UNIVERSITY OF DERBY

Transforming a Research Concept into Commercial Practice: Addressing the 'Hurdles' of Single-Species eDNA-based Detection

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Abbreviations

AIC	Akaike information criterion
ANOVA	Analysis of variance
ART	Artificial refuge trap
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
CEN	European Committee for Standardization
COI	Cytochrome C Oxidase Subunit 1
COST	Co-Operation in Science & Technology Program
CPUE	Catch per unit effort
Ct	Cycle threshold
ddPCR	Droplet digital PCR
DEFRA	Department for Environment Food & Rural Affairs
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
FAQ	Frequently asked questions
HSI	Habitat suitability index
ISO	International Organization for Standardization
IUCN	International Union for Conservation of Nature
LOD	Limit of detection
LOQ	Limit of quantification
MIQE	The Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NTC	No template control
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
SOP	Standard operating procedure
UV	Ultra violet

Preface

The research and writing contained within this thesis has been solely authored by the doctoral candidate, with guidance and thesis direction advice only given by those stated within the supervisory package. Guidance for the statistical analysis and R coding was provided by Dr Alfred Burian and Dr Mark Bulling. Prior to commencement of the project, all research was considered and approved by the University of Derby College of Life and Natural Sciences ethical committee.

All activities relating to surveying and handling white-clawed crayfish is required to be conducted under license from Natural England. All ecological survey work within this thesis was therefore conducted by licensed surveyors from external project partners. In Chapter 3, traditional crayfish surveys in France were carried out by licensed ecologists from Fédération de Pêche et de Protection du Milieu Aquatique du Loir-et-Cher. In chapter 4, mesocosm experimental set up and ark site experiments were conducted through Jen Nightingale at Bristol Zoological Society as part of their captive breeding and release programme for white-clawed crayfish in the UK. In the commercial applications described within Chapter 4 traditional ecological surveys were conducted by external parties. Before commencement of each project, detailed plans were submitted to, considered and approved by the University of Derby's research ethics board.

Abstract

The use of environmental DNA (eDNA) for measuring and monitoring biodiversity has been identified as a novel molecular based method to complement more commonly utilised traditional ecological sampling techniques. It is a time and cost-efficient technique, which is rapidly advancing due to the capabilities of low eDNA detection levels. As the efficiency of the technique has increased, commercial organisations and end-users have gained a greater interest in its application. Despite this, the technique is currently only commercially available from a select few service providers. In the UK, the main target species for commercial scale eDNA-based detection is the great crested newt (*Triturus cristatus*). Interest has now been sparked for the development of eDNA assays to detect various other species, both for use as a regulated informative tool and a conservation aid. However, many recent studies have highlighted various limitations associated with the use of eDNA-based detection and this appears to be hampering commercialisation of this tool. eDNA-based detection methods remain relatively underdeveloped and un-validated for use as reliable and accurate widespread monitoring programs and other such applications. Here, the so called 'hurdles' associated with the development and validation of eDNA-based methods and its use as a fully available commercial service are reviewed and addressed, in order to develop and validate a commercially applicable eDNA assay for the endangered white-clawed crayfish, *Austropotamobius pallipes,* as a target organism. When designing novel species-specific assays, detailed validation steps need to be undertaken, ensuring they perform under various conditions, habitats, and which sampling methods should be utilised. Currently, more traditional methods used to asses populations of white-clawed crayfish (such as trapping and hand searching) are becoming increasingly more difficult to undertake as the species become rarer and populations more fragmented. Such techniques are therefore expensive (with regard to time spent surveying) and often result in low probability of detection. A new species-specific qPCR assay to detect white-clawed crayfish was developed and tested under various conditions both *ex-situ* (laboratory and mesocosms) and *in-situ* (ponds and rivers) to explore the optimum sampling strategy giving the most reliable results. Experiments were also conducted on a wider scale to determine the impact of DNA degradation and seasonal influence on eDNA persistence. Interestingly, this thesis illustrates that sample collection choice is not simple, and the 'best' methodology was shown to vary between habitat type.

This indicates that great care should be taken when designing any such assays and implementing them in the field. Furthermore, this study highlights that a 'standard operating procedure' for eDNA-based detection in the commercial sector may not be possible and this will have to be explored on an assay by assay basis. Alongside case studies from real-world application of the technique, recommendations are made on how this novel eDNA assay can be used for the commercial practice of white-clawed crayfish assessment.

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I am extremely thankful for the patience of Dr Alfred Burian and your superb support with the statistical analysis and interpretation, for which I definitely would not have been able to finish this thesis without. My thanks also to Dr Louise Robinson for your invaluable eye for detail and comments which have driven significant improvements to the structure and content of this thesis, and Dr Mark Bulling for guidance on my somewhat complicated and muddled datasets. I would like to specially thank Quentin Mauvisseau for his enthusiasm and for always being up for a chat about eDNA, providing insight, advice and opportunity to discuss new ideas.

I am thankful to the College of Life and Natural Sciences at the University of Derby and SureScreen Scientifics for my studentship and opportunities to explore research and present at various national and international conferences. I give special thanks to Troy Whyte and Jim Campbell from SureScreen for their continued financial support and employment opportunities towards the end of my PhD.

For providing my enthusiasm to study eDNA for my undergraduate dissertation in the first place and subsequently giving me the confidence to consider applying for a PhD I would like to thank Dr Mike Wheeler (University of Worcester). I would also like to thank Dr Graham Hill (University of Worcester) for his consistent assistance and interest with my research, right from the beginning in 2014.

Outside of my studies several people need to be thanked, most importantly these go to Stephanie Logue and Janie Strafford for their unrivalled life advice. Elinor Nixon-Davingoff for proof reading at such short notice (and newfound enthusiasm in eDNA). Ann Kirkman, for the emergency tea breaks and mutual breakdown support. And for being excellent PhD colleagues and friends, my thanks also go to Ivana Babicova, Abi Page, Bilal Akbar and Charitos Zapitis.

I also wish to thank Keziah Drew for the excellent crayfish illustrations which have helped present a number of figures within this thesis.

Throughout the last four years there have been a number of people and organisations who have helped with sample collection and/or funding. Most notably I wish to thank Jen Nightingale and team (Holly Thompson, Jacob Ball and Alex King) (Bristol Zoological Society), Angus Menzies (Dorset Wildlife Trust), the Environment Agency, and Christophe Mauvisseau (Fédération de Pêche et de Protection du Milieu Aquatique du Loir-et-Cher).

Finally, my parents Sara and Robert, sister Rachael and grandparents Barbara, Cecil and Evelyn. You have always believed in me and provided unconditional love and support 24/7, throughout the last four years, through every up and down.

I wish to dedicate this thesis in loving memory of 'Grampy', Cecil Jeacock.

Chapter 1: The 'Hurdles' associated with single-species eDNA-based detection methods

1.1. Introduction

Monitoring the presence and absence of a wide variety of different species and their relative abundances is a large aspect of the workload of an ecological consultant. To date, this has typically been undertaken using labour intensive methods such as trapping, hand searching, and/or torching. Over the next few years, this workload is predicted to expand, driven by an increase in infrastructure and building projects, and an ever-growing list of endangered species (Butchart et al. 2017). Indeed over 27% of known species are now listed as threatened with extinction according to the IUCN Red List (IUCN 2019). Current efforts of population monitoring, specifically those directed towards threatened and at-risk species have had varying levels of success with regard to reliably detecting individuals (Elphick 2008). Furthermore, these methods are often expensive, and can sometimes be invasive and destructive (Jones 1992; Petitot et al. 2014). Until recently, non-invasive detection methods have been rarely utilised. However, advancements in molecular techniques including DNA extraction, species-specific primers design, quantitative PCR (qPCR) and next generation sequencing have led the way for the development of a novel non-invasive molecular based method which is being utilised for an ever-increasing list of organisms.

In this chapter, the development of non-invasive molecular species detection will be reviewed, through the detection of environmental DNA (eDNA), along with addressing possible future developments which could be undertaken in order to bring this method from an established scientific concept to a sound and reliable commercial technique for single species detection.

1.1.1. Environmental DNA detection

Over the past decade, the development of eDNA-based species detection has expanded exponentially from theory into a heavily researched tool with a wide range of uses in conservation ecology and management. These have included but are not limited to: (i) species presence/absence surveys, (ii) pathogen detection (Huver et al. 2015; Schmidt et al. 2013),

(iii) population abundance estimates (Ficetola et al. 2008; Pilliod et al. 2013) and (iv) disease detection. The term eDNA is simply defined as a source of DNA which can be found within environmental samples such as water, soil, or air without sampling or even seeing the target organism (Taberlet et al. 2012). The DNA found in these environments originates from both cellular and extracellular DNA (Nielsen et al. 2007) which can be from the faecal matter, urine, blood, secretions, gametes of living organisms and the decay of dead organisms (Martellini et al. 2005; Pietramellara et al. 2009; Turner et al. 2014).

As the quantity of eDNA in these environments is usually extremely small (due to dilution within the natural environment) (Schultz and Lance 2015), mitochondrial DNA rather than genomic DNA is most commonly used as a target for qPCR detection and amplification within a sample (Thomsen and Willerslev 2015), unless in the case of hybrid species, where nuclear DNA is required to be utilised instead (Fukumoto et al. 2015). This is due to the higher copy number that mitochondrial DNA contains within each cell and the slower rate of degradation, which together provide a greater chance of detection from free cellular material within any given sample (Mills et al. 2000; Wilcox et al. 2013). Interestingly, the first reference to the term 'eDNA' was actually directed towards the detection of microbial organisms and 'ancient DNA' within soils (Ogram et al. 1987). However, since this study, it has been applied to the detection of an ever-growing list of 'macrobial' species (Thomsen et al. 2015). Currently, the need for eDNA-based detection methods in the commercial sector arguably falls with the detection of these macrobial species in aquatic environments which will therefore form the focus of this chapter and later the choice of target organism for eDNA-based method development associated with this thesis as a whole.

1.1.2. Development and history of the technique

The first study to use the underlying concept of 'eDNA detection' for non-microbial species was by Martellini et al. (2005), who aimed to isolate DNA originating from humans and farm animals in water contaminated with faecal matter. However, the term 'eDNA' was not given to the technique until the 2008 study by Ficetola et al. who targeted the assessment of populations of the American bullfrog (*Rana catesbeiana*) in both controlled and natural environments (Ficetola et al. 2008). Since then, the method has been expanded to include various targeted and untargeted approaches towards different species ranging from mammals (Foote et al. 2012; Thomsen et al. 2012), to fish (Jerde et al. 2010; Klymus et al.

2015; Takahara et al. 2013), reptiles (Piaggio et al. 2014), amphibians (Goldberg et al. 2011; Thomsen et al. 2012a), invertebrates (Goldberg et al. 2013; Thomsen et al. 2012a) and even plants (albeit using different marker genes) (Gantz et al. 2018). eDNA-based detection has not only been applied to freshwater, various marine environments have also been explored (Kelly et al. 2014; Mauvisseau et al. 2017; Port et al. 2016; Thomsen et al. 2012b). The expanding field of species detection through eDNA presence can evidently be seen in the research output; since 2008 to 2018, this has expanded from only a handful of papers to almost 1000 published articles per year. (Fig. 1.1.).

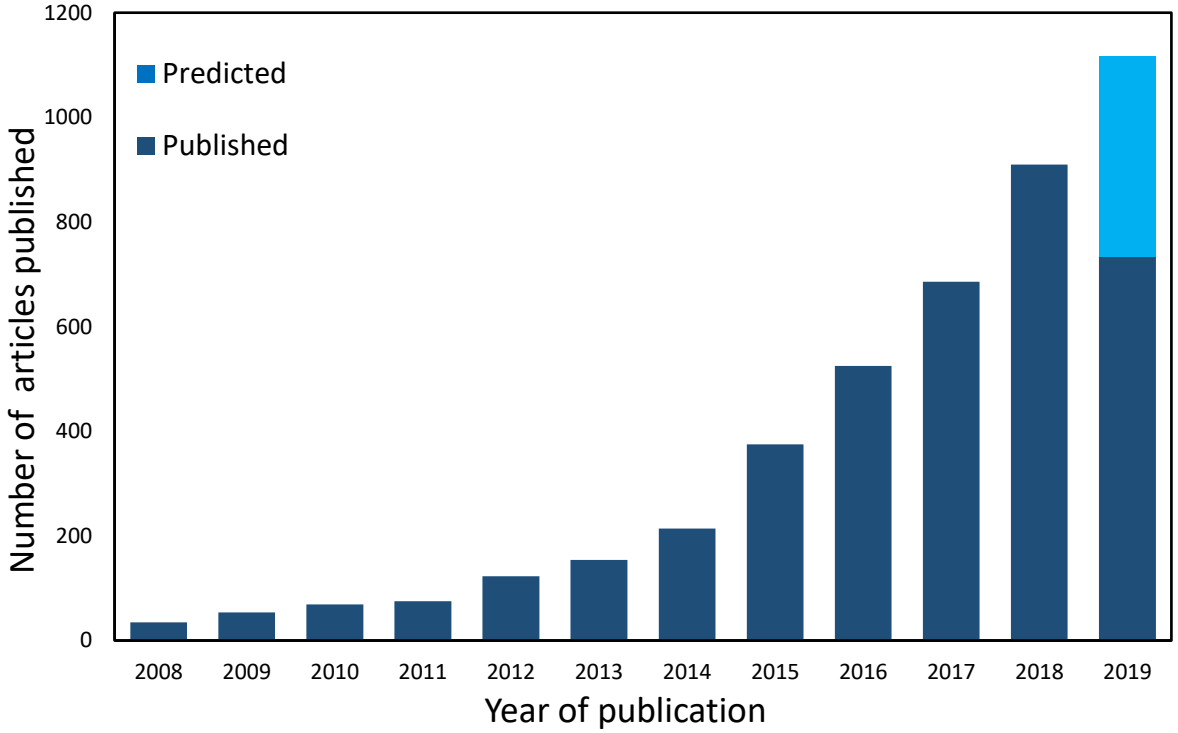

Fig. 1.1. Number of published papers each year since the first studies on aquatic eDNA in 2008, based on search term: [eDNA "environmental DNA"], using Google Scholar (Google, 2019). Predicted publications are based on the rate of papers published from 1st January 2019 to 1st September 2019.

Another emerging area of eDNA research is the detection of specific pathogenic agents. However, I recommend that using the term eDNA in this context should be avoided, as sampling for microorganisms (which most disease-causing organisms are) usually means sampling the whole organism and thus does not fit into the definition for eDNA used above and could be termed spore or microorganism detection instead. That said, some studies do still use eDNA in this context. For example, Schmidt et al. (2013) and Kolby et al. (2015) focus

on assessing the presence or absence of the wildly spread amphibian fungal pathogen, chytridiomycosis (*Batrachochytrium dendrobatidis*). Although it is agreed that identifying the presence or absence of specific pathogenic agents is a very valuable field of study, it could be suggested that the term eDNA is not used in these cases. For example, Strand et al. (2014), avoided the term eDNA when designing a method to assess the fungal mould *Aphanomyces astaci,* the causal agent of another wide spread crayfish plague disease affecting crayfish on a global scale.

1.1.3. Applied benefits of eDNA-based species detection methods

As an established scientific research technique, eDNA-based detection methods show promise as an additional option for species monitoring. eDNA assays are non-invasive, arguably more sensitive, efficient, and commercially viable than the majority of more traditional established methods in wide usage such as hand searching and net sampling for example (Davy et al. 2015; Smart et al. 2015; Wilcox et al. 2016). Although these traditional freshwater sampling methods, (Table 1.1.) are well tested and thought to be reliable, they can in some instances lead to findings which misrepresent species presence, or population abundance (Elphick 2008). This can result in the conclusion that a species is not present, even if it is, albeit in extremely low abundance, i.e. resulting in what is known as a false negative. One report on the detection probabilities of great crested newts (*Triturus cristatus*) using manual searching (such as trapping and torching) has individual success rates in the region of 75% (Biggs et al. 2015). eDNA-based detection in contrast, for the same species has been shown to identify levels of DNA down to just two copies of its genetic sequence (Schultz and Lance 2015) and detection success rates greater than 99% (Biggs et al. 2015). It is however important to note that eDNA-based detection has not always been the most reliable method, due to incidences of false negative (and false positive) detection (Tréguier et al. 2014; Lacoursière-Roussel et al. 2015).

Table 1.1. Summary of typically used sample collection methods for species presence/absence and population sampling within freshwater environments, ordered by relative cost to the end-user for application of method.

Technique	Environment	Target taxa	Invasive	Costs	Advantages/Disadvantages
Kick sampling	Lotic/Lentic	Benthic invertebrates	Invasive	High	Disturbance of habitat can cause damage to individuals. Can obtain qualitative and quantitative data.
Electrofishing	Lotic/Lentic	Fish, some invertebrates	Invasive	High	Least biased of the traditional methods. Can have high success rate, however certain habitats may limit this. Quantitative and qualitative.
Baited/cage trapping	Lentic, occasionally lotic	Invertebrates/ fish	Invasive	Medium	Success/detection rate depends on bait, trap dimensions and species size. Can obtain quantitative and qualitative data, population proportions can be biased as it is often difficult to trap smaller individuals.
Bottle trapping	Lentic	Fish/ amphibians	Invasive	Medium	Disturbance of habitat, entrapment of individuals. Useful in environments where visual inspection is impaired.
Hand sampling	Lotic/Lentic	Larger invertebrates	Invasive	Medium	Highly invasive. However, allows for detailed observational data to be taken. Low catch per unit effort (CPUE).
Egg searching	Lotic	Multiple taxa	Invasive	Medium	Difficult to find eggs, can be invasive (e.g. unfolding wrapped eggs for count) and can take a long period of time. Low CPUE.
Vacuum	Lotic/Lentic	Small invertebrates	Invasive	Medium	Highly invasive, can be time consuming to assess small sample volumes.
Bou-Rouch	Lotic/Lentic - shallow	Small invertebrates	Invasive	Medium	Invasive disturbance of organisms. Only representative sample taken at location of device.
Fyke netting	Lotic/Lentic	Fish	Invasive	Medium	Rely on fish entering the net in order to be included in the sample. Unlike gill nets, fish are released unharmed.
Gill netting	Lentic, marine	Fish	Invasive	Medium	Entrapment of fish within twine, highly invasive often resulting in harm. High degree of species selectivity.
Seine netting	Lentic, marine	Fish	Invasive	Medium	High degree of species selectivity. Can sometimes entrap larger species.
eDNA sampling	Lotic/Lentic	Any	Non-invasive	Medium	Relatively new underdeveloped technique has potential to be used as a detection and quantification tool, with further research.
Torching/ visual surveys	Lotic/Lentic	Invertebrates/ amphibians	Non-invasive	Low	Only samples visible individuals, only quantitative data can be obtained from this method of sampling.

In addition to its application as a successful habitat presence/absence survey method, studies are now attempting to use the eDNA-based detection methodologies to quantify species biomass (Takahara et al. 2012; Evans et al. 2015; Jo et al. 2017; Knudsen et al. 2019). However, this appears to be species specific and not always reliable (Dougherty et al. 2016; Deutschmann et al. 2019; Lacoursière-Roussel et al. 2015; Takahara et al. 2012). In some instances, a non-linear relationship between biomass and eDNA copy number can be identified (Kelly et al. 2014; Thomsen et al. 2012), whilst others have found a stronger relationship between eDNA copy number and the abundance of individuals in any given habitat (Baldigo et al. 2016; Doi et al. 2016). Other studies are however reporting correlations between eDNA concentrations and estimated biomass of a population (Baldigo et al. 2017; Nevers et al. 2018), with reasons for such disparity in investigations often attributed to the numerous limiting factors and variables which can affect the presence, persistence and distribution of eDNA.

1.1.4. Present commercial use of eDNA-based detection methods within the UK and Europe

An increasing number of UK species both native (Biggs et al. 2015) and invasive (Davison et al. 2017) have been successfully detected using eDNA-based detection methods. However, only one species, the great crested newt has a commercial testing service readily available and recognised by UK government (i.e. Natural England/Natural Resources Wales).

Species detection by eDNA-based methods is also available, on a more limited scale for a number of other species. For example, one company in Europe (Spygen, 2018) is offering a targeted eDNA-based detection service for a number of species, including; the red swamp crayfish (*Procambarus clarkii*), the American bullfrog (*Lithobates catesbeianus*), the European pond turtle (*Emys orbicularis),* common spadefoot (*Pelobates fuscus),* weather loach (*Misgurnus fossilis),* the apron (*Zingel asper*)*,* marbled newt (*Triturus marmoratus)* and great crested newt, along with metabarcoding analysis for amphibians, fish and mammals (SpyGen® 2018). Other companies, within the UK for example are also starting to offer such services for example, NatureMetrics and Applied Genomics. Listed on the websites of eDNA-based analysis service providers there is also indication of the development of commercially applicable targeted assays for any given species, including invasive species such as the signal crayfish (*Pacifastacus leniusculus*), zebra mussel (*Dreissena polymorpha*) and quagga mussel (*Dreissena bugensis*) as well as species of conservation concern such as the white-clawed

crayfish (*Austropotamobius pallipes*) and the freshwater pearl mussel (*Margaritifera margaritifera*). However, for these tests there is currently no accreditation or proficiency testing scheme in place.

1.2. Addressing the 'hurdles' associated with the commercial development and implementation of eDNA-based detection methods

eDNA sampling and detection methods have already shown a great deal of promise for commercialisation. However, due to an almost unlimited list of species that could benefit from the required specificity of the technique and the overwhelming number of variables associated with the topic, there is still a requirement for a large amount of detailed research to be conducted on a species-to-species basis before a reliable commercial practice can be put in place. Therefore, research and development of eDNA-based detection methods are becoming increasingly designed in a manner to take into account the requirements of potential end-users. This leads to the first hurdle in the commercialisation of eDNA-based detection assays (see Table 1.2. for the complete list). Throughout the remainder of this chapter, each hurdle is listed and discussed, offering suggestions on how hurdles can be overcome to obtain greater commercial output from existing and future eDNA research.

Table 1.2. Recommendations on how to address the 'hurdles' associated with the large-scale commercialisation of environmental DNA (eDNA) based detection methods.

	'Hurdle'	Recommendations
1	Assay validation	• For reliable and accurate commercial application of an eDNA test, validation acknowledging and addressing hurdles 2 to 7 should be conducted.
2	Detection sensitivity	• Use of an appropriate number of environmental replicates, depending on target species and predicted abundance within sample site (see sample collection method choice). • Use of MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) to develop and validate qPCR assay (Bustin et al. 2009). • Between 3 and 12 qPCR replicates, depending on the need to keep costs down (note this is for each of the environmental replicates – see sample collection method choice). • Use of statistical modelling to address the issues of sensitivity and to account for potential false positives/negatives.
3	Sample collection and preservation methodology	• Cross-environmental studies should be conducted on a species-to-species basis to ascertain the appropriate sample collection method. • A minimum of 2-3 independent environmental replicate samples should be taken from each site to ensure accurate detection.
4	Sample site	• Sample site selection guidelines should be developed for various 'types' of environment, depending on specific variables and habitat type.
5	Persistence and decay of eDNA	• Needs to be assessed on a species-to-species basis and across different environmental habitats (lotic and lentic) due to the variance in eDNA origin (faeces, skin, exoskeleton etc.) between species and therefore difference in persistence.
6	Environmental influences	• Seasonal variations in eDNA production and persistence should be investigated to enable confident use of eDNA detection over a yearly cycle. • Assessment of: flow rates, dispersal, weather at time of sampling and other environmental factors which may influence eDNA detection should always be undertaken.
7	Quantification	• For true and accurate quantification, a complete understanding of the environment, the organism, and the techniques utilised is required. It is important to consider the persistence of eDNA (see above). • This should not be attempted without a robust assessment of the environmental, biological and technical variables which may affect quantification of species biomass or abundance.
8	Inhibition and contamination	• Use of internal laboratory controls (no template control, field negative control) and the routine processing of known negative samples with each batch of samples. • Follow good lab practice guidelines (standard operating procedures) as outlined in Goldberg et al. (2016). • Use of markers during qPCR to assess for inhibition in any given sample (Biggs et al. 2015).
9	Consistency and reliability	• Improved proficiency testing scheme to a pass/fail system, where only accredited laboratories which pass a minimum level of testing can provide a service. • Protocol to allow service providers to make changes/develop services, reduce costs, make efficiency savings, whilst always maintaining accuracy and reliability.
10	Commercial accessibility	• Costs should be kept low to ensure end users (such as charities and conservation organisations) can utilise the methods routinely. • Any reduction in laboratory analysis due to advances in molecular biology (and therefore reductions in costs) should be passed on down to the end-user. • Potential regulation of cost in line with validation. • Approval and/or validation/regulation at governmental level.

1.2.1. Hurdle 1: Validation

Despite the reported early success of eDNA-based detection techniques, very little has been done to indicate the accuracy and validity of the results achieved in the majority of investigations. Presently, the only assay developed and used on a commercial scale is for the great crested newt, as a direct result of the effective and meticulous validation which was conducted (Biggs et al., 2015, 2014; Buxton et al., 2018; L. R. Harper et al., 2018; Helen C Rees et al., 2014; Rees et al., 2017). The majority of existing published assays fail to meet validation and developmental standards remotely similar to that of the paper by Biggs et al. (2015) and as such are inappropriate to use (in their current forms) to monitor populations across a large study.

For any commercial eDNA-based detection test, validation requirements should be driven from potential end-users and regulators, taking into account the latest academic research and knowledge on the technique. The following hurdles (2-7) should be assessed to enable a sound scientific basis behind each developed test. Finally hurdles 8, 9 and 10 should be considered in order to ensure that any developed test meets the needs of the end-users, transforming academic research into a more practicable technique which is useful in real-world conservation. Without addressing these hurdles, a non-validated assay would be insufficiently developed to be applied on a commercial scale, which makes validation the first, and arguably most important hurdle to be overcome before such an assay can be made commercially available. A typical approach to the commercial conception, development and adoption of an eDNA assay is depicted in Fig. 1.2. In addition to this flowchart, the levels of validation which can be applied to eDNA assays is presented as Table 1.3.

A high level of validation is required for commercial application of any PCR based assay, particularly those which could be linked with government management practices. This means that the majority of published eDNA assays require much more development for them to be at a suitable level for application as a commercially reliable detection service. It should be made clear it is not always necessary, or a requirement to validate every eDNA assay as strictly as the levels of validation demonstrated within Fig. 1.2., and Table 1.3. Most commercial eDNA-based services are driven by the needs of end-users, and it may not always be useful for the potential customer to have full scale validation, for example as part of a

small-scale non-repeating project. In these smaller applications, the validation requirements of the assay may vary depending on the needs of the customer (i.e. detecting species with less cause for conservation concern or screening smaller study areas). However, if an assay is to be applied on a national level and used as an approved survey technique for a species like the great crested newt, then validation as in Table 1.3. should be attempted at the very minimum.

Table 1.3. Levels of validation in eDNA studies and the hurdles which complement each level.

	Level of Validation
1	Design and testing of primers *in-silico.*
2	Limited testing of primers *in-vitro* and *in-vivo*.
3	Full assessment of assay sensitivity and specificity. **(Hurdle 2).**
4	Field trials across different sites, environments and conditions.
5	Assessment of the temporal, spatial influences and sample collection approaches. **(Hurdles 3,4,5,6,7).**
6	Trials in a commercial setting with real-world commercial application of developed assays. **(Hurdles 8,9,10).**

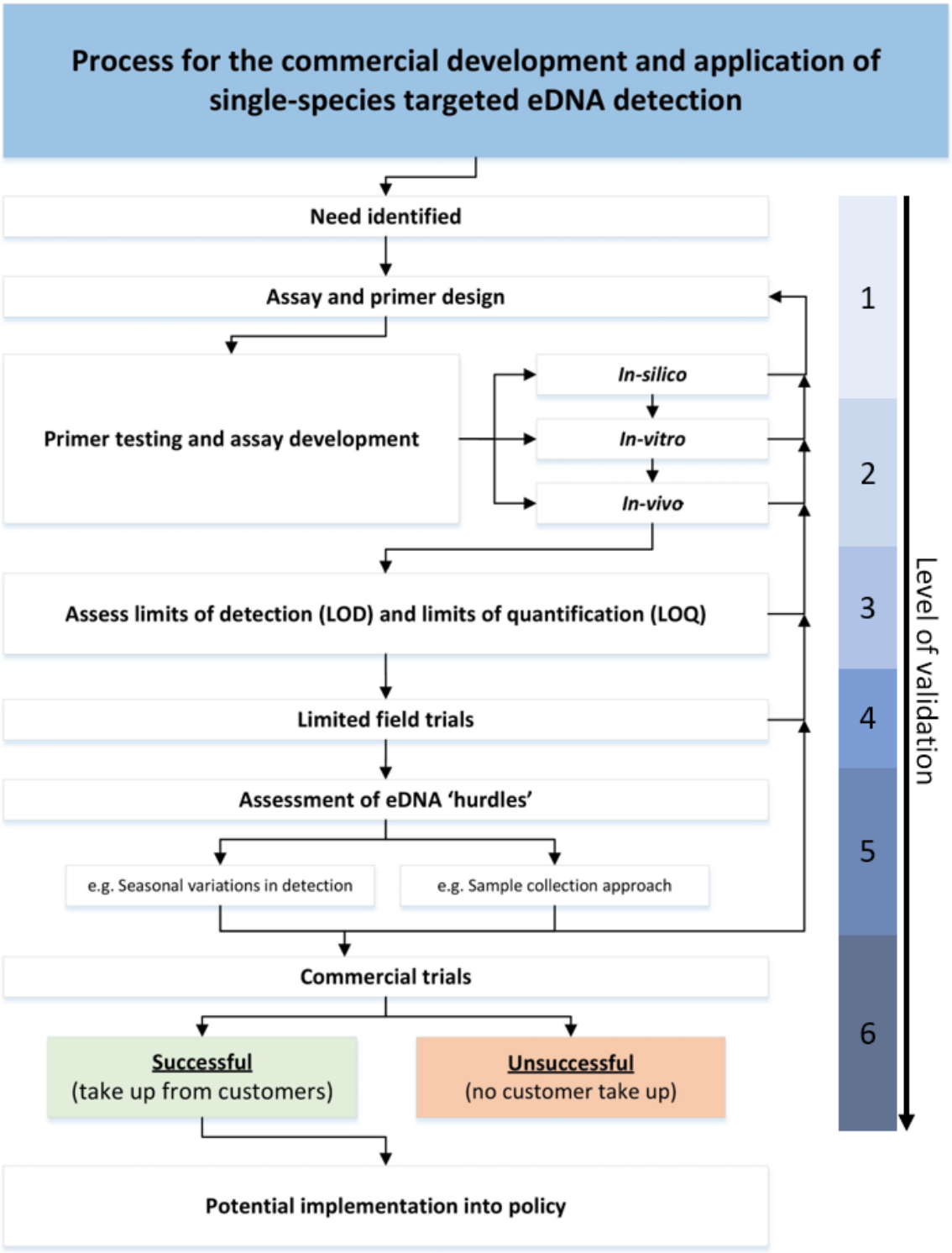

Fig. 1.2. A flow-chart to depict the process for the commercial development and application of single-species targeted DNA detection. At each stage within the flow-chart, the level of the validation conducted on the assay is demonstrated (1-6) and is described within Table 1.3.

1.2.2. Hurdle 2: Detection sensitivity

The issue of detection sensitivity is also a major concern with eDNA surveys – and illustrates the second 'hurdle' to the commercialisation of eDNA-based detection techniques. Primer design is key for an efficient qPCR assay. Guidelines have been established and published by MacDonald and Sarre (2017) which are specifically aimed for assay development of species-specific eDNA-based methods. Primer testing *in-silico, in-vitro* and *in-vivo* should all then be assessed and a good assay at a minimum should also be assessed for its limit of detection (LOD) and limit of quantification (LOQ) according to and following the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin et al. 2009; Mauvisseau et al. 2019). As with any molecular tool, qPCR assays work only as well as the original design, and often have their own LOD and LOQ which indicate how sensitive a technique the assay in question can be. The LOD is typically defined as the last standard dilution of a standard curve in which detection of target DNA with at least one qPCR replicate is achieved below an assay-specific set threshold cycle (Ct), and as such can give an idea about assay sensitivity. The LOQ is often defined as the last standard dilution in which targeted DNA is detected and quantified in a minimum of 90% of qPCR replicates of the calibration curve under that same Ct value (Mauvisseau et al., 2019a). Although increasingly being used in eDNA investigations a number of studies still fail to report these details, meaning that it is difficult to assess or understand assay sensitivity.

There are currently several steps in the process where sensitivity affecting errors could occur. Attempting to minimise these by experimental design is therefore important. According to recent consensus of the available literature (Goldberg et al. 2016), any number greater than three qPCR replicates has been suggested for eDNA-based studies. Take the current commercial application of eDNA-based monitoring for the great crested newt for example. At the time of writing, a proportionately large number of qPCR replicates (n = 12) is used (Biggs et al. 2014) in an attempt to increase the sensitivity of the technique and reduce occurrences of false negatives (be they due to extraction or technical issues). Indeed, in many instances only 1 or 2 of these 12 qPCR replicates gives a positive result (per observation). When presented with samples, stochastic in nature resulting in low positive detection, it is important that the assay design is reflected to ensure accurate identification of these samples is achieved, and thus a high number of qPCR replicates is therefore often appropriate. To

improve on this, it could be recommended to use around two or three environmental replicates (covered in more detail in the next hurdle below). The phrase 'environmental replicates' in this case indicates separate, independent replicates, i.e. the number of samples taken from any one single specific location. From each of these independent environmental replicates one would run at least six qPCR replicates to insure reliability and robustness of the results. That said, for the application of eDNA-based testing it is important to be mindful of the costs associated with such replication. The use of statistical models in the commercialisation process is one possible work around this replication issue. Such models can also deal with issues associated with the occurrence of false negatives and sensitivity (Lahoz-Monfort et al. 2015).

1.2.3. Hurdle 3: Sample collection and preservation methodology

eDNA sample collection and preservation methods can be varied (Goldberg et al. 2016) and is therefore another important 'hurdle' to consider with regard to commercialisation of any newly designed assay. Improvements in sample collection and the development of associated legislation surrounding ecological surveys may in the future reduce the licensing requirements for the sampling of some species, opening up the use of eDNA-based detection to a larger number of untrained individuals or 'citizen scientists'. The first issue to discuss when conducting any eDNA sampling is the number of environmental replicates needed. Rees et al. (2014b) evaluated this issue and suggested that a multi-replicate sampling approach is most efficient for the detection of species in aquatic environments due to the increased chance of detecting eDNA from each additionally tested replicate. Therefore at least three environmental replicates are commonly recommended to be taken at each site (where possible), in order to ensure the greatest accuracy and consistency (Goldberg et al. 2016; Pilliod et al. 2013).

The two most common sampling methods used are filtration and ethanol precipitation. However, there is a large amount of variation regarding the methods utilised by researchers and service providers. Hence there is an urgent need for standardisation of techniques. With filtration for example, the sample volume, filter type, pore size, diameter size, etc. can and does vary – which can lead to difficulties when attempting to compare success between studies (Buxton et al. 2017; Dougherty et al. 2016; Mauvisseau et al. 2017) on a species-to-species and habitat-to-habitat basis. For commercialisation purposes, the

simplification and/or standardisation of this part of the methodology would certainly improve the accessibility of eDNA-based detection to an increasing number of ecologists and citizen scientists (Biggs et al. 2015). Interestingly, research has highlighted that the majority of commonly used water sample collection techniques do not actually affect the probability of the detection of eDNA within an environment (Pilliod et al. 2013). However, a later study by Deiner et al. (2015) contradicts this statement, illustrating significant differences between the techniques used and that the results appear again to be species specific. This could have far reaching implications in the development of new assays. Further research is therefore still urgently required in this area, at least to assess the true extent of the effect that sample collection has on detection probability, both on the given species and/or habitat level. Some of which is now beginning to emerge, both in single-species assays (Spens et al. 2017) and in metabarcoding studies (Li et al. 2018).

On a similar topic of sample collection, researchers have recently developed an all-in-one eDNA sampling backpack device (Thomas et al. 2017). Dubbed the ANDe™. The device is designed to make the sampling process simpler and more replicable by achieving faster on-site sampling. However, the current system does carry a high (at least initial) financial cost to any given service provider and is therefore not likely to be taken up in its current form, particularly for commercial and citizen science-based projects.

