Population assessment of great crested newts using environmental DNA

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Author's Declaration

All chapters of this thesis were written by Andrew S. Buxton. Comments and editorial input were provided by the supervisors Richard A. Griffiths, and Jim Groombridge. Chapter 6 also received comments and editorial input from researchers at the NERC Biomolecular Analysis Facility at the University of Sheffield as listed below. Chapters 3, 4 and 5 have been published in the scientific literature, with comments received from anonymous reviewers. All research within this thesis was approved by the University of Kent, School of Anthropology and Conservation Ethics Committee.

Chapter 1. – The chapter was written by A.S. Buxton and received editorial suggestions from R.A. Griffiths and J.J. Groombridge.

Chapter 2. – A.S. Buxton and R.A. Griffiths conceived the idea. A.S. Buxton designed the study and conducted the data collection, analysis and wrote the manuscript with editorial suggestions from R.A. Griffiths and J.J. Groombridge. This chapter has been accepted for publication, with permission of all co-authors, in Citizen Science: Theory and Practice and received comments from the editor and two anonymous reviewers.

Chapter 3. – A.S. Buxton and R.A. Griffiths conceived the idea. A.S. Buxton designed the study and conducted the data collection, analysis and wrote the manuscript with editorial suggestions from R.A. Griffiths and J.J. Groombridge. This chapter is published, with permission of all co-authors, in PLoS ONE (Buxton, A.S., Groombridge, J.J. & Griffiths, R.A. (2017). PLoS ONE. 12. e0183371) and received comments from the editor and two anonymous reviewers.

Chapter 4. – A.S. Buxton and R.A. Griffiths conceived the idea. A.S. Buxton designed the study and conducted the data collection, analysis and wrote the manuscript with editorial suggestions from R.A. Griffiths and J.J. Groombridge. This chapter is published, with permission of all co-authors, in PLoS ONE (Buxton, A.S.,

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Chapter 5. – A.S. Buxton and R.A. Griffiths conceived the idea. A.S. Buxton designed the study and conducted the data collection, analysis and wrote the manuscript with editorial suggestions from R.A. Griffiths and J.J. Groombridge. Nurulhuda Zakaria, a fellow PhD student with DICE, was involved with data collection and capture mark recapture modelling for this chapter, but had no input into the text. This chapter is published, with permission of all co-authors, in Scientific Reports (Buxton, A.S., Groombridge, J.J., Zakaria, N.B. & Griffiths, R.A. (2017): Scientific Reports. 7. 46294) and received comments from the editor and two anonymous reviewers.

Chapter 6. – A.S. Buxton and J.J. Groombridge conceived the idea, with additional input from Professor Terry A. Burke, Dr Deborah A. Dawson, Dr Helen Hipperson and Dr Gavin J. Horsburgh from the NERC Biomolecular Analysis Facility where the work was undertaken. A.S. Buxton, with input from NERC staff designed the study and conducted laboratory work. A.S.Buxton undertook the data collection, analysed the data, and wrote the manuscript with editorial suggestions from R.A. Griffiths and J.J. Groombridge, as well as D.A. Dawson and H. Hipperson and G.J. Horsburgh. This chapter is in prep for submission into the scientific literature.

Chapter 7. **–** This chapter was written by A.S. Buxton and received editorial suggestions from R.A. Griffiths and J.J. Groombridge.

Summary

Targeting environmental DNA (eDNA) for species monitoring and biodiversity assessment is a newly emerged technique. Surveys targeting eDNA involve the isolation of DNA shed into the environment by an organism to identify species utilizing a particular location. Despite uncertainties surrounding the technique, eDNA has begun to be used extensively for species assessments. Using the great crested newt (*Triturus cristatus*) as a model species, we (1) determined seasonal trends in eDNA with a view to optimising survey timing; (2) estimated the detection probabilities for eDNA and their covariates; and (3) explored how abundance estimates may be made from aquatic eDNA samples.

We conclude that detection varies through the year, with most reliable detection coinciding with peak breeding. However, outside the breeding season detection is possible where larval numbers are high. Environmental and population factors may influence release of DNA from a target species or eDNA persistence in water and sediments. These include sediment type, number of both adults and larvae, changes in adult body condition, habitat variables and sampling location. As many external covariates were found to influence eDNA concentration, it would not be appropriate to use eDNA concentration as a predictor of abundance. However; we apply a modelling approach to generate estimates of abundance using genomic DNA, with a degree of accuracy deemed acceptable for ecological monitoring.

The conclusions are directly relevant to refining survey design and analysis for the assessment of great crested newt populations. The results are also applicable more generally to the eDNA survey method, its development, survey design and interpretation, whether for single species analysis or community analysis.

Key Words: Environmental DNA; eDNA; great crested newts; *Triturus cristatus;* population; abundance; detection probability; survey; monitoring

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Chapter 1 – Introduction

1.1. Emerging technologies in biodiversity assessment

Threats to global biodiversity are increasing with species extinction rates 1000 times higher than background levels (Pimm et al., 1995; Barnosky et al., 2011). This is driving the need for evidence-based conservation actions which rely on effective and rapid species monitoring. This global biodiversity crisis is coinciding with a fall in specialist taxonomists (Oliver et al., 2000). As a result, the capacity for biodiversity assessment using traditional taxonomic methods is falling at a time when the need and demand for rapid biodiversity assessments has never been higher. This has precipitated a call for new technological solutions to solve this problem. For example, virtual biodiversity assessment has emerged using digital technologies to build virtual reference collections (Oliver et al., 2000), allowing visual identification to be undertaken by a wider group of individuals. Additionally, species distribution modelling has allowed the prediction of historic, current and future species ranges and how these may change with biological invasions and changing climatic conditions (Elith & Leathwick, 2009).

In addition to methods that streamline data processing and interpretation of biodiversity assessments, technological advances have been made in species detection. Genetic techniques and DNA barcoding have been used for species identification to reduce the reliance on taxonomists, and increase the speed at which results are available for analysis (Hajibabaei et al., 2007). As well as taxonomic identification using DNA from specimens, DNA technology has been used to identify species presence and identification of individuals from non-invasively collected genetic material. This has allowed population abundance estimates to be generated by mark-recapture models, when only scat or hair samples can be collected (Waits & Paetkau, 2005; Aziz et al., 2017). However, more recently species presence has

begun to be identified by DNA isolated from samples of environmental material, and used for biodiversity assessment (Ficetola et al., 2008).

1.2. What is environmental DNA (eDNA)?

1.2.1. eDNA - Definition and history

Environmental DNA (eDNA) comprises trace amounts of DNA that have become separated from the source organism and incorporated into the environment (Jane et al., 2015), whether the environment be aquatic, terrestrial or air-borne. Detecting species presence by isolating eDNA is a relatively new and developing technique. Traces of extra-organismal genetic material are collected in samples of environmental material, DNA is then isolated from that material and used to identify the current or historic presence of organisms within that environment without observing the whole organism (Lodge et al., 2012; Jane et al., 2015; Herder et al., 2014; Barnes et al., 2014). This has commonly become used within freshwater environments (Rees et al., 2014b), marine environments, (Thomsen et al., 2012a), as well as sediments (Turner et al., 2015), soils (Andersen et al., 2012) and from airborne pollen or fungal spores (Kraaijeveld et al., 2015; Abrego et al., 2018), these are reviewed by Thomsen & Willerslev (2015).

eDNA techniques were initially developed for the survey of microorganisms (Roose-Amsaleg et al., 2001). Similar techniques were used within the field of ancient DNA, isolating paleoecological communities from sediment, permafrost and ice cores (Thomsen & Willerslev, 2015). The first aquatic eDNA study of contemporary eukaryotic organisms was undertaken in 2005, to analyse the source of organic pollution within a riverine system, distinguishing between pollution from different species of livestock (Martellini et al., 2005). The technique was then first used for the contemporary detection of a species within a waterbody in 2008 (Ficetola et al., 2008), where the distribution of American bullfrogs (*Lithobates catesbeianus*) was assessed in France. Since this time the method has been used to target a wide variety of

vertebrate and invertebrate species including amphibians, fish, insects (Thomsen et al., 2012b), reptiles (Hunter et al., 2015), crustaceans (Ikeda et al., 2016), molluscs (Xia et al., 2017), as well as plants (Newton et al., 2015) and a wide variety of pathogens (Schmidt et al., 2013; Strand et al., 2014). The technique has also been used in many aquatic environments, including small standing water pools (Schneider et al., 2016), ponds (Biggs et al., 2015), lakes (Hänfling et al., 2016), streams (Goldberg et al., 2011), rivers (Deiner & Altermatt, 2014) and marine (Thomsen et al., 2012a) and estuarine (Stoeckle et al., 2017b) environments.

1.2.2. Uses of eDNA

The overriding benefit of eDNA survey methodologies is the ability to detect species at low population densities; allowing the detection of rare and cryptic species (Goldberg et al., 2011), as well as for the detection of invasion fronts of invasive species (Jerde et al., 2011). The rapid and accurate detection of rare or cryptic species has wide-ranging applications in ecology and conservation. Survey methods of freshwater species often suffer from low detection probabilities, are logistically onerous and require highly skilled surveyors at high cost (Laramie et al., 2015). Surveys also often require multiple visits and multiple methodologies to obtain reasonable confidence of a negative result (Sewell et al., 2010), which may compromise the scale of a project. eDNA sample collection can be undertaken rapidly and cost effectively, with relatively few visits (Goldberg et al., 2011; Smart et al., 2016). This allows distribution assessments to be undertaken on a much greater scale than has been possible in the past (Biggs et al., 2015; Gibson et al., 2015). As detection with the eDNA methods does not require direct observation or capture of individuals, the risk of disturbance to target animals and their habitat is reduced, with associated ethical and welfare benefits (Santas et al., 2013).

