detection probability in species-specific detection (Mauvisseau et al., 2019a). Choosing this critically endangered organism (i.e. *M. margaritifera*) previously studied through two distinct eDNA studies was a very important step. eDNA detection of this species has been characterised by a relative low sensitivity (Carlsson et al., 2017), hence investigations within this chapter represented an opportunity to investigate this topic. Only very few studies complied with such extensive validation when designing and validating eDNA barcoding assays. Therefore, a critical comparison of different published assays for species-specific eDNA detection is often impossible. One of the first aims of this chapter was to highlight these critical aspects. Then, following these extensive validations between these two published assays, I found that the choice of assay

depended on the detection probability, offering a better detection resolution. More specifically, eDNA detection using the COI target gene was found to be more efficient than 16S targeted gene in a controlled mesocosm experiment even if both COI and 16S assays shows similar accuracy and repeatability when following the MIQE Guidelines. However, it is important to highlight that it is unclear whether these findings can be generalized to other organisms. For this reason, care must be taking when utilizing already published, or designing any new, eDNA assays. Moreover, in this chapter, the controlled mesocosm experiment conducted additionally revealed that the variability between natural replicates strongly influences the number of replicates required for a reliable species detection in the natural environment. These findings allowed a very important step forward, which was establishment of an optimal eDNA sampling and analytical protocol. By allowing the identification of key variables and assessment of the optimal number of necessary natural replicates and technical replicates, this second chapter can be considered as the baseline of this thesis. Finally, it is anticipated that the published version of this chapter will help improve the reliability of future eDNA barcoding studies.

7.3 Chapter 3

In the third chapter, the efficiency of eDNA detection was investigated and compared with traditional monitoring tools such as netting and electrofishing, for assessment of the presence of endangered and invasive fish species. Moreover, I assessed in this chapter, the optimal filter pore size allowing a maximal recovery of eDNA under controlled mesocosm conditions in ZSL, using *V. robertae* as a model organism. Surprisingly, both pore sizes tested (i.e. 0.22 µm and 0.45 µm) proved to have similar recovery level of eDNA traces. However, in order to minimize the potential effect of suspended particles present, I chose to use the larger pore size (i.e. 0.45 µm) for the field sampling. In natural systems, organic matter and abundant particles often ‘clogged’ filters, leading to reduced volumes of filtered water, high impact of inhibition factors and an overall decrease of the detection probability (Goldberg et al., 2016; Harper et al., 2018a). This potential issue was not investigated in the previous chapter. Indeed, as the second chapter focused mainly on method optimization using controlled mesocosms, suspended particles in the water were kept at a minimal level (see methods, chapter 2). In this third chapter, two field campaigns were also performed in various freshwater systems of Western Greece to compare the detection capacity of eDNA with

the established traditional methods. The first field campaign focussed on the detection of both Valencia species, while the second additionally focussed on the invasive *G. holbrooki*. Both campaigns showed that eDNA detection proved to be more efficient for detecting endangered and rare fish species and had a similar efficiency for detecting the invasive species. These findings are now adding to the increasing body of literature suggesting than eDNA detection surveys are more sensitive than traditional surveys (Smart et al., 2015; Franklin et al., 2019; Sepulveda et al., 2019; Sengupta et al., 2019; Wineland et al., 2019). I was able to show that the eDNA detection required less fieldwork and had no effect on the sampled systems in comparison to the more traditional electrofishing and netting. Despite these advantages and the optimization of the eDNA sampling protocol and analysis, some of the sites were negative for eDNA despite the fish being detected through electrofishing. This false negative highlights a potential limitation of molecular-based detection as found in other studies (Pinfield et al., 2019; Mirimin et al., 2020). Such limitation could potentially be mitigated by the use of occupancy detection models (Chen and Ficetola, 2019). In this chapter, independent single-species approaches were used due to their reduce cost compared to metabarcoding (Harper et al., 2018b). Furthermore, implementing a metabarcoding approach would have been difficult due to the absence of DNA sequences from targeted or co-occurring fish species on genetic databases. Building or filling such database would have drastically increase the costs of eDNA-based survey. Finally, there was no extensive comparison between the financial costs of the two methods (traditional fishing and eDNA-based assessment). Indeed, even if eDNA sampling was incorporated into an existing survey, this resulted from a collaboration between three different institutions: ZSL, HCMR and the University of Derby. Financial costs of the project were shared between these project partners; therefore, it was not possible to investigate the true costs of assay development in this instance.

7.4 Chapter 4

Following the findings of the previous chapters, here I aimed to compare the reliability between eDNA detection and kick-sampling for monitoring a bio-indicator and endangered invertebrate species. Very few studies have investigated the use of eDNA detection for monitoring rare invertebrates bioindicators (Mächler et al., 2014; Fernandez et al., 2018; Wei et al., 2018). In this chapter, *I. nubecula* was chosen as a model organism and two different PCR amplification