1.2.4. Hurdle 4: Sample site

The location of where a sample is collected from is another key hurdle which needs to be addressed for the commercial application of eDNA-based detection. This is particularly important to consider in lotic environments, where hydrological factors may affect the detection rate of eDNA due to increased or decreased downstream transport of eDNA (Davy et al. 2015). There are few studies which address the lotic environments and the detection issues associated (Deiner and Altermatt, 2014: Deutschmann et al. 2019). However, a recent study by Shogren et al. (2017) highlights a simple conceptual model which focuses on how DNA is likely to be transported, retained and re-suspended in streams. Detailed studies both *ex-situ* and *in-situ* will now need to be conducted in order to test this model for a variety of different species and ecological systems.

1.2.5. Hurdle 5: Persistence and decay of eDNA

eDNA is well reported to have a degradation rate at the point when it is no longer detectable in the environment (Barnes et al. 2014). This limitation is reported to range between hours (Maruyama 2014; Piaggio et al. 2015; Thomsen et al. 2012a), days (Barnes et al. 2014; Turner et al. 2015), and in some cases years (Andersen et al. 2012; Turner et al. 2015). This degradation rate has been shown to vary with regard to the environmental type and conditions at the time of sampling (e.g. freshwater, marine, sediment type, flow rates, microbial activity), and is dependent on the source and production of the eDNA (i.e. what species it originates from and whether it is faeces, skin cells, decaying matter etc.) (Pilliod et al. 2013). Under some conditions (in sedimentary and terrestrial environments for example), eDNA has been shown to persist for a number of years, much longer and at higher concentrations than when suspended within water (Andersen et al. 2012; Turner et al. 2015). Disturbances of the sediment may therefore cause an external source of eDNA release (Turner et al. 2015; Goldberg et al. 2016), potentially leading to false positive results for the presence of a species, my fifth identified 'hurdle' is that of persistence of eDNA. Degradation rates have also been investigated with regard to the levels of sunlight/UV exposure at any given site. Some studies highlight that a quicker degradation rate occurs when exposed to higher amounts of UV exposure (Pilliod et al. 2013; Pilliod et al. 2014). In contrast, there is also a body of evidence which suggests there is in fact no significant relationship between UV and detectability (Mächler et al. 2018). A further study has suggested that aquatic environments which are more favourable for eDNA persistence are those which are cooler, more alkaline and protected with shade (Strickler et al. 2015). It is therefore highly likely that different sampling protocols and possibly different assays will need to be utilised, based on the environment, at least for still ponds and fast flowing rivers for example, even if the species being detected is the same (Jane et al. 2015). Therefore, due to the contrasting literature amassed to date, further study into this issue is paramount in order to assist assay validation for commercialisation in this regard (see Barnes and Turner (2016) for a detailed review).

1.2.6. Hurdle 6: Environmental influences

Environmental variables can influence the persistence, concentration, dispersal and ability to detect eDNA (Barnes et al. 2014). The changing nature of ecological environments provides additional variables to the detection and reliability of eDNA-based methods (Jane et al. 2015).

Within lotic environments, the variability observed can be predominantly attributed to the potential for downstream flow of eDNA (Roussel et al. 2015). For example, eDNA has been detected over 12 km from the original site of DNA release (Deiner and Altermatt 2014). Detectable distance was also found to vary between different species and over different seasonal conditions in the same study (Deiner and Altermatt 2014). This decrease in detection rate would also lead to a lower detection probability of samples at a further distance due to the greater dilution and downstream loss of eDNA. Another hypothesis is that eDNA concentration would be lower at the site of the source in areas of moderate flowing water. However, this has not yet been verified with the current studies available (Laramie et al. 2015). It is often important to gain an indication of the location of a potential species within the sample site for detection probabilities to be accurately mapped against influential variables such as downstream flow. As the dispersal factor of eDNA is important, the velocity of flow of a lotic environment will almost certainly need to be factored into consideration within future studies. Recent work by Shogren et al. (2017) has now begun to fill in these blanks, by proposing a framework associated with eDNA transport and dispersal.

Further, another layer of complexity is bought about by the complex life cycles of most aquatic species for which eDNA-based detection is useful for. In this instance for example, at any given time period, a species could be more or less active than at another point in the year. Increased activity during breeding seasons, and decreased activity at dormant winter periods could therefore lead to a higher and lower amount of detectable eDNA within the environment respectively (de Souza et al. 2016; Ostberg et al., 2018). A good example of this is the study by Buxton et al. (2017) whereby an increase of detectable DNA was found around the breeding period and larval stages of the the great crested newt life cycle. Therefore, the time at which a sample is collected is highly important and could be the difference between accurate detection and false negative detection.

Further environmental influences include: pH, microbial activity, temperature (Eichmiller et al. 2016), weather conditions, UV radiation (Strickler et al. 2015) and water levels (Smart et al. 2015). All of the above represent the sixth ‘hurdle’ to the efficient, commercial use of eDNA-based detection methods and must be considered on a species-to-species, habitat-to-habitat level in order to fully understand and take variations in analysis into account.

1.2.7. Hurdle 7: Quantification

Quantitative PCR (qPCR), has the ability to enable the user to determine the concentration of target DNA within a given sample (Nathan et al. 2014). In the case of eDNA this initial concentration of DNA can (at least in theory) infer some form of abundance estimate of the target species. Such quantification has long been a goal for many studies, however the complex nature of the almost unlimited variance of a given environment and the nature of eDNA persistence have so far meant that such studies have had limited success, which leads to the seventh 'hurdle', quantification. That said, some studies have highlighted promise in this area. Quantification has at least in part been successfully demonstrated for some species including: common carp (*Cyprinus carpio*) (biomass) (Takahara et al. 2012), the Rocky Mountain tailed frog (*Ascaphus montanus*) and the Idaho giant salamander (*Dicamptodon aterrimus*) (Pilliod et al. 2013) (biomass and density), the American bullfrog (*Rana catesbeiana*), and the round goby (*Neogobius melanostomus*) (abundance) (Nevers et al. 2018). Metabarcoding sequencing reads have also been shown to infer species abundance (Evans et al. 2015). Early tests have also indicated that there is a relationship between species biomass and eDNA concentration within certain environments such as the oceans, despite the greater dilution factor associated with these environments as compared to freshwater systems (Kelly et al. 2014).

However, these studies do not fully address the issue that changes within the dynamics of a given population may also affect the eDNA-based detection rate and any subsequent quantification attempts. Individuals of a certain size, weight, age, health or diet may have an increased or decreased output of cellular material, therefore contributing to different concentrations of eDNA within the environment (Klymus et al. 2015). This makes the task of quantifying species or population abundance even more complex as a small 'stressed' population (by disturbance or environmental conditions) may be releasing the same amount of eDNA as a considerably larger healthy population. With the current techniques, full scale quantification is also likely to be unable to take external factors into account and, if attempted, would likely lead to the inaccurate recording of species biomass. Such an issue further indicates the large amount of development still required in this aspect of the eDNA topic area.

Aside from the external factors affecting the ability to quantify the number of, or biomass of, any given species present in any given habitat, there are also methodological constraints which need to be considered here as well. Limits of quantification (LOQ) (MIQE Guidelines – Bustin et al. 2009) should be addressed to assess the limit of reliable quantification of eDNA within a sample. qPCR by design is a very sensitive technique, however it has been suggested that the LOQ may not be sensitive enough for the lower spectrum of eDNA which is likely found in areas with few individuals of the target species - which in turn would result in a significant number of false negatives being reported (Cai et al. 2017). Further research is therefore required to push the technologies to their limit to achieve reliable quantification at lower species abundance.

1.2.8. Hurdle 8: Inhibition and contamination

Inhibition

The use of inefficient laboratory procedures or the presence of inhibitory substances (Foote et al. 2012) may reduce the detectability of eDNA-based monitoring methods and is the eighth 'hurdle'. Efficient and clean laboratory practices are essential to reduce the risk of inaccurate analysis and lab-based sample contamination (Wilson et al. 2015). However, due to the uncontrolled nature of the environments from which samples are obtained, many additional substances may be present which could limit or inhibit DNA extraction or the subsequent PCR procedures which follow. For example: obtaining eDNA from marine environments is thought to be more challenging than in freshwater (Díaz-Ferguson and Moyer 2014) due to the higher salinity, which contains inhibitory NA^+ ions, thought to affect the extraction and amplification processes (Foote et al. 2012; Wilson 1997). To overcome this problem, modified laboratory protocols can include additional ethanol wash steps, which allow for the effective removal of Na^+ ions from these samples, thereby reducing the chance of PCR inhibition (Foote et al. 2012). These and other molecules may be present naturally or originate from external sources - such as phenolic compounds from plant matter and heavy metal pollution (Wilson 1997). Inhibition of PCR can result in an increase in the number of false negatives achieved. Inhibitory substances originating from dead and decaying plant matter have also been found to present false negatives, even in samples which are known to have high concentrations of the target species (Jane et al. 2015).

Existing commercially available kits are spiked with artificial markers (Biggs et al. 2014, which are then tested for during the analysis. If the marker is not detected, then it is likely that the extraction process has failed due to viable sedimented blocking of spin columns or if the sample has been subjected to some form of inhibition or degradation. The sample collector is then advised to re-collect any samples from that site or collect better quality samples in cases of poor sample quality. However, the need to revisit a site (which in itself can be impractical at times) could be avoided by the collection of multiple samples per site, as recommended in section 1.2.3.

Contamination

False positives are a high risk to eDNA-based investigations and steps therefore must be put in place to reduce incidences where possible. Internal laboratory controls (No template conrol (NTC's) and field negative controls) should always be used i.e. negative samples which are processed alongside the true environmental samples. If these samples report as positive, then it can be assumed that there is some form of contamination and all samples analysed alongside that sample should be re-analysed to avoid the incidence of false positives. In terms of field sample contamination - cross contamination of water source is the most likely origin (Díaz-Ferguson and Moyer 2014). One example where this can be a major problem regards the ballast water of ships. Ballast water has been well documented to be a significant cause of species transfer over small and large spatial scales (Lavoie et al. 1999; Ruiz et al. 1997). Therefore, the threat of transfer of fragments of free-floating DNA is guaranteed. Furthermore, within freshwater environments, contamination could occur from external water sources, such as during floods, and from ditches or ground waterbodies for example and via transfer on fishing hear, sampling equipment and leisure equipment such as kayaks.

The use of positive control samples which contain high copy numbers of the exact same sequence as the target sequence is also a major risk, and along with contamination from previously analysed and amplified samples. In response to this issue, one study has integrated a synthetic control which can be differentiated between the target sequences (Wilson et al. 2015). This synthetic oligonucleotide is almost identical to the target sequence, however a slight change in its structure has allowed for contamination to be recorded on a sample by sample basis, rather than as a separate control sample (Wilson et al. 2015). The synthetic sequence is amplified by the same primers and qPCR probes as the target eDNA, but it

contains a short restriction enzyme site insert, which is absent in the naturally occurring DNA. This means that contamination from this positive control sequence can be detected when present within a sample, however would require a number of additional steps to the analysis, further increasing the sample analysis time and costs.

Good laboratory practice

'Good laboratory practice' should always be a standard in any molecular biology laboratory and should go without saying. In this regard, for eDNA-based methods it is recommended that each step of the process within the laboratory should be conducted in a separate dedicated room for example, extraction, PCR prep and running the plates, with a unidirectional flow going from areas of low contamination risk to areas of high contamination risk (Biggs et al. 2015; Goldberg et al. 2016). Here, personnel, equipment and processes would be conducted in an order as such to reduce the risk of fresh samples becoming contaminated by old previously amplified PCR samples. Finally, if service providers practice efficient laboratory isolation and cleaning protocols such as regularly autoclaving and deep cleaning equipment with 10% or 50% bleach daily and after use, and using UV cabinets, there should be minimal incidences or risk of contamination.

To summarise, the occurrence of false positives and false negatives has proven to be a challenging issue to address, particularly when it comes to interpretation of eDNA analysis results. In a commercial setting, the presence of these erroneous results can have a serious impact on both the service provider and end-user. It is therefore of high importance to ensure that on a commercial scale, the incidence of false positives and negatives is kept to an absolute minimum.

1.2.9. Hurdle 9: Consistency and reliability

Accreditation and regulation

In the case of great crested newt eDNA-based detection within the UK, those who are wishing to offer this service must currently follow the protocol outlined in the Department for Environment Food & Rural Affairs (DEFRA) methodological guidelines – report WC1067 (Biggs et al. 2014). For results to be accepted by government agencies in this instance, service providers must ensure that they take part in a national proficiency testing scheme. This is in order to ensure some level of cross-lab consistency with regard to the analysis and reporting of the results. However, under the current system there is no official laboratory accreditation

as the proficiency testing scheme does not provide a pass or fail score, as just taking part is currently enough for result acceptance. This could mean that laboratories that performed particularly poorly in proficiency testing can still continue to provide a (potentially insufficient) service to end-users. Laboratories should, at the very least be working with or towards universal standards such as ISO accreditation. Before the implementation of the proficiency testing scheme, there was a high possibility of unapproved variations and modifications to the WC1067 method (Biggs et al. 2014), however, increasing rounds of testing should have now encouraged laboratories to further improve their systems. Since commercialisation, there has been minimal scope for service providers to make changes to the assay, something which could be implemented on a regulatory level and addressed in the near future to allow for improvements in both accuracy and reliability as the science moves forward.

The ninth hurdle is related to the consistency and reliability of any given assay, analytical method or service provided. The recent introduction of the proficiency testing scheme for service providers aims in part to address this 'hurdle'. However, from the initial results it is clear there is significant variation in the reliability of the different companies and therefore end users may become unsure of the technique as a whole, whilst taking more care when deciding who to 'trust' with their samples.

Without governmental or industry wide regulation, it is essential that any service provider which offers these assays follows effective, thoroughly tested protocols and contamination reduction programmes in order to ensure that there is minimal risk of reporting false negatives or otherwise incorrect results. However, if the service for a particular species is required on a large-scale commercial level it is essential that further validation steps are conducted on a wide range of samples, environments and conditions similar to those conducted during the development of eDNA-based detection for great crested newt (Biggs et al. 2015).

Improving consistency in the results

It is vital that consistency occurs with any existing and/or proposed government approved method (such as great crested newt eDNA-based detection). Especially as these tools could have impacts on development and infrastructure projects on a regional, national, and international level. Such consistency needs to span across the different service providers in

both the methodology and the result analysis. Technical advice for the great crested newt method indicates that small changes to the methodology can be made, on the condition that they are based on evidence and do not affect the results (Biggs et al. 2014). At the time of writing, only one small change to the approved great crested newt methodology has been approved and this only occurred as a result of service providers combining evidence, thus leading to an increase in the use-by-date of the sampling kits. With the development of eDNA-based detection as a commercial product for an increasing list of species, it is important that consistent practices are ensured across laboratories for each method used so that the end user can be assured that they will achieve reliable results no-matter which service provider they use. The implementation of a proficiency testing, code of practice and agreed consistent standards across all providers of the eDNA-based analysis would allow for this to be achieved.

Standardisation

The formation of a generalised 'best practice' is one key area which can easily be implemented. This would allow for the validation and potential standardisation of certain aspects of the sample collection and processing steps enabling consistency across service providers but would need to be conducted on a species-to-species basis (Goldberg et al. (2016). Such standardisation would enable the technique to be applied in most environments whilst compensating for, and adjusting to, different experimental and environmental conditions without affecting the results. The creation of a large databank available for use in cross-study and institute comparison of results and methodology would also be a highly useful step forward and allow for tests to be conducted aimed at addressing the effects of many of the environmental parameters discussed - particularly on the end result.

Standardisation however, does come with some disadvantages, particularly within the commercial sector where any changes made to improve a method would need approval and would also need to be shared with business competitors, something which not every commercial organisation would be open to. Further, in 2016, a Co-Operation in Science & Technology Program (COST) was initiating with these exact goals. This program encourages trans-national collaboration between researchers and end–users with an interest in eDNA with the end goal of trying to establish agreed upon 'gold standard' practices for use in aquatic genetic bioassessment throughout Europe (CA COST Action CA15219 – DNAqua-Net). There are several working groups which have been associated as part of this COST Action – each

focussing on different topic areas, however as this is still in the early stages of implemented action for this initiative, further discussions cannot be conclusively made. One outcome of the initiative (which is currently publicly available) is the applicability of eDNA-based monitoring as a habitat ecological assessment tool under the European Water Framework Directive (Hering et al. 2018). This is an important step which can feed into future commercial suitability and importantly sustainability of eDNA-based detection for species groups as a standard ecological survey technique. Additionally, laboratory analysis could be conducted under CEN (European Committee for Standardization) or ISO (International Organization for Standardization) standards which would ensure consistency and that effective standard operating procedures (SOP's) are followed. Currently these have not been implemented in the field of eDNA-based analysis, however with increasing commercial demand for the technique, it is likely that within the next few years more laboratories will be incorporating approved standards into the routine analysis of samples.

As discussed within this chapter, robust methodologies and quality control measures are beginning to form a 'gold standard' procedure for analysis. However, the implementation of 'gold standard' protocols across all commercial service providers presents various challenges. Whether it be due to financial reasons, facility-based restrictions, or even unconscious disregard, some service providers may not be able to implement efficient quality control measures. It is therefore important that the end-user has a good unbiased understanding of the validation processes conducted between laboratories in order to make an informed decision on the quality and accuracy of the commercial service that they are choosing to receive.

1.2.10. Hurdle 10: Commercial accessibility

Although it has been demonstrated that sample collection for eDNA-based analysis could be successfully collected by volunteers (Biggs et al. 2015), there is still a large aspect of the process which requires specialised expensive equipment and extensive training, especially when it comes to laboratory analysis (Bohmann et al. 2014). Such equipment has relatively high start-up costs therefore making it inaccessible to the majority of small ecological consultancies and conservation driven organisations - this is the tenth and final hurdle. Keeping costs low should be a top priority. Over the past few years molecular analysis in general has become cheaper, and as such, with any reduction in the cost of analysis, savings

should be passed onto the end user. However, as it stands eDNA detection services are still more cost effective than traditional survey methods after the initial set up (Huver et al. 2015; Rees et al. 2014a). In terms of great crested newt surveys, traditional sampling methods can cost from £500 for a simple visual site inspection, to several thousands of pounds depending on the sample site and can often take many days, weeks and sometimes months. eDNA-based detection methods take only a few hours and are priced from around £140 per habitat sampled (SureScreen Scientifics 2019). However, this only takes the laboratory analysis into account, sample collection by a qualified individual would also need to be factored into this price. When taking all costs in to account, ecological consultancies charge around £400 for a full eDNA-based survey of one pond, compared to over £1500 for a full traditional trapping and assessment survey (Brindle & Green 2018 – personal communication).

It is important, when developing a commercial service that the science is accessible to the everyday end-user (i.e. those collecting the samples). This puts great importance on the development of end-user friendly sample collection methodologies. Work is required in this sector, as presently a number of the sample collection methods used require a large amount of training. This enforces the importance of end-user driven research both by academic institutions and service providing commercial organisations in order to provide to the end-user the most accessible and easy to use methodologies. Getting the right end-user to research formula here will be key in the development of all future eDNA-based detection services.

1.3. The commercial potential of eDNA-based detection methods

eDNA-based detection is less invasive and more cost effective than the more traditional first-line sampling of an environment and the species it contains. That is at least after the initial set up and design/validation of the assays have been taken into account (Goldberg et al. 2015). This gives a strong case for the commercialisation of the technique, allowing for eDNA-based detection to become commonplace in many environmental studies and reports. Although there is a lot of promise for the technique, there are many limitations or 'hurdles' (as discussed) that are currently holding back the approach and preventing it from being used as an independent or additional method for full-scale surveys. There are only a

few (but increasing number of) researchers who are attempting to address these 'hurdles' – at least in regard to the commercial use of the tool.

The success linked with the commercialisation of eDNA-based detection surveys for the great crested newt brings promise for the use of this method by improving detection of many other species both native and invasive as discussed briefly above. eDNA-based monitoring methods have huge potential, both as a scientific concept and in practice in the real world to move ecological conservation and environmental consultancy into the 21st century (Takahara et al. 2012).

eDNA-based detection has particular commercial value for the survey of species which have legal protection or are a listed priority species which needs to be regularly surveyed by a consultant. For example, as a legal obligation associated with planning processes or to assess and avoid impacts to said species during the management and development of land. Many of these UK species of conservation concern, (a selection of which are shown in Table 1.4.), are also often cryptic, exist in small populations and have extensive ranges - making them ideal candidates for eDNA-based detection. For example, the use of molecular detection methods for such species would not only provide a reduction in on-site survey time, cost and labour, but provide a less intrusive survey of any given site, limiting expensive delays to new developments (Biggs et al. 2015). The results of ecological surveys that inform planning decisions often result in the requirement of species translocation, the monitoring of species, biodiversity, diseases, and habitat management. Using eDNA-based detection as an additional or standalone survey method during such ecological actions has, in a number of cases, the potential to improve the effectiveness and accuracy of these actions whilst reducing further costs.

eDNA-based analysis could also be coupled with an assessment for habitat suitability for any given species as well as the test for said species. For example, the traditional methods of assessing habitat suitability for the great crested newt is the conduction of a habitat suitability index (or HSI) (Oldham et al. 2000). The HSI uses ten key criteria to give a measurement of the suitability of any given habitat. Three of these criteria include measuring the number of waterfowl, the water quality (based upon the invertebrate assemblage) and the number of fish. These criteria are likely to be difficult to record accurately during a single observational visit or by persons untrained in bird, fish, and invertebrate identification.

Traditional biodiversity assessment tools are also likely to underestimate the true biodiversity of the habitat being assessed and usually is only representative of the sample location (Deiner et al. 2016). Future eDNA-based species detection surveys that include testing for common waterfowl, fish, and indicator invertebrates could not only improve the accuracy of these initial habitat biodiversity assessments but also advance the analysis of the results. Some researchers (and service providers) indeed, already provide such a service through eDNA metabarcoding to screen for a wide range of taxonomic groups within habitats such as streams and lakes (see section 1.3.3.).

There has been a common misconception by some that eDNA-based techniques will begin to threaten the work of the ecologist in much the same way that DNA barcoding was thought to threaten taxonomists. However, there would still be a requirement for ecologists to collect the eDNA samples as in many cases a licence, or at the very least specific training is still needed for eDNA sample collection (at least when seeking validation for commercial purposes). Traditional sampling would also still be required under some circumstances (Thomsen and Willerslev 2015) and therefore highlight that eDNA-based detection should be considered as an additional commercial service, or a standalone primary survey technique for species presence/absence, rather than a replacement for current methods.

Table 1.4. The extent of single-species eDNA detection development and commercial availability within the UK with respect to potential key target freshwater species (such as species of conservation concern or invasive/problematic species of interest to ecologists).

Target species	Binomial name	Invasive/Native	eDNA test investigated	Commercially available in the UK	References
Amphibians					
Great crested newt	*Triturus cristatus*	Native	Yes	Yes	(Biggs et al. 2014)
Smooth newt	*Lissotriton vulgaris*	Native	Yes	No**	(Smart et al. 2015)
Common toad	*Bufo bufo*	Native	No	No	
Natterjack toad	*Epidalea calamita*	Native	No	No	
American bullfrog	*Lithobates catesbeianus*	Invasive	Yes	No	(Ficetola et al. 2008)
Pool frog	*Pelophylax lessonae*	Native	No	No	(Eiler et al. 2018)
Fish					
Sturgeon	*Acipenseridae spp.*	Native	Yes	No	(Dejean et al. 2011)
Common carp	*Cyprinus carpio*	Invasive	Yes	No	(Takahara et al. 2012).
Mammals					
Otter	*lutra lutra*	Native	Yes	No**	(Thomsen et al. 2012a)
Water vole	*Arvicola amphibius*	Native	No	No	
Invertebrates					
White-clawed crayfish	*Austropotamobius pallipes*	Native	Yes	Yes*	(Robinson et al. 2018)
Signal crayfish	*Pacifastacus leniusculus*	Invasive	Yes	Yes*	(Larson et al. 2017)
Red swamp crayfish	*Procambarus clarkii*	Invasive	Yes	No	(Tréguier et al. 2014)
Little Ramshorn whirlpool snail	*Anisus vorticulus*	Native	No	No	
Freshwater pearl mussels	*Margaritifera margaritifera*	Native	Yes	No	(Stoeckle et al. 2015)
Zebra mussel	*Dreissena polymorpha*	Invasive	Yes	No	(Penarrubia et al. 2016; De Ventura et al. 2017)
Quagga mussel	*Dreissena rostriformis bugensis*	Invasive	Yes	No	(Penarrubia et al. 2016; De Ventura et al. 2017)
Killer shrimp	*Dikerogammarus villosus*	Invasive	No	No	
New Zealand mudsnail	*Potamopyrgus antipodarum*	Invasive	Yes	No	(Goldberg et al. 2013)
Daphnia	*Daphnia spp.*	Native	Yes	No	(Deiner and Altermatt 2014)
Dragonfly larvae	*Anisoptera spp.*	Native	No	No	
Damselfly larvae	*Zygoptera spp.*	Native	No	No	
Disease causing organisms					
Chytrid fungus	*Batrachochytrium dendrobatidis*	Invasive	Yes	No	(Schmidt et al. 2013)
Crayfish plague	*Aphanomyces astaci*	Invasive	Yes	Yes*	(Vrålstad et al. 2009; Strand et al. 2014)

*Assay now commercially available as a direct result of this thesis and associated works.

**Assays advertised as available but upon contact with service provider were identified as not currently available (September 2019).

1.4. Conclusion

As development progresses, eDNA-based detection is becoming a much more advanced tool for species detection and quantification. With this, comes an increase of interest from prospective clients for the tool, giving species detection through eDNA presence a greater commercial value. Although currently available techniques show promise (as efficient and reliable species presence/absence detection protocols), with the exception of the existing great crested newt methodology, most are not quite yet developed to a suitably high level of standard required to enable its use as a stand-alone technique. Therefore, its application (outside of scientific literature), lies as an additional sampling technique for use during primary site surveys for the species, or taxonomic group of species of interest, with a requirement for follow up surveys to be conducted soon after using more established survey methods. Thus, eDNA sampling adds to the repertoire of the consultant ecologist, allowing for the commercial provision of an additional efficient and cost-effective method to be available for the planning stages and/or constant monitoring during the development stages of various industries such as construction. At this moment in time, the technique is only fully available for the great crested newt on a commercial scale in the UK. Validation of any new technique is an important area which will need to be considered alongside assay development, with researchers working with governmental bodies and end-users to drive the development and optimisation of a fully validated methodology (to the requirements of the end-users) to be incorporated within legislation.

With research beginning to delve deeper into some of the 'hurdles' which could create inconsistency with this tool, the technique will start to be much more efficient and effective and may become commonplace in environmental surveys for a whole number of species in the future. However, to achieve this, the development of SOPs and the reduction of the effect of variables and limitations such as population density, degradation of eDNA and downstream flow need to be addressed to a higher level. The progress of research into the 'hurdle' areas addressed in this review, and subsequent implementation of more accurate techniques can only have a positive impact on the capabilities of eDNA-based detection, giving the technique even greater commercial viability and reach within conservation.

Chapter 2: Introduction to crayfish in the United Kingdom

2.1. Introduction

To demonstrate the development of a commercial eDNA-based service, one which tackles the various hurdles associated with assay development identified in chapter 1, I utilised one of the UKs most endangered invertebrate species, one which is of high conservation concern (Holdich et al., 1978), the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858).

2.1.1. White-clawed crayfish

The white-clawed crayfish (Fig. 2.1.) is the only species of freshwater crayfish native to the UK found in both lotic and lentic habitats. Although white-clawed crayfish have a native home range across most of mainland Europe, from southern Spain, France, Italy upwards to Slovenia (Kozak et al., 2015), it has been estimated that 30% of the total population is present in the UK alone (JNCC, 2010). Although activity appears to differ with seasons (Barbaresi and Gherardi, 2001), the species is largely nocturnal, with foraging beginning soon after sunset, however, under some conditions the species can be found out during the day (Holdich, 2003). Like most freshwater crayfish, their general habitat requirements consist of an aquatic riverbed with a relatively low water flow rate. The presence of rocky substrate, used for the refuge of crayfish, has been identified as a critical parameter for the survival and colonisation of a specific habitat (Broquet et al. 2002). Areas of substrate which allow burrowing activity is also important for the species particularly for over-wintering when they become substantially less active. Holdich (2003) outlines that the favourable habitat conditions for white-clawed crayfish include: heterogenous flow rates (in lotic systems) with an abundance of suitable refuges, including fallen logs, leaf litter, rocky substrate (of varying sizes) and undermined/overhanging banks. Within the UK, white-clawed crayfish populations have traditionally been found in alkaline waters from the south coast up as far north as the Scottish border (Holdich et al., 1978) and is reported as an indicator of a healthy ecosystem and excellent water quality (Trouilhe et al., 2007). Despite this, the species is now known to be much more tolerant than originally hypothesised to conditions of moderately polluted environments (Trouilhe et al., 2007) and muddy and silty waters (Holdich et al., 2006).

White-clawed crayfish play an important role in the recycling of detritus and organic material (Gherardi et al., 2004) within the ecosystem, and are also an important food source for a number of species including large fish, birds, otter and mink (Arce and Alonso, 2011). Although typically a detritivorous species, individuals can often be opportunistic and exhibit carnivorous behaviour under certain conditions, this is particularly seen in the juveniles during the first period of growth (Gherardi et al., 2004).

Fig. 2.1. An adult white-clawed crayfish, Cumbria UK. Source author, ©Chris Troth.

2.1.2. Biology of white-clawed crayfish

White-clawed crayfish individuals are typically described as light to dark brown in colour, with a paler underside. They typically weigh up to 90g and have an average body length of 12cm from the tip of the rostrum to the end of the tail (Holdich, 2003), however they can be found to grow larger under near perfect habitat and environmental conditions. White-clawed crayfish are a moulting species and as such the growth of an individual typically occurs in combination with the shedding of their exoskeleton. Once the exoskeleton is released, individuals typically increase in

both length and size by roughly 10% during the short period of time whilst their new exoskeleton forms before hardening (Reynolds et al., 2010). It is important to note that it is at this stage when the crayfish are most vulnerable to predation and harsh environments due to their soft, unprotected exoskeleton (Reynolds et al., 2010). The number of times that white-clawed crayfish moult each year can depend on a number of variables including environmental conditions and food availability, however, individuals within the first two years after hatching typically moult at an increased rate when compared to larger and older adult crayfish. In the first year of life, white-clawed crayfish juveniles typically moult up to seven times (Reynolds, 1998; Rogers and Watson, 2007). On the other hand, for older crayfish: males of five years and above may only moult twice in a year (once at the beginning of summer and once at the end) and mature females may only moult once a year (Reynolds et al., 2010). For reference, a typical crayfish moult is shown in Fig. 2.2.

Fig. 2.2. A freshly moulted (less than 2hours) crayfish exoskeleton from a sub-adult white-clawed crayfish, Somerset UK. Source author, ©Chris Troth.

2.1.3. Life cycle of white-clawed crayfish

Crayfish, like many invertebrate species have a seasonally based life-cycle, with many biological processes including mating, hatching and dormancy being governed by environmental

temperatures. The lifespan of white-clawed crayfish is recorded to be of around 10 years (Holdich, 2003), with sexual maturity being reached at three to four years of age, when the length of the carapace reaches roughly 27mm for males and 23mm for females (Rhodes and Holdich, 1982).

Once sexual maturity is reached, breeding occurs during the months of September to November, when the water temperature begins to lower to a consistent average of 10°C (Reynolds et al., 2010). During mating, males produce spermatophores which are then transferred onto the underside of the female using specialised abdominal appendages, where they then externally fertilise the eggs, once produced (Holdich, 2003; Yazicioglu et al., 2016).

The female crayfish on average produces around 100 eggs (Holdich, 2003), however, this number can range from anything between 20 and 160, with a maximum number of 161 eggs observed on a large adult female by Rhodes and Holdich (1982). Each egg is within the region of 3mm in diameter (Reynolds et al., 2010) and becomes 'cemented' into a cavity formed by the bending of the abdomen on the underbody of the female using a sticky substance produced by the crayfish known as glair (Holdich et al., 1978). The eggs then remain attached to the under-appendages of the female over winter, until they hatch in the following spring – during this period the crayfish are described as 'berried' (Reynolds et al., 2010) due to the appearance of the eggs attached to the underbody - a photo of a female crayfish with recently developed eggs is displayed as Fig. 2.3. The high egg-producing fecundity of the species is typically as a result of not all eggs surviving (remaining attached) on the female up until the hatching season. It has been reported that has many as 50% of all eggs do not survive to hatching (Reynolds, 1998; Reynolds et al., 2010), with an even smaller number of individuals surviving through to their first year due to their vulnerability in the aquatic environment (Holdich, 2003).

Fig 2.3. A captively bred female crayfish carrying recently produced eggs, cemented to the underbody using 'sticky' glair (Bristol Zoo Gardens, UK). Source author, ©Chris Troth.

Once breeding is over the crayfish then begin to burrow or take shelter for a period of inactivity over the winter months when the environment is below temperatures which permit activity and as such become largely torpid (Peay, 2000). Once water temperatures increase during late spring/early summer the juvenile crayfish begin to hatch from the eggs, which are still attached to the berried females. The exact timing of hatching is dependent on temperature and as a result of this can somewhat vary geographically over a period of two months from the southern to northern extent of their populations within the UK alone (Peay, 2000). After hatching the juveniles (and adults) then begin the growth season where they moult and grow at different rates depending on their age (Reynolds et al., 2010). The growth will then continue until the breading season in the autumn. The full seasonal activity cycle of the white-clawed crayfish is depicted as Fig. 2.4.

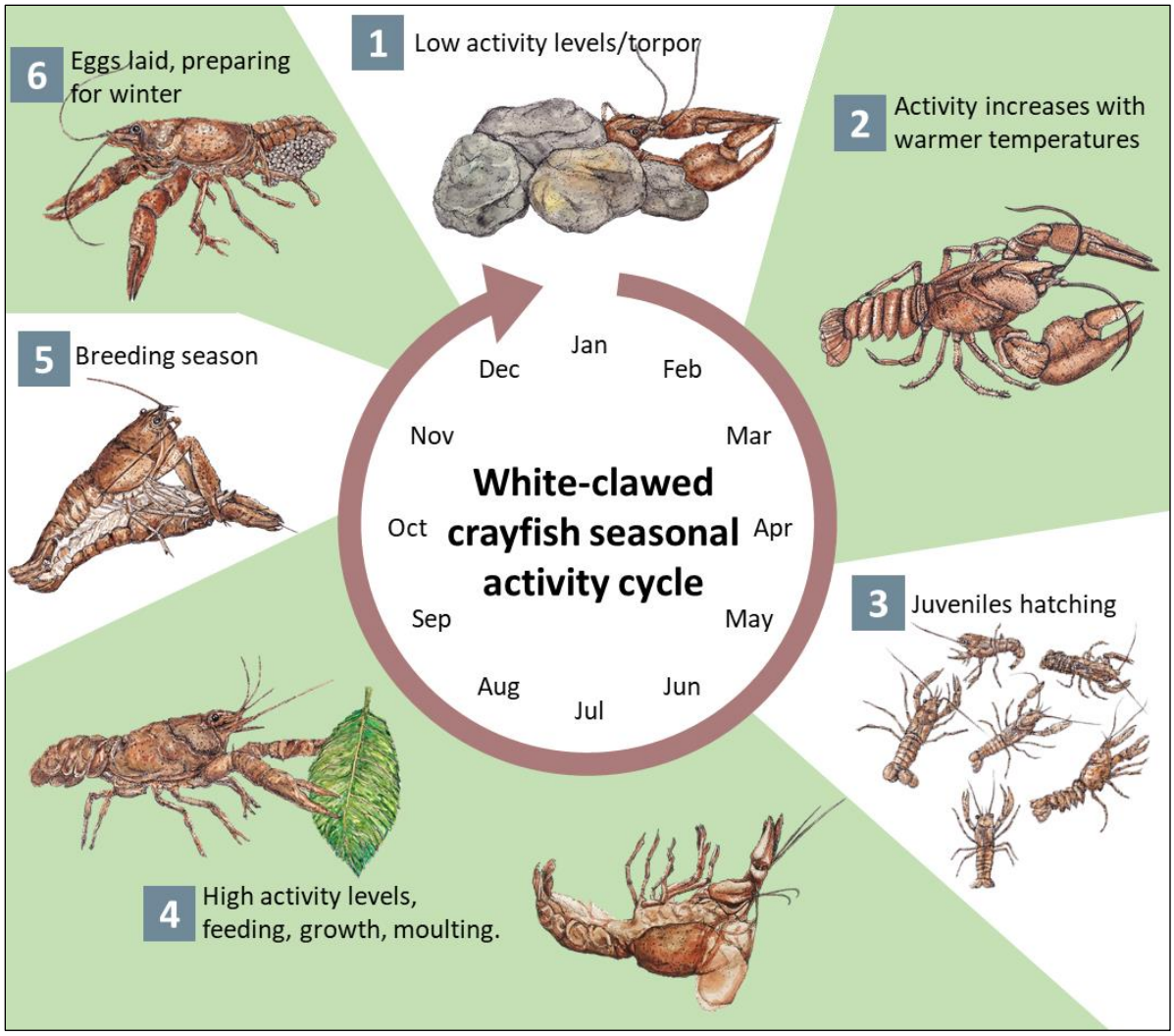

Fig 2.4. Seasonal activity cycle of the white-clawed crayfish. ©Keziah Drew, ©Chris Troth.