Invasive species are very damaging to the environment. In the USA, invasive species cause damage in excess of $120 billion annually (Pimentel et al., 2005). Early

detection of invasive species should therefore form a core component of invasive species management plans (Lodge et al., 2006; Gu & Swihart, 2004; Harvey et al., 2009; Ando et al., 1998). The detection of organisms at lower densities allows for control measures to be implemented earlier than with detection using traditional methods, increasing the effectiveness of the control measures applied (Ficetola et al., 2008; Jerde et al., 2011; Dejean et al., 2012; Darling & Mahon, 2011).

In addition to the detection of species of conservation concern, eDNA has been used in the detection of wildlife pathogens. This can be used to explain declines in populations where unexplained population crashes have been observed or to inform management options for susceptible species. eDNA methods have been applied to a wide variety of pathogens for example *Batrachochytrium dendrobatidis* (Schmidt et al., 2013) and crayfish plague (*Aphanomyces astacin;* Strand et al., 2011). Other uses of eDNA or ancient DNA can be the identification of ecological relationships (Zobel et al., 2018), or biodiversity and environmental impact assessments of anthropogenic activities such as pollution spills, uranium mining or oil drilling (Andújar et al., 2017; Klymus et al., 2017a; Laroche et al., 2016, 2017).

1.3. eDNA methodologies

The collection of eDNA requires a number of steps: collection of sample, extraction of DNA and amplification to allow detection. Efficiency of the process at each step is imperative to have the greatest chance of detecting the target species or achieving an accurate representation of the species present (Deiner et al., 2015; Eichmiller et al., 2016a; Renshaw et al., 2015; Piggott, 2016; Hinlo et al., 2017; Williams et al., 2016; Djurhuus et al., 2017; Takahara et al., 2015; Goldberg et al., 2016).

1.3.1. Sample collection

eDNA is often present in only trace amounts within the environment (Bohmann et al., 2014). As a result, compared to conventional samples for genetic analysis, a

comparatively large sample of environmental material is needed to concentrate and isolate sufficient amounts of target DNA to be detected. Within aquatic eDNA this is either achieved by (1) passing large volumes of sample water through a filter trapping the DNA, cells or cell organelles on the filter membrane; or (2) by mixing sample water with absolute ethanol and a salt firstly to preserve the DNA and then aid in the extraction process, DNA and solid material then precipitate out of solution (Rees et al., 2014b). There are drawbacks to both methods. Precipitation in ethanol is limited to a relatively small water volume (approximately 90 mL maximum; Biggs et al., 2015), but is a relatively fast process in the field. On the other hand, filters can sample a much larger volume, but may become blocked (Williams et al., 2017), particularly in samples from turbid waters or water with high algal loads, which are both common in ponds. Filters also require either returning large volumes of water to a lab for filtration or the use of a mechanical pump, hand pump or syringe in the field (Pilliod et al., 2013; Deiner et al., 2015) which can be time consuming and physically challenging. Additionally, there is a wide variety of choice of membrane type, pore size and structure when using filters (Minamoto et al., 2016; Spens et al., 2016; Turner et al., 2014a; Djurhuus et al., 2017; Eichmiller et al., 2016b; Lacoursière-Roussel et al., 2016b), with the benefits and drawbacks of each combination not well assessed.

1.3.2. Extraction

After water samples have been collected, immediate DNA extraction is not always practical and samples may need to be stored for days, weeks or even months, and the method of storage can influence the detection of DNA (Takahara et al., 2015; Renshaw et al., 2015; Majeneva et al., 2018). Filtered samples may be preserved through desiccation, stored in ethanol or lysis buffer, or samples may be frozen (Majeneva et al., 2018). With samples collected through precipitation in ethanol, the ethanol serves a dual function of both preservation of the DNA and aiding the DNA to form a precipitate and fall out of solution, thereby allowing the concentration of the

sample. Ethanol precipitation samples may also be stored at low temperatures to further reduce DNA degradation.

Precipitation in ethanol samples are concentrated through centrifugation, trapping precipitated DNA and other material as a pellet on the sample tube. This pellet is then digested using a lysis buffer to release the DNA from the cells. Samples collected through filtration are concentrated on the filter paper during sample collection, with lysis buffer directly applied to that at the extraction phase. The extraction process then isolates the DNA from the other material within the sample. The DNA is cleaned at this step to reduce PCR inhibitors, which can impact the efficiency of downstream analysis. As with collection methods, a wide variety of extraction methods have been utilised with eDNA (Djurhuus et al., 2017). Extraction methods have to be tailored to the type of environmental sample and how it was collected. For example, some kits originally designed for extraction from environmental samples have been designed with prokaryotic cell types as targets, using mechanical cell lysis steps, which have reduced efficiencies when targeting organisms with eukaryotic cells (Eland et al., 2012; Deiner et al., 2015; Hinlo et al., 2017). Commercially available DNA extraction kits such as DNeasy® Blood and Tissue Kit (Qiagen®, Inc.; Biggs et al. 2015 and others) and PowerWater® DNA Isolation Kit (MoBio® Laboratories, Inc.; Jane et al., 2015 and others) with protocol modifications are popular in eDNA studies. Commercial kits are not universally used with “homemade” protocols based on phenol-chloroform-isoamyl alcohol (Renshaw et al., 2015), or CATB buffer solution (Turner et al., 2014b) also used in eDNA studies. Some of these homemade protocols offer cost savings and have been shown to yield greater DNA extract concentrations (Renshaw et al., 2015; Turner et al., 2014b; Deiner et al., 2015; Schiebelhut et al., 2017; Djurhuus et al., 2017), but may struggle to remove PCR inhibitors.

1.3.3. Analysis

After eDNA is extracted, various approaches to the downstream analysis have been adopted to identify species presence depending on research goals. In early studies, conventional polymerase chain reaction (cPCR) was used (Ficetola et al., 2008) for species detection, with quantitative real-time PCR (qPCR) rapidly replacing this as the norm (Thomsen et al., 2012b). qPCR shows many benefits over cPCR including the removal of ambiguity surrounding the viewing of electrophoresis gels, ability to quantify the amount of DNA in a sample and multi-channel detection which can be used to identify the presence of PCR inhibitors and target DNA simultaneously. qPCR is also overwhelmingly recommended over cPCR, with qPCR one or two orders of magnitude more sensitive (Turner et al., 2014b; Qu & Stewart, 2017). PCR based analyses allow for targeted detection of a single species, but do not allow for more complex community analysis to be undertaken. High-throughput sequencing using platforms such as the Roche 454 Pyrosequncer (Mahon et al., 2014; Klymus et al., 2017b) and Illumina MiSeq (Valentini et al., 2016), have allowed metabarcoding approaches to be applied to eDNA analysis, allowing sequence reads to be returned that can be compared to reference libraries to assess wider biodiversity and address various ecological and conservation questions (Valentini et al., 2016). Targeted species detection using qPCR appears to be marginally more sensitive than using metabarcoding (Harper et al., 2018), can be conducted more quickly and does not require the use of libraries to compare returned sequence reads to. However, initial set-up costs and sensitivity aside, metabarcoding is otherwise comparable to qPCR (Harper et al., 2018), and can be used to generate much more detailed analysis of biodiversity information, potentially allowing for more effective monitoring programmes, allowing the understanding of community structure and ecosystem functioning (Harper et al., 2018).

1.4. eDNA challenges

There are a wide variety of challenges which need to be considered in relation to eDNA sampling. These challenges vary depending on the target organism, the questions being addressed by the project as well as the habitat in which the samples have been collected. Harper *et al.* (in review – Appendix I) have reviewed the application of eDNA methods to ponds, and the more prominent issues are summarised here.

1.4.1. False positives/negatives

Two types of error may persist in eDNA sampling, false positive error and false negative error. False positive error identifies the presence of a species when it is not actually present. False negative error is when the presence of a species is not identified when it is in fact present. The most likely source of false positive results will be the contamination of a sample or a pond with target DNA from an external source. This may be within the laboratory, via surveyors, water flows or the movement of non-target species between water bodies. False negative results are more likely, and would be due to the failure to collect any or sufficient target DNA within a sample or through the presence of PCR inhibitors in a sample (Nathan et al., 2014; Biggs et al., 2014b; Ficetola et al., 2014). Both false positive and false negative rates are key considerations when planning eDNA studies. Although methods of identifying error rates are beginning to be developed (MacKenzie et al., 2002, 2003; Tyre et al., 2003), so far it has not been common to report estimates of detection probability using occupancy modelling approaches.

1.4.2. Contamination

Contamination can cause false positive results and various precautions are needed to minimise its risk. Contamination is a major consideration with regard to environmental samples, with more opportunities for contaminants to be introduced into the analysis process than with conventional DNA based analysis. Contamination

can enter from a number of sources. Natural contamination could be from natural water flows between ponds or the movement of DNA by non-target animals as they visit multiple water bodies and this is unavoidable (Biggs et al., 2014b). However, contamination can also occur in both the field sampling and laboratory analysis phases. Field contamination may arrive on the surveyor's equipment or clothing, whereas laboratory contamination can be introduced by PCR products, fresh tissue, DNA residues or airborne DNA at the DNA extraction or amplification stages in the laboratory (Champlot et al., 2010; Kowalchuk et al., 2007; Biggs et al., 2014b). Such contamination risks can be minimized by using rigorous protocols and good laboratory practices, with stringent sterilisation of equipment, and the separation of amplified or concentrated target DNA from DNA extraction and PCR set up areas. Contamination can be tested for using separate negative controls at each stage; in the field, at extraction and at amplification (Goldberg et al., 2016).