strategies using qPCR and ddPCR were compared. The sampling methodology of this chapter was conducted following the previous findings of chapter two and three, by using large pore size filters (i.e. 0.45 µm) and conducting three natural replicates and six technical replicate using qPCR (Mauvisseau et al., 2019a). However, findings in this chapter showed that eDNA amplification and detection using qPCR was inefficient in that case, although ddPCR analyses resulted in a much better and clearer detection of the targeted organism. This surprising result can be explained by the water conditions during sampling, i.e. high turbidity and high flow rates, which potentially led to a decrease of the target eDNA concentration and an increase of inhibitors factors that might limit the amplification process in qPCR reactions. These combined factors can explain the difficulties of filtering large volumes of water, and the resulting inhibition occurring during the qPCR analysis. However this could also be due to a low release of DNA from the targeted organisms or insufficient volume of water sampled (Sepulveda et al., 2019). As already found in previous studies, ddPCR analysis allowed detection and quantification of low amounts of eDNA, even in the presence of inhibition factors (Doi et al., 2015a; Hamaguchi et al., 2018). In that case, the survey would have led to incorrect results using only qPCR. This extreme example is a powerful demonstration that care must be taken while conducting eDNA surveys, in order to account not only for the false positive but also for potential false negative results. In this instance, the use of relatively new technology such as ddPCR allowed me to overcome a potential limitation of molecular-based detection. Finally, an occupancy modelling approach was also utilised to assess the effects of environmental variables on the probability of detection of the targeted species. No significant effect was found, which could be explained by the relatively low number of sites sampled (n= 12) and the fact that all sites where sampled from the same river. This further highlights that sampling a unique system at various locations using eDNA methodology can lead to limited additional results when using occupancy modelling analysis.

7.5 Chapter 5

In the fifth chapter, I developed a species-specific assay for monitoring an invasive invertebrate (i.e. *D. haemobaphes*) spreading throughout Western Europe. Here, I followed the same validation guidelines as in chapter 2, 3 and 4 and assessed the efficiency of the developed assay by comparing both molecular-based detection and the species presence at several sites through kick-sampling.

However, in this chapter, I changed the eDNA sampling protocol, and instead of filtration, used the 'ethanol precipitation' method. This sampling method has been used for the early detection of an invasive crayfish species (Tréguier et al., 2014) and is currently used for commercial detection of the Great Crested Newt in UK (Biggs et al., 2015; Harper et al., 2018a). This change of sampling protocol allowed me to assess if this specific collection method can also be used for the reliable detection of an invasive species. Furthermore, samples analysed for assessing the presence of Great Crested Newt could also be used for monitoring the invasive species studied in this chapter, decreasing the cost and disposable plastic wastes associated with eDNA sampling. While the designed assay and sampling protocol proved to be efficient for detecting and monitoring the targeted species, the amount of eDNA retrieved was generally low regarding the LOQ and LOD generated in this chapter. This could have be due to a lower efficiency of the eDNA collecting method. Indeed, this method only retrieves eDNA from a limited amount of water (only 90 mL) and as only one sample per site was collected using this protocol (as per Biggs et al., 2015), both sampling method and lack of replicates could explain the low level of eDNA retrieved. Despite this, in this case at least, eDNA results between close locations were consistent. This means that if a location was positive through eDNA detection, there is a significant probability that a sample taken in the immediate neighbourhood shows a similar eDNA detection result. Regardless of these promising results, further work is needed in order to develop an optimal sampling protocol for this species. However, this chapter illustrates the fact that the sampling and eDNA extraction method utilised in the UK for the commercial detection of *T. cristatus* could be utilised for monitoring highly invasive species, and therefore, could be easily deployable for large-scale citizen science programs.

7.6 Chapter 6

In the last case study of this thesis, I focussed on the development and validation of an eDNA assay allowing the early detection of a highly invasive parthenogenetic invertebrate (i.e. *P. virginalis*) and a potential associated pathogen (Jones et al., 2009; Keller et al., 2014). The species studied in this chapter has been previously considered as a 'perfect invader', and therefore, an early detection tool is urgently needed in order to precisely map the distribution of this species (Jones et al., 2009). This fits into the potential implementation of eDNA detection for regular

monitoring and assessment of early detection and spread of invasive species. In this chapter, an efficient detection of the species at low abundance was achieved with the developed assay. However, it was shown than in a same closed system (i.e. a lake) with a known previously recorded population, not all 'sites' around the lake gave a positive eDNA detection. This led to an important recommendation for future survey aiming to detect invasive organisms with potential 'patchy' distribution in closed system. As recommended in Harper et al., (2018a), a regular sampling strategy must be followed, in order to increase the detection probability of the targeted species. Furthermore, in this chapter, the pathogen was not detected with its potential vector, contrary to other studies conducted in different countries (Keller et al., 2014). It is not clear whether the pathogen is currently not co-occurring with its potential vector at this location, or if a seasonality aspect might have led to a negative results for all sampled sites. Indeed, several other studies have highlighted the absence of the pathogen in several locations infected by *P. virginalis* (Lipták et al., 2016; Pârvulescu et al., 2017). Future research is therefore needed for investigating this aspect.

7.7 General conclusion

Following the study from Ficetola et al., 2008, eDNA detection has been increasingly used in the last few years for detecting and monitoring various aquatic organisms (Thomsen and Willerslev, 2015; Coble et al., 2019; Ruppert et al., 2019). Currently, many studies focus on method development, in order to keep a high level of confidence in the methods and assess its potential suitability for replacing or complementing traditional monitoring methods. The overall aim of this work was to focus on various methodological aspects and highlight potential limitations or benefits of molecular-based detection techniques. The novelty of this thesis relies on the establishment of a new reliable protocol allowing to decrease the error probability in species-specific molecular based detection (see chapter 2, Mauvisseau et al., 2019a). Another aspect was the first implementation of ddPCR techniques when conventional qPCR failed to amplify eDNA traces from a rare bio-indicator invertebrate species (see chapter 4, Mauvisseau et al., 2019c). Finally the development and validation of species-specific assays allowing a reliable monitoring of rare (i.e. *V. letourneuxi*, *V. robertae* and *I. nubecula*, see chapters 3 and 4) or invasive species (i.e. *G. holbrooki*, *D. haemobaphes* and *P. virginalis*, see chapters 3, 5 and 6) contribute to the novelty of this thesis.