2.1.4. Populations in decline

Populations of white-clawed crayfish across Europe have been a cause for concern for a number of years. First being recognised as a rare species in 1986, vulnerable in 1996, and more recently becoming listed as endangered on the International Union for the Conservation of Nature (IUCN) Red List in 2010 (Füreder et al., 2010). Data collected on populations across Europe over a period of 10 years found that from 2000 to 2010 the number of individuals had decreased within the region of 50% to 80% giving a serious cause for concern and resulting in the species' classification as endangered (Füreder et al., 2010). Interestingly, until recently there was debate over whether white-clawed crayfish was in fact a true indigenous species to the British Isles or not. Recent work

has now settled this, concluding that sufficient evidence is present within historical literature providing evidence to the presence of the species prior to 1500 AD (Holdich et al., 2009a; D. Holdich et al., 2009b). This ensured that the species made the criteria for listing on the IUCN Red List in the UK, cementing the importance and need for increased efforts of conservation towards the white-clawed crayfish.

In addition to being listed on the IUCN Red List (Füreder et al., 2010), the species is protected on a European level through the 1982 Bern Convention and the 1992 Habitats Directive. Within the UK, these directives were transposed into the Wildlife and Countryside Act (as amended) 1981, where white-clawed crayfish is protected under schedule 5, making it an offence to take individuals from the wild or to sell or offer to sell live or dead individuals of the species (Wildlife and Countryside Act 1981). The white-clawed crayfish has been a UK Biodiversity Action Plan (UK BAP) priority species since 1997 (JNCC, 2010), at the time of the last UK BAP update (2010) the species was recorded to have had a 21% decline in the UK over the previous four-year period.

Up until the 1970's, the native white-clawed crayfish was the only species of crayfish present within the United Kingdom (Holdich and Reeve, 1991), with relatively stable population numbers across the country. The first non-native crayfish species to be recorded in British waters was the signal crayfish, *Pacifastacus leniusculus*, introduced as a source of food stock for fishing lakes by the government in 1976, and by 1988 over 250 separate signal crayfish colonies were known to exist (Lowery and Holdich, 1988). At the time, it was unknown what impact this species would have on UK crayfish populations; however, it was soon described as a highly invasive species and the main driving force behind the decline of white-clawed crayfish (Holdich et al., 2009a). Today in the UK, signal crayfish are now much more common than the native white-clawed crayfish. There is a growing need to survey and monitor for both species, to aid with the conservation effort of white-clawed crayfish and to prevent the extent of the spread of signal crayfish.

2.1.5. Signal crayfish

Morphologically, the signal crayfish (Fig. 2.5.) is roughly 1.5 to 2 times larger than the native white-clawed crayfish when in ideal habitat conditions (Dunn et al., 2009). This size, coupled with its greater strength and aggression allows it to out-compete the native species for a habitat, resulting in the loss of native white-clawed crayfish populations soon after the introduction of signal crayfish. This invasive species to UK freshwaters generally has a higher rate of reproduction and proliferation than white-clawed crayfish (Lowery, 1988) and it is recorded that signal crayfish juveniles hatch 2-3 weeks before white-clawed crayfish young, giving them a competitive head start on growth (Peay, 2000). It has also been reported that signal crayfish can survive greater levels of stress in an environment than white-clawed crayfish, such as variation in climatic conditions and pollution events. Such high tolerance to variation of conditions is one of the reasons behind their ability to colonise and spread at such speed (Lowery, 1988). The proliferation of the species from 1980 to present, is coupled with a comparable decline in white-clawed crayfish populations (Fig. 2.6.). Despite only first arriving in 1976 (Lowery and Holdich, 1988), the species soon colonised much of the waterways across the UK.

Fig 2.5. A group of three adult signal crayfish, Worcestershire UK. Source author, ©Chris Troth.

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Accessible in an alternative version via https://nbnatlas.org/ by searching for ‘white-clawed crayfish’ and ‘signal crayfish’

Fig. 2.6. Map of the British Isles displaying all publicly available historical point records of white-clawed crayfish and signal crayfish distribution since records began up until February 2017 (NBN Atlas species ID: NBNSYS0000033009).

The negative effects that a population of signal crayfish can have on an ecosystem are well recorded. These include: damage to the habitat by the undermining of river banks (Peay, 2009), increases in bioturbation and fine sediment load (Harvey et al., 2011; Rice et al., 2014; Turley et al., 2017), declines in general biodiversity (Lodge et al., 2000) and the abundance of: macrophytes (Harvey et al., 2011), invertebrates, (Crawford et al., 2006), fish species (Guan and Wiles, 1997; Peay et al., 2006) and of course - the white-clawed crayfish. Signal crayfish are also more aggressive (Bubb et al., 2009) and often exhibit carnivorous behaviour. Although, they can be found co-habiting a given space, in most cases the signal crayfish will eventually completely outcompete the white-clawed crayfish within a few years (Holdich and Domaniewski 1995).

2.1.6. Crayfish plague

Out of all of the documented impacts that signal crayfish can have on the white-clawed crayfish, spread of *Aphanomyces astaci* (or crayfish plague as it is also known), is more often agreed as being the most devastating. Crayfish plague is an oomycete water mould originating from North America. Signal crayfish are a carrier vector and are almost completely immune to the effects of crayfish plague as a result of co-evolution, however, this is not the case with most indigenous European species, which can be and have been devastated by the fungus that is believed to have entered Europe via the ballast water of a steam ship carrying infected signal crayfish individuals in the 1850's (Holdich, 2002). Within the UK, the species was first identified as present (and subsequently wiped out a population of white-clawed crayfish) in 1981 (Alderman et al., 1984). A significant number of white-clawed crayfish populations have been recorded to have since been destroyed by the crayfish plague, including; within the Thames catchment, Derbyshire, the Severn catchment and East Anglia, this list has increased year on year, devastating populations across the UK and mainland Europe (Sibley et al., 2002).

Crayfish plague is carried by infected signal crayfish individuals within its cuticle (Oidtmann et al., 2002), and this is often the most direct cause of the transmission of the plague – through infected individuals. However, a number of other vectors and situations may also be the cause of transmission of the plague into what was a healthy or uninfected population of white-clawed crayfish. These include: through the faeces of mammals, birds and fish; through contaminated water as a result of direct infection; or as a result of being introduced by vector

means from contaminated animal skin; or from human-mediated causes (Svoboda et al., 2017) from contaminated inanimate objects such as boats and fishing gear, all of which is outlined by Oidtmann et al. (2002). In conclusion, Holdich (2003) therefore states that it is of upmost importance to follow strict biosecurity cleaning and disinfection protocols (GB Non-Native Species Secretariat, 2019).

The plague starts by infecting the muscle tissue, leading to a more opaque abdominal area (Holdich, 2003), and causing a gradual paralysation of an individual (Svoboda et al., 2017). This is often followed by atypical behaviour such as individuals seen walking as if they are on stilts or exhibiting more frequent diurnal behaviour (Holdich, 2003; Oidtmann et al., 2002). Infected individuals often die from the infection within a matter of several days (Matthews and Reynolds, 1990). Most outbreaks of crayfish plague within white-clawed crayfish populations are often identified at a stage which is too late for action to be undertaken, with the majority of individuals already infected or dead (Holdich, 2003). Due to the fast spreading abilities of this disease, it is often difficult to control or prevent the spread. Research on the longevity of the crayfish plague, when separated from a living host suggests that the spores are no longer at an infectious level after around nine days (Matthews and Reynolds, 1990) to two weeks (Oidtmann et al., 2002).

Up until 1981 there was no control over the importation or release of invasive crayfish species into the UK, which allowed for the development of populations across all areas of the UK, before the introduction of the Wildlife and Countryside Act 1981. The invasive potential of this species has now resulted in large scale destruction of white-clawed crayfish populations, resulting in their classification as endangered leading to a greater need for efforts of conservation directed at the species. The combination of the introduction of the invasive species and crayfish plague has resulted in widespread decline in white-clawed crayfish populations. Studies have indicated that even without the addition of the plague, populations of white-clawed crayfish can suffer and become eliminated from an area within a short period of ten years, simply due to the presence of signal crayfish (Holdich and Domaniewski 1995), even with the use of signal crayfish population control efforts. There is therefore often a need to survey for white-clawed crayfish,

signal crayfish and crayfish plague, due to the impact that the invasive species and the plague can have on native populations.

2.1.7. Existing ecological survey methods for crayfish

A range of methods are currently employed by ecologists for the surveying and sampling of crayfish populations, many of which are previously listed in Table 1.1., yet, there is no true consensus on the most appropriate and efficient. With an efficiency (total population detection success) of around 90%, electrofishing is recorded to achieve reliable results when surveying for crayfish; however, it is also the most harmful with high rates of cheliped or antennae loss and damage (Alonso, 2001) and in some instances death. On the other hand the method of baited trapping can be very time consuming to the ecologist, often requiring many repeated visits, days apart, to collect, count and measure individuals once the trap is in place (Bernardo et al., 2011). Hand searching (refuge searching/turning over stones and rocky substrate) and kick sampling can also be less accurate, invasive and time-consuming, and although trusted and well-established techniques they are not usually relied on for quantification and population abundance studies due to their variable rates of success. In one study, crayfish were recorded present using these methods in only 64% of habitats which were known to contain crayfish, with false negative detections being recorded in the other 36% of sites (Gladman et al., 2010). A further method used (which is less invasive than each of the methods discussed so far) is night-torching. Here, a torch is used to search a given length of river in a given time for the presence of crayfish as this is when they are most active (Hill, 2010). There is no doubt that although each of the techniques discussed above are useful in the detection of crayfish, none are consistently reliable and a combination of them is often required to increase effectiveness (Gladman et al., 2010).

All of the above techniques presently require the user, typically a fully qualified ecologist or ecological volunteer to either hold or be working under a license (License: WML – CL11 (Natural England, 2019)). In addition to this, the majority of survey methodologies: kick sampling, hand searching, baited trapping, require entry to the watercourse and the use of re-usable equipment. This can not only cause damage to the habitat being surveyed but also comes with the additional risk factor of introducing or spreading the crayfish plague, if inefficient biosecurity measures are not followed.

The most recent and comprehensive state of white-clawed (and invasive) crayfish distribution across the UK dates back to 1991 (Holdich & Reeve, 1991). Although a large amount of effort has been made to survey crayfish presence / absence, many projects attempting this have lacked the funding or resources needed to obtain a substantial national record. It is thought that the high speed of colonisation of signal crayfish (Lipták and Vitázková, 2014) and spread of the crayfish plague will have completely changed the dynamics discussed by Holdich and Reeve in the 26 years since their report. Despite the great importance in updating these records, many of the more recent efforts to survey the extent of the crayfish populations in the UK often fall short of providing enough detailed, long term data due to the high cost and time required for existing 'traditional' sampling methods. There is therefore a need for increased detection efforts, through the use of new eDNA-based technologies (as mentioned in chapter 1) which would enable an increase in monitoring efforts. The application of an eDNA-based survey methodology for white-clawed crayfish (and other invasive species) would allow for a much greater survey detection rate per unit of effort, whilst achieving accurate results and keeping costs low.

2.1.8. Crayfish and eDNA

Since the first application of an eDNA-based survey method for a crayfish species, *P. clarkii* (Tréguier et al., 2014), interest has expanded, resulting in the development and utilisation of eDNA for an increasing number of crayfish species worldwide. These include: signal crayfish (Agersnap et al., 2017; Larson et al., 2017; Mauvisseau et al., 2018), Rusty crayfish - *Orconectes rusticus* (Dougherty et al., 2016), Japanese crayfish - *Cambaroides japonicas* (Ikeda et al., 2016), Noble crayfish - *Astacus astacus* (Agersnap et al., 2017), Narrow-clawed crayfish - *Astacus leptodactylus* (Agersnap et al., 2017), spinycheek crayfish - *Orconectes limosus* (Mauvisseau et al., 2018), coldwater crayfish - *Faxonius eupunctus* (Rice et al., 2018), shasta crayfish - *Pacifastacus fortis* (Cowart et al., 2018) and the white-clawed crayfish (Atkinson et al., 2019; Robinson et al., 2018). eDNA-based methodologies have also now been investigated, developed and tested for the crayfish plague (Strand et al., 2014; Wittwer et al., 2018).

Thus far, the majority of these research studies have focussed on the detection of the species in one particular habitat or environment, with little work taking these studies further to understand the full applicability of the methodologies for their respective species in each type of

environment, habitat, condition and season. For example, although one study on *P. clarkii* has identified a high detection success rate of 73% (Tréguier et al., 2014), it does not consider any implications which may arise from seasonal crayfish life-cycle influences on eDNA persistence in the environment – something which may be a key influence on experimental success in eDNA-based studies. As the first paper on crayfish eDNA, it did however, provide an excellent example of a proof of concept for eDNA on the group of organism. However, as with many 'ground-breaking' studies it fell short a little and did not provide all of the necessary information needed in order for the method to be utilised as a commercialised tool, missing for example information on the ability to use the tool across seasons and different environments. Table 2.1. illustrates which UK priority species eDNA assays have been developed for, presenting some key findings of the research conducted to date.

Table 2.1. Table indicating the extent of the development of eDNA-based methods applied to detect native, non-native crayfish species and the crayfish plague, all of which have the potential to be present within the UK.

Species	Binomial name	Has eDNA been applied?	What is the extent of research?
Signal crayfish	*Pacifastacus leniusculus*	**Yes** (Agersnap et al., 2017; Dunn et al., 2017; Larson et al., 2017; Mauvisseau et al., 2018; Robinson et al., 2018)	• Species detection in-situ and ex-situ. • Multiplex species detection. • Investigations into species-specific eDNA degradation. • Assessment of year-round seasonal method applicability. • Assessment of the impact of behaviour patterns on eDNA species detection success.
Noble crayfish	*Astacus astacus*	**Yes** (Agersnap et al., 2017)	• Species detection in-situ and ex-situ. • Attempt at quantifying abundance with eDNA concentration.
Narrow-clawed crayfish	*Astacus leptodactylus*	**Yes** (Agersnap et al., 2017)	• Species detection in-situ and ex-situ. • Attempt at quantifying abundance with eDNA concentration.
Red Swamp crayfish	*Procambarus clarkii*	**Yes** (Cai et al., 2017; Geerts et al., 2018; Mauvisseau et al., 2018; Tréguier et al., 2014)	• Species detection in-situ and ex-situ. • Investigations into species-specific eDNA degradation. • Assessment of sampling strategy and analytical method choice on detection success rate.
Spinycheek crayfish	*Orconocetes limosus*	**Yes** (Mauvisseau et al., 2018)	• Species detection in-situ and ex-situ. • Investigations into species-specific eDNA degradation.
Virile crayfish	*Orconocetes virilis*	**No** (as of March 2019)	• N/A
White river crayfish	*Procambarus actutus*	**No** (as of March 2019)	• N/A
Red claw crayfish	*Cherax quadricarinatus*	**No** (as of March 2019)	• N/A
Marbled crayfish (Marmorkrebs)	*Procambarus fallax f. virginalis*	**Yes** (Mauvisseau et al., 2019c)	• Species detection in-situ and ex-situ.
White-clawed crayfish	*Austopotamobius pallipes*	**Yes** (Robinson et al. 2018; Atkinson et al. 2019)	• Species detection in-situ and ex-situ. • Multiplex species detection.
Crayfish plague	*Aphanomyces astaci*	**Yes** (Robinson et al., 2018; Strand et al., 2014; Wittwer et al., 2018)	• Species detection in-situ and ex-situ. • Multiplex species detection. • Assessment of year-round seasonal method applicability.

2.1.9. White-clawed crayfish and eDNA

It is clear that eDNA-based detection would be highly beneficial as a survey technique, if available on a large scale for the detection of white-clawed crayfish. However, with the research conducted so far it is clear that there are several 'hurdles', limitations and affecting variables which could impact the success of the tool as a widely available and approved additional survey technique.

Since the commencement of this research investigation, two eDNA-based methodologies for white-clawed crayfish have been concurrently developed by Robinson et al., (2018), and Atkinson et al. (2019). These two papers, although successfully demonstrating the applicability of eDNA for the species by taking novel and relevant cost-saving approaches, fail to address many of the questions, or hurdles outlined within chapter 1. All of which are necessary for the application of eDNA-based detection on a commercial end-user level. Due to the shear complexity of the current eDNA picture, it would be difficult to address all of these 'hurdles' within one single research paper – therefore there is a requirement for more research around this topic, to develop eDNA as a reliable technique for white-clawed crayfish.

Since the introduction of the great crested newt, *Triturus cristatus* eDNA-based service, a number of organisations have called for a similar method for the detection of crayfish species including: The Environment Agency, The Wildlife Trusts (including: Dorset Wildlife Trust, Hampshire Wildlife Trust and Derbyshire Wildlife Trust), The Canal and River Trust, Bristol Zoological Society, The South West Crayfish Partnership and a number of ecologists and conservation/study groups. At the beginning of this research project (November 2015), although listed on a number of UK eDNA service provider websites as 'in-development' there was no commercially available technique for the detection of any UK present (native or invasive) crayfish species using eDNA (SureScreen 2016; NatureMetrics 2016), however, crayfish eDNA is now available as an output of this thesis, see chapter 5. There is therefore, a clear demand for the service. However, it is important that any eDNA-based survey methodology for white-clawed crayfish should be fully examined to address and examine as many hurdles, limitations and pitfalls as possible. This will enable its success as an additional survey technique for the detection of the species.

2.2. Rationale

White clawed crayfish are therefore an ideal focus species for this PhD both due to the increasing number of calls from end-users for eDNA to be available for the species and their important status as an endangered species. There is a need for the conservation of this species, not only as a result of its importance ecologically (Gherardi et al., 2004; Trouilhe et al., 2007) but because the decline of the species has been documented as a direct impact of anthropogenic activities over the last century (Peay and Füreder, 2011). It is widely reported that the existing legal protection for white-clawed crayfish within the UK and Europe is not sufficient to ensure the successful conservation of the species (Peay and Füreder, 2011). More 'hands on' conservation actions are therefore urgently needed to ensure the species survival. As monitoring efforts are often expensive, exhaustive of time and species/environmentally invasive, there is also an urgent need for a more novel approach to identifying populations – the application of eDNA here has the potential to address this, should it be developed in an efficient and methodological manner.

As already discussed in length, there are many methodologies, techniques, tests, assays and research articles available addressing 'eDNA based' detection of an ever-increasing list of species. However, the majority of these fail to consider all of the limitations, variables and 'hurdles' which must be addressed before an assay can be used as an accurate, reliable, widely applicable commercial tool, available to everyone. Through careful step-by-step assessment of each of the 'hurdles' and limitations as previously described, this PhD research project will enable eDNA detection of white-clawed crayfish and ensure it addresses the hurdles producing a reliable commercially viable tool. There is a real call from ecologists for cheaper and time effective large-scale monitoring of crayfish populations, in order to increase the ability of these end-users and organisations to provide more conservation efforts and actions towards the species on an ever-decreasing budget.

In this study, whilst working alongside a commercial eDNA service provider, stakeholders, governmental agencies, voluntary organisations, 'grass-roots' ecologists, and 'citizen science' end-users, a real-time PCR assay is designed and applied a as an additional cost effective and

efficient commercially viable and validated molecular method for the detection of white-clawed crayfish within lentic freshwater systems.

2.3. Thesis Aims

To assess the limiting variables or 'hurdles' and effectiveness of the commercial application of eDNA-based assays as a species presence/absence survey tool, using this knowledge to develop and validate an eDNA-based assay for white-clawed crayfish. Ensuring that such an assay is tailored for suitable for application as a commercially available, viable and end-user accessible product for the non-invasive and reliable detection of white-clawed crayfish in all known inhabited aquatic environments.

Chapter 3: To develop a reliable eDNA assay for the detection of white-clawed crayfish by designing and critically evaluating a primer set for sensitivity and specificity before conducting in-depth laboratory mesocosm and field-based testing across different habitat types and examining and comparing the effectiveness of existing eDNA sampling approaches.

Chapter 4: Using the assay developed in chapter 3, assess i) the impact of seasonal variations in the life histories of white-clawed crayfish on detection probability, ii) the impact of spatial and temporal factors on detection probability, and iii) the persistence and degradation rate of white-clawed crayfish eDNA.

Chapter 5: To demonstrate the application of the white-clawed crayfish assay in real-world conservation activities, enabling the research to be transformed into a commercial product, by developing standard operating procedures for the laboratory analysis and sample collection.

Chapter 3: The development and application of an eDNA-based methodology for the detection of white-clawed crayfish

3.1. Abstract

The use of eDNA based methods for species monitoring is a promising conservation tool and is now applied to an increasing number of species. Benefits such as cost and time effectiveness and increased detection sensitivities provide the opportunity for an increase in species monitoring and survey effort. However, a significant amount of thorough method development is still required for the reliable application of eDNA-based methods. Using the endangered white-clawed crayfish, *Austropotamobius pallipes* as a target organism (see chapter 2), the reliable development of an eDNA qPCR assay is demonstrated by following a thorough validation process through a number of stages of method and field testing. *In-silico* and *in-vitro* (laboratory) testing of primer and assay specificity and sensitivity was first implemented before *in-vitro* comparison of eDNA-based and classical sampling approaches. Further, differences in eDNA sample collection methods were assessed in mesocosm populations of white-clawed crayfish as well as in the field (both pond and river systems) to find the most appropriate sampling approach. The assay performed well across different field sites providing reliable presence/absence detection. Attempts at quantification of species biomass was, however, less reliable. In the comparison of eDNA sampling methods, the optimal sampling approach was found to vary across different environments. Overall, the analysis highlights the importance of thorough methodological development of eDNA-based assays. Only a critical evaluation of methodological strengths and weaknesses will allow for the ability to capitalise on the full potential of eDNA-based methods and use them as a decision support tool in environmental monitoring and conservation.

3.2. Introduction

Since its initial conception as a method for aquatic ecological surveys in the late 2000s, the use of environmental DNA or eDNA is becoming ever popular (see chapter 1, Biggs et al. 2015; Harper et al. 2019; Jerde et al. 2013; Spear et al. 2015). Clear methodological advantages and higher cost effectiveness of non-invasive sampling compared to many other established survey techniques have been excessively outlined (Goldberg et al., 2015; Huver et al., 2015; Helen C. Rees et al., 2014; Takahara et al., 2012). With a number of untested uncertainties there remain some questions over the reliable application of eDNA assays and metagenomic approaches (Mauvisseau et al., 2019a) as species survey tools. Although eDNA-based methods are frequently highlighted for improved detection rates to traditional surveys (Dejean et al., 2012; Hering et al., 2018), there are often numerous inconsistencies between the results obtained and those from the comparable traditional method used in that system or for any given species or taxa (Davis et al., 2018; Rheyda. Hinlo et al., 2017).

In the case of species-specific eDNA assays, many of these inconsistencies are contingent on the design and validation of the assay (Geerts et al., 2018; Helen C. Rees et al., 2014). Systematic method testing requires several coordinated steps, as outlined in chapter 1, addressed as hurdle 1 (validation). Initially to address hurdle 2 (detection sensitivity) the primers must be designed to ensure a high target specificity and the amplification of a suitably short sized fragment (Bylemans et al., 2018) through *in-silico* testing. *In-vivo* laboratory validation should then ascertain that the assay complies with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009) i.e. the minimum information for publication of quantitative real-time PCR experiments identifying limits of detection (LOD) and quantification (LOQ). Comparisons can then be conducted with field surveys (Smart et al., 2015). However, as mentioned above, both traditional survey approaches and eDNA-based detection are affected by various error sources creating inconsistencies (Rheyda. Hinlo et al., 2017), thus requiring careful interpretation. Further, sampling methodology should also be assessed (see chapter 1, hurdle 3), because choice of method (ethanol precipitation vs. filtration approaches) can substantially affect results (Hinlo et al. 2017).

Currently, there is contrasting evidence for which eDNA sampling method is optimal (Rees et al., 2014; Deiner et al., 2015; Dickie et al., 2018). Sampling methods (and therefore the quantity and quality of the DNA collected), may also be affected by the environment where the sample will be taken. For example, the 'optimal' method for collecting eDNA may vary between lentic (i.e. ponds or lakes) and lotic (i.e. rivers and canals) systems (Geerts et al., 2018; Harper et al., 2019). Despite reporting clear advantages of filtration for eukaryotic detection, it is still recommended that comparisons are made between sample collection strategies to ensure the most appropriate is selected for the target species (Deiner et al., 2015), due to the high level of variations in detection sensitivity that sample collection method can present on a species-to-species basis. Various factors associated with these systems have indeed been shown to influence the amount of DNA which is freely available, including; temperature, pH, flow rates and vegetation for example (Jane et al., 2015; Jo et al., 2019), all of which will vary considerably from location to location. The sampling methods (for any new eDNA assay), need to be tested under both controlled conditions (in a mesocosm for example) and under different field conditions where the target organisms are likely to be found (lotic and lentic).

The white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), is an endangered and important umbrella species in the U.K. and Western Europe as mentioned in chapter 2 (Füreder et al., 2010). Range reduction of white-clawed crayfish began in the 1860s, with declines rapidly accelerating in the UK (Fig. 2.5, chapter 2) after the introduction of invasive crayfish from north America in the 1970s (Lowery and Holdich, 1988). Moreover, the spread of crayfish plague *Aphanomyces astaci* (Schikora 1906), an oomycete pathogen carried by the North American crayfish, has greatly exacerbated the negative impact of invasive competitors, pollution and habitat degradation (Holdich et al., 2009b). Despite its legislative protection (EU Habitats Directive), white-clawed crayfish has continued to decline by as much as 50-80% over the last decade (Füreder et al., 2010). Traditional survey methods are having unsatisfactory success in monitoring crayfish populations (Gladman et al., 2010; Holdich and Reeve, 1991) highlighting the urgent need of further method development.

Consequently, the aim of this chapter was to develop a reliable eDNA assay for the detection of white-clawed crayfish. A primer set was designed and critically evaluated for

sensitivity and specificity before conducting in-depth laboratory mesocosm and field-based testing across different habitat types, whilst examining and comparing the effectiveness of existing eDNA sampling approaches.

3.3. Methodology

3.3.1. Primer design and in-silico tests

Primer/probe design and validation followed guidelines established by MacDonald and Sarre (2017) aimed for assay development of species-specific eDNA methods. The primers and probe, targeting the Cytochrome C Oxidase Subunit 1 (COI) mitochondrial gene of white-clawed crayfish, were designed *in-silico* using the Geneious Pro R10 Software (Kearse et al., 2012). The forward primer WC2302F 5' -GCTGGGATAGTAGGGACTTCTTT - 3', reverse primer WC2302R 5' – CATGGGCGGTAACCACTAC - 3' and probe WC2302P 5' - 6-FAM-CTGCCCGGCTGCCCTAATTC-BHQ-1 -3' amplified a 109bp fragment. To ensure specificity, *in-silico* tests were run against published sequences of closely related and/or co-occurring crayfish species.

3.3.2. In-vitro validation

The DNA of species which are taxonomically similar, or co-occurring were selected, and used to test the specificity of the assay *in-vitro*. These included; *Faxonius limosus* (Rafinesque, 1817), *Pacifastacus leniusculus* (Dana, 1952), *Astacus astacus* (Linnaeus, 1758), *Astacus leptodactylus* (Eschscholtz, 1823), *Procambarus clarkii* (Girard, 1852), and *Procambarus virginalis* (Lyko, 2017). DNA was extracted from crayfish tissues using the Qiagen DNeasy® Blood & Tissue kit, following manufacturers' instructions. PCRs were performed using the primers and methods from Folmer et al. (1994) and sequenced by Eurofins Genomics (Germany) to confirm species identity of all specimens. Specificity of the newly designed assay was then assessed using qPCR.

The reactions for both tissue and all eDNA samples contained; 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5µl DH_20, 1µl (10µm) of each primer, 1µl (2.5µm) of probe with the addition of 3µl template DNA. qPCR's were performed with 6 replicates of each sample on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). qPCR conditions were as follows: 50°C for 5 min, denaturation at 95°C for 8 min, followed by 50 cycles of 95°C for

30 s and 55^{o}C for 1 min. Six no template controls (NTC's) were prepared using RT-PCR Grade Water (Ambion™) alongside a duplicated serial dilution of positive control white-clawed crayfish DNA (10^{-1}-10^{-3} ng uL^{-1}) for each qPCR plate that was run.

3.3.3. Limits of detection (LOD) and quantification (LOQ)

The reliability of the assay was also assessed, following the MIQE Guidelines, which recommend the establishment of a calibration curve to determine LOD and LOQ (Bustin et al., 2009). A serial dilution of DNA extracted from white-clawed crayfish was prepared starting from 0.79ng μl^{-1} to 7.9x10^{-8} ng μl^{-1} with 10 qPCR replicates per dilution analysed. The LOD was defined as the last standard dilution that resulted in a detection of target DNA with at least one qPCR replicate at a threshold cycle (Ct) of <45. The LOQ was defined as the last standard dilution in which targeted DNA was detected and quantified in a minimum of 90% of qPCR replicates of the calibration curve under a Ct of 45 (Mauvisseau et al., 2019c).

3.3.4. In-situ validation

The reliability of the assay was further field tested by comparing eDNA-based and traditional capture-mark-recapture sampling techniques at six sites of confirmed white-clawed crayfish presence (2017) in the Centre-Val de Loire region, France. Each site was visited at least twice in subsequent nights between 22nd June and 1st of August 2018 (Table 3.1). Individual white-clawed crayfish were surveyed using a torching approach, counted and marked using a white waterproof marker stain. In the second night the survey was repeated and marked, and non-marked crayfish were differentiated. Additionally, eDNA samples (two environmental replicates, i.e. true environmental replication) were collected at each site using the 0.22µm Sterivex filters (see below for detailed description). All surveys were conducted by licensed ecologists from Fédération de Pêche et de Protection du Milieu Aquatique du Loir-et-Cher. eDNA samples were collected between the 22nd and 29th June 2018. The water volume filtered varied due to cases of high turbidity (consistent minimum volume of 150ml, see Table 3.2 for list of all sample volumes). Further to the method below, eDNA filters were fixed with 2ml of ethanol to accommodate for the longer storage and transport time between the field and the laboratory. All sampled locations are part of an extensive monitoring programme for white-clawed crayfish population studies and

due to conservation reasons, locations of sites are not reported. The water temperature, and the volume of water filtered (which varied due to turbidity) were recorded at each site.

3.3.5. Ex-situ comparison of eDNA sampling methodologies

Another objective of this chapter was to assess the impact of eDNA sampling methodology on both the probability of eDNA detection and the signal strength (i.e. Ct) of its detection. Differences between the most common eDNA sampling methods (utilised to date) were tested, including (i) ethanol precipitation (Biggs et al., 2015), (ii) 2µm pump-based filtration (Strand et al., 2014), (iii) 0.45µm pressure filtration and (iv) 0.22µm pressure filtration (Spens et al., 2017). All methods were assessed in two mesocosms, housed at Bristol Zoological Gardens, Bristol, UK, during autumn 2018. Both mesocosms were designed to the same specifications but contained different water volumes and crayfish numbers. Mesocosm 1 had a volume of 3000L and contained 249 individual adult white-clawed crayfish and sub-adults (between 17 months and four years old) with a total biomass of 1.3kg. Water parameters of mesocosm 1 were; pH 8, temperature 11°C, and under natural light conditions. Mesocosm 2 contained a volume of 1000L of water with the same pH (8) but with higher water temperatures (16°C) and under artificial light conditions. This mesocosm held a larger number of crayfish (379) but all were juvenile (five months old). The sensitivity of juveniles to handling did not allow an exact biomass of this mesocosm to be recorded, but biomass was estimated as 250g. Both mesocosms were set up as recirculating 'flow through filtration systems', ensuring high water quality at all times. Six samples for each method and mesocosm combination were collected from both mesocosms.

eDNA samples classified hereafter as 'precipitation' samples were collected following the protocol outlined in Biggs et al. (2014). 1L of water (20 x 50ml subsamples) was collected from ~20cm below the surface and after homogenization, a subsample of 90 ml (6x 15ml) was aliquoted into sterile tubes containing a pre-mixed buffer solution of 100% ethanol and sodium acetate 3M pH 5.2 (Biggs et al., 2014). Samples were stored at -20^{o}C prior to extraction and extracted using the protocol of Tréguier et al. (2014).

Table 3.1. Crayfish survey data and sample collection dates at each site as part of the *in-situ* validation project, collected from six sites (undisclosed for conservation reasons) of confirmed white-clawed crayfish presence (2017) in the Centre-Val de Loire region, France.

Site	Survey 1	No. captured 1	Survey 2	No. captured 2	No. recaptured 2	Survey 3	No. captured 3	No. recaptured 3	Survey 4	No. captured 4	No. recaptured 4
1	26/06/2018	3	28/06/2018	5	0						
2	26/06/2018	9	28/06/2018	10	0						
3	05/07/2018	33	11/07/2018	62	2	31/07/2018	68	0	01/08/2018	110	5
4	03/07/2018	0	21/07/2018	0	0	01/08/2018	0	0			
5	04/07/2018	40	06/07/2018	45	17						
6	09/07/2018	84	12/07/2018	75	11						

Table 3.2. eDNA sampling collection dates and volume filtered at each site as part of the *in-situ* validation project, collected from six sites (undisclosed for conservation reasons) of confirmed white-clawed crayfish presence (2017) in the Centre-Val de Loire region, France.

Site	Date	Time	Filtered volume	Water temp °C
1	22/06/2018	09:50	500ml	12.4
2	22/06/2018	11:05	1000ml	12.5
3	22/06/2018	15:50	500ml	14.1
4	22/06/2018	18:06	150ml	14.8
5	29/06/2018	11:40	1000ml	19.0
6	29/06/2018	15:00	250ml	16.7

eDNA samples collected with a 2µm pump-based filtration consisted of 2L of water collated by the same sub-sample method outlined above but were then filtered through a Millipore Glass fibre filter AP25, 47mm (2µm pore size) using a peristaltic pump (Masterflex E/S Portable Sampler, Cole-Parmer, USA). The filter was housed in an In-Line Filter Holder 47mm (Merck) connected by silicone tubing. The combined use of a peristaltic pump and a larger filter pore size resulted in an increase in the volume of water filtered. The filter was then removed from the pump system and stored at -20ºC before extraction. Equipment was soaked and cleaned with 10% bleach between samples. Filters were extracted following Spens et al. (2017). eDNA sample collections for 0.22µm and 0.45µm pressure filtration were undertaken in the same manner. 20 sub-samples were collected and collated and a 50ml syringe (BD Plastipak™, Ireland) was then used to pressure filter 250ml of water through a sterile enclosed filter (Sterivex™, Merck®, Germany) with either a pore size of 0.22µm (Polyethersulfone membrane) or 0.45µm (Polyvinylidene fluoride membrane). All filters were stored at -20°C, and extracted following Spens et al. (2017).

3.3.6. In-situ comparison of eDNA sampling methodologies

Complementary to the tests in the mesocosm experiment, sampling methodologies under natural conditions were also evaluated. However, only pairwise method comparisons were performed in order to contain sampling effort in the field. As a test in a lentic system, eDNA samples were collected from a 1000m^2 pond in the South West of England after the release of 40 white-clawed crayfish individuals (equal juvenile-adult and male-female ratios, total biomass of 436g). Here, ethanol-precipitation (sample volume: 90ml) was compared against 0.22µm pressure filtration (sample volume: 250ml). Sampling started on the 20th April 2018 and was repeated two hours, seven days, 14 days and 35 days after crayfish release. At each sampling time, three environmental replicates were taken from four sites around the pond for each method. Additionally, 20 50ml sub-samples taken from the entire pond perimeter were pooled, homogenised and sampled with three environmental replicates per method.

The second field test was conducted in a lotic system. 10 sites were sampled (situated approx. 1km apart) along a chalk stream river in Dorset (UK), during September 2017, and 4 sites along a river in Derbyshire (UK). Here, ethanol precipitation was used in comparison to pump-

based filtration (2µm, sample volume: 2L), using three environmental replicates at each site per method (*n* = 42). Samples collected in the river system (20 pooled sub-samples as described above) were taken in an interval of 1-2m along a diagonal downstream-to-upstream transect across the river. In this field test, the ability to screen for crayfish plague using both sampling methods was also assessed. qPCRs in this instance were run using the primers and probe developed by Strand et al. (2014), which is an assay approved for use for national crayfish plague monitoring in Norway (Strand et al., 2019).