1.4.3. eDNA distribution

The distribution of eDNA within the environment is also an important consideration. Movement of waters such as in rivers and streams can lead to eDNA being transported long distances. This results in interpretation of positive eDNA detection limiting the location of a species to "upstream of the sample location" only (Deiner et al., 2015). In large water bodies, wind action and stratification may influence the distribution of eDNA (Hänfling et al., 2016; Boehrer & Schultze, 2008; Matsui et al., 2001), similarly tides and currents in the marine environment do the same (Thomsen et al., 2012a). However, pond water is largely stagnant, with microhabitat occupancy differing in different species (Skei et al., 2006). Consequently, within ponds eDNA is less likely to be dispersed by water flow and more likely to remain in the microhabitats occupied by the target species (Brys, 2017). Sample collection strategies therefore need to be targeted towards the environmental conditions being sampled. Additionally, sample collection strategies need to account for study goals. For

example, if a single species is being targeted, subsampling all the ecologically suitable microhabitat for that species will allow for greater detection probability than if a single point sample is collected from the pond. As a result a single sample of surface water may be sufficient in some circumstances, however sample collection may require multiple subsamples to be taken and then combined to generate a truly representative sample of a pond (Biggs et al., 2015).

1.4.4. Persistence

Accumulation rate for eDNA is influenced by environmental conditions. For example, temperature may impact the rate at which an organism sheds DNA and cells into the environment (Klymus et al., 2015). Likewise, the rate of spore release of the crayfish plague increases with temperature (Strand et al., 2012). Weakened or stressed individuals may also exhibit higher spore release rates (Strand et al., 2012), which may also be true for eDNA. Density of individuals and the metabolic rates can influence DNA shedding rates and therefore the rate at which eDNA accumulates in a waterbody (Klymus et al., 2015). The removal of aquatic eDNA from the environment is influenced by a number of processes. In lotic or marine environments water currents and flows disperse eDNA, diluting to levels that are undetectable (Pilliod et al., 2014; Thomsen et al., 2012a). eDNA can be lost from the aquatic environment, settling out of suspension or binding to the sediments (Turner et al., 2014a). Finally, eDNA can be degraded into undetectable short sections by a range of biotic processes, including microbial action and extracellular enzymes (Barnes et al., 2014); as well as abiotic factors such as temperature, UV light, biological oxygen demand, chlorophyll *a* concentration and pH (Barnes et al., 2014; Pilliod et al., 2014; Seymour et al., 2018).

Persistence of eDNA in the environment is highly variable. eDNA can persist for thousands of years if bound in sediments (Levy-Booth et al., 2007); this is widely used in the field of ancient DNA (Thomsen & Willerslev, 2015). However, it may become

undetectable within a few hours (Thomsen et al., 2012a). Persistence time can therefore lead to issues with interpretation. If eDNA persistence time is too low, the value of eDNA in detecting recent presence is reduced. On the other hand, if persistence is very long the origin of the DNA is uncertain, and the ability to infer contemporary presence of the species is compromised. In order to interpret results accurately, it is therefore important to understand eDNA persistence, accumulation, detection and degradation rates under different environmental conditions (Turner et al., 2015; Stoeckle et al., 2017a).

1.4.5. Inhibition

PCR inhibitors interact with the PCR amplification process reducing the efficiency of the reaction and in extreme circumstances can prevent amplification even if the target sequence of DNA is present in large amounts within a reaction (Jane et al., 2015). The presence of PCR inhibitors can therefore cause what appears to be a false negative result. PCR inhibitors are common within environmental samples (Jane et al., 2015), are diverse in origin and inhibit samples through a range of mechanisms (Alaeddini, 2012; Schrader et al., 2012). One of the key causes of inhibition in environmental samples are humic compounds (Matheson et al., 2010; Alaeddini, 2012; Stoeckle et al., 2017a), which are created through the decay of organic material (Alaeddini, 2012). Organic matter can be introduced into pond water through leaf litter or the breakdown of submerged and emergent vegetation. Humic compounds can also enter ponds through surface runoff, bringing soils and other organic material into ponds. The stagnant nature of ponds means that the humic compounds build up and are not removed from the system (Alaeddini, 2012; Albers et al., 2013). Humic acids inhibit PCR reactions with phenolic groups oxidising and binding to the DNA polymerase, rendering the enzyme inactive. As little as 0.08 μgmL^{-1} of humic acid can completely inhibit the PCR reaction (Tebbe & Vahjen, 1993). To avoid reporting inhibited samples as negative it is imperative to test for PCR inhibition, which can be

achieved through the use of internal positive control or internal amplification control, introducing a known amount of DNA into a reaction and testing for amplification of that sequence (Hoorfar et al., 2004). Methods for reducing PCR inhibitors include additional steps during DNA extraction or DNA purification, or the inclusion of substances such as bovine serum albumin to coagulate inhibitors. Diluting the extract to reduce the concentration of inhibitors is also used (Alaeddini, 2012), but is not advisable for use with environmental samples due to dilution effect on already low DNA extract concentrations. Additionally heat soaked PCR, hot start PCR or the inclusion of extra polymerase enzyme can all help to reduce the impact of inhibitors (Alaeddini, 2012).

1.4.6. Limits of detection and quantification

Limit of detection and limit of quantification are used to show the sensitivity of qPCR analysis. Limit of detection is the minimum DNA extract concentration which can reliably be detected by the qPCR protocol, whereas limit of quantification is the minimum concentration that yields a reasonable level of accuracy during quantification using qPCR (Tréguier et al., 2014; Biggs et al., 2014b; Smith et al., 2012; Díaz-Ferguson, 2014; Pilliod et al., 2013, 2014; Goldberg et al., 2013). Understanding the limits of detection and quantification provide confidence in the sensitivity and accuracy of the results. These limits are identified through a serial dilution of a tissue extract creating a standard curve. Limit of quantification is identified through consistency of amplification threshold value in qPCR replicates, whereas limit of detection is the minimum concentration to show any amplification. Limits of detection have ranged from concentration in the region of 10^{-11} ngµL^{-1} to 10^{-4} ngµL^{-1} (Wilcox et al., 2013; Pilliod et al., 2014; Biggs et al., 2014b; Jerde et al., 2011; Tréguier et al., 2014), with limits of quantification in the region of 10^{-4} ngµL^{-1} (Tréguier et al., 2014). It is not uncommon within environmental studies for the majority of samples to

fall below the limit of quantification for a study (Tréguier et al., 2014; Biggs et al., 2014b).

1.4.7. Population abundance

Although the assessment of presence and absence data is very informative, the ability to produce an assessment of abundance or density can allow trends in population size to be assessed, with major benefits to conservation and ecological management (Bohmann et al., 2014; Joseph et al., 2006). With the exception of capture mark recapture (Kröpfli et al., 2010) and N-mixture models (Ward et al., 2017), at present most methods for assessing abundance of British amphibians are based on traditional practices involving simple visual counts. These have little grounding in scientific rigor, and are framed within rule of thumb analysis and quasi-quantitative methods (Griffiths et al., 2015). Some studies have suggested that either eDNA concentration or the proportion of qPCR replicates that amplify may be a suitable proxy for relative abundance (Takahara et al., 2012; Biggs et al., 2014b; Pilliod et al., 2014). However, using eDNA concentration as a proxy for a population estimate would need to account for water volume, and making such a calculation is difficult to achieve accurately for natural ponds.

The use of eDNA concentration or equivalent as a proxy for abundance assumes that the release and subsequent build-up of DNA is correlated with abundance (Bohmann et al., 2014), and is constant within a species, habitat and across a sampling timeframe. eDNA concentration and accumulation rate has been shown to have a positive relationship with abundance in mesocosm experiments (Takahara et al., 2012; Thomsen et al., 2012b; Goldberg et al., 2013; Kelly et al., 2014; Iversen et al., 2015) and relative abundance in more natural environments (Takahara et al., 2012; Lacoursière-Roussel et al., 2016a; Doi et al., 2017). However, adapting these correlations to yield estimates of abundance or density assessment is problematical. It is still unclear how eDNA release rates vary both within and between species in

relation to environmental conditions. Additionally, if eDNA concentration were only a function of abundance of the target species, and DNA build-up and degradation rates, a low concentration of eDNA may relate to either a small number of individuals or a large number of individuals that have left the system, with no way to distinguish between the two (Barnes et al., 2014; Pilliod et al., 2014). Due to the wide variety of influences on the concentration of eDNA within a sample, it is therefore unlikely that eDNA concentration will be able to provide reliable estimates of abundance.

1.5. Amphibians and great crested newts

Freshwater habitats are hotspots for biodiversity but are globally under very extensive and diverse threats (Strayer & Dudgeon, 2010). Worldwide, 60% of wetland habitat was lost within the 20th century (Innis et al., 2000) with the threats faced being anthropogenic in nature (Jackson et al., 2001). In excess of 6400 freshwater species were listed as vulnerable or worse on the IUCN Red List of threatened species (search conducted January 2018), which represents 24.2% of all assessed species (IUCN, 2018b). Amphibians, a group dominated by species that rely on freshwater, are arguably the most threatened group of vertebrates, with 41% of amphibians threatened with extinction (IUCN, 2018a). Threats faced by amphibians include habitat loss, UV-B radiation, emerging diseases, the introduction of alien species, direct exploitation, climate change or a combination of factors (Beebee & Griffiths, 2005; Blaustein & Kiesecker, 2002; D'Amen & Bombi, 2009; Stuart et al., 2004). These threats are leading to declines within amphibian species throughout the world (Blaustein et al., 1994; Blaustein & Kiesecker, 2002; Stuart et al., 2004; D'Amen & Bombi, 2009).