According to these findings and the increasing body of literature concerning eDNA detection, indicate that molecular-based methods are strong candidates as alternative methods for assessing species presence, and therefore, habitat quality in freshwater systems. However, this thesis also highlights several critical limitations of eDNA-based detection. The method is prone to the occurrence of false negative and false positive results (Ficetola et al., 2016; Pinfield et al., 2019), and critical considerations are essential when choosing the targeted gene or designing the sampling and analysis protocol, especially concerning the number of natural and technical replicates. In this thesis, I investigated two different sampling methods (i.e. filtration and ethanol precipitation) associated with various collected volumes, number of replicates and amplification strategies (i.e. qPCR and ddPCR). This allowed to highlight positive and negative aspects of such methods. Despite being associated with potential bias, these different approaches were proven to be efficient for assessing the presence of aquatic species in freshwater systems. Furthermore, despite the method showing similar or better efficiency than traditional monitoring tools, several false negatives were obtained in chapter 3 and 4, highlighting again a limitation of the method. These limitations must be carefully taken into consideration before any deployment of eDNA detection as a monitoring tool for effective conservation plans. Furthermore, care must be taken in an event of negative results using qPCR amplification when monitoring rare species. Future research for species-specific eDNA detection should focus on the implementation of ddPCR, due to higher efficiency, lower LOD, analytical costs and inhibition resistance. In addition to these findings, it is interesting to show that the commercial sampling kits utilised for the eDNA detection of the Great Crested Newt can be utilised for retrieving eDNA traces from other species such as invasive. However, proper investigations should be undertaken to assess if this sampling design is optimal for eDNA surveys. Finally the design and implementation of eDNA detection throughout the various chapters led to the development of a new assay, allowing a reliable detection of a ‘perfect invader’.

This thesis is particularly important for a various range of end users, such as research scientists aiming to develop future studies, ecologists monitoring aquatic species, policy makers or businesses specialised in species assessments or eDNA analyses. Indeed, the methodological advancements underlined earlier will be beneficial when developing new research studies focussing on species-specific detection and quantification. Particularly, the implementation of a

sampling protocol including a sufficient number of natural and technical replicates, or the use of ddPCR technology will increase the reliability of such studies. If possible, new studies should always investigate the optimal number of natural and technical replicates needed. However, this is not always possible due to limited funding or technical issues. Moreover, this thesis further highlights the complementary between eDNA-based assessments and traditional monitoring approaches. Indeed, eDNA-based assessments were proven to be a valuable complementary tool when monitoring elusive aquatic organisms present in low density using traditional survey methods. Their use could therefore be generalised by ecologists to support results of such surveys, or to generate a broad understanding of a species presence before conducting traditional monitoring. This could further facilitate biological surveys in remote or dangerous locations. Additionally, this thesis is important for policy makers, as this work contributes to the growing scientific literature highlighting eDNA-based monitoring as reliable alternative method readily implementable to facilitate biodiversity assessments in freshwater systems. As specified by the European Habitats Directive (European Commission, 1992) and Water Framework Directive (European Commission, 2015), biodiversity monitoring is a cornerstone for the evaluation of ecosystem health and status. Therefore, molecular-based assessments could be added in the official toolkit utilised for monitoring aquatic species in Europe. Finally, an increasing number of businesses now propose services allowing to monitor aquatic species used DNA-based detection, such as Great Crested Newt (Biggs et al., 2015). As a large range of species-specific assays were developed, validated, and tested within this thesis, this provide a unique opportunity for such companies to advertise new services for commercial eDNA-based monitoring of these species. However, it should be noted that no temporal studies were performed within this thesis. As a result, it is unknown if the targeted species studied within this thesis would be detectable across seasons. As eDNA persistence or shedding rates are expected to vary following seasons, further work is necessary to assess these effects on eDNA-based monitoring.

To conclude, the most important and principal findings and therefore the take-home message of this thesis is that care should be taken when designing new monitoring tools, and that strict validation steps should be adhered to, particularly with respect to minimising the probability of false positives and negatives.

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APPENDICES

Appendix 1. List of articles using eDNA barcoding techniques referring or not to the MIQE Guidelines

Abbreviated references	Reference to the MIQE Guidelines	Full article references
Williams et al. 2018	No	Williams, K. E., Huyvaert, K. P., Vercauteren, K. C., Davis, A. J., & Piaggio, A. J. (2018). Detection and persistence of environmental DNA from an invasive, terrestrial mammal. Ecology and Evolution, 8(1), 688–695. doi:10.1002/ece3.3698
Takahashi et al. 2018	No	Takahashi, M. K., Meyer, M. J., Mcphee, C., Gaston, J. R., Venesky, M. D., & Case, B. F. (2018). Seasonal and diel signature of eastern hellbender environmental DNA: Temporal Signature of Hellbender eDNA. The Journal of Wildlife Management, 82(1), 217–225. doi:10.1002/jwmg.21349
Seymour et al. 2018	No	Seymour, M., Durance, I., Cosby, B. J., Ransom-Jones, E., Deiner, K., Ormerod, S. J., … Creer, S. (2018). Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. Communications Biology, 1(1). doi:10.1038/s42003-017-0005-3
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Xia et al. 2017	No	Xia, Z., Zhan, A., Gao, Y., Zhang, L., Haffner, G. D., & MacIsaac, H. J. (2017). Early detection of a highly invasive bivalve based on environmental DNA (eDNA). Biological Invasions. doi:10.1007/s10530-017-1545-7
Wittwer et al. 2017	No	Wittwer, C., Stoll, S., Strand, D., Vrålstad, T., Nowak, C., & Thines, M. (2017). eDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia. doi:10.1007/s10750-017-3408-8