3.3.7. Statistical analysis

Samples measured for the establishment of a standard curve were analysed using a linear regression to evaluate the relationship between DNA concentration and Ct. A log-log data transformation decreased the models Akaike Information Criterion (AIC) and was therefore used for downstream analyses. Residuals were tested for autocorrelation, normal distribution and any remaining patterns (same procedure applied in all regression analyses). A logistic regression analysis was also applied to test the relationship between DNA concentration and binomial detection data, assessing the change of detection probability with DNA concentrations. For the mesocosm and field samples, the relationship between (i) the population density established by traditional sampling methods and (ii) the Ct values and detection probability (calculated as the fraction of qPCR replicates that resulted in positive detection) of eDNA measurements were examined in a linear regression model. Differences in sample volumes between locations (due to turbidity) were accounted for by including sample volume as a predictor in regression models, and log-log and untransformed models were compared using AIC. Further, Ct and detection probability of different sampling methods were compared using ANOVA analyses followed by Tukey's HSD post-hoc tests, and t-tests or nested ANOVA's (lotic and lentic systems, where only two methods were compared). Prior to ANOVAs, heteroscedasticity was evaluated, and data transformed if necessary. All described statistical analyses were performed using R version 3.4.1 (R Core Team 2018).

3.4. Results

3.4.1. Assay development and in-silico and in-vitro validation

Primers and probe were highly species-specific as *in-silico* and *in-vitro* tests did not reveal any matches with non-target species (Table 3.3). Analysis of the standard curve (Fig 3.1A) revealed a strong dependency of Ct values on DNA concentrations (y=-1.73x+20.8, $p<0.001$, $r^2= 0.993$). Likewise, the detection probability was also positively related to DNA concentration in the sample (y=-0.18x+1.39, $p=0.0016$, $r^2=0.804$; Fig 3.1B), highlighting the possibility of a quantifiable assay being developed. Method sensitivity analyses revealed a LOD of 7.9 x 10^{-5}ng and a LOQ of 7.9 x 10^{-4}ng crayfish DNA extract per μl^{-1}.

3.4.2. In-situ validation

Populations of white-clawed crayfish were found in five out of the six surveyed sites using traditional survey methods. eDNA-based detection indicates the presence of white-clawed crayfish in all six sites, though the site with no visual white-clawed crayfish sightings was characterised by a very low detection probability. The Ct values from the six river sites were converted into DNA concentrations using the calibration curve, which allowed comparisons to be made on the relationship between detection probability and DNA concentration in laboratory and field samples (Fig 3.1B). Four out of the six field sites lay outside of the 95% confidence interval of the standard curve, indicating systematic differences between *in-vitro* validation and field samples. The relationship between the mean number of crayfish detected using traditional survey methods (torching) and detection probability of eDNA measurements (Fig 3.1D) was significant, but only when water temperature was included ($y=0.0118x_1-0.117x_2+1.77$; x_1=mean survey count, x_2=temperature, $p=0.035$, $r^2=0.82$). The relationship between Ct and the mean number of crayfish detected using torching was marginally non-significant but showed a reasonable model fit (Fig 3.1C; y=-0.00067log(x)+3.76, $p=0.079$, $r^2=0.47$). Differences in filtered sample volume did not significantly influence results.

Table 3.3. Table showing mismatches between the species-specific primers (WC2302) and the respective COI targeting sequence in white-clawed crayfish and species closely related or likely to co-occur within UK water systems. Base pair mis-matches are illustrated with a * and highlighted in grey and matches in bases are highlighted in white.

Forward Primer		GCTGGGATAGTAGGGACTTCTTT
Probe (reverse compliment)		GAATTAGGGCAGCCGGGCAG
Reverse Primer (reverse compliment)		GTAGTGGTTACCGCCCATG
		1 23 43 62 91 109
AB443445.1	**A.pallipes**	**GCTGGGATAGTAGGGACTTCTTT GAATTAGGGCAGCCGGGCAG GTAGTGGTTACCGCCCATG**
EU921148.1	P.leniusculus	GCTGGTATAGTGGGAACTTCTCT GAATTAGGTCAACCTGGAAG GTTGTAGTCACGGCACATG
GU727619.1	A.astacus	GCTGGGATAGTAGGAACCTCTTT GAACTCGGTCAACCTGGGAG GTAGTAGTAACTGCTCATG
KP205431.1	F.limosus	GCTGGCATAGTAGGAACTTCATT GAGTTGGGTCAGCCGGGAAG GTGGTAGTTACAGCTCATG
JQ421465.1	A.leptodactylus	GCTGGAATAGTGGGAACCTCTTT GAACTAGGTCAACCAGGGAG GTCGTAGTAACTGCTCATG
KC499604.1	P.clarkii	GCTGGTATAGTCGGAACTTCATT GAGTGCAGTCAACCAGGAAG GTGGTAGTTACAGCTCATG
LC228303.1	P.fallax	GCTGGTATAGTAGGGACTTCATT GAGTTAGGTCAACCTGGTAG GTAGTAGTTACAGCTCATG
KY745779.1	C.quadricarinatus	TCCGGTATAGTAGGCACTTCCCT GAACTTGGTCAACCAGGAAG GTAATCGTCACAGCCCACG
MF744674.1	F.virilis	GCTGGGATAGTAGGGACTTCATT GAGTTAGGTCAGCCAGGAAG GTGGTAGTTACAGCTCATG
MH235946.1	P.acutus	GCTGGGATAGTAGGGACTTCATT GAGTTAGGTCAGCCAGGAAG GTAGTAGTTACAGCTCATG
		* * * * * ** ***** * * * * ** * * * * *

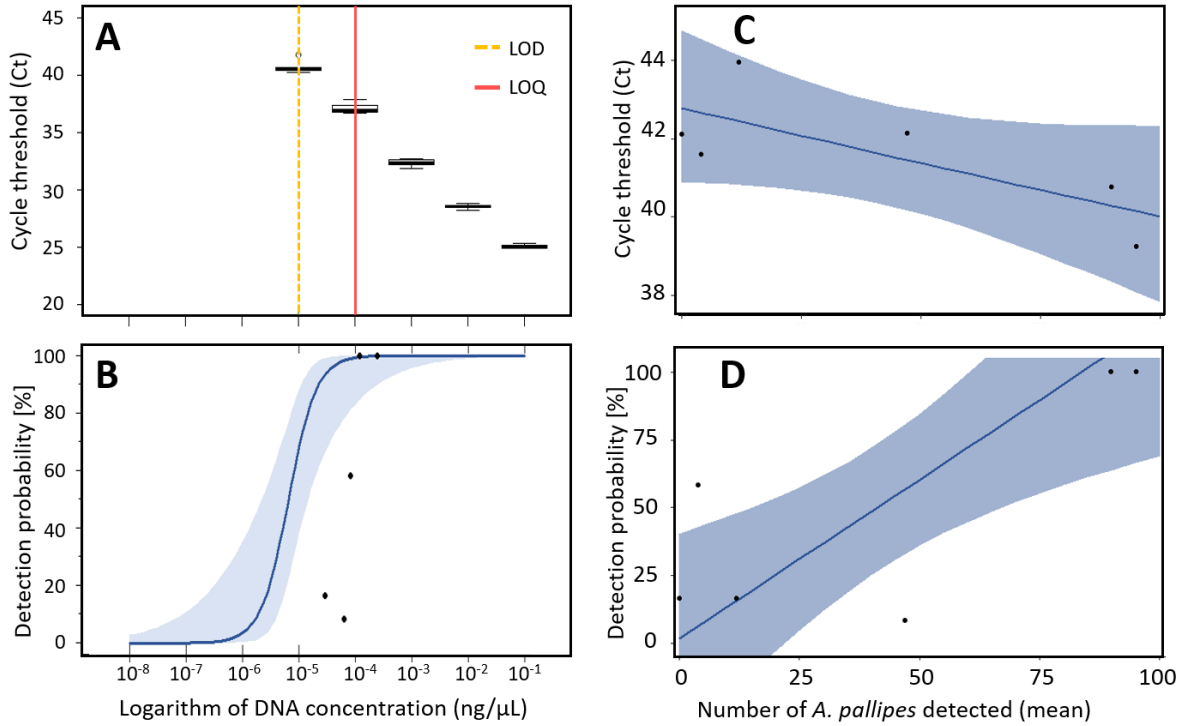

Fig 3.1. (A) Relationship between cycle threshold (Ct) and DNA concentration from white-clawed crayfish qPCR calibration curve. Limit of detection (LOD) and limit of quantification (LOQ) are illustrated by vertical lines (dashed-yellow and red respectively). **(B)** Change in detection probability with increasing DNA concentration and calibration curve data. **(C)** Relationship between Ct values and white-clawed crayfish population monitored using traditional method. **(D)** Relationship between detection probability of eDNA and traditionally evaluated mean crayfish population numbers. The blue line and the light-blue shaded area reflect the results of a logit regression and its 95% confidence interval, respectively. The black points represent data from the *in-situ* or *ex-situ* validation experiment. Four out of six data points were outside the established confidence interval in **(B)**, indicating discrepancies between field and laboratory-based data sets.

3.4.3. Comparison of eDNA sampling methods

In mesocosm experiments, sampling methodology had a significant impact on detection probability (ANOVA $F_{(3,44)}$=74.48, p<0.001). Pairwise comparisons revealed that detection probabilities of all three filtration-based methods (2µm, 0.22µm and 0.45µm) were comparable (p>0.05) but differed significantly from the precipitation method (p<0.001, Fig 3.2A). However, the p-value for the comparison between 0.45µm and 2µm was marginally non-significant (p=0.051). Similarly, methodologies also differed significantly in Ct (ANOVA $F_{(3,178)}$=90.1, p<0.001). However, in contrast to detection probability, pairwise tests indicated a difference between the 2µm filtration method and all the other approaches (p<0.001; Fig 3.2B; only samples with positive detection were included in the analysis).

In-situ comparisons of sampling methods in a lentic system were highly comparable to the mesocosm experiment (Fig 3.3 A-B). The precipitation method showed a significantly lower detection probability (T-test, t=3.55, df=75.37, p<0.001) and a significantly higher Ct (t=-2.46, df=15.72, p<0.05) than the filtration-based method (0.22µm). However, contrasting results were attained in lotic systems. Here, the method was assessed for both, white-clawed crayfish and the crayfish plague (not present in mesocosms or ponds). The detection probability of crayfish plague mirrored findings from other systems showing significantly higher detection probabilities for the 2µm filtration method (nested ANOVA; $F_{(1,69)}$=4.92, p<0.05; Fig 3.3E). Ct values were not significantly different, but also indicated a better performance of the filtration-based method (Fig 3.3F). However, the results for white-clawed crayfish contrasted all other results. In lentic systems, precipitation resulted in a higher detection probability (nested ANOVA $F_{(1,69)}$=13.77, p<0.001, Fig 3.3C) and accordingly, lower Ct values (nested ANOVA; $F_{(1,34)}$=5.24, p=0.028; Fig 3.3D). Consequently, filtration-based methods performed consistently better except in lentic systems where eDNA from white-clawed crayfish was more reliably assessed with the precipitation method.

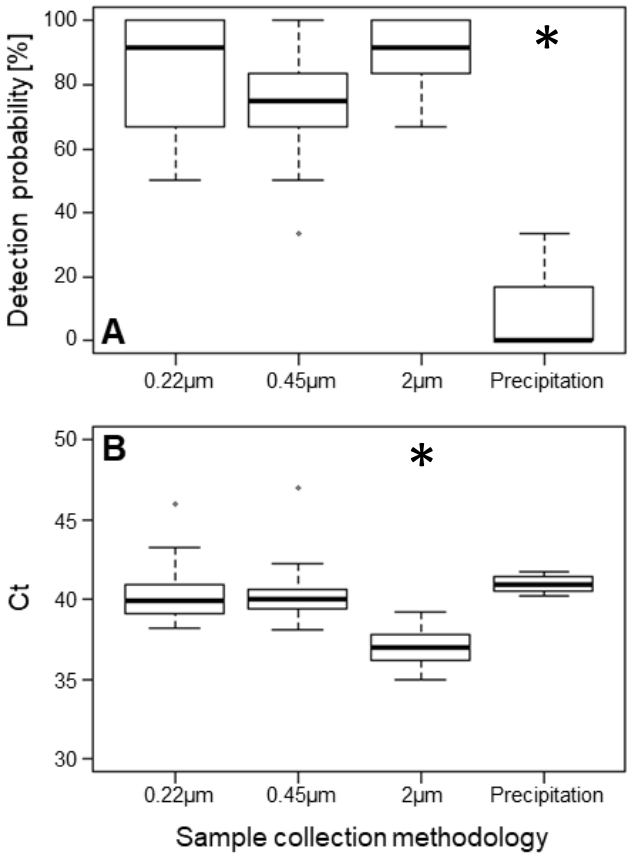

Fig 3.2. Comparison of the detection probability **(A)** and Ct values **(B)** of different white-clawed crayfish eDNA sampling methods (0.22µm filtration, 0.45µm filtration, 2µm filtration and ethanol precipitation) in a controlled mesocosm experiment (* indicates statistical significance).

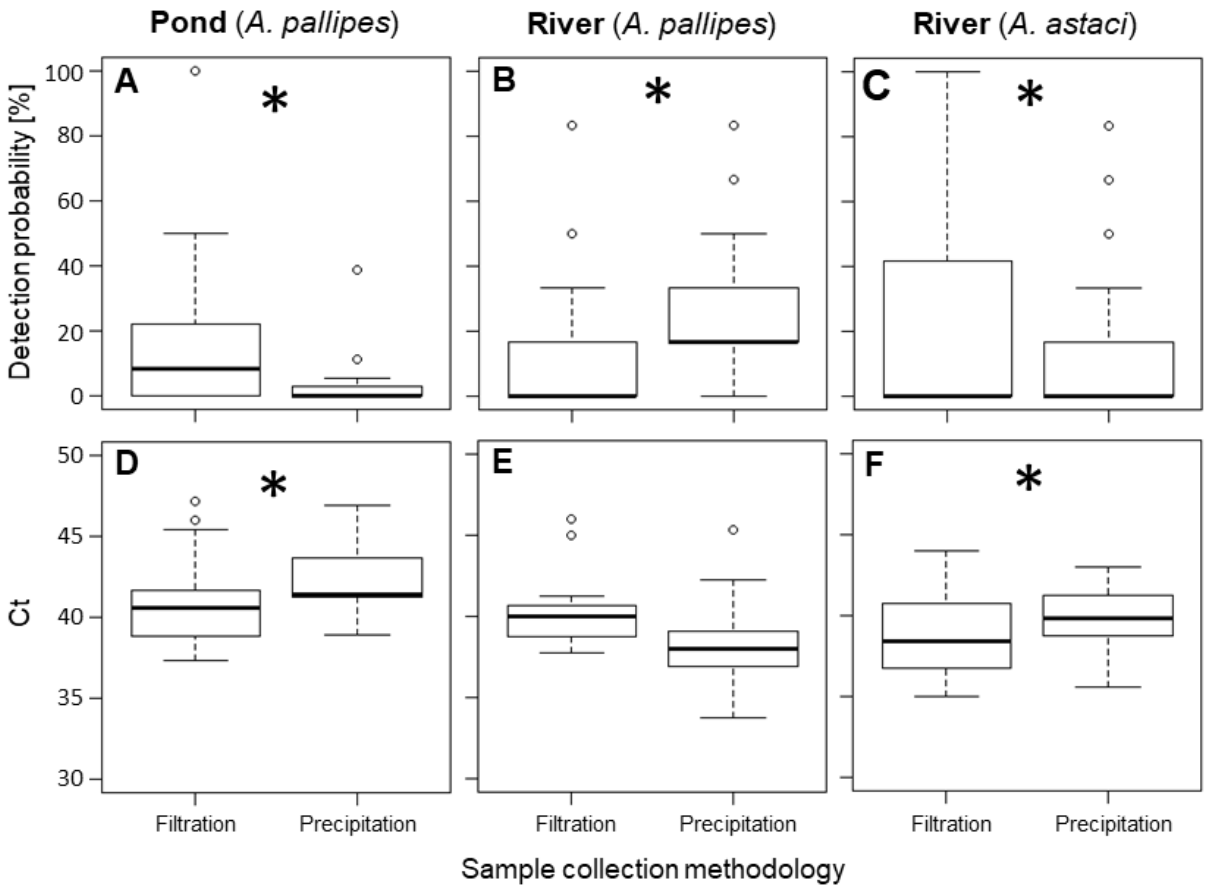

Fig 3.3. Comparison of the detection probability **(A, B, C)** and Ct values **(D, E, F)** of different eDNA sampling methods (filtration and precipitation) for white-clawed crayfish in a lentic system **(Pond: A, D)** (filter pore size 0.22 µm) and for both white-clawed crayfish **(River: B, E)** and crayfish plague **(River: C, F)** in the same lotic system (filter pore size 2 µm) (* in panels signifies significant differences between pairwise method).

3.5. Discussion

Native crayfish species across Europe are threatened by invasive competitors and the jointly introduced crayfish plague, resulting in a downward trajectory of native species' abundance and distribution (Holdich et al., 2009b). This chapter presents the development of a novel assay for the detection of white-clawed crayfish, a flagship conservation species in Western Europe. In rigorous *in-vitro* and *in-situ* tests, the reliability of the assay is evaluated under various environmental conditions. The assessments demonstrated that the assay was highly reliable for detecting the presence and absence of white-clawed crayfish but preformed less well for quantifying species abundance, illustrating the challenged posed by hurdle 7, 'quantification' (see chapter 1). Further, it is reported that the optimal choice of sampling method was dependent on habitat type, highlighting the necessity to incorporate multiple independent validation steps in method design before the practical application of assays.

Field comparisons indicated a higher sensitivity of the eDNA assay compared to traditional surveys, which only resulted in positive detection in five out of six sites. Whilst higher sensitivity is frequently reported for eDNA assays (Dejean et al., 2012; Jerde et al., 2011; Smart et al., 2015), such results should be interpreted with caution as eDNA-based approaches are associated with a risk of providing false positive results (Furlan et al., 2016). One possible cause of false positives is the downstream transport of eDNA within river networks (Pont et al., 2018). Moreover, false positives may result from historic eDNA, which is still present after the extinction or emigration of the target species (Turner et al., 2015). This represents a valid hypothesis as all field sites were populated by white-clawed crayfish a year before these field surveys (C. Mauvisseau, *personal communication*). Consequently, it remains inconclusive whether the developed eDNA-based approach truly has a higher sensitivity (i.e. false negative of torching method) or, more unlikely that white-clawed crayfish was not present at the field site in question or has recently become locally extinct.

Despite the differences in sampled water volumes in the *in-vivo* experiment, a minimal effect is observed between the detection probability of the calibration curve and the detection probability of these samples. However, despite showing a similar pattern, four out of six eDNA samples lay outside the 95% confidence interval of the model which could be explained by the presence of inhibitory substances delaying the qPCR amplification (Foote et al., 2012; McKee et al., 2015; Schrader et al., 2012).

Currently, many species-specific eDNA assays only cover *in-silico*, *in-vitro* and sometimes basic *in-vivo* validation steps (Baldigo et al., 2017; Dickie et al., 2018; Egan et al., 2017; Lacoursière-Roussel et al., 2016), addressing only in part hurdles 1 and 2. Already published white-clawed crayfish eDNA assays have yet to go through the thorough level of *in-vivo* evaluation required (Atkinson et al., 2019; Robinson et al., 2018). In addition, multiple validation steps are required in order that an assay may be utilised in commercial or government sanctioned monitoring programmes, which need to yield consistent results under different environmental conditions largely independent of weather or seasons. A key step of method validation is assessing the effect of sample collection approach (precipitation/filtration) on reliability i.e. detection probability. Indeed, the importance of sampling method (chapter 1, hurdle 3) has been highlighted for a number of eDNA assays with the goal of ascertaining the 'optimal' method to be used (Deiner et al., 2015; Rheyda Hinlo et al., 2017). In this study the effect of a number of eDNA sampling methodologies had on the ability to detect populations of white-clawed crayfish is explored, both in the field and in mesocosms. In the controlled mesocosms, filtration approaches clearly out-performed ethanol precipitation of eDNA. However, there appeared to be no 'optimal' filtration method as each performed similarly well. That said, in this instance a large volume of 2 litres of water was filtered through the larger filter used (2µm) and this resulted in a lower Ct value compared to when filtering with either 0.22µm or 0.45µm. This is in accordance with other studies which have shown filtration to be more reliable (Eichmiller et al., 2016b; Vörös et al., 2017). One factor which may have impacted comparisons and possible reason for the lower detection rates of crayfish using filtration within the river system study may have been the absence of a preservative solution (Spens et al. 2017), which if added to the filtered samples may have prevented any immediate DNA degradation between sampling and DNA extraction.

An alternative explanation for these differences in method suitability between different environments is linked to inhibition of eDNA amplification (see chapter 1, hurdle 8 'inhibition and contamination'). Inhibitor compounds (that interfere with qPCR processes), have been shown to affect target DNA amplification in a non-linear way (Goldberg et al., 2016). If inhibitor concentration is low, amplification will not be strongly impacted. However, if concentrations surpass a certain threshold, inhibitors may suppress the amplification of even high concentrations of target eDNA (Mauvisseau et al., 2019b). Sampling methods that differ in their water collection volumes and in the amount of concentrated target eDNA, will also concentrate

inhibitors to different degrees (Fig 3.4). Consequently, sampling methods that reach higher target eDNA concentrations may show a lower overall performance due to the non-linear relationship between inhibitor concentrations and DNA amplification. This scenario will occur when inhibitors are present in high concentrations and efficiently concentrated. Therefore, different ratios between target eDNA and inhibitors in different environments can cause a shift in the relative performance of sampling methods across habitats (Fig 3.4). In this case, tests for inhibition were not included, however, these could have included the addition of synthetic DNA to qPCR reactions (i.e. failure to detect synthetic DNA indicates inhibition; (Goldberg et al., 2016; Mauvisseau et al., 2019b). Such inhibition tests should therefore be included in future field method comparisons.

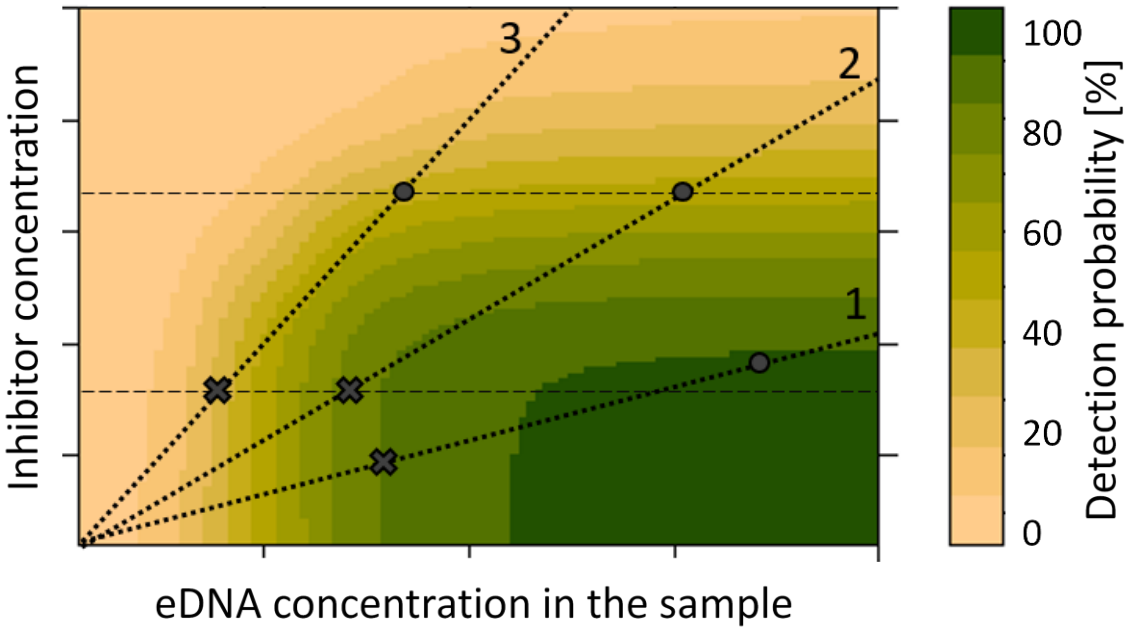

Fig 3.4. Schematic of the co-dependency of detection probability on the concentrations of target eDNA and of inhibitors in water samples. Detection probability increases with eDNA concentration and decreases with inhibitor concentrations but is low when both variables are high. Each water body is characterised by a certain ratio between inhibitor and target eDNA concentrations represented by black dotted lines (**1**-**3**). A change in sampling methods accompanied by a change in the sampled water volume will result in different concentrations of target eDNA and inhibitors in the sample and in shifts along dotted lines (grey crosses and dots). An increase in sampled water volume will therefore in some water bodies increase (Line 1) and in others decrease (Line 2) detection probability. The same is true when different eDNA assays in the same water body are considered. While eDNA concentrations of two targets may differ, inhibitor concentrations will be the same. Consequently, samples with the same water volume will have the same inhibitor concentrations (horizontal dashed lines). Nevertheless, changes in sampling volume and method can result in increased detection probability for one target (Line 3) but not for the other (Line 2).

Both inhibition and variation in target-eDNA size distributions may also explain the differences in method comparisons observed between any given species within the same environment, e.g. for both white-clawed crayfish and crayfish plague in the lotic habitats sampled in this study (Fig 3.3). A fundamental distinction between the two species is that crayfish plague depends for its proliferation on the frequent and abundant release of encapsulated spores (~8 µm in diameter). It seems likely that these spores, which are designed for transport along large distances, will show lower sensitivity to degradation than white-clawed crayfish DNA, which potentially could explain the species-specific results.

The contrasting results between the sampling methods, highlight that no single 'universally optimal' method can be identified, supporting previous work accrediting such variation down to the heterogeneity of different water systems (Dickie et al., 2018). These findings highlight the necessity to test each assay under different field conditions. Testing is recommended in both lotic and lentic habitats of varying conditions to ensure that the appropriate sample collection method is chosen for the target species, thereby gaining the highest probability of detection should the species be present. Indeed, eDNA within such systems will also likely be governed by seasonal variations (Buxton et al., 2018), persistence/degradation rates (Goldberg et al., 2018), turbidity (Williams et al., 2017), downstream flow, transport, pooling and dilution effects (Jane et al., 2015). Transport of eDNA can be difficult to model and interpret (Deiner and Altermatt, 2014) due to the aforementioned habitat heterogeneity, with frameworks to understand eDNA movement only now beginning to develop (Shogren et al., 2017). Further detailed research is necessary in this area to address the effect that these variable factors can have on the detection probability on a species-to-species basis.

3.5.1. Conclusion

To conclude, the heterogeneity of different water systems and stochasticity of eDNA can significantly affect eDNA detection probabilities and therefore the success of each method utilised. Rigorous testing of an eDNA assay to ascertain 'optimum' sampling strategies should be conducted to account for the variability that different environmental conditions can have on each methodology. It should also be noted, that even when a method shows higher performance (i.e. increased detection probability or lower Ct scores), it may not always be the most practical. Such practicality needs to be taken into account if eDNA is to be utilised to its full potential i.e. by end users such as ecological consultants and government agencies (Lugg et al., 2018). The

development and full-scale validation of eDNA assays for white-clawed crayfish paves the way for more detailed ecological studies to improve crayfish management and understanding of the environmental factors affecting the species. With further research into the importance of other eDNA concentration affecting factors, such as flow, inhibition, eDNA degradation and seasonality, validated eDNA detection for white-clawed crayfish could positively transform the conservation effort for the species.

Chapter 4: Seasonality, DNA degradation and spatial heterogeneity as drivers of eDNA dynamics

4.1. Abstract

In recent years, eDNA-based assessments have evolved as critical tools for research and conservation. Most eDNA-based applications rely on comparisons across time or space. However, temporal and spatial dynamics of eDNA concentrations are shaped by a number of drivers that can affect the reliability of such comparative approaches. Here in this chapter, (i) seasonal variability, (ii) degradation rates and (iii) micro-habitat heterogeneity of eDNA concentrations were assessed, representing three key factors that potentially inflict increased measurement uncertainty. Again, using white-clawed crayfish, *Austropotamobius pallipes*, as the model species (chapter 2), this chapter highlights that seasonal variation in detection probabilities can range from 20 to over 80% in controlled mesocosm studies. Further, slow degradation rates resulted in eDNA detection 14-21 days after the removal of the target species. Finally, substantial small-scale *in-situ* heterogeneity of eDNA detection between sites was recorded in a pond of merely 1000m^2 in size. Hence, all three tested drivers of spatial and temporal variations may severely impact the reliability of eDNA-based applications and need to be accounted for in sampling design and data analysis.

4.2. Introduction

Environmental DNA assays are now being published for an expanding range of species with an increasingly more diverse range of technological advancements and approaches being applied (e.g. CRISPR; Williams et al. (2019). However, several questions remain regarding the reliability of the application of such assays, due mainly to variations in eDNA concentrations within the water, both across space and time. Spatio-temporal factors (representing hurdles 4, 5 and 6, see chapter 1) including seasonal variations (Buxton et al., 2017; De Souza et al., 2016), eDNA degradation (Goldberg et al., 2018; Jo et al., 2019; Mauvisseau et al., 2018) and sampling site location (O'Donnell et al., 2017; Tillotson et al., 2018) are often reported to significantly drive the amount of eDNA in a given system. This in turn effects the ability to detect and quantify the success rate of any eDNA-based survey method (Goldberg et al., 2018; Kamoroff and Goldberg, 2018; Lawson Handley et al., 2019). While the importance of factors determining the distribution and concentration of eDNA is widely acknowledged, researchers are still at the very beginning of understanding these variable factors and applying them to species-specific assays (Collins et al., 2018; Dejean et al., 2011).

Temporal influences on eDNA, such as seasonal conditions (weather, food availability, changes in hydrology) and species-specific life histories (mating, spawning, moulting) can impact the detection probability and ability to quantify population size at different times of the year (Buxton et al., 2018; De Souza et al., 2016; Erickson et al., 2017; Wacker et al., 2019). Studies into the eDNA of the great crested newt (*Triturus cristatus*), report that detection probabilities vary year round, with the most reliable detection during the spring and summer months (Buxton et al., 2018; Rees et al., 2017). A similar result has been observed with the freshwater pearl mussel (*Margaritifera margaritifera*) where a 20-fold increase in eDNA concentrations was observed from late spring to late summer, during their reproduction period (Wacker et al., 2019). Indeed, eDNA detection probability has been linked to different life stages of other species such as signal crayfish (*Pacifastacus leniusculus*) with an increase occurring when eggs are present (Dunn et al., 2017). Despite such temporal variation, the sensitivity of eDNA-based methods, allow for the detection of any given species over longer seasonal survey periods than traditional ecological methods and eDNA also benefits from being non-invasive in its approach (Buxton et al., 2018; Dejean et al., 2012).

Another influencer of eDNA concentration is the degradation rate of eDNA (hurdle 5), and therefore the time for which it persists within the environment. This can have a significant impact on the detection or quantification of a species using eDNA-based methods (Goldberg et al., 2018), and as such if not assessed and interpreted effectively can lead to inaccurate (false positive or false negative) species detection probabilities (Barnes et al., 2014). Degradation of eDNA is reported to vary across species, habitat, and environmental conditions (Collins et al., 2018; Shogren et al., 2018; Williams et al., 2018) and can be influenced by factors such as temperature and UV (Goldberg et al., 2018), microbial community composition (Barnes et al., 2014), pH (Seymour et al., 2018) and conductivity (Barnes et al., 2014). Degradation rate of eDNA within aquatic environments has been shown to occur in hours or days in some instances (Piaggio et al. 2015; Thomsen et al. 2012a; Barnes et al. 2014; Maruyama et al. 2014; Turner, Uy and Everhart 2015), to as much as weeks or months (Dejean et al., 2011; Goldberg et al., 2013). Even longer degradation rates have also been shown to occur in sedimentary environments (Mauvisseau et al., 2018; Turner et al., 2015). Therefore, care is needed when interpreting results to eliminate the possibility of false positives. Due to variation in degradation rates across taxa, assessing such in any species where a new assay is designed should be mandatory.

Finally, spatial factors (which are linked to hurdle 4) are also important to consider when utilising eDNA-based detection for a species, especially for rare species of low abundance (Moyer et al., 2014). eDNA is often stochastically distributed within water systems, for example through unequal distributions of target organisms (Lacoursière-Roussel et al., 2016), flow constraints (Deiner and Altermatt, 2014; Jane et al., 2015), and stratification in lakes (Moyer et al., 2014). It is therefore key to select the most appropriate strategy when sampling a site, to ensure reliable species detection (Furlan et al., 2016; Goldberg et al., 2018). In particular, an inappropriate sampling design in this regard (i.e. insufficient spatial location of sample collection points) may lead to inaccurate results. However, it is not always possible to collect a 'gold-standard' sample from a site (e.g. overgrowth blocking access to a proportion of pond), so there is a requirement for more understanding of the effect of non-optimal sample collection design on eDNA detection probability.

Populations of the white-clawed crayfish, (*Autropotamobius pallipes*) have declined between 50 and 80% across Europe since 2000 making the species classified as endangered by the International Union for Conservation of Nature (IUCN) (chapter 2, Füreder et al. 2010). Three

independent eDNA assays have now been developed for white-clawed crayfish (see chapters 2 and 3, (Atkinson et al., 2019; Robinson et al., 2018), and these will hopefully benefit conservation efforts for this species. However, there is currently little understanding on temporal and spatial variations in eDNA concentrations and how these will impact the applicability of these assays. Using white-clawed crayfish as a model species, assessments were made on: i) the impact of seasonal variations in the life histories of white-clawed crayfish on detection probability and ii) the impact of spatial and temporal factors on detection probability. Further, the persistence and degradation rate of white-clawed crayfish eDNA was examined.

4.3. Methodology

4.3.1. eDNA degradation experiment

The degradation rates of crayfish eDNA were examined in a controlled mesocosm experiment. Mesocosms were set up as three independent tank systems, each containing a water volume of 52L and a sediment layer consisting of fine gravel and a few large pebbles. The three tanks were installed outside, under shelter (protection from rain and birds; see Fig. 4.1 for temperature) on the roof of Bristol Zoo and housed 16, 14 and 15 adult white-clawed crayfish with a total biomass of 190, 172 and 183g, respectively. Crayfish were kept for two months (as part of a licensed captive breeding programme at the zoo) and removed from the tanks on the 19th October 2017. eDNA samples were collected 20 hours and 1 hour before crayfish removal and at 18 time points after their removal (0.1h, 1h, 6h, 24h, 32h, 48h, 3d, 4d, 5d, 6d, 7d, 8d, 14d, 21d, 28d, 35d, 42d and 56d). eDNA sampling was based on the ethanol precipitation method outlined in Biggs et al. (2014) and in chapter 3. In brief, two environmental replicate samples were taken from each system. Each one of these environmental replicates consisted of 20 collated subsamples of 50 ml (equating to a total of 1L of mesocosm water). After homogenisation, 90ml of each replicate were distributed among 6 x 50ml tubes containing a pre-mixed molecular grade ethanol and sodium acetate 3M, pH 5.2 solution (Biggs et al., 2014). Samples were stored at -20°C until extraction. At the last sampling event 56 days after the crayfish removal, the sediment at the bottom of the tank was intentionally disturbed and an additional sample was taken afterwards. This allowed us to test whether remnant (ancient) DNA that became undetectable in the water column was still present in the sediments as sediment disturbance could lead to a positive detection in the absence of the target species.