Globally, the monitoring of amphibian populations is urgently needed to inform and evaluate evidence-based conservation efforts. eDNA surveys have been demonstrated with a wide range of amphibian species including American bullfrog (*Lithobates catesbeianus*; Ficetola et al., 2008; Dejean et al., 2012), Rocky Mountain

tailed frog (*Ascaphus montanus*; Goldberg et al., 2011; Pilliod et al., 2013), Idaho giant salamanders (*Dicamptodon aterrimus*; Goldberg et al., 2011; Pilliod et al., 2013, 2014), eastern hellbenders (*Cryptobranchus a. alleghaniensis*; Olson et al., 2012; Santas et al., 2013), Chinese (*Andrias davidianus*) and Japanese (*Andrias japonicus*) giant salamanders (Fukumoto et al., 2015), common spadefoot toad (*Pelobates fuscus*; Thomsen et al., 2012b), the European olm (*Proteus anguineus*; Vörös et al., 2017) and great crested newts (*Triturus cristatus*; Thomsen et al., 2012b; Rees et al., 2014a; Biggs et al., 2015; Rees et al., 2017; Harper et al., 2018).

Great crested newts, like other amphibians, face a wide variety of threats and are known to be declining in numbers across their range (Beebee, 1997; Arntzen et al., 2009; Denoël & Ficetola, 2008; Denoël, 2012; Edgar & Bird, 2006). The species is one of nine in the genus *Triturus*, seven of which form the crested newt species complex of which *T. cristatus* has the widest distribution and is the most northerly. *T. cristatus* are found throughout much of northern Europe, extending from the UK and France in the west into Russia in the east (Jehle et al., 2011; Wielstra & Arntzen, 2016; Wielstra et al., 2013; Wielstra & Arntzen, 2011; Figure 1.1). The UK is a stronghold for the species, which is found within all three mainland countries England, Scotland and Wales (Inns, 2009; O'Brien et al., 2015, 2017; Jehle et al., 2011; Edgar & Bird, 2006; Beebee, 1981).

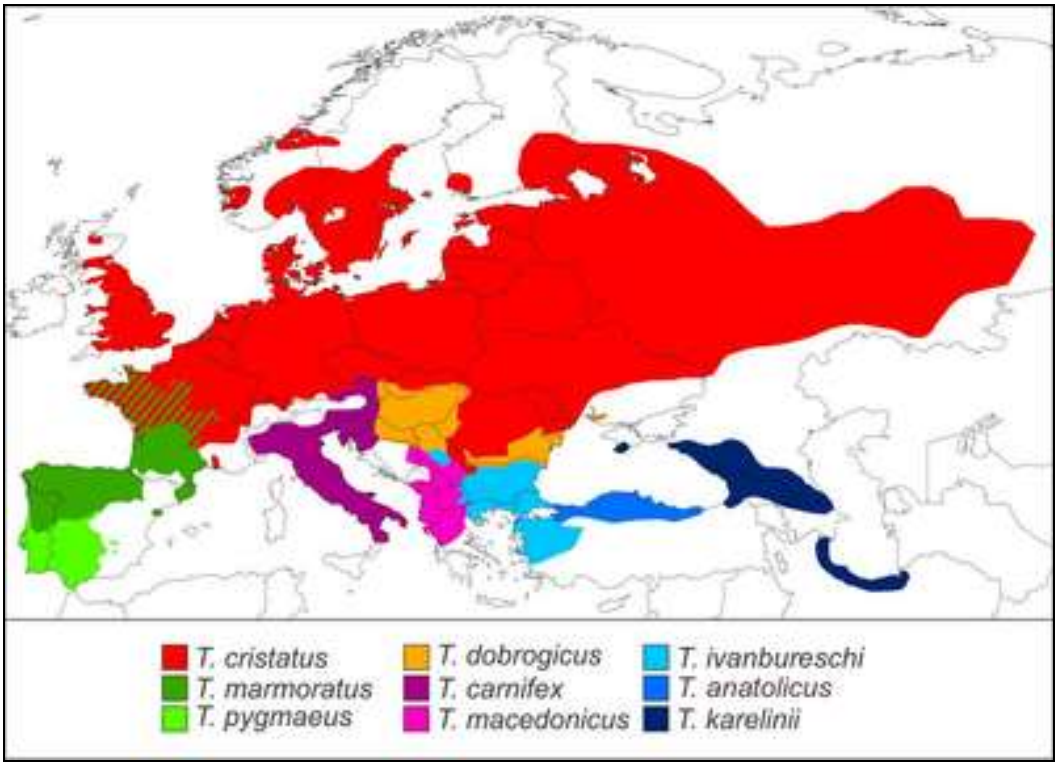

Figure 1.1 - The distribution of the nine species from the genus *Triturus* within Europe. The red/green cross-hatched area represents the area in which *T. cristatus* and *T. marmoratus* co-occur (Taken from Wielstra et al., 2013).

Great crested newts are large compared to other native newts in the UK, with males reaching a maximum size of 180 mm with females slightly larger reaching a maximum 200 mm (Jehle et al., 2011) in length. When in breeding condition adult great crested newts can exceed 20 g in weigh (unpublished data) but are usually smaller. They have a warty or rough skin which is dark brown or black on the flanks and dorsal surface, both with black blotches. White speckling under the chin and on flanks is usually present and the ventral surface is bright orange or yellow, with well-defined, irregular black spots (Jehle et al., 2011). The ventral yellow and black patterning, often referred to as the belly pattern is unique to individuals and is often used as a tool for individual recognition as part of recapture studies (Arntzen, J. and Teunis, 1993). Breeding males grow a ragged crest along the length of the body, however females do not. Breeding males also develop a silver or white streak through the

centre of the sides of the tail again absent in females. Outside the breeding season males lose much of the distinctive crest and white stripe (Jehle et al., 2011).

Great crested newts are a semi-aquatic amphibian, which over-winter on land with adults entering an aquatic phase habiting ponds in late winter or early spring, where breeding then occurs (Langton et al., 2001). The species is largely nocturnal, with the core breeding season runs from mid-March to mid-May (Langton et al., 2001), with adults often returning to the terrestrial environment in the early summer. Eggs are concealed in submerged folded leaves, and the larvae are fully aquatic, reaching 70 mm before metamorphosing in the late summer or autumn and emerging from the ponds, although some remain in the water over winter (Langton et al., 2001; Jarvis, 2016).

Great crested newts utilise networks of ponds within a landscape, forming a metapopulation structure (Griffiths et al., 2010; Jehle et al., 2005), with some individuals moving in excess of 1500 m within a season (Haubrock et al., 2016). However, most individuals remain within 20 m of the pond edge (Jehle & Arntzen, 2000). Great crested newts favour lowland river valleys or spring line ponds (Inns, 2009) which do not flood, but are often also found on brown-field sites (Baker et al., 2011). Great crested newts prefer large, fish-free ponds as breeding sites, but are also sometimes found breeding in ditches, garden ponds and shallow scrapes. The terrestrial habitat is as important for great crested newts as the aquatic breeding sites; areas of deciduous or mixed woodland, mature hedgerows (Jehle & Arntzen, 2000; Skei et al., 2006) and undisturbed grassland are favoured (Inns, 2009).

Great crested newts are protected under both European and UK legislation. The species is listed under Appendix II of the Convention on the Conservation of European Wildlife and Natural Habitats (1979) referred to as the Bern Convention, Annexes II and IV of the EC Natural Habitats Directive (1992; EC 92/43/EEC), this is ratified into law in England and Wales through The Conservation of Habitats and

Species Regulations 2017 where they are listed under Schedule 2. Additional protection is given to the great crested newt under Schedule 5 of the Wildlife and Countryside Act 1981 (as amended) in England and Wales. In Scotland the European legislation is ratified by the Conservation (Natural Habitats, and Countryside) Regulations 1994 (as amended). The great crested newt is also a priority species under the UK Biodiversity Action Plan (BAP), and listed under the NERC Act 2006 as a Species of Principal Importance (England - Section 41; Wales – Section 42). In Scotland great crested newts are Species of Principal Importance under the Nature Conservation (Scotland) Act 2004.

The combination of the above legislation makes it an offence (with certain exceptions) to do the following:

- Disturb, capture, kill or injure great crested newts;
- Damage, destroy, disturb or obstruct access to, a place used for shelter or protection by great crested newts;
- Damage, destroy or disturb a breeding site or resting place of great crested newts;
- Possess a great crested newt alive or dead, or any part of it, unless acquired lawfully; and
- Sell, barter, exchange, transport or offer for sale great crested newt or any parts of them.

An act which would otherwise be illegal can be made lawful by the issue of a licence by the appropriate licencing authority, for England (Natural England), Wales (Natural Resources Wales) and Scotland (Scottish Natural Heritage).

Under the EC Habitats Directive there is a requirement to maintain ‘favourable conservation status’ (FCS) of the species. This is of relevance to both statutory agencies as well as the planning industry. The primary impact of this legislation is that

proposals to change land use have a requirement to undertake surveys, and to identify the presence and population status of great crested newts. If an impact on a population of great crested newts is predicted following surveys, mitigation must be put in place to prevent a reduction in overall population status within the area. Mitigation often involves lengthy habitat enhancement or creation schemes with trapping and translocation of all individuals from the site of the new development to newly created or enhanced habitat. All mitigation must be undertaken under a development licence issued for the project by the licencing authority (Edgar et al., 2005; Lewis et al., 2007; Lewis, 2012; Lewis et al., 2017). However, to meet the requirements of the Habitats Directive member states also need to undertake surveillance of the conservation status of species listed under the directive, to ensure favourable conservation status is maintained.

Traditionally surveys for great crested newts involve a combination of methodologies, initially a habitat suitability index (HSI) assessment (Oldham et al., 2000), followed by a combination of bottle trapping, torchlight counts, hand searches for eggs and sweep netting for adults or larvae (English Nature, 2001; Langton et al., 2001; Griffiths & Inns, 1998; Sewell et al., 2013; Cresswell & Whitworth, 2004; Griffiths et al., 1996). To identify presence/absence of the species, three of the four methods need to be employed at a pond, on four separate occasions, in suitable weather conditions, between the middle of March and the middle of June. At least two of the visits must fall between mid-April and mid-May (English Nature, 2001).