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Takahashi et al. 2017	No	Takahashi, M. K., Meyer, M. J., Mcphee, C., Gaston, J. R., Venesky, M. D., & Case, B. F. (2017). Seasonal and diel signature of eastern hellbender environmental DNA: Temporal Signature of Hellbender eDNA. The Journal of Wildlife Management, 82(1), 217–225. doi:10.1002/jwmg.21349
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Hutchins et al. 2017	No	Hutchins, P. R., Sepulveda, A. J., Martin, R. M., & Hopper, L. R. (2017). A probe-based quantitative PCR assay for detecting *Tetracapsuloides bryosalmonae* in fish tissue and environmental

		DNA water samples. Conservation Genetics Resources. doi:10.1007/s12686-017-0812-3
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Gorički et al. 2017	No	Gorički, Š., Stanković, D., Snoj, A., Kuntner, M., Jeffery, W. R., Trontelj, P., … Aljančič, G. (2017). Environmental DNA in subterranean biology: range extension and taxonomic implications for *Proteus*. Scientific Reports, 7, 45054. doi:10.1038/srep45054
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Carlsson et al. 2017	No	Carlsson, J. E. L., Egan, D., Collins, P. C., Farrell, E. D., Igoe, F., & Carlsson, J. (2017). A qPCR MGB probe based eDNA assay for European freshwater pearl mussel (*Margaritifera margaritifera* L.). Aquatic Conservation: Marine and Freshwater Ecosystems. doi:10.1002/aqc.2788
Carim et al. 2017	No	Carim, K. J., Dysthe, J. C., Young, M. K., McKelvey, K. S., & Schwartz, M. K. (2017). A Noninvasive Tool to Assess the Distribution of Pacific Lamprey (*Entosphenus tridentatus*) in the Columbia River Basin. PLOS ONE, 12(1), e0169334. doi:10.1371/journal.pone.0169334

Cai et al. 2017	No	Cai, W., Ma, Z., Yang, C., Wang, L., Wang, W., Zhao, G., … Yu, D. W. (2017). Using eDNA to detect the distribution and density of invasive crayfish in the Honghe-Hani rice terrace World Heritage site. PLOS ONE, 12(5), e0177724. doi:10.1371/journal.pone.0177724
Buxton et al. 2017	No	Buxton, A. S., Groombridge, J. J., Zakaria, N. B., & Griffiths, R. A. (2017). Seasonal variation in environmental DNA in relation to population size and environmental factors. Scientific Reports, 7, 46294. doi:10.1038/srep46294
Buxton et al. 2017	No	Buxton, A. S., Groombridge, J. J., & Griffiths, R. A. (2017). Is the detection of aquatic environmental DNA influenced by substrate type? *PloS One*, *12*(8), e0183371.
Brozio et al. 2017	No	Brozio, S., Manson, C., Gourevitch, E., Burns, T. J., Greener, M. S., Downie, J. R., & Hoskisson, P. A. (2017). Development and Application of an eDNA Method to Detect the Critically Endangered Trinidad Golden Tree Frog (*Phytotriades auratus*) in Bromeliad Phytotelmata. PLOS ONE, 12(2), e0170619. doi:10.1371/journal.pone.0170619
Bastos Gomes et al. 2017	No	Bastos Gomes, G., Hutson, K. S., Domingos, J. A., Chung, C., Hayward, S., Miller, T. L., & Jerry, D. R. (2017). Use of environmental DNA (eDNA) and water quality data to predict protozoan parasites outbreaks in fish farms. Aquaculture, 479, 467–473. doi:10.1016/j.aquaculture.2017.06.021
Baldigo et al. 2017	No	Baldigo, B. P., Sporn, L. A., George, S. D., & Ball, J. A. (2017). Efficacy of Environmental DNA to Detect and Quantify Brook Trout Populations in Headwater Streams of the Adirondack Mountains, New York. Transactions of the American Fisheries Society, 146(1), 99–111. doi:10.1080/00028487.2016.1243578

Balasingham et al. 2017	Yes	Balasingham, K. D., Walter, R. P., & Heath, D. D. (2017). Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem. Molecular Ecology Resources, 17(3), 523–532. doi:10.1111/1755-0998.12598
Ardura et al. 2017	No	Ardura, A., Zaiko, A., Borrell, Y. J., Samuiloviene, A., & Garcia-Vazquez, E. (2017). Novel tools for early detection of a global aquatic invasive, the zebra mussel *Dreissena polymorpha*: Detection of *Dreissena polymorpha* from eDNA. Aquatic Conservation: Marine and Freshwater Ecosystems, 27(1), 165–176. doi:10.1002/aqc.2655
Andruszkiewicz et al. 2017	No	Andruszkiewicz, E. A., Sassoubre, L. M., & Boehm, A. B. (2017). Persistence of marine fish environmental DNA and the influence of sunlight. PLOS ONE, 12(9), e0185043. doi:10.1371/journal.pone.0185043
Agersnap et al. 2017	No	Agersnap, S., Larsen, W. B., Knudsen, S. W., Strand, D., Thomsen, P. F., Hesselsøe, M., … Møller, P. R. (2017). Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. PLOS ONE, 12(6), e0179261. doi:10.1371/journal.pone.0179261

Appendix 2. MIQE Guidelines

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Appendix 3.A List of fish species and related GenBank accession numbers utilized when developing and validating the three sets of species-specific primers and probes.