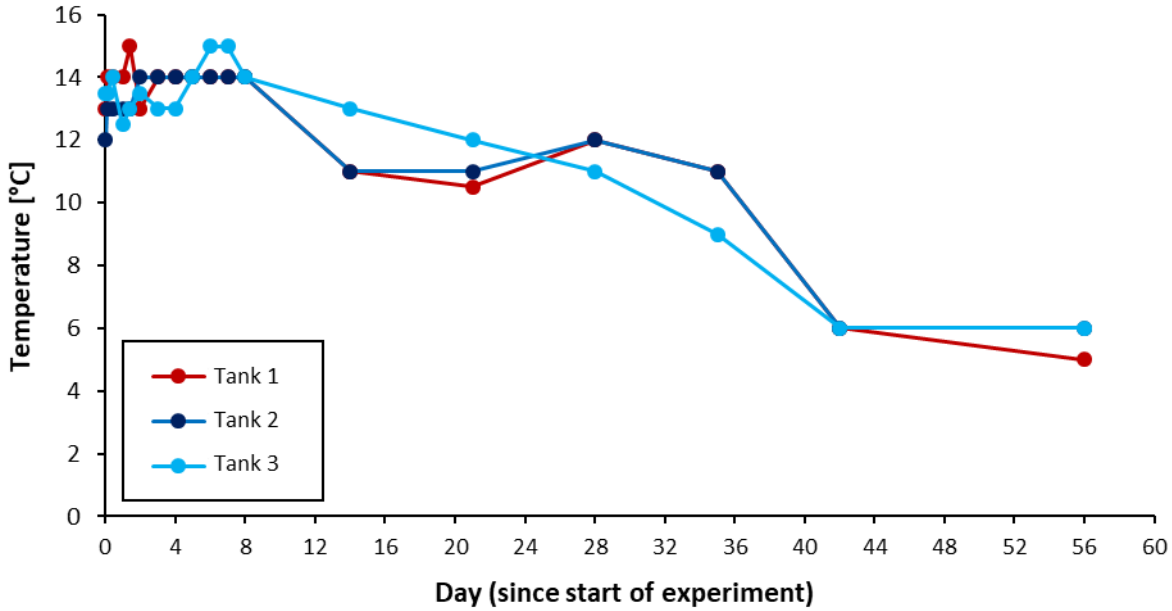

Figure 4.1. Mesocosm temperature changes over the experimental period.

4.3.2. Seasonal dynamics in eDNA concentrations

In the second experiment, the impact of seasonal changes in crayfish behaviour and physiology was assessed on the temporal dynamics of eDNA concentrations under controlled laboratory conditions. The experiment was set up on the 29th September 2017 in a 3000L tank designed for the captive breeding of white-clawed crayfish. Tank design is described in Nightingale et al. (2017) and has been developed as a suitable environment for white-clawed crayfish cultivation. In brief, it consists of 13 interconnected polyurethane tanks, each with a gravel bedded area of 0.46m^2, set up outside with protection against birds and rain. Temperature was controlled (Nightingale et al., 2017) to reflect in-situ variations in natural habitats (seasonal cycles ranged between 5°C and 20°C). Water quality was maintained through mechanical and biological filtering and UV treatments. Additionally, 25% of the water volume was exchanged with fresh water every week. The tank system contained a large breeding population (as part of a licensed captive breeding programme at Bristol Zoo) of 198 adult individuals (75 male, 124 female) with a total initial biomass of 924.75g. Adult mortality rates throughout the experiment were below 6% per month. For optimising breeding success, 12 egg carrying females were removed from the system on 6th March 2018. Decreased biomass was compensated by adding 81 juveniles (39 male, 42

female) resulting in an increase of total biomass by 223g. At the end of the experiment (16th October 2018) the population consisted of a mixed adult/juvenile population of 249 individuals with a total biomass of 1263.1g (Table 4.1; the increase reflects breeding success).

The behaviour and development of crayfish was recorded throughout the experiment. eDNA samples (n = 3 environmental replicates, each pooled across the 13 interconnected tanks) were taken at monthly intervals and additionally during or shortly after specific life-history events (e.g. breeding). This resulted in two extra sampling dates in November and December 2017 and one in October 2017 and January 2018.

Table 4.1. Recorded changes of crayfish population and biomass during the duration of the tank-based experiment. Changes were recorded after death, removal and addition of crayfish within the system.

Date	Number of crayfish in system	Biomass of crayfish in system (g)
29/09/2017	198	924.75
18/12/2017	191	892.05
09/01/2018	188	878
23/03/2018	257	1107.55
19/04/2018	337	1174.5
17/05/2018	357	879.9
06/06/2018	379	998.4
09/07/2018	326	908.7
22/08/2018	275	844.7
16/10/2018	249	1264.1

4.3.3. Spatial and temporal in-situ variation

In-situ spatial and temporal variation of eDNA detection and quantification was evaluated in the course of a captive breeding ark site release programme. An isolated site free of crayfish and crayfish plaque (*Aphanomyces astaci*) was selected in the South West of England to become a crayfish ark site (exact location not disclosed for conservation reasons; crayfish ar site release conducted under licenses held by Bristol Zoological Society) and 40 white-clawed crayfish individuals (20 male, 20 female, with a total biomass of 436g) were released into a pond of around 1000m^2. Half of the individuals were adults, half of them were juveniles, and crayfish were released at opposite sides of the pond (sites B and D; Fig. 4.8.) to create a natural population structure.

Samples for eDNA analyses were collected from four distinct $1m^2$ sampling locations distributed around the pond (sites A, B, C, D; see Fig. 4.8.). Additionally, a pooled sample was taken consisting of sub-samples from the entire pond perimeter (sample P). Samples were collected following the filtration based method outlined in Mauvisseau et al. (2019). In brief, each sample was collated from 20 individual scoops and after homogenisation, 250ml of water was pressure-filtered through a 0.22µm (Polyethersulfone membrane) sterile filter (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Germany). For each sample, three independent environmental replicates were collected. Sampling began on the 20th April 2018 and was performed before release (negative control) and 2 hours, 7 days, 14 days and 35 days after the crayfish release. All filters were stored at -20°C. DNA extraction followed protocols outlined in chapter 3 (Spens et al., 2017; Tréguier et al., 2014). In addition to eDNA sampling, 40 artificial refuge traps (ARTs; roughly spaced in 1m distances) and 12 traditional crayfish traps (Green et al. 2018; Fig. 4.2) were placed around the perimeter of the pond and crayfish captures were recorded every week after crayfish release (traditional crayfish traps were only placed over night and removed after sampling).

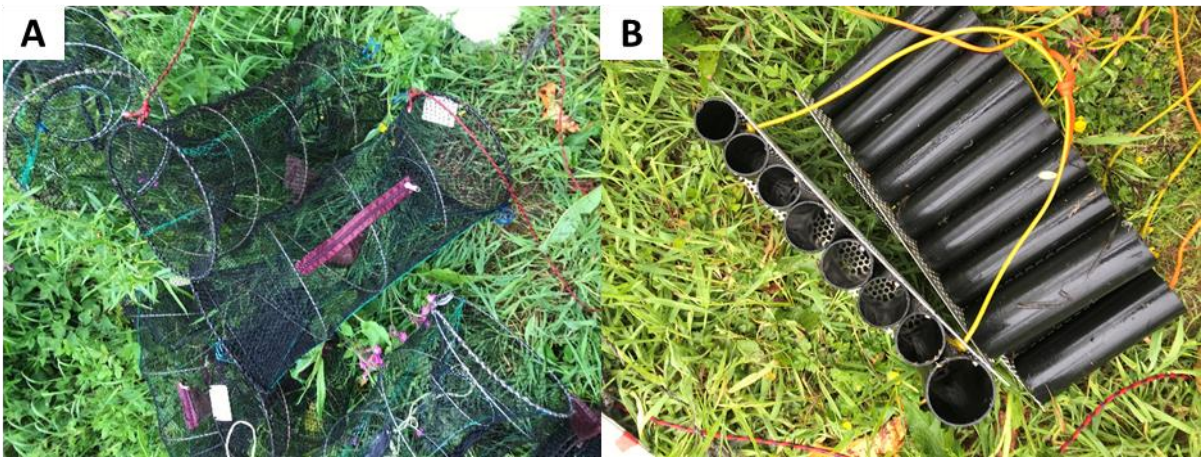

Fig. 4.2. (A) Crayfish traps. **(B)** Artificial refuge traps (ART's). Source author, ©Chris Troth.

4.3.4. Sample analysis and qPCR

A qPCR-based assay with species-specific primers and probe (targeting white-clawed crayfish) was utilised for all eDNA samples (chapter 3). The forward primer WC2302F and reverse primer WC2302R were used to amplify a 109bp fragment of the white-clawed crayfish mitochondrial COI gene (see chapter 3). All qPCR assays were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems) with the following protocols; 50°C for 5 min, denaturation at 95°C for 8 min,

followed by 50 cycles of 95°C for 30 s and 55°C for 1 min. Each sample was split into six qPCR replicates, each consisting of a 25µl qPCR reaction containing: 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5µl DH20, 1µl (10µm) of each primer, 1µl (2.5µm) of probe and 3µl of template DNA. All eDNA samples were analysed with six no template controls (NTC's) (nuclease free distilled water instead of extracted DNA). Further, a duplicated serial dilution of standard white-clawed crayfish DNA (10^{-1} to 10^{-3} ng ul^{-1}) was also run for each qPCR plate as a positive control. Field negative control (nuclease free water) samples were also analysed to assess for contamination following the same protocol as sample analysis. In terms of analysis, for each of the experiments, Ct (cycle threshold) values were recorded for each qPCR replicate, and detection probabilities were calculated as the fraction of qPCR replicates which resulted in a positive detection for a given environmental replicate. The LOD (limit of detection) was set at a Ct value of 45 as indicated by previous research (chapter 3).

4.3.5. Statistical analysis

The degradation experiment was analysed using a linear regression to evaluate the relationship between the number of hours elapsed since the beginning of the investigation and (i) detection probability and (ii) Ct. All qPCR replicates which did not amplify target DNA were assigned a Ct value below the LOD at 45. Potential tank effects were tested for by incorporating mesocosm identity into the model. Further, non-linear relationships between detection probability/Ct and time were also tested for by log-transforming variables. All possible model combinations were established, and the most parsimonious model was identified using the Akaike Information Criterion (AIC). Normal distributions were tested for, alongside autocorrelation and any remaining patterns in residuals. In the analysis of Ct values, data was analysed at both a technical replicate (using all data from qPCR replicates) and environmental replicate level (using means of all 6 qPCR replicates for each environmental replicate). However, analyses resulted in the same fundamental conclusions, and therefore only the analyses at the qPCR replicate level are presented. In analyses of the ark-release program, spatial differences among sample collection locations were assessed using ANOVA analyses, followed by Tukey's HSD post-hoc tests. Prior to ANOVAs, heteroscedasticity was evaluated, and data transformed if necessary. All described statistical analyses were performed using R version 3.4.1 (R Core Team 2018).

4.4. Results

4.4.1. eDNA degradation experiment

After the removal of crayfish from the mesocosms, eDNA was present in the water column for a number of days. Comparisons between different regression models revealed that a logarithmic relationship best described the decrease of white-clawed crayfish eDNA over time (Fig. 4.3). This was evident for both analyses in detection probability ($y=-0.15\log(x)+1.05$, $p<0.001$, $r^2 = 0.75$) and Ct ($\log(y)=0.03 \log(x) +3.6$, $p<0.001$, $r^2 = 0.64$), where models based on log-transformed data resulted in the lowest AIC (Fig. 4.3A-B). Tests were also conducted to assess whether detection probability differed between the three tanks and found that inclusion of tank effects on the intercepts marginally improved AIC by 1 unit (intercept range: 1.01 to 1.10, increases r^2 to 0.76). In terms of Ct, tank effects were more substantial and affected both slope (slope range: 0.006 to 0.018) and intercept (intercept range: 3.597 to 3.671) raising model r^2 to 0.81. It should be noted that the regression lines in Fig. 4.3. do not reach detection probability of 0 or a Ct value of 45 (LOD) within the duration of the experiment. This is a direct result of log transformations, which result in a better overall fit (especially in the first half of the experiment) but in slight deviations during the second half of the time series.

The last reliable detection of white-clawed crayfish eDNA (i.e. detection in all three experimental replicates) occurred at 14 days post removal of all individuals. Further, there was no eDNA detected at the next time point (21 days), which indicates that eDNA of white-clawed crayfish dropped below detectable levels between 14-21 days post removal. However, the disruption of sediment at day 56 revealed that eDNA was still present and detectable in all of the mesocosms, albeit at low concentrations and detection probabilities. This indicates that historic eDNA can remain in sediments longer than 56 days after the departure of the organisms.

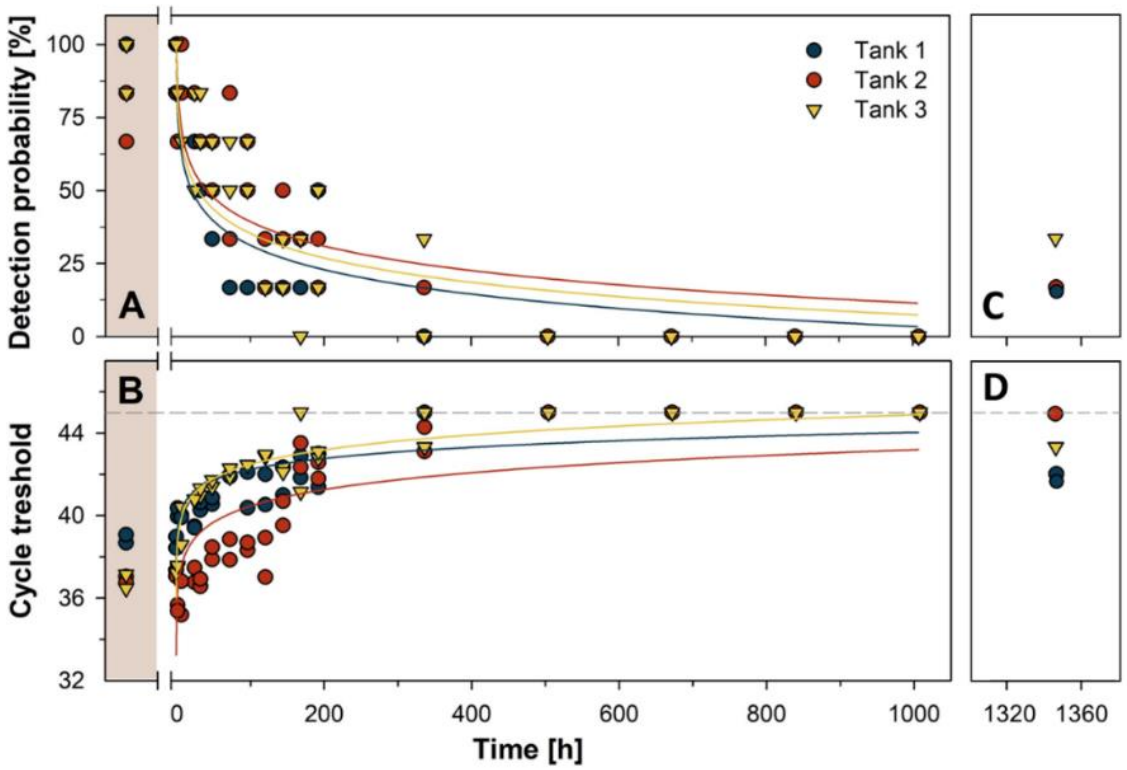

Fig. 4.3. Temporal changes in detection probability (**A**) and cycle threshold (**B**) of white-clawed crayfish eDNA in mesocosm experiments (3 tanks, 2 environmental replicates, 6 qPCR replicates). The shaded area represents the time before crayfish were removed from the tanks (time 0). The dashed line marks the LOD, i.e. a Ct of 45. All samples that did not result in a detection of white-clawed crayfish DNA were set to a Ct of 45 (LOD). The coloured curves represent the logged trend of Ct and detection probabilities over the duration of the experiment. Although there was an overall better fit of data, the log transformation resulted in an overestimation of the time for detection probability to reach 0 and for the Ct values to reach the LOD, as is highlighted within the curves. (**C**) Detection probability and (**D**) Ct values at day 56 when the sediment was manually disturbed and eDNA was re-detected within the water column. Data before the removal of crayfish was not used when creating the linear regression equation.

4.4.2. Seasonal dynamics in eDNA concentrations

Over the 13-month period of the experiment, both detection probability (Fig. 4.4A) and Ct values (Fig. 4.4B) varied substantially in the tank system. In principle, detection probability and Ct values both followed a similar but inverted trend (i.e. high detection probability was paired with low Ct values; Fig. 4.4.). Lowest detection probability (<20% in December) and highest Ct values were recorded during low white-clawed crayfish activity and periods of torpor. Highest detection probabilities (>80%) and lowest Ct values were recorded in May during egg hatching. After the end of the hatching period, detection probabilities between June and November remained relatively constant, varying around 50%.

Further, it is important to note that in the course of the experiment an egg loss event occurred (2nd March 2018). Premature loss of eggs was triggered by an extreme cold weather event (publicly referred to as the 'beast from the east') causing a substantial drop in system temperature and a failure of heating systems (Fig. 4.5.). To sustain breeding populations, 12 egg carrying females were permanently removed from the system and protected against further cold spells. The biomass loss in the tanks was compensated by the introduction of 81 juveniles, which resulted in a 26% net increase of crayfish biomass. Both the egg loss event, which is also likely to occur in natural systems, and the addition of juvenile crayfish resulted in an intermediate peak of detection probabilities and a drop of Ct values on the 6th March 2018 (Fig. 4.4).

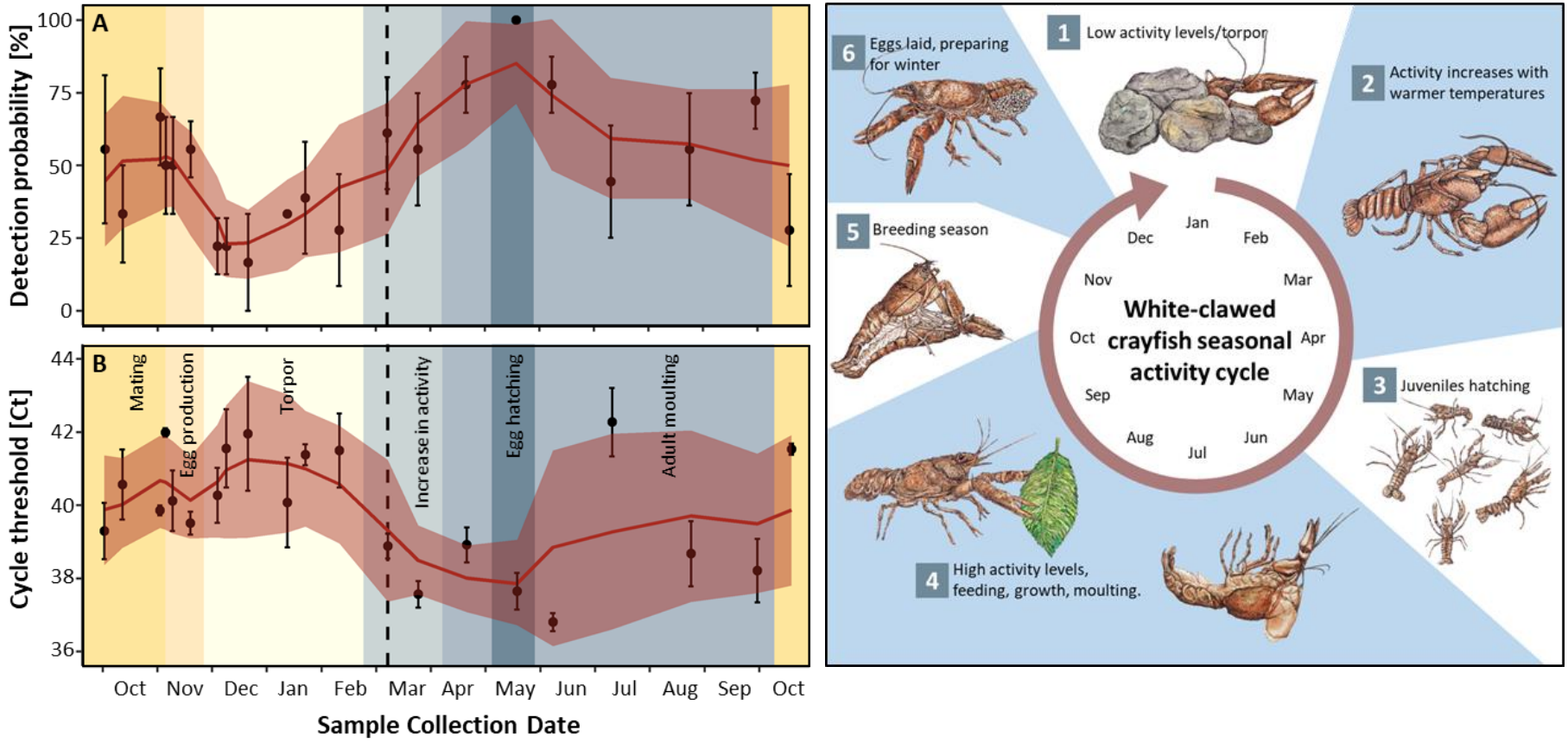

Fig. 4.4. Seasonal variation of white-clawed crayfish eDNA in a mesocosm experiment. Variation in **(A)** detection probability and **(B)** Ct values across the 13-month experimental. Each vertical colour section represents a different observed stage in the seasonal cycle of white-clawed crayfish. Error bars for detection probability represent standard deviation. For Ct the error bars are represented by standard error (uncertainty associated to means (taken from three replicate samples at each time point) of means). Solid red lines represent rolling means of three neighbouring sampling time points and the shaded red area represents rolling means of upper and lower range of standard deviation/ standard error. The dotted line represents the date of the egg loss event, triggered by extreme weather conditions. **(C)** white-clawed crayfish seasonal activity pattern cycle. ©Keziah Drew, ©Chris Troth.

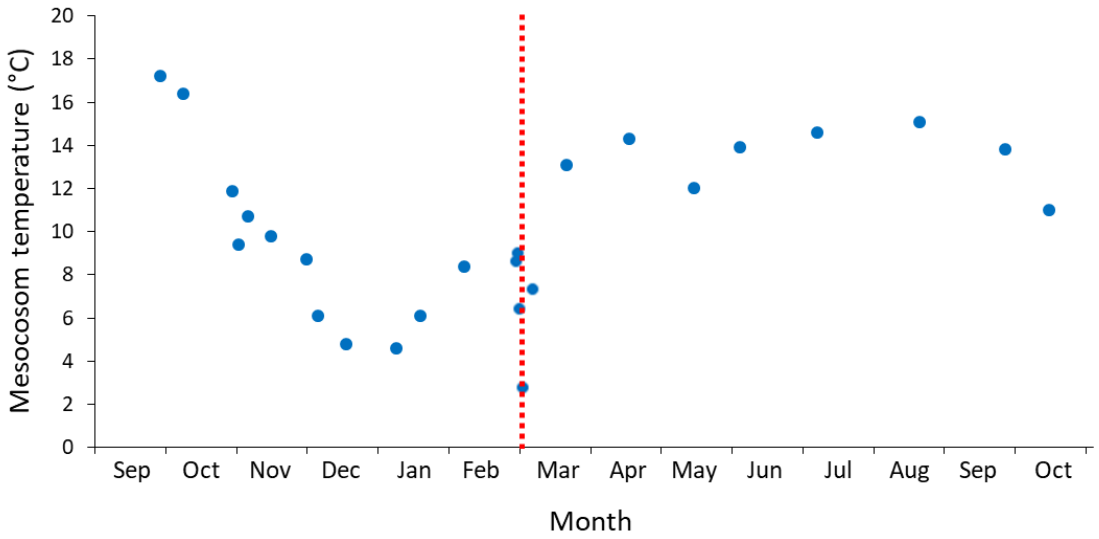

Fig. 4.5. Tank system temperatures measured over the study period. The dashed line indicates the egg loss event on the 2nd March 2018, which coincided with a substantial drop in tank temperature.

4.4.3. Spatial and temporal in-situ variation

The effect of sampling strategy on detection probability and Ct values and the degree of small-scale heterogeneity in eDNA distribution was assessed in a pond habitat. Site comparisons revealed substantial differences in the detection probabilities between sampling locations which were less than 40m apart (all site data pooled; ANOVA $F_{(4,55)} = 3.6$, $p=0.011$; Fig. 4.6). Pairwise comparisons revealed significant differences between site A and D and between site A and perimeter sample P ($p<0.045$).

In depth analyses of site-specific detection probability and Ct values showed large temporal (Fig. 4.7.) and spatial variation (Fig. 4.6.), which could partly be explained by trapping data (Fig. 4.8T). The majority of sampling events were characterised by detection probabilities below 50%. However, some sites varied substantially over short time periods including changes from 0% detection to 100% in just 14 days at site D (Fig. 4.8D). This increase over time at site D matched with the increase of trapped crayfish at this site. While crayfish were released at sites B and D, they were exclusively captured at site D (exception is one individual caught on day 7 at site C, see Fig. 4.8T for temporal patterns of captures), indicating crayfish migration within the habitat after their release. Such migration and micro-habitat preference would also explain the decrease of eDNA concentrations at site B over time (Fig. 4.8B)

A number of eDNA sampling events at specific sites were not successful in detecting white-clawed crayfish despite there being a known population in the pond. Likewise, the excessive traditional surveys (13 traps per site) did not always achieve positive detection and were mostly successful at site D. The most consistently reliable sampling approach was the perimeter sampling, which almost always resulted in higher detection probabilities than individual sites (Fig. 4.7P, Fig. 4.5.). Consequently, a pooled sampling approach was crucial to achieve high method reliability even in this small scale and seemingly homogenous ecosystem.

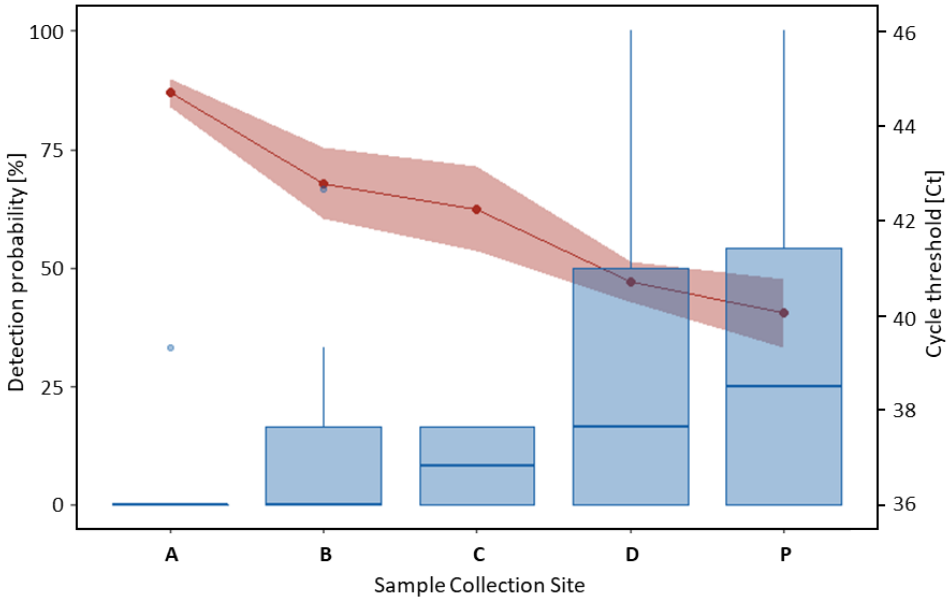

Fig. 4.6. Combined plot overlay all site data (boxplot illustrating detection probability; scatterplot illustrating Ct). Comparison of the detection sensitivity of the 0.22µm pressure filtration sample collection method used at 4 isolated 1m^2 sampling areas (sites A, B, C, D) and a representative sample collected from the entire perimeter of a pond system (P) containing a captive released white-clawed crayfish population.

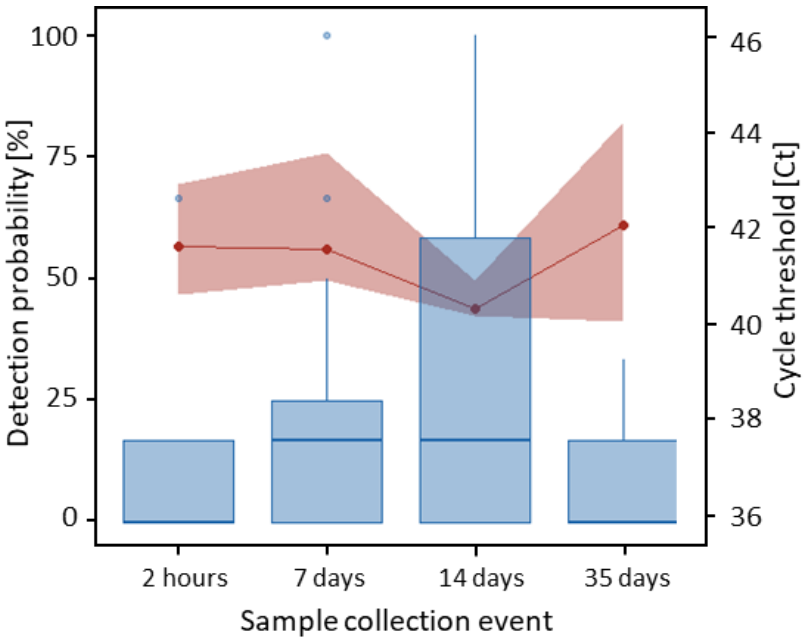

Fig 4.7. Combined plot overlay all site data (Fig. 5: A-E; (boxplot illustrating detection probability; scatterplot illustrating Ct) using three environmental replicate samples for each site (A-E) and sample collection event **(1)** 2 hours; **(2)** 7 days; **(3)** 14 days; and **(4)** 35 days after the initial population of the site on 20/04/2018).

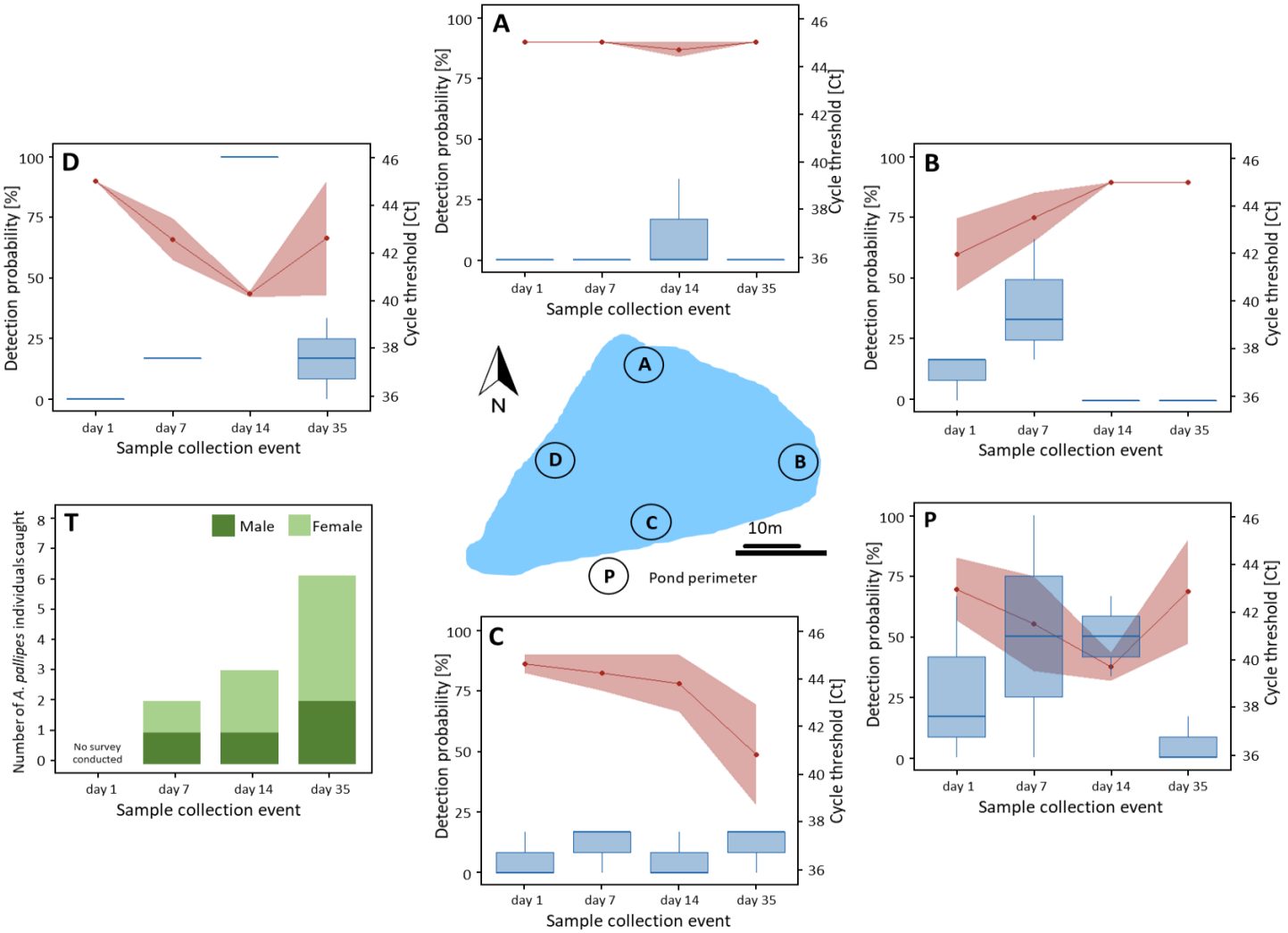

Fig. 4.8. Detection probability (blue boxplots) and Ct (red points, shaded area indicating standard deviation) of eDNA sampling in a 1000m^2 pond after the introduction of white-clawed crayfish at each sample collection event after the initial population of the site on 20/04/2018). Each plot overlay **(A, B, C, D and P)** represents a sample collection site on the map (**A-D**: 1m^2 sampling area; **P**: subsampling from entire perimeter). **(T)** 'Traditional' detection of white-clawed crayfish within the pond using extensive trapping using crayfish traps and ART's. Each individual crayfish found within a trap or ART was recorded on each visit. A schematic of the pond is also included displaying the location of each of the sampling points.

4.5. Discussion

Comparisons across sites sampled at different time points represent a key application of eDNA-based methods used in research and conservation management planning (Lacoursière-Roussel et al., 2016). Perquisites for such across site comparisons are detailed information on temporal dynamics and small-scale within habitat heterogeneity affecting eDNA-based species detection and quantification (Goldberg et al., 2018). In this study, the impact of various drivers of spatio-temporal variation on the reliability of eDNA-based approaches were critically assessed in order to address the hurdles of eDNA (hurdle 4, 5 and 6, chapter 1) using white-clawed crayfish as model species. The results demonstrate that both spatial and temporal drivers of variation in eDNA concentrations can have substantial consequences reducing detection probabilities and altering Ct values. Based on this, recommendations on how to account for such variability in sampling protocols can now be employed in order to increase the robustness of eDNA-based surveys.

The degradation experiment highlighted that white-clawed crayfish eDNA persisted for 14-21 days post species removal and that slow degradation processes may trigger false positive results in field surveys. Previous measurements of degradation rates show substantial variation between species and studies (e.g. <72hrs for common carp, Barnes et al. (2014); 8 to 18 days for Idaho giant salamander, Pilliod et al. (2014); 21 and 25 days respectively for American bullfrog tadpoles and Siberian sturgeon, Dejean et al. (2011)). Such variations in degradation rates can partly be explained by differences in environmental conditions between studies. Natural sites will tend to increase eDNA degradation due to water inflow and exchange (Shogren et al., 2018), a factor that also needs to be incorporated when extrapolating results from mesocosm experiments to the field. Further, important determinants of eDNA decay rates are pH (degradation occurs faster in acidic environments in Seymour et al. (2018)), temperature (increased degradation at >25°C in Goldberg, Strickler and Fremier (2018a)), and UV-B radiation (range of 1 to 58 days in Strickler, Fremier and Goldberg (2015)). Long degradation times as measured in this experiment increase the risk of a false positive result, either; (i) after the target species has migrated or become locally extinct (Stoeckle et al., 2016) or (ii) due to downstream

transport of eDNA in river sections (Deiner and Altermatt, 2014; Jerde et al., 2011). However, specific environmental conditions need to be considered when evaluating these risks in the field.

Further, eDNA persistence was found to be much higher in the sediment than in the water column of the mesocosms. Hence, resuspension of sediment and historic eDNA after extinction or emigration of target species represent a potential source of error for eDNA-based assessments (Turner et al., 2015). However, measurements of eDNA concentrations in aquatic but also terrestrial sediments have been recently a focus of several studies highlighting the potential of historic eDNA to reconstruct past species occurrence (Bálint et al., 2018; Thomsen and Willerslev, 2015). In the case of endangered species such as white-clawed crayfish, simultaneous measurements of eDNA in sediments and water columns could represent a valuable tool to assess the impact of environmental threats (e.g. spread of crayfish plague) on species distributions.