Detection probabilities are often low and highly variable for amphibians (Griffiths et al., 2015; Tanadini & Schmidt, 2011); with failure to take into account the variation leading to false negative results (MacKenzie et al., 2002; Schmidt, 2003). In the core of the range of great crested newts, to achieve 95% detection using either three or four of the methods, four survey visits would be required (Sewell et al., 2010).

However, outside the core range of the species this increases to six visits if four methods are used or seven visits if bottle trapping is not used (Sewell et al., 2010).

As great crested newts often come into conflict with development, and given the legal protection they receive, it is imperative that information relating to the location and status of populations is reliable, easily accessible, and cost-effective to derive. At present survey methods are seasonally-dependent, highly labour-intensive, expensive to conduct and have limited accuracy. In addition to reducing the burden on industry and statutory reporting of conservation status, the diverse and increasing threats to global amphibian species and other freshwater taxa is such that rapid distribution assessments are becoming increasingly necessary. As such the development industry, licencing authorities and conservation organisations require methods to be updated with new technologies to reduce the complexity, cost and time associated with species distribution assessments. The use of a tool to speed up and reduce the cost of assessments could lead to substantial benefits to nature conservation.

1.6. eDNA and great crested newts

The potential for the use of eDNA with great crested newts was realised early in the development of the technology. qPCR primers and probes were developed and tested for use with great crested newts by Thomsen et al. (2012b), along with a suite of other rare or threatened freshwater species from across Europe. The qPCR primer probe combination has been tested and is specific to great crested newts, but may also amplify DNA from *Melanotaenia splendida* (a warm water fish native to Australia), *Taricha torosa* (a newt endemic to California in the western United States of America), *Triturus carnifex* (The Italian crested newt), *T. karelinii* (the southern crested newt, found within south-eastern Europe) and the other members of the species complex (Biggs et al., 2014b). None of these species are native to the UK, although all may be found within the pet trade and the Italian crested newt has been released and is

persisting in isolated populations (Inns, 2009; Jehle et al., 2011). Primers have been tested and amplify DNA collected from great crested newts from around the UK (Biggs et al., 2014b).

A naïve detection rate of 91% for great crested newts has been reported from ponds with known occupancy, with an eDNA persistence of less than two weeks in mesocosm experiments (Thomsen et al., 2012b). Following the study by Thomsen et al. (2012b) the use of eDNA in surveys for great crested newts was identified as having application for presence or absence surveys within the UK, particularly for those required as a result of the legal protections afforded to the species. This has prompted interest from both government and industry (Rees et al., 2014a; Biggs et al., 2014b, 2015). These studies attempted to assess the use of eDNA with great crested newts for commercial purposes (Rees et al., 2014a) and with national monitoring schemes involving citizen science (Biggs et al., 2014b, 2015). They report naïve detection rates between 60% and 99.3%, under slightly varying conditions, and demonstrate the applicability of the methodology in undertaking distribution assessments with both the use of professionals and volunteers (Rees et al., 2014a; Biggs et al., 2014b, 2015). However, the upper estimates for naïve detection suffer from non-independence of samples, both in terms of repeated visits to the same ponds and ponds chosen being in close proximity to one another. The ponds were therefore likely to be part of the same metapopulation and experiencing similar environmental conditions. Additionally, site selection was weighted towards larger great crested newt populations. Nevertheless, ponds known to be negative showed no signs of detection of great crested newts using eDNA (Biggs et al., 2014b).

In March 2014 following the publication of Biggs et al. (2014b), eDNA was added to the suite of methods acceptable for detecting great crested newts in relation to planning applications (Natural England, 2014). However, statutory guidance stipulates that the methodology used must directly follow that used by Biggs et al.

(2014b), and sample collection must be conducted between the 15th of April and the 30th of June, the peak breeding time for newts. This has been met with a very large uptake in participation and a proliferation of commercial laboratories offering an eDNA service, but equally a great deal of debate within the professional sector (Appendix II – Advantages and disadvantages of eDNA surveys for great crested newts: perceptions from practitioners).

More recently, a controversial district licencing approach is being trialled by Natural England. Recent research has shown that site-specific mitigation for great crested newts is high cost, introduces delays into the development process and is often ineffective at protecting great crested newt populations (Lewis et al., 2017). The new strategic licensing approach embraces eDNA surveys and species distribution models, with the twin goals of improving conservation outcomes at the landscape level, while simultaneously reducing the costs and delays for developers. Results from eDNA surveys are used to construct predictive models of the distribution of the species (Bormpoudakis et al., 2016). In turn, the model outputs are being utilised to assess risk of likely impact if an area is developed, with site-by-site mitigation replaced with a regional or local conservation strategies for the species. Conservation of the species would be funded through compensation payments from the development industry and would remove the current requirement for traditional site-specific surveys and mitigation. eDNA surveys are therefore a fundamental part of this new approach to great crested newt mitigation (Freshwater Habitats Trust, 2017b). It is clearly important that the new strategic licensing initiative embraces new developments in eDNA technology and model development as they unfold, to ensure the maximum benefit of new initiatives to species conservation.

1.7. Aims of the project

At the eDNA working group meeting held at the University of Hull, in September 2014 (and coinciding with the start of this project) Dr Pete Brotherton (now Director,

Specialist Services and Programmes for Natural England) delivered a presentation on Natural England's priorities for eDNA research on great crested newts. The presentation focused on the commercial use of eDNA with great crested newt surveys and how to improve eDNA techniques with four specific priorities:

1. Improve the detection of small populations,
2. Estimate population size,
3. Extend the survey window, and
4. Develop proficiency testing for eDNA on great crested newts and other European protected species.

Building on these requirements and refining them within the framework of the wider literature reviewed above, we generated the aims of the project which are as follows:

1. Assess the currently accepted commercial eDNA collection protocol for great crested newt eDNA within the UK and evaluate protocols that might recover greater amounts of eDNA (Chapter 2),
2. Identify the probability of detection of great crested newts using the currently accepted environmental DNA survey protocol (Chapters 3 and 4),
3. Evaluate the appropriateness of the commercial sample collection window in relation to newt phenology (Chapters 4 and 5),
4. Determine whether an estimate of abundance of great crested newts can be made from eDNA samples (Chapters 5 and 6),
5. Identify environmental influences on great crested newt eDNA and its detection (Chapters 3, 4 and 5).

Chapter 2 – Comparison of two methods for collecting pond water samples in citizen science environmental DNA studies

A version of this chapter has been accepted for publication to the peer reviewed journal *Citizen Science: Theory and Practice*

2.1. Abstract

The use of environmental DNA (eDNA) for the survey of aquatic species offers a wide range of benefits over conventional surveys and has begun to be used by citizen scientists. One advantage of eDNA over conventional survey protocols is the comparative ease with which samples can be collected over a wide geographic area by citizen scientists. However, eDNA collection protocols vary widely between different studies, promoting a need to identify an optimum method. Collection protocols include ethanol precipitation and various filtration methods including those that use electronic vacuum or peristaltic pumps, hand pumps or syringes to capture eDNA on a membrane. We compare the effectiveness of two eDNA collection methods suitable for use by citizen scientists: glass-microfiber syringe filtration and ethanol precipitation. Paired samples of water were analysed for great crested newt (*Triturus cristatus*) DNA using (1) a laboratory tank experiment using different dilutions of water inoculated with newt DNA; and (2) by sampling naturally colonised ponds. Although syringe filters consistently yielded greater DNA extract concentrations in the tank experiments, this was not the case in samples collected from the field where no difference between the two methods was identified. Clearly, properties within the water – such as algae and particulate matter - can influence the amount of DNA captured by the two methods, so the sampling protocol of choice will depend on the design and goals of the study.

2.2. Introduction

With threats to biodiversity increasing (Pimm et al., 1995; Barnosky et al., 2011), rapid biodiversity assessment and the ability to reliably detect rare species and species with patchy distributions is imperative for effective evidence-based conservation actions to be implemented (Ficetola et al., 2008; Magurran, 2004). Citizen science generated data is widely used for species distribution assessments and other ecological research (Van Strien et al., 2013; Bonney et al., 2009). However, various challenges exist in working with ecological citizen science data, most notably error and bias due to variation between observers (Dickinson et al., 2010). Accuracy of visual based species identification may be as low as 60% for non-experts (Austen et al., 2016).

Citizen science schemes for the monitoring of amphibians are active within the UK, for example the National Amphibian and Reptile Recording Scheme (NARRS; Wilkinson & Arnell, 2013). Surveys for amphibians, particularly the great crested newt (*Triturus cristatus*) within the UK, require the use of multiple methods and multiple survey visits to achieve a reasonable probability of detection (Wilkinson & Arnell, 2013; Langton et al., 2001; Griffiths et al., 1996). Surveys require a combination of torchlight visual searches of ponds at night, overnight bottle trapping, sweep netting to catch individuals, and searches of vegetation for eggs. Each of these survey methods requires a considerable amount of time at a pond; has health and safety implications for surveyors (for example working at night); can have variable reliability of detection influenced by environmental factors; and may require extensive training and licencing to ensure that it is carried out with minimum risk to target and non-target organism (Langton et al., 2001). Using all four of these methods combined in a single survey visit yields a probability of between 0.41-0.68 of detecting the presence of great crested newts. To improve confidence that a site is unoccupied to the 95% level requires between 3-6 visits using traditional methods (Sewell et al., 2010). As a result,

a regional or landscape-wide survey programme can be logistically difficult, prohibitively expensive and require multiple visits and skilled surveyors with taxonomic training (Sewell et al., 2010; Biggs et al., 2015). The intensity of the survey methodologies and the number of visits required to achieve such high levels of confidence in the results has the potential to lead to low volunteer retention (Pers. comm. Dr John Wilkinson).