Species	Accession number
Anguilla anguilla (Linnaeus, 1758)	KX870809.1, KX870787.1
Aphanius fasciatus (Valenciennes, 1821)	KJ552453.1, KJ552597.1
Barbus peloponnesius (Valenciennes, 1842)	KJ552764.1, KJ552848.1
Carassius gibelio (Bloch, 1782)	KJ553172.1, JQ979145.1
Cobitis arachthosensis (Economidis & Nalbant, 1996)	KJ553181.1, KJ553088.1
Cobitis hellenica (Economidis & Nalbant, 1996)	KJ552940.1, KJ553094.1
Cobitis trichonica (Stephanidis, 1974)	KJ553170.1
Cyprinus carpio (Linnaeus, 1758)	KC500446.1, KR861880.1
Economidichthys pygmaeus (Holly, 1929)	KX673894.1, KX673900.1
Gambusia holbrooki (Girard, 1859)	JQ979158.1, HQ600731.1, JN026707.1
Gasterosteus gymnurus (Cuvier, 1829)	KR862808.1, KR862823.1, KR862816.1
Knipowitschia milleri (Ahnelt & Bianco, 1990)	KJ553398.1, KJ553527.1
Luciobarbus albanicus (Steindachner, 1870)	KJ553876.1, KJ553979.1
Pelasgus stymphalicus (Valenciennes, 1844)	KJ554374.1, HM560279.1
Pelasgus thesproticus (Stephanidis, 1939)	KJ554467.1, KJ554096.1
Salaria fluviatilis (Asso, 1801)	KJ554695.1, KJ554615.1
Squalius cephalus (Linnaeus, 1758)	KU302617.1, KR477123.1
Squalius peloponensis (Valenciennes, 1844)	KJ554940.1, KJ554769.1
Telestes pleurobipunctatus (Stephanidis, 1939)	KJ554599.1, KJ554784.1
Tropidophoxinellus hellenicus (Stephanidis, 1971)	KJ554628.1, KJ554709.1
Valencia hispanica (Valenciennes, 1846)	KF767510.1, KF767517.1, KF767528.1, KF767523.1, KF767525.1
Valencia letourneuxi (Sauvage, 1880)	KF767527.1, KF767511.1, KF767522.1, KF767518.1, KF767526.1, KF767520.1, KF767515.1
Valencia robertae (Freyhof et al. 2014)	KF767524.1, KF767516.1, KF767519.1, KF767509.1, KF767514.1, KF767521.1, KF767513.1, KF767512.1

Appendix 3.B. Additional information on primers and probes design

As specified in chapter 3, the species-specific primers and probes were designed in this case study using the Geneious Pro R10 Software (https://www.geneious.com; Kearse et al., 2012). More specifically, for each targeted species (i.e. *V. letourneuxi*, *V. robertae* and *G. holbrooki*), the COI sequences for each species (see sequences in Appendix 3.A) were aligned to create a consensus sequence for each targeted species. Then, the consensus sequences were utilised for designing the assays (primers and probe) for each targeted species. All assays were designed using the 'primers' design function from Geneious, and their specificity was assessed *in-silico* using the primer-blast tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The results were further confirmed by visual alignment of the assays against COI sequences from closely related or co-occurring fish species listed in appendix 3.A. The visual alignment was performed using the 'multiple alignment' function on Geneious. After *in-silico* validation, the specificity of each assay was tested *in-vitro* with PCR and qPCR using DNA extracted from the co-occurring species mentioned in the main manuscript. DNA from these fish species was collected during the two fieldtrips conducted during this study. Fin clips were sampled on at least one specimen of each fish mentioned in the assay development section of the main manuscript. DNA was extracted using the Qiagen DNeasy® Blood and Tissue Kit, following the manufacturer's instructions, then a PCR targeting the COI gene was conducted following (Ivanova, Zemlak, Hanner, & Hebert, 2007). PCR products were visualised on a 2% agarose gel stained with 3 µL of GelRed™ Nucleic Acid Gel Stain, Biotium and sent for sequencing to the Eurofins Genomics company in UK. Sequences obtained were blasted in GenBank for confirming the visual identification from the field and further aligned with the sequences reported in Appendix 3.A, in order to confirm the source material from GenBank. Primers and probe were designed using the sequences reported in Appendix 3.A, as these sequences were identical to the one obtained from DNA collected in the field. Finally, PCR were conducted with the developed assays targeting *V. letourneuxi*, *V. robertae* and *G. holbrooki* using DNA extracted from these targeted fish. Then, PCR products were visualised and sequenced as previously described in order to confirm the successful amplification of the targeted COI fragment of each species.

References:

Ivanova, N. V., Zemlak, T. S., Hanner, R. H., & Hebert, P. D. N. (2007). Universal primer cocktails for fish DNA barcoding: BARCODING. Molecular Ecology Notes, 7(4), 544–548. doi: 10.1111/j.1471-8286.2007.01748.x

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647–1649. doi: 10.1093/bioinformatics/bts199

Appendix 4. Variables collected during the mesocosm experiment in ZSL.

Tank	Fish	Adult	Juvenile	Total biomass (g)	Volume (L)	pH	TC
A	40	40	-	10	500	8.22	23.6
B	22	10	12	19.8	626	7.96	23.2
C	66	-	66	101.5	723	8.03	22.2

Table combining the temperature, pH, number of adult, juvenile and total number of *V. robertae* in each mesocosm. The total biomass was estimated after weighting 10 individuals of each stage of life in each aquarium. For ethical reason, only ten fish per mesocosm were weighted to avoid disturbance in the populations. All measurements were performed after eDNA sampling, to avoid any potential increase of eDNA release due to stressful conditions.