In the second experiment, large seasonal variation of crayfish detection probability and Ct values were demonstrated in a controlled mesocosm experiment. Compared to eDNA degradation, the impact of seasonal changes in environment and species' activity patterns on eDNA concentrations is largely underexplored. A number of studies investigate *in-situ* eDNA concentrations at different times of the year (Buxton et al., 2018; Furlan et al., 2016; Rheyda. Hinlo et al., 2017; Ostberg et al., 2018; Takahashi et al., 2018), revealing differences of up to 20 times higher eDNA concentrations during spawning seasons (Wacker et al., 2019). However, seasonal variations in the field can also be triggered by changes in population densities and currently, there is only one study that simultaneously measured seasonal cycles in eDNA concentrations and species densities (Buxton et al., 2017). In this experiment, not only were substantial changes in eDNA concentration (Ct values) observed, but the changes also resulted in a 4-fold increase of detection probability between winter and summer seasons. Variation in detection probability might even be amplified in the field when eDNA concentrations are much lower and close to the limit of detection, carrying large implications for the choice of sampling design and season.

The large changes observed in this experiment likely reflect the impact of three main factors. First, abrupt changes in temperature triggered an egg loss event in early spring and the degradation of disposed eggs most probably contributed to the observed increased eDNA concentration in March 2018 (Fig. 4.4). Second, while the aim was to keep crayfish biomass constant (coefficient of variation of monthly measurements was 0.14), some changes occurred due to natural mortality, juvenile growth and necessary breeding-related management (Table 4.1). Finally, white-clawed crayfish shows pronounced seasonal activity patterns (Holdich, 2003), potentially influencing eDNA shedding rates and consequently concentrations in the water column. Indeed, there was a strong accordance between torpor and low concentrations as well as egg hatching and high eDNA concentrations, underlining the potential importance of changing animal physiology and behaviour over seasons.

Seasonal dynamics in eDNA concentrations present a challenge for accurately quantifying biomass (Buxton et al., 2017) and for cross-system comparisons (Wacker et al., 2019) as environmental dynamics may vary across sites. One possibility to account for such variation is to standardise time points of sample collection. In accordance with previous studies (Dunn et al., 2017), this chapter highlights that species detection is most reliable before and during egg hatching between April to June and should be the most preferable time for field surveys. For presence/absence surveys this time period might be extended to late October before white-clawed crayfish enters torpor and detection probabilities drop. However, it is important to note that optimal sampling periods, especially for quantification might vary between altitudes and locations and show strong interannual variations. Consequently, investigators need to apply site-specific adjustments of sampling strategies.

Finally, the field surveys demonstrated striking differences in detection probabilities between sites that were less than 40m apart. Further, sampling at some sites almost always failed to achieve positive detection despite the presence of the target species in proximity of the sampling site. In the presence of such large small-scale spatial heterogeneity potentially caused by micro-habitat preferences, a pooled sampling approach was most effective in eDNA-based species detection. Pooled sampling approaches in both lotic and lentic systems have been suggested before (Biggs et al., 2015; Tréguier et al., 2014). Nevertheless, one-point sampling

procedures are still commonly applied in field studies (Agersnap et al., 2017; Rheyda. Hinlo et al., 2017; Smart et al., 2015; Strand et al., 2019). The results to this chapter clearly demonstrated that these approaches are linked to a high risk of sample bias and that pooled samples are instrumental to increase the robustness of eDNA-based applications.

In-situ eDNA concentrations also showed a high temporal variability at most sites. In contrast to the tank-system study, released crayfish did not carry any eggs excluding egg hatching as potential cause of short time variation. However, a large number of moults were observed during and after the release of crayfish, which likely represented a stress response to handling and transport. Degradation of exoskeletons and increased shedding rates of freshly moulted crayfish might have played an important role in the increase of eDNA concentrations over time (Tréguier et al., 2014). Complementary to such effects, variation in environmental conditions and the accumulation of eDNA during the sampling period (reaching dynamic equilibrium between shedding and degradation) may have contributed to observed temporal patterns.

4.5.1. Conclusion

Across site comparisons represent a fundamental aspect of eDNA applications but need to incorporate temporal dynamics and spatial heterogeneity of sampled habitats (Goldberg et al., 2018; Tillotson et al., 2018). In this chapter, critical assessments were made on; (i) time scales of eDNA degradation, (ii) seasonality in environmental conditions and species behaviour, as well as (iii) within habitat variation of eDNA concentrations affect the reliability of eDNA-based surveys. This indicates that all of these three factors can have considerable effects on the probability of detecting a target species at occupied sites. However, these factors can be at least partly accounted for, and when respective mitigation strategies are implemented, rates of false positive and false negative results can be controlled (Table 4.2). Yet, such mitigation strategies are most effective when site-specific environmental and ecological drivers are considered and consequently require adaptive applications instead of being followed like recipes in a cook book.

Table 4.2. The effect each factor assessed can have on the reliability of eDNA detection.

Factor	Explanation	Effect	Recommendations
Temporal			
Long term	Seasonal changes in environmental conditions and species activity can influence eDNA concentrations.	20-80% variation in detection probability. Risk of false negatives during winter months. Complicates eDNA-based quantification.	Sample between April and late October. If sampling in winter, increase sample replication number.
Short term	Detection probabilities can vary over time due to variation in habitat, environmental and/or biological factors.	Short term changes in conditions and species activity could lead to differences in method sensitivity and false negative results.	Combine eDNA-based methods with classical species presence absence surveys. Repeat sample collection over time.
Degradation of eDNA	eDNA persistence in the environment can lead to false positive or negative detection.	Depending on management objectives consequences can be positive or negative. Past presence of e.g. migrating individuals passing through can be detected but may also lead to false positive results.	Avoid sediment disruption during water sampling. See also short-term recommendations.
Spatial			
Sampling location	Environmental factors and species habitat preference can lead to heterogenous distribution of eDNA.	Substantial systematic differences in detection probability across sites in a small ecosystem. Risk of false negatives.	Collect a representative sample for each habitat. Sample several sites in larger ecosystems.

Chapter 5: The commercial application, adaptation and implementation of eDNA assays for the management of freshwater systems: white-clawed crayfish, signal crayfish and the crayfish plague

5.1. Abstract

Whilst there are now a significantly large and ever-increasing number of published single-species qPCR assays available for eDNA detection, still only a handful have been applied to multiple conservation projects or are available on an accessible commercial level. Although a robust methodological development is important for the success of an eDNA-based species detection method, it is also essential for its application in conservation management and decision making that the assay can be proven reliable in 'real-world' settings. However, to enable the application of eDNA-based methods in such settings often requires modifications to be made, in order to make the methods more accessible, affordable and appropriate for their target environment or consumer. Within this chapter, I take the method designed within chapters 3 and 4 and apply it to answer three case-study style questions, demonstrating examples of real-world use of the methodology. The application of the white-clawed crayfish eDNA assay is demonstrated alongside additional eDNA assays for signal crayfish and crayfish plague, which are currently also available in literature, but not on a commercial scale. Such trial experiments allowed for full consideration of the final 'hurdles' required for the full commercial application of these methods. A number of enhancements and changes are made to the assay, including simplification of the sample collection approach, to make eDNA-based species detection more accessible to the general end-user, ensuring its success as an additional ecological survey method for white-clawed crayfish.

5.2. Introduction

There are now a number of eDNA assays developed for the detection of the white-clawed crayfish, *Austropotamobius pallipes* (Atkinson et al. 2019; Robinson et al. 2018, chapter 3, chapter 4), signal crayfish, *Pacifastacus leniusculus* (Agersnap et al. 2017; Harper et al. 2018a; Larson et al. 2017; Mauvisseau et al. 2018; Robinson et al. 2018) and crayfish plague, *Aphanomyces astaci* (Strand et al. 2014; Wittwer et al. 2018). However, there is little translation of these assays into the commercial or end-user sectors. Within chapter 3 and 4 of this thesis, I developed and validated an eDNA assay for the detection of white-clawed crayfish. This addresses many of the important associated variables, limitations and 'hurdles' (identified at the beginning of this thesis; chapter 1) which is a requirement for the commercialisation of eDNA-based methods (Atkinson et al. 2019; Robinson et al. 2018). However, whilst the assessment of these hurdles is a massive step in providing a suitable end-user applicable assay with appropriated reliability and accuracy. Once developed on a theoretical basis and tested within the controlled research environment, there are still many more aspects that need to be considered before such an assay can be applied on a wide-scale commercial level.

The validation of an assay is not only important from a scientific perspective (as demonstrated within chapters 3 and 4) but is also highly valuable when transforming a scientific technique into a commercial product. Within industry diagnostic approaches and tests are conducted to a common standardised level within each business, across each facility, and employee, in order to maintain reliable, repeatable and accurate results. To achieve this, standard operating procedures (SOPs) are often required, providing a description of the entire work process. This is to ensure any trained member of staff can pick up the SOP and complete the test without any uncertainty on the quality of the produced results.

For eDNA-based surveys, this starts with ensuring the general end-user, citizen scientist or ecologist has a simple sample collection kit, complete with detailed, yet simple instructions on how to collect a sample. Such kits should be designed in an easy to use manner, which reduces any biosecurity risk in order to present further spread of the crayfish plague (e.g. use of single use, disposable kits). Further, due to the varied nature of field sites and the conditions of sites

where crayfish may be present (e.g. access to the water edge, vegetation, differences in turbidity etc. (Naura et al. 1998)), it is important to provide guides and frequently asked questions (FAQ's) for sample collection. This insures appropriate and reliable sample collection as a basis for an accurate species detection.

It is important to bear in mind that there also needs to be a demand and practical use for any commercial service in order to ensure its viability. A customer base needs to be built up through trust in the service. One way of ensuring this is to obtain governmental support for a new method such as the approved great crested newt, *Triturus cristatus* eDNA-based methodology (Biggs et al. 2014, 2015). Currently within the U.K. both Natural England and the Environment Agency are the two key governmental organisations which are responsible for regulating and monitoring native and invasive species. Both have shown substantial interest in the development of eDNA-based methods, with Natural England representing the agency which have approved the use of the great crested newt eDNA-based methodology for land planning purposes. Both organisations also show interest in utilising eDNA for other species detection. For example, they set up and organise the U.K. DNA Working Group (a group of researchers, Universities, conservation organisations, government agencies and potential end-users of eDNA). Along with tendering small-scale contracts for exploratory eDNA-based survey development for crayfish for example (Environment Agency - see Study 2 and 3 (below) and Natural England; appendix 5.1 – Natural England, Request for Quotation). However, only the Environment Agency currently offers advice on the levels of validation required on an assay before it can be considered for use in their environmental monitoring and decision-making programmes (appendix 5.2 – Environment Agency, Using DNA-based methods for environmental monitoring and decision-making). Although limited in content this document briefly states that a certain level of validation is required for eDNA-based methods to be applied confidently within regulatory frame works. On a regulatory level (within wider Europe), CA COST Action CA15219 – DNAqua-Net (see section 1.2.9.3) is now reported to be working towards recommendations for the levels of validation required for such assays, with end-users and regulatory bodies such as Natural England and the Environment Agency in the U.K (Kat Bruce, *iBOL 2019 Conference presentation*). There are reasons to be hopeful that this will bring clarity to the field of eDNA-based method validation,

increasing confidence in well validated methods and ultimately leading to a simpler end-user adoption process for well-developed methods.

For an assay to be utilised with high confidence by an end-users, it should be well developed, validated and tested under a number of conditions and for different types of project. This list of recommended validation steps can include measures to address and develop the assay to consider all of the associated 'hurdles' (Table 1.2), but it is also important for such protocols (sample collection protocols, frequently asked questions, detailed laboratory analysis instructions (Table 5.1)) to be provided in an accessible form. This is so the protocols can be easily understood and correctly interpreted by a sample collector with little experience or knowledge within the field.

Table 5.1. Requirements for a commercially applicable assay.

Aspect of eDNA	Requirements
Sample collection	• Instructions for collecting a sample. • Frequently asked questions for different situations which may be experienced during sample collection (i.e. related to access, habitat variations, issues with turbidity etc.). • Expert advice readily available to deal with additional queries.
Laboratory analysis	• Standard operating procedures (SOPs) for each aspect of analysis. • Trained and experienced staff for sample collection. • Laboratory testing to ensure reliability and repeatability of results. • Constant feedback, development and research to increase effectiveness of methodologies used with improvements to the field. • Trained and experienced staff for laboratory analyses.
Result reporting and follow up	• Easily interpretable presentation of results.

For end-user confidence, it is important that clear examples of the techniques success are reported. Hence it is essential to conduct commercial-scale trials of the assay under different conditions and with different conservation or species monitoring goals in mind. In this chapter the assay developed here (chapter 3 and 4) and the two published assays (Mauvisseau et al. 2018; Strand et al. 2014) are applied in various customer led projects, each with the goal of testing the commercial use of the assay. This informs on the suitability and applicability of the assays in order to transform the science, from principle to practice. From these field trials I was able to turn the research into a commercial product, in doing so I have developed standard operating procedures for the laboratory analysis and sample collection guidelines of these assays.

5.3. Study outlines

Within this chapter, I apply eDNA-based detection for white-clawed crayfish, signal crayfish and crayfish plague in three separate case studies, working with end-users, including the Environment Agency, Wildlife Trusts and regional ecological consultancies. Below I outline a brief aspect of the end user involved with each case study, and the goal or question that users wanted to address. I then present a general overview of the methods, (however this is described in more detail in chapters 3 and 4), then tackle the findings and discussion points on a case by case basis.

Study 1: The River Allen, Dorset

End-user: Dorset Wildlife Trust

A charitable organisation who primarily focusses on protecting wildlife and habitats. They are responsible for monitoring and maintaining over 2000 nature reserves nationally, managing and protecting habitats for the conservation of species, education of the public to improve wildlife awareness and are actively involved with research (Wildlife Trust, 2019).

End-user goal: (1) To determine if white-clawed crayfish are still present within the river Allen as surveys with traditional methods have failed to detect its presence. (2) To determine if signal crayfish have entered the water systems and if so at which point. (3) To determine if the crayfish plague is still present, after a significant outbreak in 2014.

Full report located in Appendix 5.3.

Study 2: The River Ecclesbourne, Derbyshire

End-user: The Environment Agency

A UK government agency responsible for supporting sustainable development and working to improve and create environments for people and wildlife. Their main responsibilities include: water quality and resources, fisheries, regulating waste, treatment of contaminated land, management of inland rivers and supporting conservation and ecology (Environment Agency 2019).

End-user goal: (1) To determine the presence of white-clawed crayfish, signal crayfish and the crayfish plague within the river Ecclesbourne, upstream and downstream of a weir. (2) To inform on the potential impact to the crayfish populations with the removal of the weir. The end-user was working with a limited budget and was unable to provide the time and financial backing which would be required to survey this river using a comparable traditional trapping approach.

Full report located in Appendix 5.4.

Study 3: Fish Stocking Project, Lincolnshire

End-user: The Environment Agency

End-user goal: (1) To determine the presence of an unidentified crayfish species (thought to be signal) within a pond designed to be used for stocking fish. (2) To determine the presence or absence of signal crayfish within the River Long Eau – identifying possible threats of signal crayfish or crayfish plague to the fish farm situated within the headwaters.

Full report located in Appendix 5.5.

5.4. Methodology

The sample collection and DNA extraction methodologies used for each case study are those outlined within chapter 3 (see specifics in each case study below). The detection of white-clawed crayfish, signal crayfish and the crayfish plague was achieved by implementing three separate qPCR assays, each one specific to the intended target species. Assay development and analysis protocols for white-clawed crayfish follows protocols presented within chapter 3 and chapter 4. Previously published eDNA assays for signal crayfish and crayfish plague were each utilised in accordance to Mauvisseau et al. (2018) and Strand et al. (2014), respectively. For ease of understanding the methodology for each is briefly summarised below.

5.4.1. White-clawed crayfish eDNA assay

A real-time quantitative PCR (qPCR) assay was set up in a 25µl reaction containing: 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5µl DH20, 1µl (10µm) of each primer (forward WC2302F and reverse WC2302R), 1µl (2.5µm) of probe WC2302P with the addition of 3µl template. qPCRs were performed with 6 qPCR replicates of each eDNA sample on the ABI 7500 qPCR System (Applied Biosystems) under the conditions: 50°C for 5 min, denaturation at 95°C for 8 min, followed by 50 cycles of 95°C for 30 s and 55°C for 1 min. 6 x NTC's (no template controls) were prepared using RT-PCR Grade Water (Ambion™) alongside a 10x serial dilution of white-clawed crayfish positive control DNA standard for each qPCR plate that was run.

5.4.2. Signal crayfish eDNA assay

A qPCR assay was set up using the same reagent concentrations and conditions as above, with the altered annealing temperature of 56°C and primers CO1-Pl-02-F (5'-TGAGCTGGTATAGTGGGAACT-3'), CO1-Pl-02-R (5'-AGCATGTGCCGTGACTACAA-3'), and probe (5'-FAM-CGGGTTGAATTAGGTCAACCTGGAAG-BHQ1-3'). Full protocol and primers including method development can be found in Mauvisseau et al. (2017).

5.4.3. Crayfish plague eDNA assay

Analysis for the crayfish plague was conducted using primers and conditions designed by Vrålstad et al. (2009). A (qPCR) assay was set up in a 25µl reaction containing: 12.5µl TaqMan®

Environmental Master Mix 2.0 (Life Technologies®), 4.5µl DH20, 1µl (10µm) of each primer (AphAstITS-39F (5'-AAGGCTTGTGCTGGGATGTT-3') and AphAstITS-97R (5'-CTTCTTGCGAAACCTTCTGCTA-3')), 1µl (2.5µm) of probe (AphAstITS-60T (5'-FAM-TTCGGGACGACCC-MGBNFQ-3') with the addition of 5µl template. qPCRs were performed with 6 qPCR replicates of each eDNA sample on the ABI 7500 qPCR System (Applied Biosystems) under the conditions: 50°C for 5 min, denaturation at 95°C for 8 min, followed by 50 cycles of 95°C for 15 s and 58°C for 1 min. 6 x NTC's were prepared using RT-PCR Grade Water (Ambion™) alongside a 10x serial dilution of crayfish plague positive control DNA standard for each qPCR plate that was run.

For all assays, species presence within a site was inferred by the positive amplification of target species eDNA within at least one of the qPCR replicates out of at least one of the environmental replicate samples collected from each site.

5.5. Study 1: <u>The River Allen, Dorset</u>

The River Allen is a chalk stream river, fed by an aquifer in Dorset, England. It begins near the village of Monkton Up Wimborne and travels for around 14 miles before it has its confluence with the River Stour in Wimborne Minster. It was once known for its large population of native, white-clawed crayfish – one of only three populations remaining in Dorset (Dorset Wildlife Trust, Environment Agency 2014). However, survey methods since 2016 using torching and trapping were unable to detect presence of white-clawed and/or signal crayfish.

History of white-clawed crayfish populations in the River Allen:

2012 A crayfish population survey was conducted along the River Allen. Hundreds of white-clawed crayfish were subsequently identified at several sites along the river. 200 individuals were taken from the river to an ark site in the Purbecks (Dorset Wildlife Trust).

2013 River restored with added reed banks to slow down flow (Fig. 5.1), making habitat improvements for white-clawed crayfish and other species of ecological and conservation importance.

Figure 5.1. Reed bank restoration at points along the River Allen, put in place to create more habitat for white-clawed crayfish and to slow down river water flow. Source author, ©Chris Troth.

2014 Crayfish plague was thought to be the cause of a mass crayfish die off within the River Allen and this was confirmed by the Centre for Environment, Fisheries and Aquaculture Science CEFAS (UK). Individual deceased white-clawed crayfish were found floating down the river. Almost all individuals appeared to have been wiped out by the plague, based on attempts to trap (Environment Agency 2014).

2015 A further trapping survey was conducted by the Environment Agency which found eight individuals. These were found at Site 9 (Fig 5.2) (Environment Agency, 2015).

2016 In an attempt to provide habitat for any remaining individuals, 54 crayfish artificial refuge traps (ART's; Fig. 5.3) were introduced to 10 sites (Fig. 5.2) across the river (20m lengths) (Dorset Wildlife Trust), in the hope that any remaining crayfish would use these for refuge to support population growth, post-plague outbreak. Surveys were undertaken in 2016 by checking each of the 54 ART's for the presence of white-clawed crayfish; however, no individuals were seen.

2017 Dorset Wildlife Trust considered the option of screening for white-clawed crayfish, signal crayfish and crayfish plague by using eDNA. The University of Derby were contacted and the crayfish eDNA assays outlined above were applied to determine if white-clawed crayfish were in fact still present within the river system, or if an invasion of signal crayfish accompanied by crayfish plague was the potential causal factor which has led to the loss of the once prominent white-clawed crayfish inhabitation.

To achieve this, traditional ecological surveys (trapping and torching) were also planned to be conducted alongside the environmental DNA survey/analysis using the assays described above.

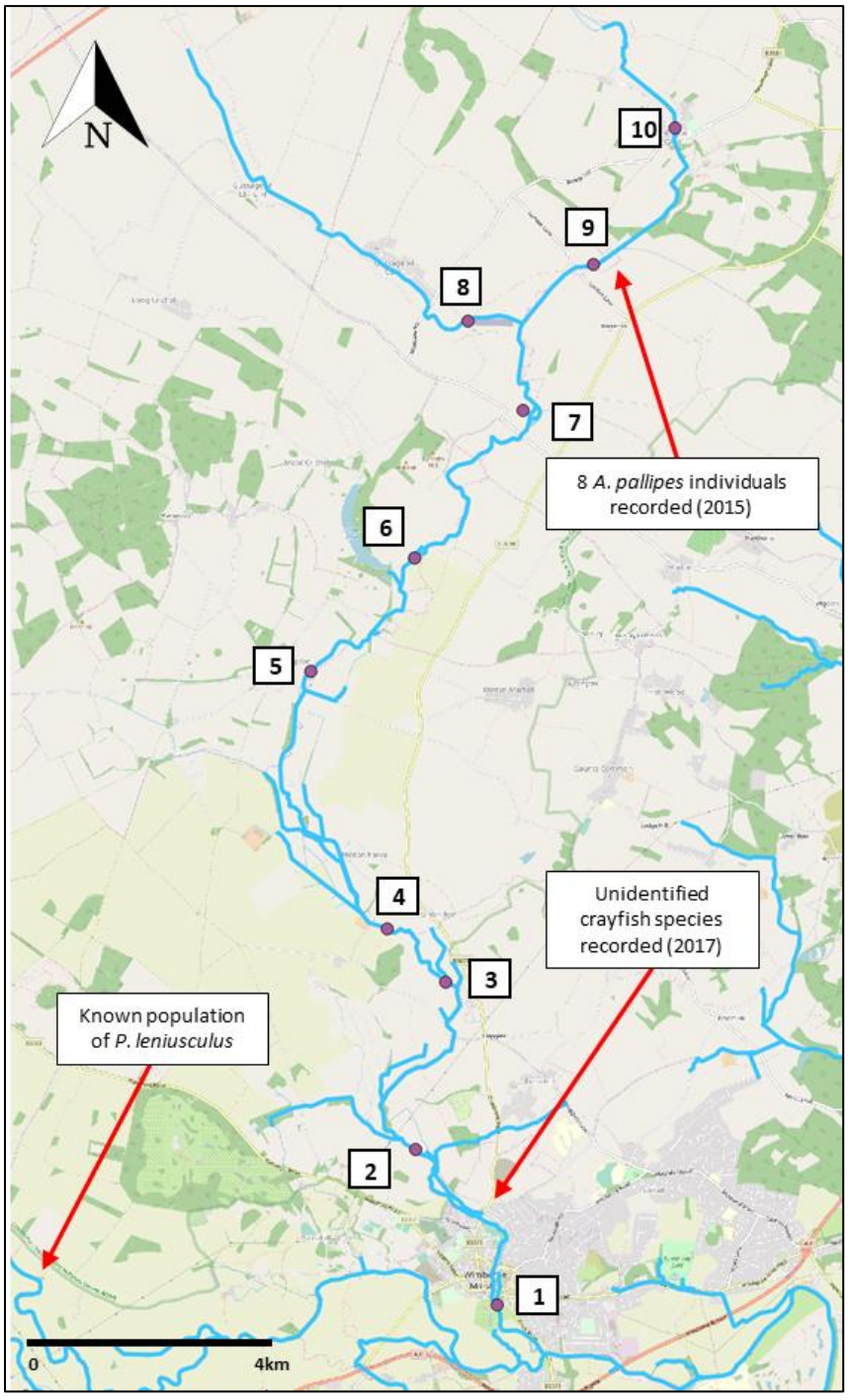

Fig. 5.2. Indicative map of sample site location along the River Allen including historical crayfish records and sightings since 2015. ©OpenStreetMap contributors https://www.openstreetmap.org/copyright.

Fig. 5.3. Photograph displaying one of the panpipe refuges which had been placed within the river Allen during summer 2016. Source author, ©Chris Troth.

5.5.1. Sampling strategy

10 individual sites were selected along the river, 1km to 4km apart for where eDNA sampling would be conducted (Fig. 5.2). These sites were selected on the basis of their interest to the end-user and were either; 1) sites which have been regularly monitored in previous years, 2) had recent crayfish sighting reports or 3) contained particular habitat features which were beneficial to white-clawed crayfish. Sampling commenced on the 6th September 2017 and was completed within 48 hours, beginning at site 1 (Fig. 5.2), in a downstream to upstream sampling direction.

Two sample collection methods for eDNA were tested which contributed to aspects of the method development in chapter 3. These were ethanol precipitation (Fig. 5.4) (90ml per sample) and filtration (2µm pore size, 2L per sample) using a battery powered peristaltic pump-based method (Fig. 5.5). For each eDNA-based method, three environmental replicates were taken per site. eDNA samples were then frozen at -20°C and then stored until extraction.

Three additional sites were assessed using ethanol precipitation for the presence of white-clawed crayfish, signal crayfish and crayfish plague as part of an initial screening of potential suitable white-clawed crayfish ark sites.

Further, once eDNA sampling had been completed, trapping and visual surveys were employed in an attempt to detect crayfish individuals via more traditional methods. At each of the 10 sites (Fig. 5.2), two crayfish traps were set overnight and checked the following morning for the presence of crayfish species. To accompany this, the 54 artificial panpipe refuge traps (placed within the river in 2014) were also examined for crayfish presence. All white-clawed crayfish ecological surveys were conducted under license, by trained and licensed individuals of the Environment Agency and Dorset Wildlife Trust.

Fig. 5.4. Disposable single use kit for the ethanol precipitation eDNA sample collection method. Source author, ©Chris Troth ©SureScreen Scientifics.

Fig. 5.5. Re-useable battery powered peristaltic pump and accessories used for the filtration sample collection approach. Source author, ©Chris Troth.

5.5.2. Results and discussion

Each eDNA sample from the River Allen was analysed for the presence of white-clawed crayfish DNA, signal crayfish and the crayfish plague. All three species were detected by both methodologies within the course of the river system (Fig. 5.6). However, the results were not always consistent between the different methods used for sampling (Fig. 5.7, Fig. 5.8, Fig. 5.9). For example, white-clawed crayfish eDNA was detected at every site using ethanol precipitation method, yet filtration failed to give a positive detection at sites 3 through to 6 (Fig. 5.7). Similar results were attained in analyses of signal crayfish eDNA. Of the four positive sites, neither were positive with both sample collection methods (Fig. 5.8). This suggests that there are large variations in the detection success of both sample collection methods, and that both methods used were providing results close to their limits of detection sensitivity. One reason for this could be the stochastic distribution of both species and the eDNA within the system (see chapter 4). A complementary explanation is that very low population densities are present within the system, leading to the observed low concentrations of eDNA.

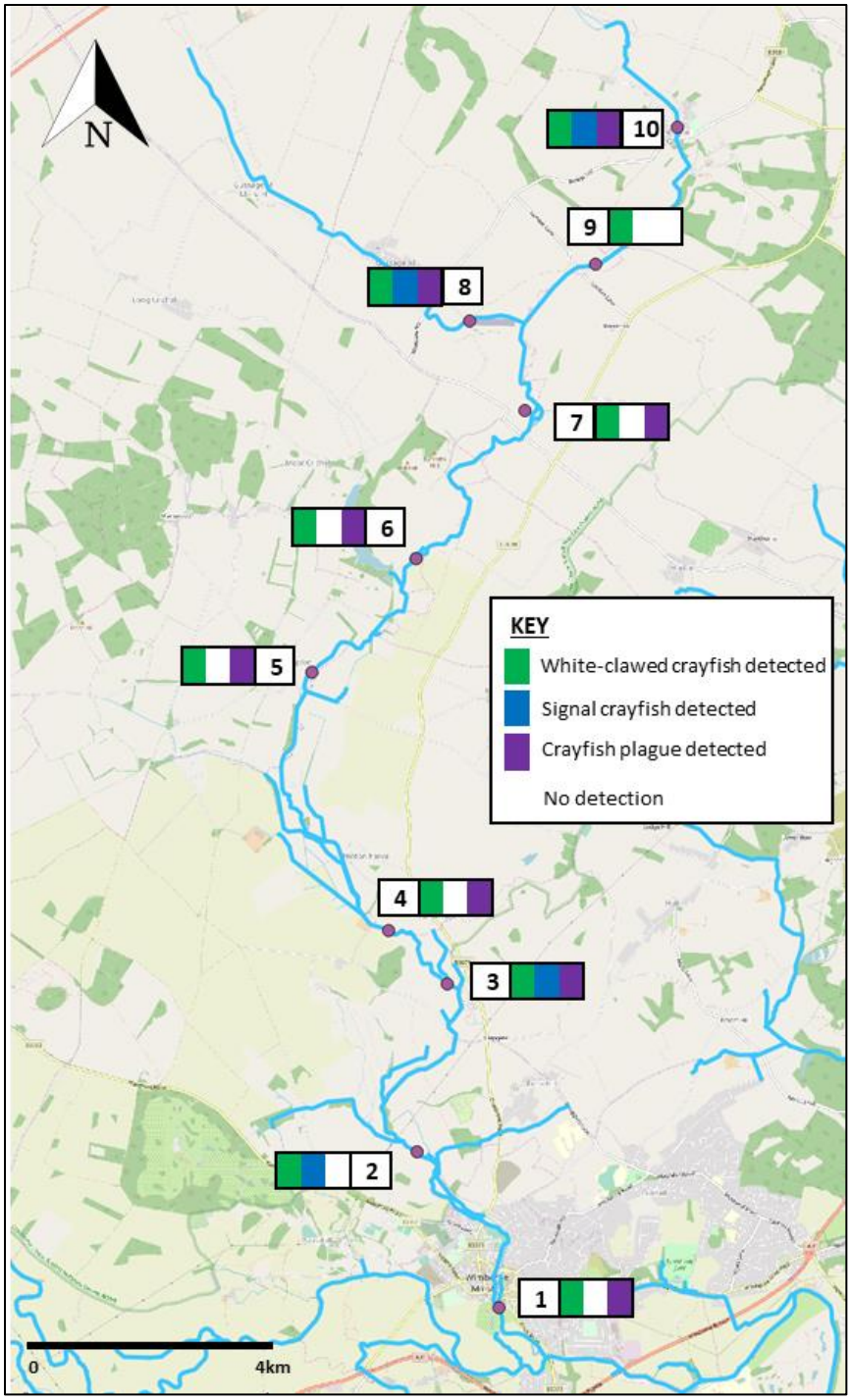

Fig. 5.6. Summary of species *(*white-clawed crayfish*,* signal crayfish*,* crayfish plague*)* presence/absence detection at each site along the River Allen using combined data from both sample collection approaches (ethanol precipitation and filtration (2L, 2µm pore size)). Site 10 was the furthest point upstream, with downstream flow occurring to site 1. ©OpenStreetMap contributors https://www.openstreetmap.org/copyright.

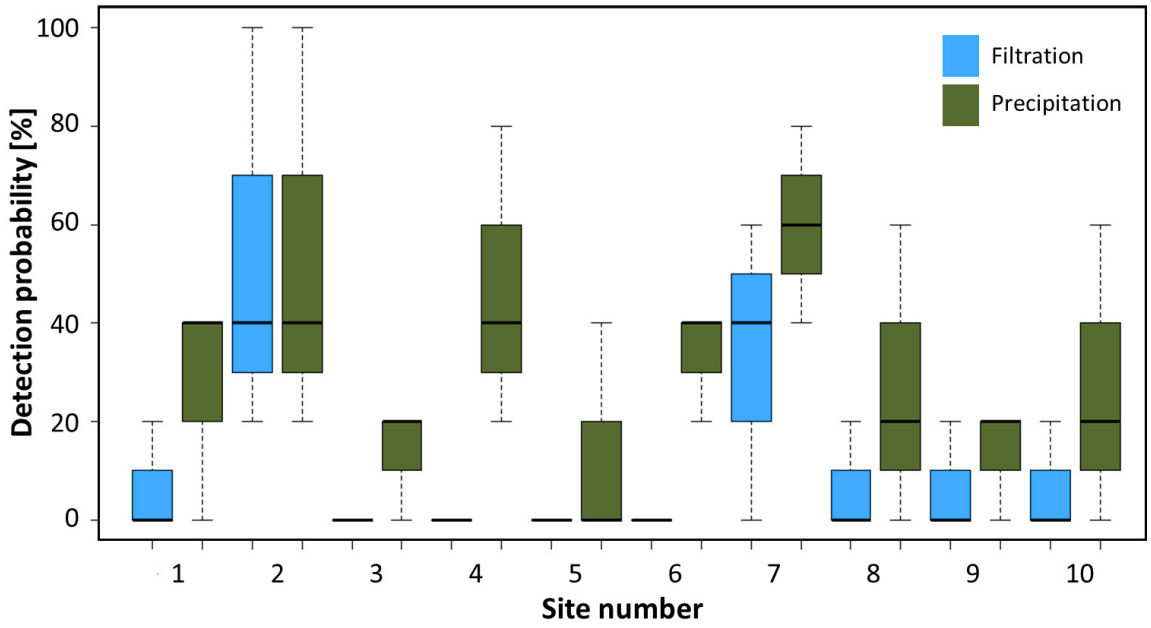

Fig. 5.7. Comparison of the detection probability of the filtration and ethanol precipitation methods on ability to detect white-clawed crayfish eDNA in the River Allen. At each site (with the exception of site 2), precipitation outperformed filtration in terms of detection probability. As at least one method was successful at each site, it is indicative of a population of white-clawed crayfish across the entire system. Site 10 was the furthest point upstream, with downstream flow occurring to site 1.

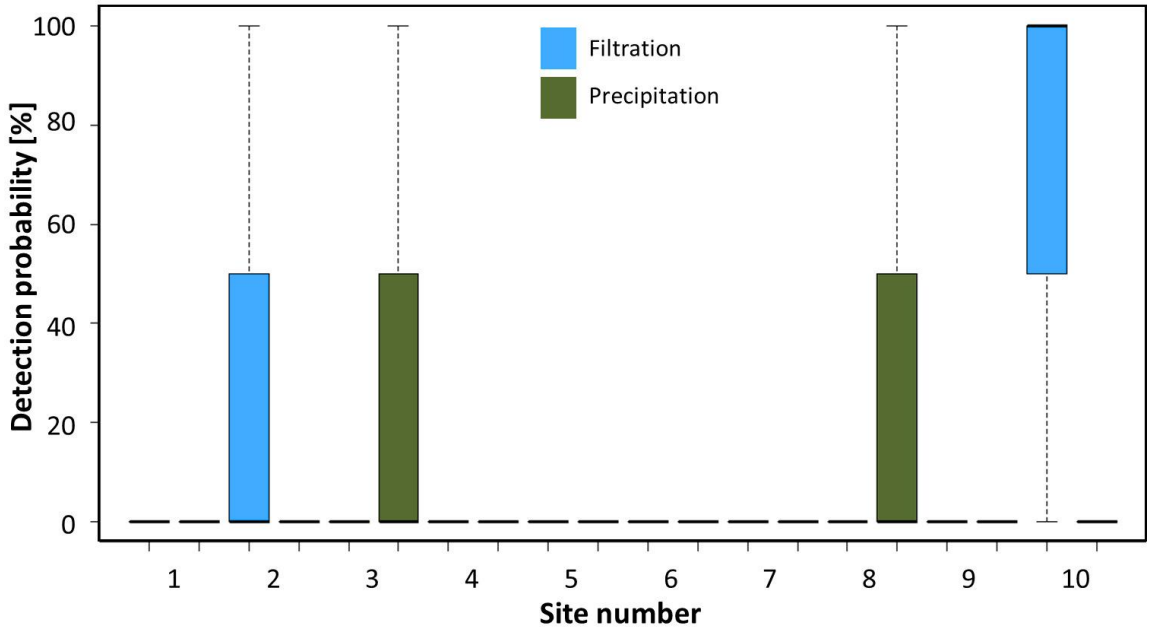

Fig. 5.8. Comparison of the detection probability of the filtration and ethanol precipitation methods on ability to detect signal crayfish eDNA in the River Allen, displaying the inconsistency in results between sample collection approaches at the sites with positive detection. Site 10 was the furthest point upstream, with downstream flow occurring to site 1.