Given the issues with traditional survey methods for amphibians, there is a demand for developing simple yet reliable survey methods that can be carried out by citizen scientists. Environmental DNA (eDNA) surveillance is a technique where DNA is isolated from a sample of environmental material and used to identify the presence of a species or community of species through detection of DNA shed into the environment by the target species (Jane et al., 2015). Since 2008, eDNA has become a widespread tool for the detection of invasive aquatic species (Jerde et al., 2011; Wilson et al., 2014; Ficetola et al., 2008) and species of conservation importance (Biggs et al., 2015). Laboratory analysis based on DNA circumvents variation in species identification between surveyors and should reduce inaccuracies in data sets contributed to by many individuals.

Despite wide use by the research community there has been limited uptake of eDNA within citizen science studies. However, its utility with citizen scientists, for the detection of great crested newts, has been demonstrated by the Freshwater Habitats Trust Pond Net scheme (Freshwater Habitats Trust, 2017a; Biggs et al., 2015) and the “Great Crested Newt Detectives” project of Amphibian and Reptile Conservation in Scotland (Minting, 2016) both within the UK. The method has wide applicability with citizen science based studies, allowing assessments of species distribution at scales that would make conventional or commercial surveys prohibitive (Biggs et al., 2015; Gibson et al., 2015). Collecting environmental samples for eDNA analysis requires little training and can be carried out quickly. Samples can be collected by citizen

scientists in the field at a time that suits them, with water samples returned to a central location for shipment to a laboratory for analysis. DNA is then isolated and identified using molecular techniques such as real-time quantitative PCR (qPCR; Thomsen et al., 2012b) or metabarcoding (Valentini et al., 2016).

eDNA sample collection requires a much shorter time at the pond than traditional methods, and is often conducted using only a single sample collected during one visit, with detection probabilities much greater than for a single visit using conventional methodologies (Chapter 4) . The reduced number of visits required and lower intensity of sample collection will increase the number of sites a single volunteer can survey for the same effort, and may increase volunteer retention, facilitating an increase in scale for the study as a whole, and increasing statistical rigor (Wilkinson and Arnell 2013). Additionally, surveys targeting eDNA reduce disturbance to the studied species by reducing the number of visits required to the pond, removing the need to disturb the structure of a pond with sweep netting and removing any animal welfare concerns associated with trapping. eDNA also increases the accuracy of results by removing any ambiguity arising from visual species identification. As such eDNA offers a new tool for use by NGOs and other bodies to work with citizen scientists to generate large, accurate species distribution data sets. Nevertheless, if managed inappropriately, removal of contact with the target species in the field may reduce participant engagement.

A variety of methods have been used for the collection of aquatic environmental samples and when using eDNA it is vital to choose sampling methodologies that are appropriate for the goals of the research. To date, most studies have focused on how best to detect a target species from samples rather than the reliability of the sample collection protocol itself (Deiner et al., 2015; Goldberg et al., 2016). The most popular protocols use one of two approaches. Firstly, precipitation in ethanol, where a sample of water is preserved within a large volume of absolute ethanol and a small volume

of a salt (Ficetola et al., 2008; Biggs et al., 2015; Deiner et al., 2015; Eichmiller et al., 2016b; Spens et al., 2016). Alternatively, a sample of water is passed across a micropore membrane to concentrate and preserve the DNA; however, the volume of water, membrane substrate and membrane pore sizes used vary considerably between studies (Goldberg et al., 2016). A summary of methods used by different studies has been collated by Rees et al. (2014b). The different sample collection and extraction methodologies may have advantages and disadvantages, but few studies have assessed how they perform against each other, or within different environments i.e. in ponds, lakes, rivers and the marine environment. However, some studies have found filtration recovers more DNA than ethanol precipitation (Deiner et al., 2015; Spens et al., 2016; Eichmiller et al., 2016b); these however, focus on stream and lake environments and do not take into account environmental conditions unique to ponds.

Likewise, not all sampling protocols are suitable for citizen science initiatives (Biggs et al., 2015). For example, many filtration protocols require the transport of large volumes of unpreserved sample water, on ice, to a central location for filtration (Pilliod et al., 2013) while others use expensive electronic pumping equipment in the field (Pilliod et al., 2013). Neither approach is easily adopted by - nor practical for - citizen science studies, where volunteers may each be expected to collect a small number of samples in a time frame fitted around other commitments. Due to the very low concentrations of target DNA, it is imperative that eDNA sample collection and extraction methods recover the highest amount of DNA possible in a sample. Here we assess two eDNA collection methods that would be applicable to a citizen science study with a large number of surveyors each collecting a small number of samples. We evaluated these methods for their suitability for citizen science studies as they do not require bulky, expensive or electronic pumping equipment or the transport of large volumes of water to a central location for filtration, and can be supplied as individual sealed kits for each sample.

We test the precipitation in ethanol method (Biggs et al., 2015), and syringe filtration (Deiner et al., 2015) with glass microfiber syringe filtration method, aiming to identify whether eDNA extract concentrations varied between the two methods as they would be used in the field. We did this in both laboratory tanks and ponds, using water volumes applicable to the different methods. We target the great crested newt, a semi-aquatic amphibian that has been widely used as a study species within eDNA research and citizen science (Thomsen et al., 2012b; Biggs et al., 2015; Rees et al., 2014a, 2017; Biggs et al., 2014b; Minting, 2016), and one in which commercial eDNA analysis has been pioneered (Natural England, 2014).

2.3. Methods

2.3.1. Field samples

Two eDNA collection methods, precipitation in ethanol (hereafter referred to as 'ethanol precipitation'), and glass-microfiber filtration using syringe filters (hereafter referred to as 'filtration') were compared using water samples from a naturally colonised pond system. A network of eight small (600 L, 1 m by 2 m and up to 0.6 m deep) ponds used by great crested newts, at the University of Kent, Canterbury Campus (UK) were utilised (Lewis, 2012). Sixty-one pairs of eDNA samples were collected from the eight ponds, by experienced researchers, using the ethanol precipitation and filtration methods between March and September 2015, covering the period when adults and larvae are in their aquatic phase.

Prior to field collection all equipment was sterilised in 10% bleach and thoroughly rinsed with water, sterilised in an autoclave or UV-Crosslinker, and then sealed prior to transport to the study site. Due to the small size of the ponds each sample consisted of a single 1 L surface water sample from the pond centre. The order the two samples were taken in was randomised so as to remove sampling bias. A fresh set of disposable gloves were used for each of the samples to prevent contamination.

To collect the filtration samples a sterilised 1 L water bottle was unsealed and rinsed with pond water prior to being filled. A disposable 100 mL syringe was used to pass the sample water across a Sterlitech Corporation® glass-microfiber syringe filter (0.7 µm pore size, 30 mm diameter), refilling the syringe until 1 L had been filtered or the 2 filter units had become blocked. Two syringes of air were then passed through each filter to reduce the amount of residual water in the sealed unit. Filters were sealed in plastic bags and transported to the laboratory; the maximum time between sample collection and reaching the laboratory was three hours, with samples then maintained at -20 °C until extraction.

Ethanol precipitation sample collection followed a protocol originally from Biggs et al. (2015). In brief, six, 50 mL centrifuge tubes, each containing 33 mL of absolute ethanol and 1.5 mL of 3 M sodium acetate solution were filled to the 50 mL gradation with sample water using a disposable plastic pipette. This volume equates to approximately 15 mL of sample being placed into each of the 6 sample tubes and a total sample volume of approximately 90 mL. The lid to each tube was sealed, and the tube contents mixed by inversion. All six sample tubes were then placed in a sealable bag for transport to the laboratory, the maximum time between sample collection and reaching the laboratory was three hours, with samples then maintained at -20 °C until extraction.

2.3.2. Serial dilution of tank water

A laboratory experiment was carried out using great crested newts under controlled conditions. Plastic boxes, dimensions 490 mm x 360 mm x 240 mm deep, were set up in a temperature controlled room (18 ºC ± 2 ºC), containing 10 L of tap water. The water was allowed to stand for a minimum of 24 hours to allow the water to naturally dechlorinate. Great crested newts were collected using the standard bottle trapping method (Langton et al., 2001) from a pond within the campus of the University of Kent and taken into captivity under licence from Natural England (Licence number 2015-

10937-SCI-SCI). The newts were allowed to acclimatise to the temperature of the room in tanks containing water from their original pond before one newt was introduced to each experimental tank. The individuals were left in the study tanks for 24 hours before being removed and released into their original pond. Five replicate tanks were used between the 28th and 29th of April 2015, and an additional three replicates between the 14th and the 15th of May 2015.

Prior to sample collection all equipment was sterilised in 10% bleach and thoroughly rinsed with water, sterilised in an autoclave or UV-Crosslinker, and then sealed. Once the individuals had been removed from the tanks, a 1 in 2 dilution series was performed on the tank water to create samples at 100%, 50%, 25%, 12.5%, 6.25% and 3.125%, of the starting concentration of the tank water. Dilution was undertaken with tap water, making 1 L of sample water at each dilution for each tank. The dilutions were made using the lowest concentration first in order to prevent contamination between levels. An ethanol precipitation eDNA sample of 0.09 L was initially taken, with the remainder of the water, totalling 0.91 L, then passed through two Sterlitech Corporation® glass-microfiber syringe filters (0.7 µm pore size, 30 mm diameter), in equal proportions, following the protocols described above. In total 48 pairs of samples were collected. Samples were then stored at -20ºC until DNA extraction.

2.3.3. Extraction protocols

DNA extractions were conducted in a UV sterilisable work station, with dedicated equipment, and were based on the Qiagen® DNeasy® Blood & Tissue kit with amended protocols as outlined. Periodic extraction blanks for both methods were undertaken through the laboratory phase of the project to check for equipment contamination.