Appendix 5. *In-situ* validation trial 1 (A) for *V. letourneuxi* and *V. robertae* .

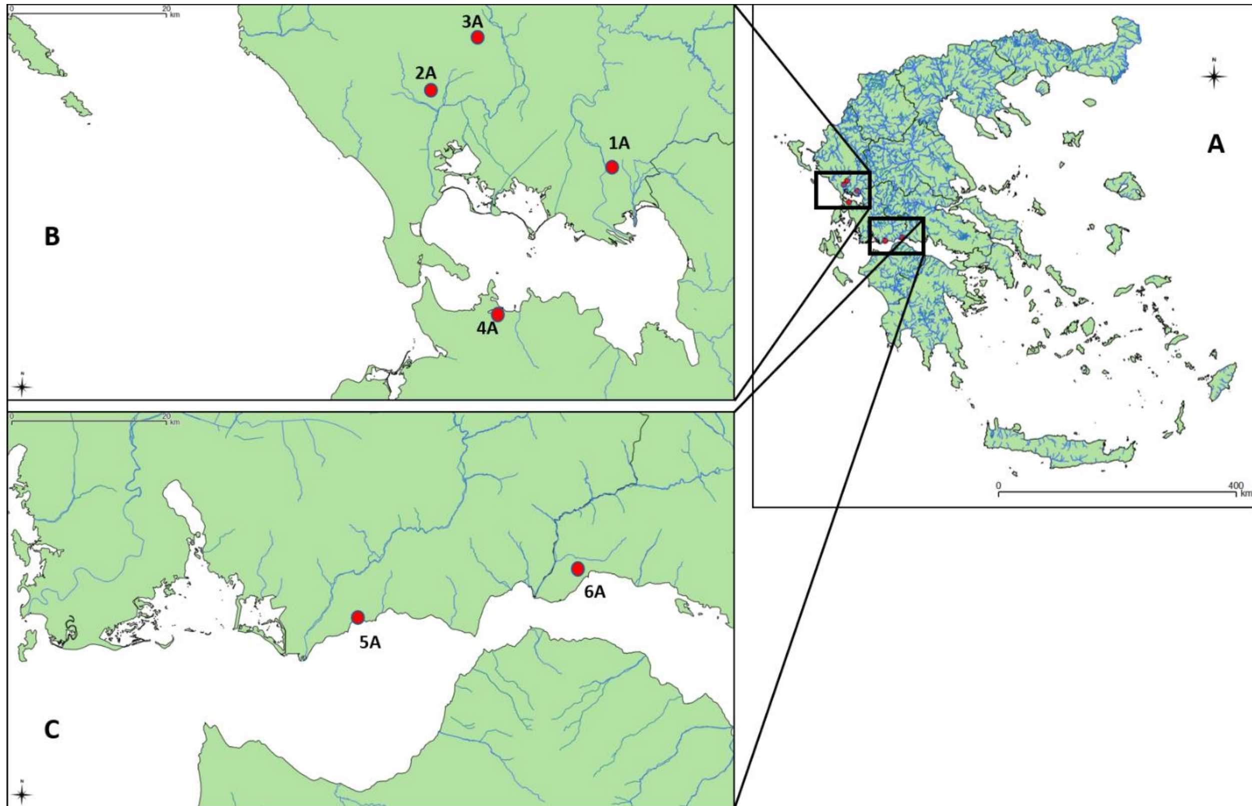

Figure S1. Map showing the freshwater locations sampled in Western Greece during the first *in-situ* survey conducted from 26th to 27th September 2017. Eight samples from six sites at six aquatic systems (stream, wetland, canal) were sampled over two days (see also Table **3.2.**). A single water sample was collected at sites 1A, 2A, 3A and 4A. At two sites (5A and 6A), two water samples were collected, 20 meters apart from each other. Sub panel A represents the map of Greece, sub panel B represents the sites in the geographical area of *V. letourneuxi,* and sub panel C represents the sites in the geographical area of *V. robertae*.

Site	Region	Habitat type	Fresh/Brackish	***V. letourneuxi* historical presence**	***V. robertae historical presence***	**Sampling method**	***V. letourneuxi* abundance (%)**	***V. robertae* abundance (%)**
1A	Ipeiros	Spring-fed stream	Freshwater	YES		D-net, small nets	0,00	
2A	Ipeiros	Spring-fed stream	Freshwater	YES*		NS		
3A	Ipeiros	Spring-fed canal	Freshwater	YES		D-net, small nets	0,00	
4A	Aitoloakarnania	Spring-fed stream	Brackish	YES		D-net, small nets	1.52	
5A	Aitoloakarnania	Spring-fed wetland	Freshwater		YES**	NS		
6A	Aitoloakarnania	Spring-fed stream	Freshwater		YES	Seine net, small nets		87.92

Table S1. Fishing data of sites sampled for eDNA in 2017. Information is provided on historical presence *of V. letourneuxi, V. robertae* (data from the 1980s, the earliest available for the full set, unless otherwise stated), fish sampling method, confirmed presence/absence through fishing with relative abundance data (percentage contribution) of the target species. NS: not sampled. * Translocation conducted. ** Enhancement conducted.