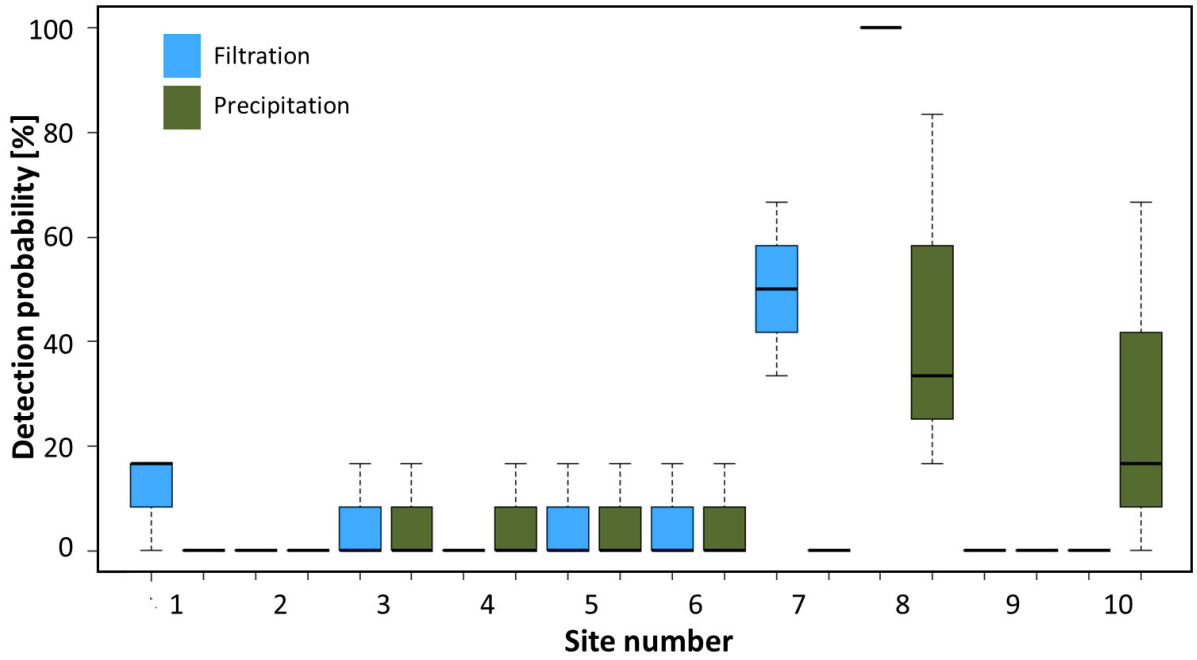

Fig. 5.9. Comparison of the detection probability of the filtration and ethanol precipitation methods on ability to detect crayfish plague eDNA in the River Allen. The highest concentrations of crayfish plague eDNA were found directly downstream of site 9 (the location of the fish farm), with plague eDNA consistently detected at a number of additional sites downstream in lower concentrations, highlighting the potential of the fish farm as a source of plague in the river system. Site 10 was the furthest point upstream, with downstream flow occurring to site 1.

For signal crayfish, the eDNA assay indicated that they were indeed present within the River Allen, despite a lack of previous physical evidence of the species being present. This species was found at four sites across the river, all be it at similarly low detection sensitivities to that of the white clawed crayfish. There is therefore the possibility that at least one small population has been introduced into the River Allen. Although eDNA was detected at multiple sites for this species it could be that there is one single population upstream. In this scenario, the downstream detection would be just the result of downstream transportation of eDNA (Deiner and Altermatt 2014; Jane et al. 2015). Another explanation for this result, could be that the positive detection is due to introduced eDNA, from sources such as fishing gear and bait (Hänfling et al. 2016), although this is thought to be highly unlikely due to regulations and restrictions around using and cleaning fishing gear at this site.

Traditional survey methods were also utilised in order to assess if both species of crayfish were indeed present at the sites. However as in previous years, no crayfish were caught in either the traps or refuges. Nevertheless, the eDNA assays results are indicative of populations or a population still existing within the stream, apparently below levels detectable by 'traditional' established survey methods. The detection success of all the crayfish eDNA assays also varies considerably, with detection probability at some sites being much higher than others. The difference in detection probability across different sites along the river could be explained by several hypotheses. First, a larger population of the crayfish are present at sites with higher detection probability (either white clawed or signal). Or, alternatively that the eDNA sample was taken in close proximity to a population of crayfish (by chance). In this system, the filtration method, using the Millipore Glass fibre filter AP25, 47mm was the least successful at detecting white-clawed crayfish, with lower detection sensitivity occurring in the majority of the samples, and no comparable detection in sites 3, 4, 5 and 6. The limited data obtained on signal crayfish eDNA within the system could not lead to conclusions on the most appropriate survey method here, as neither method was successful at the same sites (both methods reported positive detections at different sites – Fig. 5.9).

It should be noted that the presence of eDNA yet not being able to catch the animals themselves might be indicative of a false positive. False positives could occur due to a number of reasons such as contamination, laboratory errors or due to faulty qPCR assay design and can be costly if wrongly interpreted (Ficetola, Taberlet and Coissac 2016). That said, it is unlikely that the disparity between traditional survey methods and eDNA-based detection in this case can be explained by false positives, in particular historical DNA (Mauvisseau et al. 2018; Turner, Uy and Everhart 2015). Throughout the development, assessment and use of this assay negative controls have been implanted at each stage of the analysis, each successfully yielding true negative results, indicating that no contamination was likely present. Within the degradation mesocosm experiment in chapter 4, crayfish DNA was found to persist within the water column for a maximum of 21 days. Although the decay time was much longer within the sediment, the concentrations detected within the River Allen are at an increased detection probability to those

detected within the final sedimentary disruption stage of the degradation mesocosm experiment (chapter 4) suggesting the presence of an active population.

It was suggested (by the customer) that crayfish plague might have been introduced via a fish farm at the source of the river system (Fig. 5.6, site 9). This was the first time an eDNA-based assay was used to detect plague since the reported outbreak in 2014. Indeed, the eDNA assay did indicate the presence of the plague. It is important to note that this test is thought to be highly sensitive (Strand et al. 2014) and may therefore highlight that small traces of the plague is indeed being introduced in an upstream location (Fig. 5.6, site 9) and/or via transfer from fishing gear. Fish have been identified to carry viable crayfish plague spores within their faeces after ingestion of food sources containing spores (Oidtmann et al. 2002). This could therefore lead to the transmission of crayfish plague spores to a new site such as through the movement of fish between fish farms. It may also, however, be indicative of an existing plague outbreak within the system. It is possible that upon the introduction of signal crayfish to the River, as suggested by the results of this case study, the plague may have also been introduced, causing the decline of white-clawed crayfish to undetectable levels (with traditional surveys).

Caution should be applied when drawing conclusions on the effective comparison of eDNA-based methods when traditional ecological and physical surveys have failed to detect any individuals of either species. In these instances, it is important that the eDNA-based methods utilised have been developed effectively and that quality assurance steps have been implemented. These include the use of field negative controls, NTC's, regular cleaning and decontamination of laboratory workspace and unidirectional laboratory flow (chapter 1, Goldberg et al. 2016). All of these steps were implemented during this investigation, providing confidence that any results were subjected to an minimum contamination risk, supporting the confidence of the detection results observed. Conversely, traditional surveys are notorious in failing to detect species presence or absence in areas of extremely low population levels (chapter 1, Gladman et al. 2010). The disparity in results here is therefore most likely due to very low population levels of the two crayfish species and the plague. This leads to the inability to detect them with traditional ecological methods.

Finally, as expected the three potential crayfish ark sites proved negative for white-clawed crayfish eDNA (Table 5.2). However, two of the proposed sites, were positive for both signal crayfish and plague. This would render these two sites inappropriate for use as crayfish ark sites. Further testing is, however, still recommended using a more extensive survey effort before any conclusions are made on ark site suitability in reference to the presence of other harmful and invasive fish species.

Table 5.2. Percentage of eDNA detection rate (detection probability %) of white-clawed crayfish, signal crayfish and crayfish plague at each of the three potential crayfish ark sites.

Site ID	**Name**	White-clawed	Signal	Plague
13	Lulworth	0	0	0
14	Fonthill	0	25	17
15	Jordan	0	17	17

5.5.3. Conclusions

This investigation has demonstrated that although a population of white-clawed crayfish has not been identified through traditional ecological survey methods in recent years, it is likely to still be present, albeit in low numbers. However, there is also the presence of signal crayfish (accompanied by crayfish plague) within the river giving concern to any future protection or conservation of the population. This report has only provided a snapshot of the crayfish species and crayfish plague presence/absence situation during the sampling period (September 2017) and provides little evidence of the stability of these populations or the changing population dynamics. In order to get a better idea on the crayfish situation within the river eDNA sampling would be recommended at regular intervals to track changes and assess if the situation improves.

5.6. Study 2: The River Ecclesbourne, Derbyshire

The River Ecclesbourne is a small river located in Derbyshire. With its source near Wirksworth, it flows southwards through farmland and villages for roughly 9 miles eventually meeting its confluence with the River Derwent in the village of Duffield, just north of Derby. Historically, there have been records of white-clawed crayfish across the river. However, recent visual survey and trapping survey efforts have failed to find a single individual across the river in the last few years (Fig. 5.10B). There are also currently no records of the presence of any invasive crayfish species, despite large populations of signal crayfish known to be present within the River Derwent.

There is a large weir less than a mile upstream of the rivers confluence with the Derwent. It has been proposed that this weir may be preventing fish migration within the catchment to headwaters. Free movement of the fish would allow for new spawning grounds to be established increasing the health of the river system. Consequently, a solution to allow fish passage at this weir is sought. This could either be the construction of a fish pass or easement on the weir. However, as there are known populations of signal crayfish within the Derwent (below the weir) there is a concern that construction of a fish pass may allow these non-natives to migrate upstream. This would be a threat to any populations of white-clawed crayfish upstream, potentially leading to the loss of these populations. Hence, some explorative surveying was required to assess the presence or absence of signal crayfish within the catchment; this is critical as the presence or absence of signal crayfish will influence the design of the fish pass solution. The use of eDNA sampling methods to identify signal crayfish presence or absence within the catchment may reduce the need for an extensive traditional based survey methodology trapping campaign.

Fig. 5.10. (A) Location of the River Ecclesbourne sample collection sites and the weir. (**B)** Historical white-clawed crayfish presence evaluated via traditional hand searching and trapping surveys was mapped onto the River Ecclesbourne catchment.

To assess for the presence of white-clawed crayfish, signal crayfish and crayfish plague eDNA within the river Ecclesbourne, 14 sample collection sites (Fig. 5.10A) were selected across the course of the river. Each site was selected to obtain data from areas across the entire site, focussing on areas which may have potential crayfish presence in different sections and tributaries. eDNA samples were also collected from seven distinct sites across the Derwent catchment where the presence or absence of either species has been recorded as part of a blind trial for the validation of this eDNA detection methodology for white-clawed crayfish and signal crayfish.

5.6.1. Methodology

eDNA samples were collected from the river Ecclesbourne during October 2017 at 14 distinct sites (Fig. 5.10). Across the Derwent catchment, six additional sites were also sampled. At each of the sites, eDNA samples were collected in duplicate, using the ethanol precipitation method as described in chapter 3 and in Biggs et al. (2014). Analysis was conducted as outlined above for white-clawed crayfish, signal crayfish and crayfish plague.

5.6.2. Results and discussion

Despite recent 'traditional established' trapping and visual crayfish surveys finding no evidence of either crayfish species present within the River Ecclesbourne, the eDNA-based analyses indicated the presence of populations. By collecting samples at regular distances, the areas within the river which are inhabited by each species has been determined. The majority of white-clawed crayfish were identified towards the southern end of the river near to the weir and the confluence with the river Derwent. This can be identified as between sites 4B and 8 (Fig. 5.11/5.12). The results are indicative of a population within the area immediately upstream of the weir. The greatest detection rates were found between sites 6A and 8 suggesting that the majority of the population may lie within this region. Despite white-clawed crayfish eDNA being at detectable levels below the weir, it is difficult to determine if the population is present below the weir as this result may be due to the effect of downstream transport of eDNA fragments (Deiner and Altermatt 2014; Jane et al. 2015; Pont et al. 2018). A smaller localised population was also detected (via eDNA) at site 2B (Fig. 5.11/5.12). Further monitoring of this population is

recommended as signal crayfish and crayfish plague (see below) continue to spread in the catchment.

eDNA-based analysis has provided strong evidence for the presence of signal crayfish (sites 1 through to 3A inclusive) within the River Ecclesbourne, approximately 8km upstream of the weir, at the northern reaches of the river. At site 7A just one qPCR replicate was positive for signal crayfish. This positive sample appears to be right in the middle of the white-clawed population. There is a potential that this could indicate that signal crayfish are present within this population of white-clawed, or within a tributary near this population. However it is more likely that it is indicative of the population upstream and a direct result of eDNA material that has been transported downstream (Deiner and Altermatt 2014). Further analysis consisting of both additional eDNA samples taken from this region and manual searching is recommended to test this hypothesis. An alternative possibility is that a small population of signal crayfish has already established at this site – if so, such a population could be managed effectively.

The amplification of crayfish plague eDNA within several of the samples taken from the River Ecclesbourne, confirms the presence of the plagues pathogen within the river. The plague was most persistent around the downstream sites with higher detection of crayfish, particularly around the weir, and with a high site-specific detection at site 2A (50% detection probability). The detection of plague at this site may also be indicative of the small population of carrier species in this area. The higher detection rate of plague further downstream is however more alarming and could indicate the possibility of an outbreak. Although, only a small number of samples were taken for this part of the study, the results indicate the presence of the plague pathogen throughout the river catchment and therefore populations of the white claws are at high risk. However, it is unknown to what extent that this 'outbreak' is affecting the population of white-clawed crayfish without further monitoring. Further study is recommended, in order to monitor the plague across the whole river and catchment.

Not every site tested positive for target DNA, neither white-clawed crayfish, signal crayfish nor crayfish plague were detected at sites 3B, 4A and 5B (Fig. 5.11/5.12).

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Fig. 5.11. Summary of species *(*white-clawed crayfish, signal crayfish, crayfish plague*)* presence/absence detection at each site along the River Ecclesbourne using the ethanol precipitation sample collection approach.

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Fig. 5.12. eDNA based presence/absence detection of white-clawed crayfish, signal crayfish and crayfish plague mapped onto the River Ecclesbourne catchment. The number of positive (shaded) and negative (unshaded) qPCR replicates (out of six, for each of the duplicate samples – 12 in total per species/site) within the qPCR are indicated.

Derwent catchment

Analysis of the Derwent catchment samples indicate that white-clawed crayfish are not present in any of the sites visited. However, it should be noted that populations may remain but at very low abundances, beyond the limit of detection of this assay (chapter 3) and/or environmental constraints or sample inhibition (Jane et al. 2015). That said, all control samples (conducted during analysis) resulted in the expected outputs i.e. negative controls were negative and positive controls were positive.

Signal crayfish, however, were detected within three of the six sites (Table 5.3). Both environmental replicate samples taken at site D have a high proportion of positive qPCR reads - indicating the potential of a high-density population (or the collection of a sample local to a population). Site A was found to have an extremely low detection of eDNA from species, which could be indicative of a small population which has only recently been introduced and is only beginning to establish itself within the sample site.

Table 5.3. eDNA presence/absence results of signal crayfish in the Derwent catchment, including the detection probability and the accompanying Ct value.

Site ID		Present / Absent	Detection probability [%]	Average Ct* value
A	a	Absent	0	n/a
	b	Present	17	44.921
B	a	Absent	0	n/a
	b	Absent	0	n/a
C	a	Absent	0	n/a
	b	Absent	0	n/a
D	a	Present	67	39.093
	b	Present	83	39.484
E	a	Present	50	40.143
	b	Absent	0	n/a
F	a	Absent	0	n/a
	b	Absent	0	n/a

*Ct = Cycle threshold – the number of cycles it takes for the amplification signal to become distinguishable form the background amplification signal. i.e. the point at which the sample is distinguishable from the negative controls (NTC's). A lower Ct value indicates a higher starting concentration of eDNA within a sample (a/b = environmental replicate).

5.6.3. Conclusions

Despite confirming that the population of white-clawed crayfish are still present, this report has also confirmed the presence of both signal crayfish and the crayfish plague within the system. It was previously thought that signal crayfish were not yet present above the weir, with only records of them being found within the Derwent. Although this report did not identify the presence of signal crayfish at the single sample site directly downstream of the weir it has resulted in the confirmation of the presence of a small population in the upper reaches of the river. The presence of the invasive species and the crayfish plague could result in the loss of the white-clawed crayfish population in the future.

This report has only provided a snapshot of the crayfish species and crayfish plague presence/absence situation during the sampling period (October 2017) and provides little evidence of the stability of these populations or the changing population dynamics. To get a greater idea on the crayfish situation within the river eDNA sampling would be recommended at regular intervals to track these changes (as in chapter 4).

The removal of the weir could pose a risk to the white-clawed crayfish population by encouraging the further spread of signal crayfish and crayfish plague within the system. Despite this risk, the population is already under serious threat from the signal crayfish population present upstream, and under stress from the outbreak of crayfish plague which was detected in this study.

5.7. Study 3: Lincolnshire fish stocking project

Dunston coaching pond was dug approximately 40 years ago and within a couple of years, an unknown species of crayfish was introduced. Water surface area covers 1.4 acres in total with a maximal depth of over 6ft. It has historically been stocked with trout and now contains a few crucian carp (*Carassius carassius*), tench (*Tinca tinca*) and rudd (*Scardinius erythrophthalmus*) (Environment Agency 2018, *personal communication*). Within recent years, the landowner has not seen any crayfish present within the water, however, no real effort has been made to detect or survey for the species. In the future, the landowner was discussing the potential of utilising the site as a stock brooding pond for a number of freshwater fish species as part of a fish farm. However, due to the risks of spreading the plague or the invasive crayfish itself the site must be confirmed signal crayfish free before used as a brood stock site, if the species present are to be translocated to sites containing white-clawed crayfish.

Additionally, the end-user was interested in surveying a nearby river – the Long Eau for the presence of signal crayfish using eDNA-based detection. In the river headwaters there is a Trout farm with close links to the river and as such there is a risk of plague or invasive crayfish transfer between waterways with fish stock transfers. Twice annual trapping surveys at two of the sites included in this investigation (E3, E4) have resulted in no detection of any species. However, due to the presence of a significantly large population of signal crayfish in a nearby river (the upper Lud), there is a substantial risk signal crayfish introduction to the River Long Eau. This nearby population, coupled with the risk of plague transfer into the river Long Eau via the fish farm, means that it is therefore important to monitor the Long Eau. As such, the end-user was interested in incorporating an eDNA-based approach to increase this surveillance effort.

5.7.1. Methodology

eDNA samples were collected from 4 independent sampling locations across Lincolnshire on 15/10/2018. These included one still water site, consisting of two isolated ponds (E1, E2 – Fig. 5.13) and three additional river sites (E3, E4 and E5). At each of the river sites, duplicate samples were collected following the filtration (Sterivex syringe filter, 0.22µm, volume: 200ml) protocol outlined above (Fig. 5.14). However, only one sample was taken from each pond site. In terms

of analysis, each sample was prepared with 6 qPCR replicates and assessed for the presence of signal crayfish eDNA only (Mauvisseau et al. 2018).

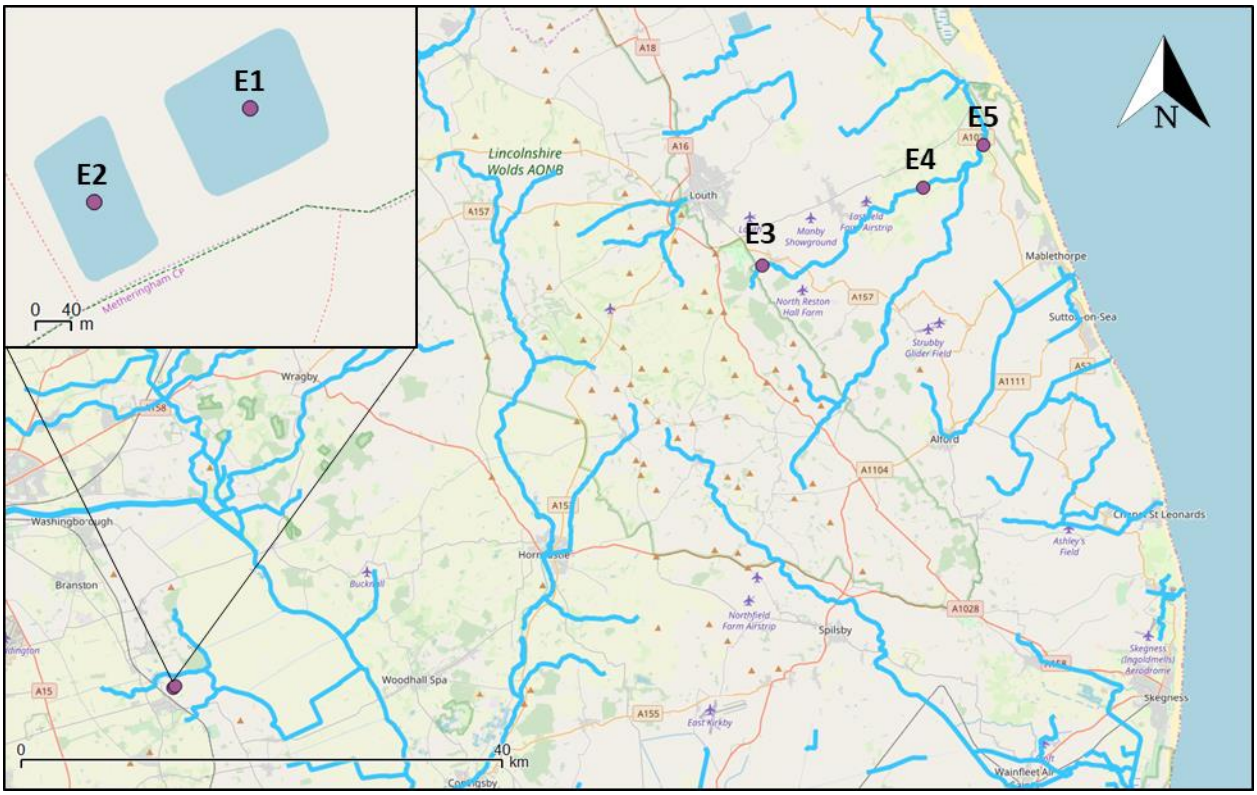

Fig. 5.13. Indicative locations of each sampling site (E1 to E5), within Lincolnshire, UK. ©OpenStreetMap contributors https://www.openstreetmap.org/copyright.

Fig. 5.14. Disposable single use kit for the Sterivex filtration eDNA sample collection method. Source author, ©Chris Troth, ©SureScreen Scientifics.

5.7.2. Results and discussion

Out of all of the sites visited and sampled, only site E2 (Dunston reservoir 2) resulted in a positive detection for signal crayfish eDNA (Table 5.4). This is indicative of the presence of signal crayfish individuals or a population within the reservoir and reveals the identity of the previously unknown crayfish species. The other reservoir (currently in use for farm water storage) was not positive for crayfish presence. No signal crayfish were also detected in any of the river samples, indicating that the Long Eau in these locations does not support a population of signal crayfish. This confirms the longstanding trapping effort results. Further monitoring is, however, still recommended within the river sites due to the significant risk posed from the transport of invasive species, particularly crayfish plague (Oidtmann et al. 2002).

Table 5.4. Results from the qPCR analysis for the detection or signal crayfish eDNA.

ID	Replicate	Site Location	eDNA detected	Detection probability [%]
E1	A	Dunston – main farm reservoir	No	0
E2	A	Dunston – reservoir 2	Yes	66.67
E3	A	Upstream of fish farm	No	0
	B	Upstream of fish farm	No	0
E4	A	Downstream – Long Eau	No	0
	B	Downstream – Long Eau	No	0
E5	A	Beneath A1031 Bridge	No	0
	B	Beneath A1031 Bridge	No	0

5.8. Discussion

5.8.1. Case studies

Within this chapter three case studies are described, which were completed as part of the development and validation of the white-clawed crayfish eDNA assay. Each case study demonstrated how such an eDNA assay would be used by end-users to assist with species monitoring, presence/absence detection or to assist with a conservation programme.

Each project demonstrates different ways in which end-users could utilise an eDNA service for crayfish species and/or crayfish plague, from large scale river and catchment projects (Study 1, Study 2) to smaller scale projects focussing on individual sites (Study 3). The reasons behind an end-user selecting to utilise crayfish eDNA-based detection (instead of traditional ecological survey options) can vary depending on the needs of the end-user. These include: the speed of result turn-around required, the ease of use, survey licensing requirements and costs. The service would most likely be used as either a first line species screening test (before more in-depth surveys are conducted (i.e. Study 3)) or as a last resort when all other survey methods have been exhausted (as is the case within Study 1 and Study 2). The first 'type' of use would typically be the most common used by an average ecologist and citizen scientists, and therefore would be the most common application commercially. The more in-depth projects would likely only be

conducted by larger organisations and government agencies. With each progressive study, methodological improvements as assessed within chapter 3 and 4 were implemented.

5.8.2. Commercial development

Concluding from the development of the white-clawed crayfish eDNA assay (chapter 3 and chapter 4) and the case study application discussed within this chapter, the assay was further developed and incorporated into a commercially available product (SureScreen Scientifics, 2018). To achieve this on a suitable, effective and affordable scale (for both industry and end-users) a few modifications were made to the sample collection process and analytical stages, although the principles of analysis remain largely the same (Table 5.65.

In chapter 2, a comprehensive comparison was made of four different sample collection approaches – ethanol precipitation, and filtration with pore size 2µm, requiring a pump, pore size 0.22µm, single use and pore size 0.45µm, single use. After some disparity in results across different environmental situations (i.e. between ponds and rivers), from a commercial point of view, filtration using 0.45µm, single use sterile disposable filters were selected as most appropriate. When proposing a specified sample collection method, it is also important to assess the accessibility of the methods in question. On an end-user applicability basis, this method is most suitable due to its ease of use (single use), limited risk of contamination and cost effectiveness, compared to filtration using the pump. Whilst the use of the pump may result in a higher volume of water collected, it is also heavy, relies on battery power and as a result of this can be difficult to transport between sites. In comparison with 0.22µm filtration, despite little apparent differences in the resulting DNA concentrations, the larger pore size (0.45µm) allows for larger volumes of water to be collected, something which is important, particularly when collecting samples in murky conditions. Although ethanol precipitation did on average have a greater detection success rate than filtration in the river-based experiments, it failed to achieve acceptable levels of detection in either the mesocosm or pond comparison experiments.

Table 5.5. Post developmental modifications made to the white-clawed crayfish (and also therefore signal crayfish and crayfish plague) eDNA assays to allow for the provision of these assays on a commercially accessible basis.

Stage	Modification	Method used during development	Reason for modification
Sample Collection	Choice of sample collection method (filtration 0.45µm).	Combination of methods (precipitation and filtration (0.22µm, 0.45µm, 2µm).	Most appropriate in a commercial setting.
	Use of single environmental 'site' replicate.	3 environmental replicates.	For cost savings, allowing the provision of the test at an affordable level.
	Provision of 'spike DNA' within sample collection kit as a degradation and inhibition control marker.	N/A	To detect incidences of inhibition of degradation of sample.
Sample Analysis	Use of 12 qPCR replicates.	6 qPCR replicates.	To offset for the use of a single environmental 'site' replicate.
	Multiplex of qPCR to allow for testing of DNA spike.	N/A	To detect incidences of inhibition of degradation of sample. Multiplex used for cost and time savings.
Result interpretation	Results presented as score out of 12 (i.e. 0/12, 6/12 etc.)	N/A	In line with current report for GCN eDNA (Biggs et al. 2014).

Although not always as expensive as traditional survey methods, the cost from sample collection to report writing can be as high as £90 for the analysis of a single sample (Table 5.6). This is only the cost of analysis associated to the service provider, with additional costs later added for service provider profit, VAT and costs associated with the end-user's time for sample collection. When studying large survey areas, it is clear that the costs to the end-user can run into thousands of pounds. To promote the adoption of the methodology, it is therefore important to ensure that any eDNA-based service made commercially viable is economically

viable, both to the customer and service provider. To keep the costs down, several cost-saving changes were made to the designed crayfish assays mentioned in this thesis. A reduction in environmental replicates (from two to three as recommended in chapter 1), to a single replicate is one such example. The cost of the analysis of three samples from a single site to the end-user would render eDNA-based detection methods highly expensive and unaffordable. To counteract the potential effect such a change had on the reliability or repeatability of the assay, I also altered the assays recommended number of qPCR replicates run (from an original six used within the study examples to 12) (see Table 5.6).

Table 5.6. Calculated cost (to the service provider per sample) of crayfish eDNA kits and analysis for different levels of analysis (single, double or triple analysis of a sample for different target species, i.e. white-clawed crayfish, signal crayfish and/or crayfish plague).

Stage of analysis	Kit	1 Target	2 Target	3 Target
material cost	£12	£20	£40	£60
labour	£3	£25	£35	£45
shipping	£3	-	-	-
overheads	£0	£25	£25	£25
Total Cost	**£18**	**£70**	**£100**	**£130**

Further, in line with existing commercial eDNA-based methods (Biggs et al. 2014), an inhibition and degradation control marker or 'DNA spike' was added ('SureScreen Scientifics GCN eDNA Spike'). This allows the assessment of each sample for any inhibition which may be affecting results, increasing confidence in the obtained signal. I also developed a multiplex qPCR application combining white-clawed crayfish with the DNA spike within the same qPCR run. This reduced the level of reagents now required for the same analytical procedures.

The way data are presented to the end user also needs to be explored. Currently, most eDNA providers send results as presence/absence or positive and negative (Biggs et al. 2014; Rees et al. 2017). However, in the scientific literature, results from eDNA-based methods

usually provide more detail such as Ct values (Stoeckle et al. 2017), detection probability [%] (Erickson et al. 2017; De Souza et al. 2016) and/or DNA concentration (Tillotson et al. 2018). At the current time, I have recommended the service provider to produce the standard report (without additional data). However, it should be noted that providing more data would also increase the value generated through the assay for the end-user.

5.8.3. Commercial application

To enable SureScreen Scientifics to launch the white-clawed crayfish assay, alongside assays for signal crayfish and crayfish plague (validated separately) a number of SOP's, consumer instructions and further informative documents were composed (Table 5.7). It is essential that for successful end-user application of a comprehensive and sensitive test (such as the one developed here), such documents are provided containing detailed instructions on sample collection for example. For effective provision of an eDNA-based test it is also important that the service provider has thorough and detailed step-by-step laboratory analysis instructions detailing the entire process. Before the launch of any commercial eDNA-based product, each organisation must also make a number of additional considerations including: pricing strategy, website additions and design, product launch and additional customer service requirements relevant to the new product. However, these points are not discussed here because it is specific to the commercial organisation and their product launch strategy.

Table 5.7. Documents required and developed for the commercial application of the white-clawed crayfish using the methodologies outlined within chapter 3, 4 and 5.

Document	Description	Location
End user requirements		
Sample collection form	- Designed to collect information from the end-user; name, contact details, site and sample details. - Contains brief sample collection instructions.	Appendix 5.6
Sample collection instructions	- Detailed guide on sample collection. - Contains step-by-step instructions with photo guide.	Appendix 5.7
FAQ's and detailed sampling advice	- Additional information provided based on common end-user questions. - Provides advice on sample collection under different environments and conditions.	Appendix 5.8
- Service provider-based requirements:		
Kit manufacturing	- Standard operating procedure for the manufacture sample collection kits. To ensure consistent and reliable kits are provided to the end user.	Appendix 5.9
DNA extraction	- Standard operating procedure for DNA extraction from crayfish eDNA samples. - To ensure that standards are maintained within the lab and that each sample is treated with the same analytical process.	Appendix 5.10
qPCR	- Standard operating procedure for qPCR set up for crayfish eDNA samples (white-clawed crayfish, signal crayfish and crayfish plague). - To ensure that standards are maintained within the lab and that each sample is treated with the same analytical process.	Appendix 5.11
General result report format	- Report format containing space for results and an explanation on how a result should be interpreted.	Appendix 5.12

5.8.4. Conclusion

It is well noted that the majority of scientifically published eDNA assays cannot immediately be applied on a commercial scale for conservation and monitoring purposes. Here, I used the assays developed during my thesis and applied them in three separate field trials to illustrate how a commercial application of such assays could be packaged, and what results may be expected. Although each of the case studies were successful in fulfilling their objectives, it should be noted that effective application of any assay within the end-user community can only occur with the additional development of SOP's, sample collection instructions and effective staff training as indicated in this chapter.

Chapter 6: General discussion of thesis findings and future directions

6.1. Introduction

End-user adoption of eDNA-based detection remains relatively small-scale, for a small selection of invasive species or those of conservation concern. Despite the huge potential of eDNA-based detection methods, the level of validation required to transform any given assay into a viable commercial service as outlined within chapter 1 of this thesis, presents the biggest hurdle to the adoption of the approach for a wider list of species. This thesis was designed to develop and validate an eDNA-based species detection test for the white clawed crayfish, *Austropotamobius pallipes*, which could be applied upon a commercial scale. By first outlining the 'hurdles' which would need to be considered and addressed before any designed assay could be provided to end-users, I was then able to validate the methodology to an effective level. Thus, transforming the assay to a commercially available, viable and end-user accessible product.

6.2. A commercially available white-clawed crayfish eDNA assay

In September 2018, once assay validation was completed (chapters 3 and 4), the white-clawed crayfish eDNA-based service outlined within chapter 5 was launched by SureScreen Scientifics. During the first sample collection season of the service becoming available (1st September 2018 – 31st October 2018), 41 samples were analysed (SureScreen Scientifics 2018). For the first half of the 2019 season, (1st April 2019 – 31st July 2019) a further 23 samples were analysed.

Since the initial launch, there has been an increasing amount of interest in its use. However, in order to achieve further end-user confidence and potential subsequent government agency approval the assay now needs to be made publicly available and peer reviewed. Chapter 3 of this thesis is in review (Molecular Ecology Resources), with the addition of some of the ecological data found within chapter 5. This is titled "Development and application of eDNA-based tools for the conservation of white-clawed crayfish". Chapter 4 is also due to be submitted for publication

post submission of this thesis. Once the applicability of this assay has been proven on both a commercial level and through peer reviewed scientific publication, end-user adoption is predicted to increase – providing a basis for future adoption of the assay into standardised species survey and monitoring regulatory framework within the U.K.

6.3. Addressing the hurdles associated with the commercial development and implementation of eDNA-based detection methods

6.3.1. Hurdle 1: Validation

Utilising the white-clawed crayfish as a model organism, this thesis demonstrates the application of thorough validation and assessment of limiting variables or 'hurdles' to the full-scale commercial implementation of eDNA-based detection methods (Table 1.2). For such applications it is important to assess and address the effect that variations in methodology, experimental and environmental conditions can have on the outcome of an eDNA-based species detection survey.

The remainder of this final chapter briefly summarises the hurdles which were introduced within chapter 1, giving indication on how each hurdle was considered and addressed in the case of the development of the commercially applicable eDNA-based assay for white-clawed crayfish (Table 6.1). The strengths and weaknesses of the approach taken are identified and outlined, giving further recommendations for future research and development which could improve the commercial application of eDNA-based techniques.

Table 6.1. Summary of how each of the 'hurdles' associated with the commercialisation of eDNA have been addressed, in regard to the white-clawed crayfish eDNA assay developed for this thesis.