2.3.3.1. Syringe filtration

In a fume hood sterilised with a 10% bleach solution and UV-light the filter paper was removed from the sealed syringe filter holder using sterilised wire cutters and sterilised forceps. Once removed the filters were cut into strips approximately 3 mm in width with each filter placed into a separate 1.5 mL microcentrifuge tube. Thus, in the digestion step each sample consisted of two microcentrifuge tubes, one for each of the two filters. 675 µL of the ATL buffer from the DNeasy® Blood & Tissue kit (Qiagen®) was added to each tube; it was then vortexed for 15 seconds to mix before 20 µL of Pro K was added and again vortexed. The samples were then incubated on a rotating block, for 3 hours at 56 °C or overnight at 37 °C. Following incubation the two digestion reactions for a sample were combined in a fresh microcentrifuge tube. DNA extraction continued as per the DNeasy® Blood and Tissue kit manufacturers' protocol, eluting into 200 µL of the elution buffer.

2.3.3.2. Ethanol precipitation

eDNA extraction from ethanol precipitation samples was undertaken using a modified protocol from Biggs et al. (2014). The six centrifuge tubes were centrifuged at 10,020 g, (8500 rpm) for 35 minutes and the supernatant discarded. The remainder of the extraction protocol followed the modified Qiagen® DNeasy® blood and tissue kit protocol, from Biggs et al. (2014).

2.3.4. qPCR analysis

Following extraction, samples were stored at -20°C until real-time qPCR could be undertaken. qPCR plate set up was conducted in a separate dedicated laboratory, also within a separate UV-sterilisable work station. qPCR was performed using qPCR primers and hydrolysis probe and assay designed by Thomsen et al. (2012) and validated by Biggs et al. (2014, 2015), using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems®). Samples were run on a BIO-RAD® CFX Connect Real-Time PCR detection system, under thermal cycling conditions from Biggs et al. (2014,

2015). Eight qPCR replicates were performed on each sample (Ficetola et al., 2014). qPCR standards were created from a serial dilution of a great crested newt tissue extract, quantified using a Qubit® 2.0 fluorometer (Life Technologies®) with the double stranded DNA high sensitivity kit following manufacturers' instructions. Three standards were used in each assay, acting as positive controls and to allow quantification using a standard curve, negative qPCR controls were also included.

2.3.5. Limit of quantification and limit of detection

The limit of quantification (LOQ) and limit of detection (LOD) were assessed using a serial dilution of a DNA extract from great crested newt tissue. DNA from the tissue of a dead great crested newt (kept under licence from Natural England; Possession Licence Number: 2015-7591-SCI-SCI) was extracted using Qiagen® DNeasy® Blood and Tissue Kit following manufacturer's instructions. The extract concentration was quantified using a Qubit® 2.0 fluorometer (Life Technologies®) with the double stranded DNA high sensitivity kit following manufacturers' instructions. The extract was diluted, with double distilled water, creating 1/10 dilution series, to a minimum concentration of 1/10 million of that of the tissue extract. The LOQ and the LOD were calculated through qPCR from this serial dilution, the lowest level of concentration that exhibited a high degree of conformity between the eight PCR replicates and the minimum concentration of target DNA that can be detected in a sample respectively (Tréguier et al., 2014).

2.3.6. Analysis

All statistics were undertaken using R version 3.1.3. (R Development Core Team, 2016), and the tests used are indicated within the results section. Median values were used in the analysis over arithmetic mean to prevent outlying qPCR replicates from affecting the extract concentration. Linear regression was conducted for both of the eDNA collection methods comparing dilution level and extract concentration. An analysis of covariance (ANCOVA) was then performed on the linear regression

models to compare the effect of collection method on the DNA extract concentration. Wilcoxon-Pratt signed rank tests were then used to compare filter and ethanol precipitation samples to ascertain whether there was a statistically significant difference between the two methods. Wilcoxon-Pratt signed rank tests were also used to identify any difference between the sampling methodologies between paired samples from the real ponds, in terms of eDNA concentration and eDNA score (i.e. the proportion of positive qPCR replicates).

2.4. Results

2.4.1. Limit of quantification and limit of detection

The LOQ for great crested newt eDNA within this study was determined to be 10^{-5} ngµL^{-1} (Figure 2.1). At greater extract concentrations all qPCR replicates tightly clustered around a similar threshold value: at concentrations below this the number of positive replicates decreases and the conformity of threshold value between replicates is reduced. The dilution level for this study only went as low as 10^{-7} ngµL^{-1}. However, at this level, three PCR replicates were still positive, indicating the LOD achieved here was less than 10^{-7} ngµL^{-1}.

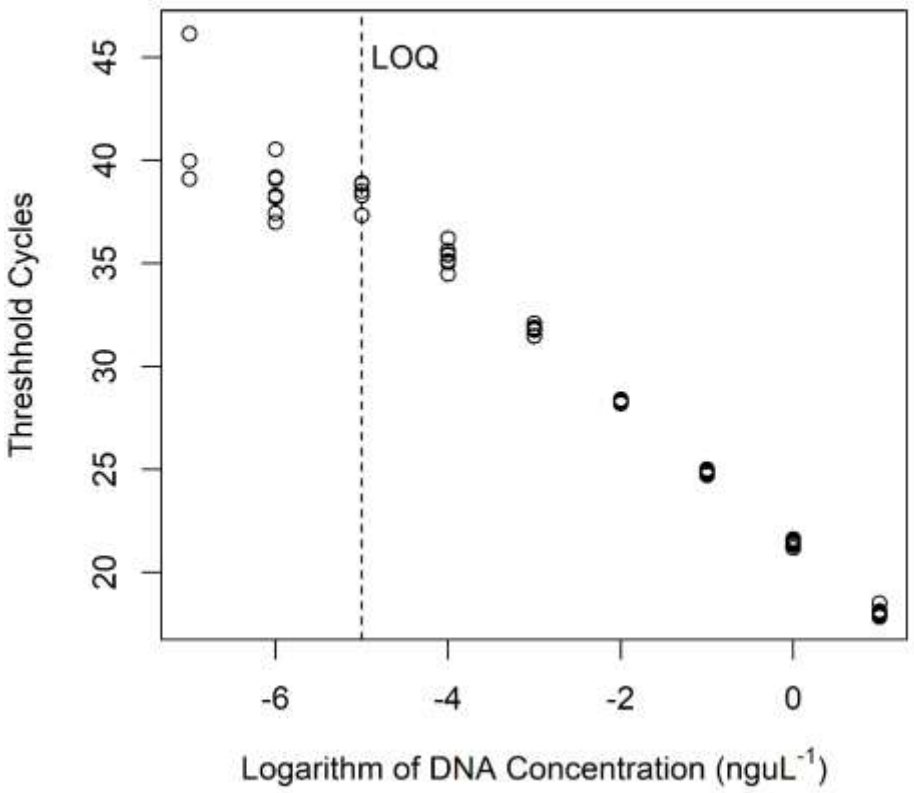

Figure 2.1 - Limit of detection and quantification. Limit of detection (LOD) and limit of quantification (LOQ) for great crested newt eDNA. A dilution series of known amounts of great crested newt DNA was used to identify these limits, calculated across eight qPCR replicates at each concentration. The PCR cycle number at which positive amplification is first identified is known as the Threshold Cycle.

2.4.2. Samples from naturally colonised ponds

All positive field samples were found to be above the limit of quantification. There was no difference in extract concentration from filters and ethanol precipitation paired samples collected from ponds (Wilcoxon-Pratt signed-rank test z = -1.03; p = 0.30; Figure 2.2).

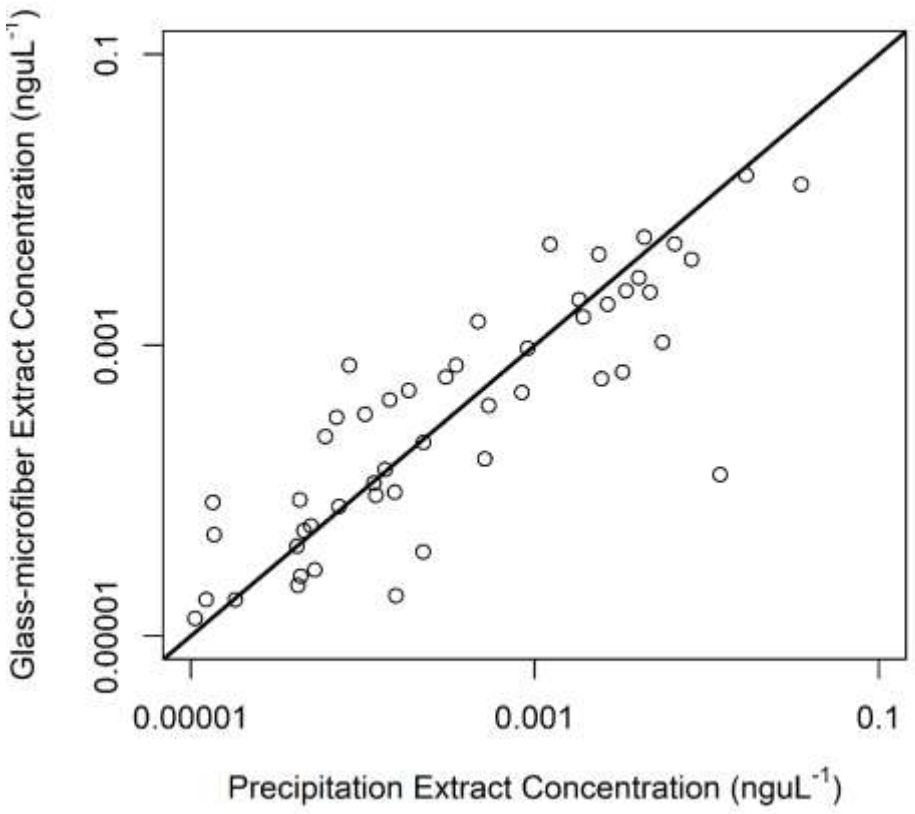

Figure 2.2: Paired eDNA samples collected from natural ponds. The black line indicates the point at which the two extract concentrations have equal DNA concentration. Sample pairs with greater extract concentration from the glass-microfibre filter collection method appear above the line and sample pairs with greater extract concentration from the precipitation method appear below the line. Due to logarithmic scale where one or both of a pair are a negative sample, no result is shown.