Appendix 6. *In-situ* validation trial 2 (B) for *V. letourneuxi*, *V. robertae* and *G. holbrooki*

Site	Region	Type	Fresh/Brackish	Visible depth turbidity	Salinity	Temperature	Sampling method	Sampling Strategy
1B	Corfu Isl.	Spring fed stream	Freshwater	Slight turbid >1m	0.37	16.8	Electrofishing	whole
2B	Corfu Isl.	Spring fed stream	Freshwater	Clear	0.9	16.7	Electrofishing	whole
3B	Corfu Isl.	Spring fed stream	Freshwater	Slight turbid >1m	0.78	19.5	Electrofishing	whole
4B	Corfu Isl.	Canal in lagoon	Brackish	-	1.17	19.5	-	
5B	Ipeiros	Spring fed canal	Freshwater	Clear	0.51	16.7	Electrofishing	one bank
6B	Ipeiros	Spring fed canal	Freshwater	Clear	0.52	16.7	Electrofishing	one bank
7B	Ipeiros	Spring fed canal	Brackish	Clear	5.28	18.5	D net	
8B	Ipeiros	Spring fed canal	Brackish	Clear	2.56	18.9	D net	
9B	Ipeiros	Spring fed canal	Freshwater	Clear	0.22	16.5	Electrofishing	one bank
10B	Ipeiros	Spring fed canal	Freshwater	Clear	0.28	16.7	Electrofishing	one bank
11B	Aitoloakarnania	Spring fed canal	Brackish	Clear	2.65	16.87	D net	
12B	Ipeiros	Spring fed canal	Freshwater	Clear	0.5	16.8	Electrofishing	whole
13B	Aitoloakarnania	Spring fed canal	Brackish	Slight turbid >1m	8.99	18.2	D net	
14B	Aitoloakarnania	River (tributary)	Freshwater	Clear	0.51	16.7	Electrofishing	one bank
15B	Aitoloakarnania	Canal	Freshwater	Slight turbid >1m	0.53	17.2	Electrofishing	whole
16B	Aitoloakarnania	Wetland	Freshwater	Clear	0.94	17	Electrofishing	whole
17B	Peloponnese	Spring-fed stream	Freshwater	Clear	0.65	17.6	Electrofishing	one bank
18B	Aitoloakarnania	Spring fed canal	Freshwater	Clear	0.74	17.2	Electrofishing	one bank
19B	Aitoloakarnania	Spring-fed stream	Freshwater	Clear	0.27	18	Electrofishing	one bank
20B	Aitoloakarnania	Canal	Freshwater	Slight turbid >1m	0.29	19.1	Electrofishing	whole

Table S2. Table depicting additional information on the locations sampled during the second field trial in 2018. Additional information includes the region of Greece where the site is located, the type of system sampled, visible depth and turbidity, salinity, water temperature, fishing method used and sampling strategy for electrofishing.

	V. letourneuxi **historical presence**	***V. robertae historical presence***	***G. holbrooki*** **historical presence**	***V. letourneuxi*** **abundance (%)**	***V. robertae*** **abundance (%)**	***G. holbrooki*** **abundance (%)**
1B	yes*		no	25.49		0.00
2B	yes		unknown	0.00		0.00
3B	yes		yes	0.00		96.98
4B	yes		yes	-		-
5B	yes		yes	0.00		58.06
6B	yes		yes	0.00		45.57
7B	yes		yes	0.00		89.70
8B	yes		yes	0.00		37.00
9B	yes		yes	0.00		148
10B	yes		yes	1.67		52.84
11B	yes		yes	13.79		13.79
12B	yes			0.00		0.00
13B		yes	yes		2.61	36.10
14B		yes	yes		1.76	17.60
15B		yes	yes		0.00	1.69
16B		yes**	no		0.00	0.00
17B		no	yes		0.00	45.45
18B		yes	no		0.00.	0.00
19B		yes	no		3.65	0.00
20B		yes	no		0.89	0.00

Table S3. Fishing data of sites sampled for eDNA in 2018. Information is provided on historical presence of *V. letourneuxi*, *V. robertae* and *G. holbrooki*, (data originates from the 1980s, the earliest available for the full set, unless otherwise stated) and confirmed presence/absence through fishing in 2018, with relative abundance data (percentage contribution) of the target species. * First record 2009; ** First record 2008.

Appendix 7. List of invertebrate species and the related GenBank accession number utilized when developing and validating the species-specific primers and probe used in this chapter.

Species	**Accession number**
Isogenus nubecula (Newman, 1833)	MF801622.1
Amphinemura standfussi (Ris, 1902)	JX460920
Amphinemura sulcicollis (Stephens, 1836)	JX495637
Brachyptera risi (Morton, 1896)	KF492801
Capnia atra (Morton, 1896)	KF809153
Zwicknia bifrons (Newman, 1838)	KF144842
Capnia vidua (Klapálek, 1904)	JQ736348
Chloroperla tripunctata (Scopoli, 1763)	HQ705654
Cotesia acuminate (Reinhard, 1880)	AY333870
Dinocras cephalotes (Curtis, 1827)	KF492802
Diura bicaudata (Linnaeus, 1758)	KJ675053.1
Heptagenia longicauda (Stephens, 1836)	LN734744
Isoperla grammatica (Poda, 1761)	KU955895
Isoperla obscura (Zetterstedt, 1840)	KJ675043
Kageronia fuscogrisea (Retzius, 1783)	JN299122
Leuctra fusca (Linnaeus, 1758)	KT807840.1
Leuctra hippopus (Kempny, 1899)	KF809176.1
Nemoura avicularis (Morton, 1894)	JX905857
Nemoura cinerea (Retzius, 1783)	JX495661
Nemurella pictetii (Klapálek, 1900)	KF492804
Protonemura meyeri (Pictet, 1841)	KF492803
Sterrhopterix standfussi (Wocke, 1851)	HM873931
Nemoura lacustris (Pictet, 1865)	MF801623.1