	'Hurdle'	Addressed in:	Summary
1	Assay validation	Chapter 3, Chapter 4, Chapter 5.	• Validation addressed each of the hurdles 2 to 7 (see below). • Submission of research paper to a scientific journal for peer review and publication to begin the process of validation of methods for regulatory use.
2	Detection sensitivity	Chapter 3	• MIQE guidelines consulted during the development and validation of the assay. • Limits of detection (LOD) and quantification (LOQ) were assessed.
3	Sample collection and preservation methodology	Chapter 3	• Four sample collection methodologies were compared (filtration 0.22µm, filtration 0.45µm, filtration 2µm and ethanol precipitation) to determine the most suitable. • Interestingly it was highlighted that different sample collection approaches may yield different results across different types of habitat, with no one method optimal for all.
4	Sample site	Chapter 3, Chapter 4.	• Sample collection approaches were trailed in a number of different environments in which white-clawed crayfish can be found (ponds, lakes, rivers, etc.). • Within a lentic site there was significant variation in the detection probability of white-clawed crayfish less than 10m apart.
5	Persistence and decay of eDNA	Chapter 4	• Within a controlled environment white-clawed crayfish eDNA degrades below detectable levels within the water column 14-21 days post removal of individuals. • Sedimentary (ancient) eDNA persists for much longer, with detection observed 56 days after removal of individuals. • Likely to vary across habitat type and environment.
6	Environmental influences	Chapter 4	• The detection success of eDNA-based survey methods can vary significantly across different seasons. • A recommended survey season is proposed for white-clawed crayfish eDNA surveys (1st April – 31st October) to coincide with the increased activity of crayfish during this period, and subsequent increases in field eDNA concentrations.
7	Quantification	Chapter 3	• The relationship between estimated crayfish population size (estimated capture-mark-recapture methods) and detection probability of eDNA was significant, but only when water temperature was included. • For reliable quantification, assessment and consideration of environmental variables is required.
8	Inhibition and contamination	Chapter 3	• Use of internal 'spike DNA' degradation and inhibition control marker within sample collection kits.
9	Consistency and reliability	N/A	• Not currently applicable – methodology is currently only applied within one laboratory and is not part of a national monitoring scheme.
10	Commercial accessibility	Chapter 5	• Standard operating procedures and instructions have been developed for laboratory staff and end-users for analysis and sample collection. • Methodology now available through a commercial service provider.

6.3.2. Hurdle 2: Detection sensitivity

Ensuring a high detection specificity and sensitivity is key to a reliable and reproducible eDNA assay (MacDonald and Sarre, 2017; Mauvisseau et al., 2019a), only with an effective assessment of the suitability of an assay across different habitats, conditions and non-target species can it be truly considered appropriate for use. The sensitivity of the eDNA assay developed and demonstrated within this thesis has been continuously challenged in several respects (see chapter 3). For example, sensitivity was confirmed using *in-silico*, *in-vitro* and *in-vivo* approaches as outlined within the MIQE guidelines (Bustin et al., 2009). Such validation also followed the approaches adapted specifically for eDNA assays by MacDonald and Sarre (2016). Throughout assay testing and development qPCR replicates were assessed with a minimum of six per sample, however to increase detection sensitivity further in commercial field samples this was increased to 12 environmental variables (see chapter 4). This was especially necessary when I trialed the assay under different field conditions (chapter 5), and the majority of samples had detection probabilities of lower than 30%.

6.3.3. Hurdle 3: Sample collection and preservation methodology

The existing consensus among researchers is that filtration is the most appropriate methodology employed for eDNA sample collection (Spens et al., 2017). Indeed, in the majority of cases this method returns the greatest eDNA concentrations compared to alternative methods such as ethanol precipitation (Eichmiller et al., 2016b; Spens et al., 2017). However, in chapter 3, I highlight, that although filtration does appear to provide certain benefits including a greater detection probability and lower Ct values, this is only true in particular environments. Therefore, at least for the assay developed in this thesis, it is also not always the most appropriate method of choice, and that the most effective, reliable and repeatable sample collection method varies between both habitats sampled and species surveyed. Further research should therefore be conducted on optimal sampling methods for any newly designed species-specific assay.

6.3.4. Hurdle 4: Sample collection site

Choice of sample collection sites is an important factor affecting success of species detection using eDNA-based methods (Furlan et al., 2016; Goldberg et al., 2018; Moyer et al., 2014).

Within chapter 4, a significant difference was observed in the detection probability of white-clawed crayfish eDNA between sites less than 40m apart (in this instance within a pond). In some instances, false negatives were also reported as no eDNA signal was detected at some of the sites sampled despite the release of 40 white-clawed crayfish individuals. This experiment emphasised the importance of effective sampling methodological design to ensure maximum repeatability and effectiveness of eDNA-based methods. Interestingly, despite these key findings, there is very little information on sample collection site bias, with only a handful of studies assessing this in either lotic (Deiner and Altermatt, 2014; Jane et al., 2015) and/or lentic (Goldberg et al., 2018) sites.

I conclude that to ensure the greatest chance of detection, the whole perimeter should be sampled (where possible). It should be noted that at the current time, the majority of published studies only collect a sample from one single location of a pond, river or lake (Agersnap et al., 2017; Rheyda. Hinlo et al., 2017; Smart et al., 2015). This would, based on findings in chapter 4 indicate these studies would suffer from a large number of false negatives and the results should be taken with caution.

In a river system, sampling the whole perimeter is likely to be impossible and so I indicate that a representative sample can be collected by conducting a transect across the river, or by collecting samples from the river's edge in as many locations as possible. However, it may be the case that samples collected from specific locations within the river (such as the bank, within riffles, slow flowing areas or areas with rocky substrate for example) may yield higher concentrations. Such micro-scale environmental variation and influences on eDNA abundance should be explored in further studies.

6.3.5. Hurdle 5: Persistence and decay of eDNA

Degradation of eDNA is another factor, which is important to account for when designing and validating an assay (Goldberg et al., 2018; Murakami et al., 2019), especially if that assay is to be applied across a number of different environments. The determination of the time that eDNA remains detectable for (once individuals have left the system) was identified in this thesis at between 14-21 days for white-clawed crayfish in controlled environments. This is at the

longer end of the spectrum. It is vitally important to get an understanding on degradation rates of eDNA for any newly designed assay or target species, in order to appropriately interpret the results. For example, shifts in community dynamics can occur in weeks or even days with regard to the invasion of signal crayfish and the subsequent loss of native white claws.

Interestingly, the extended period of detection as shown within the sediment (chapter 4) not only highlights the importance of effective sample collection design i.e. to avoid disruption of historic eDNA but it could be targeted to give a historic view of any given river and its previous occupants.

6.3.6. Hurdle 6: Environmental influences

Environmental variables, factors and influencers are one topic which has been repeatedly investigated assessed and discussed throughout this thesis (chapters 1, 3 and 4). The reported significant impact that changes in conditions and environment can have is important to understand and assess across all eDNA-based detection studies for assurance in reliability and repeatability (Harper et al., 2019). Indeed, a number of eDNA studies have reported the influence of environmental conditions such as temperature (Eichmiller et al., 2016a; Goldberg et al., 2018; Jo et al., 2019; Tsuji et al., 2017), UV (Mächler et al., 2018; Strickler et al., 2015), pH (Seymour et al., 2018; Strickler et al., 2015), yet the full impact of these remains relatively unknown. However, it is agreed by most, that such conditions or factors should be assessed before an assay can be applied in a sensible level for species monitoring purposes (Goldberg et al., 2016; Harper et al., 2019).

In each of the field studies described within this thesis, variables including water temperature, pH, sediment type, habitat type and the presence of other species, were all collected. However, due to the complex interaction effect that such variables may have on the success of eDNA-based detection methods (Strickler et al., 2015), and the varied or stochastic nature of sample collection sites, species population numbers and habitat features, it was difficult to draw any conclusions between which variables may be affecting the detection probability of white-clawed crayfish using eDNA. In order to pick apart the specific role each has

on eDNA detection, one would need to conduct more tests *ex situ* in controlled mesocosms as developed in this study and so further work could focus on this aspect.

Interestingly, in my field tests (chapter 3) I was able to show the influence of temperature on eDNA detection for white-clawed crayfish. In brief, there was only a relationship in physical capture data (collected via mark-recapture methodology) and eDNA detection when water temperature was factored in. This result highlights the importance of collecting environmental data when conducting eDNA assessments, particularly if one of the goals is to quantify species presence or biomass (see section 6.3.7.).

In terms of seasonal detection of white-clawed crayfish (chapter 4), I outline a recommended survey period between April and October. This coincides with both observed crayfish activity levels and the concentrations of eDNA detected. Within the commercial application of white-clawed crayfish eDNA the recommended survey dates, based upon this research have been set from April 1st until October 31st for reliable and repeatable detection. Existing traditional survey recommendations in the U.K. for white-clawed crayfish suggest that ideally surveys should only be conducted between July and September in order to avoid disturbance of the breeding and hatching periods. The extended eDNA survey period highlighted here can increase the crayfish survey window by over 100% and improve the species monitoring effort.

Although a key aspect to the assessment of the 6th hurdle, the effect of downstream flow and dispersal of eDNA was not assessed within this thesis, despite being reported by other studies as having a clear influence on the eDNA-based detection (Deiner and Altermatt, 2014; Wacker et al., 2019). I recommend further investigations are conducted into the effect of downstream flow, particularly when attempting to estimate population numbers or biomass within lotic systems. Downstream flow is a complex model (Shogren et al., 2017), and to be effectively interpreted would require a number of additional large-scale projects in rivers of different sizes, flow rates and of differing habitat features, something which would be difficult to orchestrate, particularly as a result of increasingly rare and declining populations of white-clawed crayfish.

6.3.7. Hurdle 7: Quantification

The challenge of quantifying species biomass and/or population numbers from eDNA-based methods is well known (Nathan et al., 2014; Pilliod et al., 2013). However, the value such data could provide to conservation studies is significant and therefore worthy of exploring when validating any new assay. That said, as demonstrated throughout this thesis (chapter 3 and 4), it is not a simple task, again likely due to the strong driving influences environmental factors have on eDNA (Klymus et al., 2015; Strickler et al., 2015).

Although quantification of white-clawed crayfish via the use of eDNA shows promise (chapter 3 and 4), there are limiting factors which still present 'hurdles' (chapter 1) necessary to overcome. As such, I have recommended that at the current time the assay is only used to detect the presence or absence of white claws until further work can be undertaken to improve the reliability of quantification.

6.3.8. Hurdle 8: Inhibition and contamination

Although rarely reported by commercial laboratories or for that matter, within scientific literature, contamination can be a real risk factor and sometimes a regular occurrence when working with samples which naturally contain low DNA concentrations. Therefore, it is important that any service providing eDNA laboratory testing is implementing strict de-contamination and cleanliness protocols. It is important that the laboratory is open about their procedures to assure end-user confidence in the service which they provide.

The use of spiked DNA (as demonstrated within chapter 5 of this thesis), provides an additional level of quality assurance to the end-user. Such practice provides knowledge that the sample has (in most cases) not been subjected to degradation factors or containing excessive amounts of inhibitory substances. Going forward, it is key that measures such are these are implemented in all commercial eDNA services.

6.3.9. Hurdle 9: Consistency and reliability

As the eDNA-based detection field moves along, and services become ever increasingly used (on a commercial level), it is inevitable that further check-points and quality assurance steps will need to be applied. Such quality assurance may also be a requirement by regulators, should

any given assay be used on a similar scale to that of the great crested newt for example. The European CEN standards are only just being considered a necessity for eDNA monitoring on a commercial level and this is being explored as part of multi-national working group, DNAqua-Net and EU COST Action CA15219 (as discussed in chapter 1). It is expected that guidelines will soon be made available describing certain standards for laboratory analysis and management. This work group is developing additional guidelines on single-species qPCR assay design and development (DNAqua-Net, *personal communication*), similar to those demonstrated within this thesis.

6.3.10. Hurdle 10: Commercial accessibility

Through the creation of standard operating procedures (SOP's) such as the DNA extraction process (appendix 5.10) and qPCR process (appendix 5.11), step-by-step sample collection instructions (appendix 5.7), and end-user FAQ's (appendix 5.8), the research outcome from this thesis has been disseminated into a commercial product which is accessible to the general ecologist, conservationist or citizen scientist. The development and design of this assay has been conducted in such a way to allow for future changes and adaptations to be made in order to incorporate future methodological developments and advancements in the field.

6.4. Future directions

6.4.1. Using citizen science for eDNA sample collection

Since the development of the standardised DEFRA protocol for the molecular detection of great crested newt, *Triturus cristatus* populations (Biggs et al. 2014), eDNA-based detection of this species has become increasingly popular. Five years since the method was approved it is now offered by several consultancies and diagnostic laboratories and is now a widely recognised method across the field. Interestingly, the success associated with eDNA-based detection commercialisation of great crested newt has resulted in some exploring how the technique can be more widely utilised; for example, an increasing trend associated with 'citizen science' projects (Biggs et al. 2015). This citizen science led approach attempts to remove any barriers which would limit the usability of the technique, therefore making it available for the educated

public to use. In Biggs et al. (2015) volunteers were utilised to collect eDNA samples in the place of trained individuals. Interestingly and somewhat surprisingly, the results showed there was minimal risk of contamination and incorrect collection if a small amount of training was made available, highlighting that citizen science may prove an important aspect of conservation and biodiversity monitoring in the future (Biggs et al. 2015). In the case of sample collection for white-clawed crayfish a number of citizen scientists may already be employing eDNA-based detection through the now commercially available service.

6.4.3. Occupancy modelling

Occupancy modelling is an additional application which could easily be applied to eDNA investigations in order to improve detection, accounting for imperfect detection and allowing for a determination of the optimum number of samples needed for a reliable result (Schmidt et al. 2013). Occupancy modelling could therefore be applied to ensure consistency and reliability to eDNA investigations through the use of statistical modelling (Lahoz-Monfort et al., 2016; Wineland et al., 2018). Although not reported in this thesis, modeling was attempted on some parts of the data, though omitted due to insufficient replication to make any effective comparisons. However, if a sufficiently large number of samples can be collected, occupancy modelling would allow for a greater level of detail to be brought into any assessment. Such modeling would effectively give a reliability percentage of any false negative (and possibly as the tools develop false positives too), which can be used to improve the design of eDNA surveys (Lugg et al., 2018). Applying this to a commercial setting would be desirable but would incur additional costs and may be seen as unnecessary by either the suppliers and/or the end users for their purposes. That said, a more detailed study focusing on the minimum requirements to deliver occupancy modeling on eDNA based projects would be a sensible next step.

6.4.2. Metabarcoding

The development of a one-for-all commercially applicable and affordable metabarcoding test for the detection of multiple species from one sample would provide greater commercial value to eDNA-based detection services. At the moment, it would require a drop in the costs of sequencing technologies for this to be achieved for a single sample on a commercial scale similar to that which is currently implemented for the existing great crested newt targeted assay. Large

sequencing runs of many pooled samples can bring the costs significantly down to an affordable level, however, in the commercial sector it may often be a number of weeks before a sufficient number of samples are collected for a run, presenting potentially significant delays in sample analysis. However, the approach has already been demonstrated for a selection of taxonomic groups including: freshwater metazoans (Lim et al. 2016), fish (Hänfling et al 2016; Miya et al. 2015), amphibians (Valentini et al. 2016), invertebrates (Blackman et al. 2017; Deiner et al. 2016) and aquatic mammals (Port et al.2016). For this to work, specifically designed barcoding primers (designed to bind to conserved regions of a gene) are used to amplify a target gene (the sequence of which will vary between species) of species within the same target taxa. The resulting amplicons are then sequenced, enabling multiple different species to be identified at the same time, within the same sample. This is in contrast to using the more targeted approach using species specific primer pairs which formed the basis of this review previously. Despite a number of apparent downfalls associated with this meta-species approach as listed by Thomsen et al. (2015), eDNA metabarcoding does show great potential with regard to a commercial tool. However, more research is required within this area to assess the limitations of this particular area of commercialisation.

6.4.2. Further development of single-species eDNA detection

Regarding the more targeted approach as applied within this thesis for white-clawed crayfish, an alternative to qPCR has been developed and offers great promise in the commercial practice of eDNA-based surveying. Droplet digital PCR (or ddPCR) promises to provide a cheaper and quicker method of assessing eDNA samples, when compared to qPCR (Nathan et al. 2014). ddPCR appears to be more sensitive for targeted assays as reported in one of the first initial eDNA-ddPCR studies (Doi et al. 2015). ddPCR works similarly to qPCR, with the difference being that the reaction (rather than being conducted as a whole) is fractioned into thousands of droplets. Each of these are then subjected to amplification and measured separately, providing a consensus of many individual amplification events, something which cannot be easily determined using qPCR. Research is now proving that ddPCR can be much more sensitive than qPCR (Wood et al., 2019), and further investigations and development of technologies such as this, will allow the technique to become much more sensitive and reliable.

The simplification of eDNA-based detection in the laboratory and/or in the field is also a potential area for growth. Products are now available to conduct processes such as DNA extraction and qPCR in the field, using mobile devices (Biomeme, 2019), specifically designed for eDNA applications. The further development of even smaller handheld devices which can conduct all stages of the diagnostic procedure may soon become a reality in this regard. So-called 'lab-on-a-chip' devices have already been developed for clinical applications with uses in diagnostics (Srinivasan et al. 2004; Tudos et al. 2001). These have been successfully used to detect small amounts of DNA from a selection of species of interest, however more in the microbiological field rather than eDNA as of yet (Diakité et al. 2012). More recently qPCR has even been achieved on similar hand-held devices (Ahrberg et al. 2017), highlighting the very real possibility of this being used in eDNA practices in the not too distant future. Indeed, one cross industry report into handheld 'lab-on-a-chip' devices has suggested that there may be a worldwide commercial value of $350million to environmental testing (Rainina 2010). Bohmann et al. (2014) went one step further and suggested that sampling devices linked with data transmitting software could also possibly examine difficult to access locations or work automatically without the need for human intervention, increasing the ease and accessibility of the technique even further.

6.4.2. Future applications of eDNA surveys

It is important to note that moving forward the applications of eDNA-based species detection lie much further than within the aquatic environment. As research gaps are beginning to be filled, the application of eDNA-based detection is now beginning to be applied in alternative environments, i.e. within sediment: another key habitat linked to rivers and lakes which can provide additional valuable data for species detection (Nelson-Chorney et al. 2019). With sediment having a slower DNA degradation rate and therefore higher concentration of eDNA to aquatic suspensions (Turner et al. 2015), species detection within these habitats could hold vital information about previous species inhabitation when looking back in time at local extinction events or introductions (Nelson-Chorney et al. 2019). Here, sediment cores could be taken, for example ice cores to determine species inhabitation at the time when each individual ice or

sediment layer was formed (Díaz-Ferguson and Moyer, 2014), providing useful knowledge on past species presence or absence and historical ecological processes.

6.5. Conclusions

The overall aim of this thesis was to assess the validation requirements for commercially applicable eDNA assays and then utilise this information to develop an eDNA assay for white-clawed crayfish, suitable for commercial, end-user application. Through careful assessment of each of the ten hurdles outlined in chapter 1, the white-clawed crayfish assay has now been validated to a level where it is able to be offered to ecologists as an additional crayfish survey tool. This output will considerably increase the number of crayfish presence/absence detection surveys to be conducted, increasing the accessibility of crayfish survey methods to a wider body of organisations, ecologists and citizen scientists. The application of the eDNA-based approach will allow for time and cost-saving surveys to be implemented on a wider scale than is currently available using traditional survey methods.

However, despite this success with the validation of the white-clawed crayfish eDNA assay, I wish to highlight that eDNA development and validation is not a simple task, with many aspects requiring a multipronged approach in order to effectively assess the numerous discrepancies and variable factors. It is also important, for long term success of an eDNA assay, that a constant cycle of adjustment should be implemented, incorporating advancements in the field, to enable the provision of an up-to-date service. Of course, eDNA can only offer a snapshot of an ecological picture, and the level of validation required can vary depending on the intended use; further validation is always possible and should not be over-looked, ensuring that all future commercially available eDNA assays are supported by a strong backbone of validation and 'hurdle' assessment.

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Appendices

Appendix 5.1. Natural England, request for quotation. (signal crayfish, crayfish plague eDNA).

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Appendix 5.2. Environment Agency, using DNA-based methods for environmental monitoring and decision-making.

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Available here: https://www.gov.uk/government/publications/using-dna-based-methods-for-environmental-monitoring-and-decision-making-position-statement

Appendix 5.3. Study 1: The River Allen, Dorset. Full report as provided to end-user

Assessing Crayfish Distribution in the River Allen (Dorset) Using Environmental DNA

Thursday 2nd May 2018

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1. Introduction

1.1. Crayfish in the UK

The White-Clawed Crayfish *Austropotamobius pallipes* is the only freshwater crayfish species indigenous to the United Kingdom and until recently, it was commonly found across an extensive range over most of England and Wales. Over the last 40 years its population numbers have seen a dramatic decline, subsequently becoming listed as endangered on the IUCN Red List (Füreder et al., 2010). One of the main reasons attributed to this decline has been the introduction of non-indigenous crayfish species, for commercial purposes during the 1970s (Holdfch et al., 1997). These species include the signal crayfish, *Pacifastacus leniusculus*, a more dominant species and a carrier of the crayfish plague *Aphanomyces astaci*, a water mould which has little effect to the invasive species, yet can have a devastating impact on the native *A. pallipes* (Holdich et al., 2009b), often completely wiping out populations.

Crayfish, particularly at low abundance can be notably difficult to find using existing survey efforts. This makes current survey techniques expensive and time exhaustive, often resulting in small pockets of isolated data with little large-scale implication.

1.2. eDNA

Over the past decade, the emergence of molecular environmental DNA (eDNA) based species presence/absence detection has proven to be a valid non-invasive, additional and cost-effective method to traditional surveys for a whole range of aquatic based species. The term eDNA is simply defined as a source of DNA which can be found within environmental samples such as water, soil, sediment, and air (Taberlet et al. 2012). The DNA found in these environments originates from both cellular and extracellular DNA which can be from the faecal matter, urine, blood, secretions and gametes of living organisms and the decay of dead organisms (Pietramellara et al. 2009). eDNA in aquatic environments is usually found in extremely small abundances (Schultz and Lance 2015), therefore often requiring highly sensitive techniques such as qPCR as a method for the detection of these quantities.

As an established scientific research technique, eDNA shows promise as an additional option for species detection in the environment as the method is less invasive, more sensitive, efficient, and commercially viable than current species detection methods such as hand searching.

1.3. Applied benefits of eDNA

Since its first use, eDNA analysis has become a more heavily researched field and subsequently popular method for the presence/absence surveys of many species. Sample collection and analysis methods have developed into competent survey methods for species including the great crested newt, *Triturus cristatus*, which is now commercially available in the UK.

eDNA methodologies can be cost-effective, after initial set-up (Rees et al. 2014a) and less invasive than traditional survey methods (Goldberg et al. 2015). Better presence/absence detection rates have also been reported when compared to traditional methods survey methods (Dejean et al. 2012) and depending on the habitat, in many cases the detection probability per unit of effort is much greater when using eDNA detection techniques as opposed to traditional methods (Jerde et al. 2011). More recently correlations between eDNA copy number and species abundance have been identified (Lacoursiere-Roussel et al. 2015), however, as it is still in its infancy, eDNA detection

should be used with some caution as an additional tool in combination with traditional survey methods for a true representation of species presence (Rees et al. 2014b).

1.4. eDNA for crayfish

Over the last few decades several efforts have been made to track the decline of *A. pallipes* and spread of *P. leniusculus* with varying levels of little success. The application of an eDNA survey methodology for this task would allow for a much greater survey detection rate per unit of effort, whilst achieving accurate results and keeping costs relatively low.

eDNA has been applied successfully to a number of crayfish species found within Europe, *P. clarkii* (Tréguier et al., 2014), and *Orconectes rusticus* (Dougherty et al., 2016), *P. leniusculus* (Larson et al. 2017; Agersnap et al. 2017; Mauvisseau et al. 2017) and *A. leptodactylus* (Agersnap et al. 2017). eDNA methodologies are now also available for the crayfish plague.

An eDNA methodology for *A. pallipes* has now been developed and is now used within this study to detect and locate populations of white-clawed crayfish, alongside methods used for signal crayfish and the crayfish plague.

1.5. Summary of recent work

1.5.1. Development

- Development of primers for the detection of white-clawed crayfish DNA within eDNA water samples, tested in-silico and on tissue and controlled eDNA samples.
- Determination of the limits of detection and quantification of this methodology.
- Assessment of sample collection techniques to determine usability and reproducibility on a citizen science/commercial based use.
- Use of and further development of methodologies which have been developed for other crayfish species, particularly the assessment of sample collection technique required for the crayfish plague.

1.5.2. Assessment of method-based limitations

- Seasonality experiment to determine the presence of eDNA within the environment over the course of a year. This is to see if there should be a 'season' of when best to sample for *A. pallipes* eDNA as determined by eDNA concentration because of active and inactive periods of the crayfish life cycle.
- Determining the persistence and longevity of *A. pallipes* eDNA within the environment and the implication that this may have on historically present but now absent populations.

1.5.3. 'Real world' applications

- Detection of populations of *A. pallipes, P. leniusculus* and *A. leptodactylus* populations across the Birmingham and West Midlands canal network.

- Detection of populations of *A. pallipes, P. leniusculus* and *A. astaci* along the River Ecclesbourne, Derbyshire and the Wyre Forest, Worcestershire to inform conservation work.
- Monitoring of *A. pallipes* crayfish ark sites to inform on success of introductions.
- Methodology used in combination with an ecological consultancy to inform species presence/absence on a commercial level.

2. Study Site

2.1. Site description

The River Allen is a chalk stream river, which is fed by an aquifer in Dorset, England. It begins near the village of Monkton Up Wimborne and travels for around 14miles before it has its confluence with the River Stour in Wimborne Minster. It was once known for its large population of native, white-clawed crayfish.

2.2. Timeline:

2012 Crayfish survey on River Allen, hundreds of white-clawed crayfish identified in sites along the river. 200 individuals taken from site to Ark site in the Purbecks.

2013 River restored with added reed banks to slow down flow, making habitat better for white-clawed crayfish and other species of important.

2014 Outbreak of the crayfish plague confirmed by CEFAS, individual deceased white-clawed crayfish found floating down the river. Almost all individuals were appeared to have been wiped out by the plague.

2015 Survey conducted by Environment Agency found just 8 individuals across the whole system, these were found at SU0209410647.

2016 Summer 2016, 54 crayfish panpipe refuges were introduced to 10 sites across the river (20m lengths), in the hope that any remaining crayfish would use these for refuge to support population growth, post-plague outbreak.

Surveys were undertaken at the 54 panpipe refuge traps, however, no individuals were seen.

2017 Proposed re-survey of sites, adding additional refugia in another 10 sites, taking the site number up to 20 sites, with approximately 1km between each site. Check each site with hand searching, some trapping etc.

It is intended that this will be supported with environmental DNA survey/analysis.

2.3. Site map

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2.4. Indicative sample site map

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3. Methodology:

3.1.1. Ethanol precipitation

eDNA samples were collected from the River Allen during September 2017 using the sample collection protocol based on Biggs et al. (2014). Using a 50ml ladle and a collection bag, 20 sub-samples were taken at regular intervals along a 5-10m section of river, with the length depending on access and site constraints (***Figure 1.***). Care was taken to ensure that each sample was taken in a consistent manner with minimal disruption of sediment to avoid the disturbance of historical DNA. Subsamples were taken in a downstream to upstream direction to avoid the collection of any disturbed sediment. The collected water was then homogenized, with 15ml distributed into six ethanol filled tubes (filled with 35ml ethanol/sodium acetate buffer solution). This process was repeated at each site to obtain triplicate samples. Samples were then refrigerated until arrival in the lab at which point they were stored at -20°C until extraction.

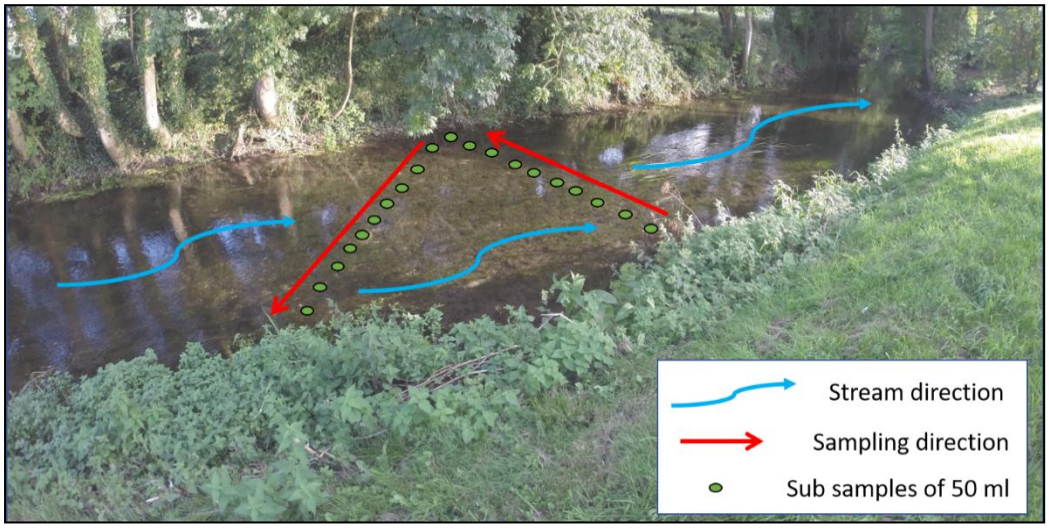

Figure 1. Sample sampling strategy indicating the direction of sampling and indicative subsample collection points. Source author, ©Chris Troth.

3.1.2. Filtration

Filtration of water was also conducted at each of the sites in triplicate. This was achieved by collecting a 2L sample from the river using the methodology as described in section 3.1.1. This water was then passed through a portable battery powered peristaltic pump containing Millipore Glass fibre filter AP25, 47mm. The filter was then removed from the pump system and stored in a falcon tube at -20ºC and then -80ºC before extraction.

3.2. DNA extraction

For ethanol precipitation, the six subsamples were subjected to centrifugation at 14000g (30 min at 4ºC). The eDNA samples were then extracted following the protocol outlined for eDNA from invertebrate species (namely *Procambarus clarkii*) by Treguier et al. 2014 using the Qiagen DNeasy Blood & Tissue kit. DNA was also extracted from the filters following the same kit. Samples were then stored at -20ºC until analysis.

3.3. Analysis by quantitative PCR

The detection of *A. pallipes, P. leniusculus* and *A. astaci* was conducted using three separate qPCR protocols, each one specific to the intended target species.

3.3.1. White-clawed crayfish

A real-time quantitative PCR (qPCR) assay was set up in a 25µl reaction containing: 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5µl DH20, 1µl (10µM) of each primer (forward and reverse), 1µl (2.5µM) of probe with the addition of 3µl template. qPCRs were performed with 6 replicates of each eDNA sample on the ABI 7500 qPCR System (Applied Biosystems) under the conditions: 50ºC for 5 min, denaturation at 95ºC for 8 min, followed by 50 cycles of 95ºC for 30 s and 55ºC for 1 min. 6 x NTC's (no template controls) were prepared using RT-PCR Grade Water (Ambion™) alongside a 10x serial dilution of *A. pallipes* control DNA standard for each qPCR plate that was run.

Primers:

Forward:	WC2302F	GCTGGGATAGTAGGGACTTCTTT
Reverse:	WC2302R	CATGGGCGGTAACCACTAC
Probe:	WC2302P	FAM-CTGCCCGGCTGCCCTAATTC-BHQ1

3.3.2. Signal crayfish

For *P. leniusculus* detection, A qPCR assay was set up using the same reagent concentrations and conditions as in section 3.3.1. with the altered annealing temperature of 56ºC. Full protocol and primers including method development can be found in our colleague's study - Mauvisseau et al. (2017) – PhD student at the University of Derby.

Primers:

Forward:	CO1-Pl-02-F	TGAGCTGGTATAGTGGGAACT
Reverse:	CO1-Pl-02-R	AGCATGTGCCGTGACTACAA
Probe:		FAM-CGGGTTGAATTAGGTCAACCTGGAAG-BHQ1

3.3.3. Crayfish plague

Analysis for the crayfish plague was conducted using primers and conditions designed by Vrålstad et al. (2009). A (qPCR) assay was set up in a 25µl reaction containing: 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 4.5µl DH20, 1µl (10µM) of each primer (forward and reverse), 1µl (2.5µM) of probe with the addition of 5µl template. qPCRs were performed with 6 replicates of each eDNA sample on the ABI 7500 qPCR System (Applied Biosystems) under the conditions: 50°C for 5 min, denaturation at 95°C for 8 min, followed by 50 cycles of 95°C for 15 s and 58°C for 1 min. 6 x NTC's were prepared using RT-PCR Grade Water (Ambion™) alongside a 10x serial dilution of *A. astaci* control DNA standard for each qPCR plate that was run.

Primers:

Forward:	AphAstITS-39F	AAGGCTTGTGCTGGGATGTT
Reverse:	AphAstITS-97R	CTTCTTGCGAAACCTTCTGCTA
Probe	AphAstITS-60T	FAM-TTCGGGACGACCC-MGBNFQ

Species presence within a site was inferred by the positive amplification of target species eDNA within at least one of the replicates out of the two duplicate samples collected from that site.

4. Results/Discussion

4.1. eDNA analysis

Each of the water samples from the River Allen were analysed for the presence of *A. pallipes* DNA, *P. leniusculus* and *A. astaci* DNA. The results from this analysis are presented in ***Table 1.*** indicating where either of the species were detected as present and for how strong this detection was (detection %), shown as a percentage.

All species were detected by both methodologies, however it is clear that the methodologies are not always consistent with each other – in some cases reporting conflicting results.

4.1.1. White-clawed crayfish

Despite unsuccessful bait-trapping and panpipe refuge searching, the eDNA survey has indicated the presence of white-clawed crayfish within the River Allen at every site studied. This may be indicative of a population still existing within the stream, potentially below levels detectable by 'traditional' established survey methods. At some sites, the detection sensitivity is much higher than others, this could indicate one of a number of situations including: a larger population of crayfish is present here, or the sample was taken in close proximity to a population of crayfish. Filtration using the Millipore Glass fibre filter AP25, 47mm filter was less successful at detecting white-clawed crayfish, with lower detection sensitivity in most samples, and no comparable detection in sites Allen03-06.

4.1.2. Signal crayfish

Signal crayfish were found within the river, despite no previous records of the species being present. This species was found at four unique sites across the river, at low detection sensitivity. There is the potential that at least one small population has been introduced into the river Allen. Although eDNA was detected at multiple sites for this species it could be the case that there is one single population upstream, with the downstream sites presenting with a positive detection due to downstream transportation of eDNA. It could also be the case that the positive detection is due to introduced eDNA, from sources such as fishing gear, bait, etc., although this is highly unlikely.

4.1.3. Crayfish plague

At a number of sites, the crayfish plague was also detected using eDNA based methods. It is important to note that this test has been shown to be much more sensitive and may be the result of detecting extremely low abundance of the plague within the system. This may be because of direct transfer i.e. fishing gear – or through the re-stocking of the fishing farm on the northern reaches of the river.

Table 1. Percentage detection rate (detection %) of each species at each site along the River Allen, Dorset (and additional test sites). Where a percentage detection is present is indicative of the positive detection of a species at a site. The higher the percentage value, the greater the concentration of eDNA within a sample.

Site Information			% Presence (ethanol tube)			% Presence (Filtration)		
No.	Site ID	Site name	White-clawed	Signal	Plague	White-clawed	Signal	Plauge
1	Allen 01	Dean's Court	22.22%			5.56%		11.11%
2	Allen 02	Knobcrook	44.44%			44.44%	5.56%	
3	Allen 03	Honeybrook	11.11%	5.56%	5.56%			5.56%
4	Allen 04	Hinton Parva	38.89%		5.56%			
5	Allen 05	Witchampton	11.11%		5.56%			5.56%
6	Allen 06	Didlington	27.78%		5.56%			5.56%
7	Allen 07	Stanbridge Mill	50.00%			27.78%		50.00%
8	Allen 08	Gussage All Saints	22.22%	5.56%	100.00%	5.56%		44.44%
9	Allen 09	Brockington Farm	11.11%			5.56%		
10	Allen 10	Wimborne St Giles	22.22%		27.78%	5.56%	11.11%	
13	Allen 13	Lulworth						
14	Allen 14	Fonthill		25.00%	16.67%			
15	Allen 15	Jordan		16.67%	16.67%			

5. Notes

This report has only provided a snapshot of the crayfish species and crayfish plague presence/absence situation during the sampling period (September 2017) and provides little evidence of the stability of these populations or the changing population dynamics. To get a greater idea on the crayfish situation within the river eDNA sampling would be recommended at regular intervals to track these changes.

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Appendix 5.4. Study 2: The River Ecclesbourne, Derbyshire. Full report as provided to end-user

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Appendix 5.5. Study 3: Fish Stocking Project, Lincolnshire. Full report as provided to end-user

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Appendix 5.6. Sample collection form used in the commercial application of crayfish eDNA assay

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Available here: https://surescreenscientifics.com/edna/crayfish/

Appendix 5.7. Sample collection instructions used in the commercial application of crayfish eDNA assay

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Available here: https://surescreenscientifics.com/edna/crayfish/

Appendix 5.8. FAQ's provided for the commercial application of crayfish eDNA assay

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Available here: https://surescreenscientifics.com/edna/crayfish/

Appendix 5.9. SOP for kit manufacture

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Appendix 5.10. SOP for DNA extraction protocol

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Appendix 5.11. SOP for qPCR analysis protocol

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Appendix 5.12. Example results report for commercial application of crayfish eDNA assay

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