Appendix 8. ddPCR detection of *Isogenus nubecula*

site	sample	pcr1	pcr2
W1	1	0	0
W2	1	0	0
W3	1	0	0
W4	1	0	1
W5	1	1	0
W6	1	0	0
W7	1	0	0
W8	1	0	0
W9	1	0	0
W10	1	0	0
W11	1	0	0
W12	1	0	0
W1	2	0	0
W2	2	1	0
W3	2	0	0
W4	2	0	0
W5	2	1	0
W6	2	0	0
W7	2	0	1
W8	2	0	0
W9	2	0	0
W10	2	0	0
W11	2	0	0
W12	2	0	0
W1	3	0	0
W2	3	1	0

W3	3	0	0
W4	3	1	1
W5	3	1	1
W6	3	0	0
W7	3	0	0
W8	3	0	0
W9	3	0	0
W10	3	0	0
W11	3	0	0
W12	3	0	0

Appendix 9. Variables collected from the field survey targeting *Isogenus nubecula*. 'kick' means kick-sampling and 'abs' means absence.

site	volume	pH	O2	time	kick
W1	350	7.48	12.5	60	possible
W2	200	7.53	11.9	60	possible
W3	700	6.69	11.9	60	possible
W4	1000	6.52	11.8	120	possible
W5	750	7.83	11.4	45	abs
W6	300	7.82	12.5	60	abs
W7	750	7.67	11.6	90	possible
W8	750	7.8	10.7	90	possible
W9	750	6.75	11.8	90	possible
W10	300	7.74	13	60	possible
W11	500	6.63	11.6	45	possible
W12	750	7.69	10.9	90	possible

Appendix 10. Additional eDNA and kick-sampling validation

Six locations were surveyed using both traditional method (i.e. kick-sampling) and the novel eDNA assay. Presence of the targeted species was assessed after visual identification and DNA sequencing of sampled *D. haemobaphes* specimens following the method outlined in (Folmer et al., 1994). eDNA detection results were obtained following the sampling and qPCR methods detailed in the main manuscript. The results of both the kick sampling and eDNA assay are displayed in the following Table. A "blank sample", whereby tap water was extracted and analysed in the same manner as all environmental samples and at the same time as the other samples was undertaken to ensure the absence of any contamination during the extraction process. All qPCR technical replicates of this "blank sample" and all negative qPCR controls (no extracted template added – run on all qPCR plates for the entire sample set) showed no amplification of *D. haemobaphes* as expected.

Table depicting kick-sampling results, eDNA assay results, the number of positive qPCR replicates (Slope= -3.351, Y inter= 24.45, R^2= 0.961, Eff%= 98.805), the mean Ct, the kick-sampling and eDNA collection date, the type of site and the GPS coordinates of each sampled locations.

Locations	Kick Sampling detection	eDNA detection	Number of positive replicates	Mean Ct	Collection date	Type of site	Latitude	Longitude
1	Yes	Yes	4/6	38,425930	26/09/2018	Canal	52,7526	-2,0978
2	Yes	Yes	6/6	36,829502	26/09/2018	Canal	52,7380	-2,0942
3	No	Yes	5/6	37,628826	26/09/2018	River	54,3523	-2,9388
4	Yes	Inconclusive	1/6	39,394210	26/09/2018	Canal	52,4639	-2,2010
5	Yes	Yes	6/6	35,360614	26/09/2018	Pond	52,4746	-2,1276
6	Yes	Yes	6/6	38,529853	26/09/2018	Canal	52,4906	-2,0668

Appendix 11. Table showing mismatches between the species-specific primers and the respective COI targeting sequences in *D. haemobaphes* and various other species closely related or likely to co-occurring species.

Base pair matches are highlighted in yellow for G, red for A, blue for C and green for T. Differences in bases are highlighted in white.

Appendix 12. Variables collected from the positive locations targeting *P. virginalis.*

Sites	Volume (mL)	Known presence /absence	eDNA detection	Natural replicates	Technical replicates	Mean Ct	Mean SD	Collection date	Site	Latitude	Longitude
1	1000	Yes	No	0/2	0/12	-	-	10/05/2018	Lake	49.293929	8.546733
2	1000	Yes	Yes	2/2	9/12	34.67	0.86	10/05/2018	Lake	49.294786	8.544093
3	1000	Yes	Yes	2/2	11/12	34.86	1.09	10/05/2018	Lake	49.295521	8.545458
4	600	Yes	Yes	2/2	12/12	34.18	0.95	20/06/2018	Lake	49.299823	8.545879
5	1000	Yes	Yes	2/2	6/12	34.47	1.32	15/06/2018	Lake	51.056863	9.312571
6	1000	Yes	No	0/12	0/12	-	-	15/06/2018	Lake	51.057900	9.313470
7	1000	Yes	Yes	1/2	6/12	29.17	0.14	15/06/2018	Lake	51.059368	9.313730
8	1000	Yes	Yes	2/2	12/12	29.86	0.12	15/06/2018	Lake	51.060908	9.311718

Table showing the volume of water sample for each natural replicate, the known presence/absence of each sampled sites, the eDNA detection results, the number of natural replicates positive to eDNA detection of *P. virginalis*, the number of technical replicates positive to eDNA detection of *P. virginalis*, the mean Ct of the technical replicates, mean SD of the technical replicates, the collection date, type of site and GPS coordinates or each sampled location found to be positive for the presence of *P. virginalis*.