We analysed the eDNA score (proportion of positive qPCR replicates) for the two collection methods for all 61 paired eDNA samples. Again we found no significant difference between the sample collection methods (Wilcoxon-Pratt signed-rank test $z = -1.0$; $p = 0.319$).

2.4.3. Experimental serial dilution

All samples from the experimental serial dilution fell above limit of quantification. Regression analysis of the level of dilution on the final extract concentration was highly significant for both the ethanol precipitation ($t = 5.0$; $df = 46$; $p < 0.0001$) and filter ($t = 6.3$; $df = 46$; $p < 0.0001$) collection methods. A significant interaction (ANCOVA: $F = 33.3$; $df = 1, 93$; $p < 0.0001$) was found between the collection method and the sample dilution level. In addition to the significant difference in slope between the two eDNA collection methods, the intercept was also found to be different (precipitation = 2.541×10^{-5} $ngul^{-1}$; glass-microfiber filter = 0.003892 $ngul^{-1}$). This indicates that the effect of initial sample concentration on the final extract concentration depends on the collection method used (Figure 2.3).

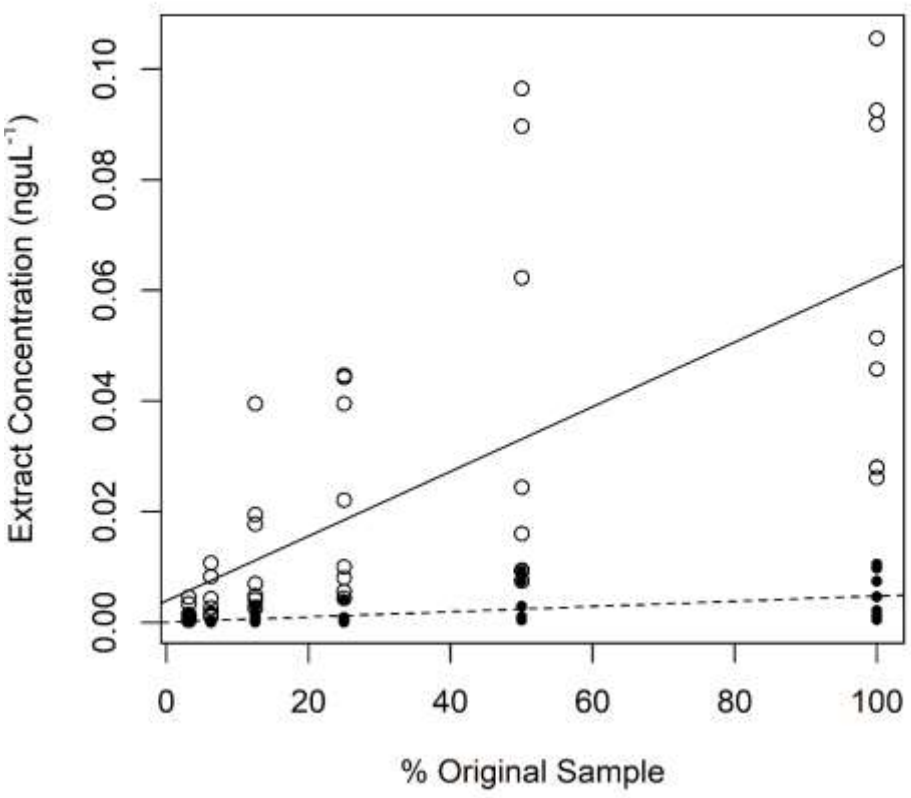

Figure 2.3: Relationship between DNA extract concentration and the level of dilution. Each point represents the median qPCR concentration value for an eDNA sample for two collection methods at the different dilution levels. Open circles represent samples collected via filtration, closed circles represent samples collected via ethanol precipitation.

For each of the paired samples at all dilution levels the filtered samples yielded a greater extract concentration than the corresponding sample collected using the ethanol precipitation method (Figure 2.4). This result was highly significant (Wilcoxon-Pratt signed-rank test: $z = 6.03$, $p < 0.0001$).

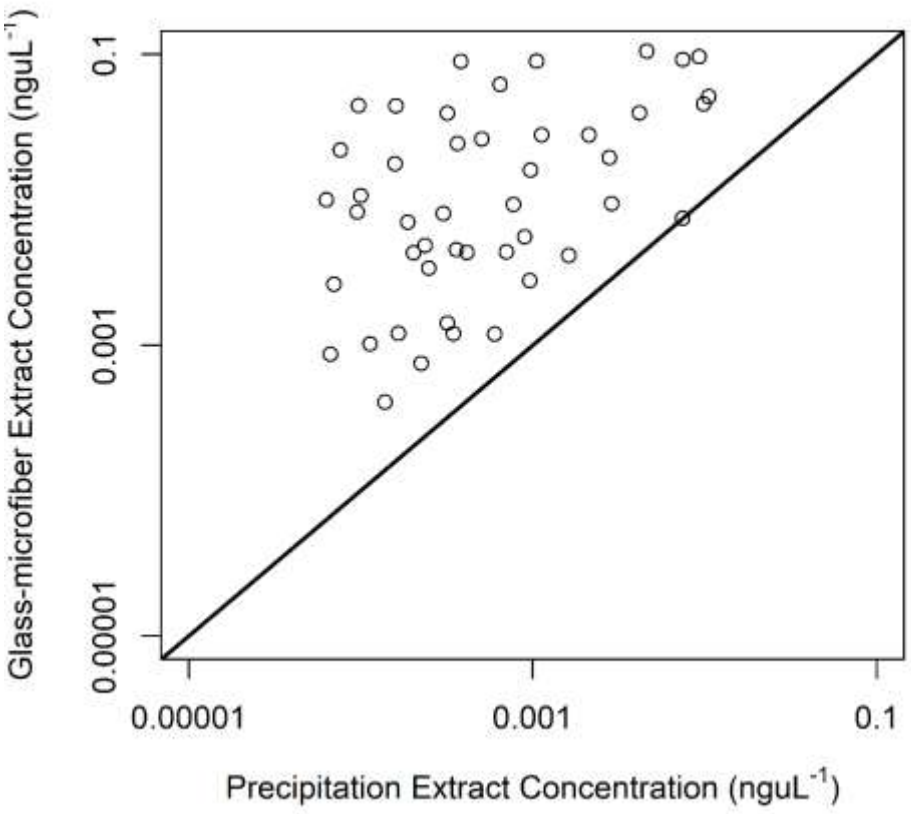

Figure 2.4: Paired eDNA samples collected from serial dilution of tank water. The black line indicates the point at which the two extract concentrations would have equal DNA concentration. Sample pairs with greater extract concentration from the glass-microfibre filter collection method appear above the line and sample pairs with greater extract concentration from the precipitation collection method appear below the line. Note the logarithmic scale for clarity.

2.5. Discussion

The limits of detection and quantification achieved >10^{-7} ngµL^{-1} and >10^{-5} ngµL^{-1} respectively: these thresholds are similar to those achieved in other studies (Tréguier et al., 2014). In experimental tanks where water contained no organic or particulate matter, filtration of 0.91 L of sample water using 0.7 µm glass-microfiber syringe filters

recovered larger amounts of eDNA than ethanol precipitation with a sample volume of 0.09 L across the range of sample water concentrations tested. However, when assessed in the field with real pond water no significant difference between the collection methods was observed.

We compared two methodologies considered to be suitable for use within widespread citizen science projects, where provision of equipment such as peristaltic pumps would not be logistically or financially viable (Biggs et al., 2015). However, we recognise that other filtration methodologies are available that require the use of pumping equipment, which may allow for increased eDNA capture rates (Spens et al., 2016; Minamoto et al., 2016).

The concentrations of target DNA in the extracts from the laboratory tanks were within or above the range of that observed in the natural ponds. Our results from the laboratory tanks support previous work on lake water, which showed that filtration recovers greater amounts of total and target eDNA than the ethanol precipitation method (Spens et al., 2016). However, Spens et al. (2016) used different filters and sampling volumes from the present study. Increased sample volumes used in the filtration method are likely to have been responsible for the greater concentrations of eDNA recovered, when compared with the ethanol precipitation method, in the tank experiment.

The difference in extract concentrations between the two sampling methods observed in the laboratory tanks was not repeated in the field samples. This result may reflect the composition of pond water compared to tap water. When processing natural pond water filters may become blocked by suspended solids and algae which were not in the samples from laboratory tanks. We found that that 0.91 L of water from laboratory tanks could easily be passed through two filters. In contrast, in some field samples it was not possible to pass 500 mL of pond water through two glass-microfiber filters disks. The lower water volume is likely to reduce the amount of eDNA captured and

therefore the quantity available for extraction. In contrast, during the initial precipitation and centrifugation step with the ethanol precipitation samples, suspended solids and algae precipitated out of solution with the eDNA collecting as a pellet on the side of the tube. This additional material may have assisted in securing the eDNA in the pellet, preventing it being discarded with the supernatant and increasing the amount of DNA within the extract.

Pond water can differ from water found in rivers, lakes or the marine environment. Pond water is more stagnant, allowing the build-up of algae and suspended solids to a greater extent than lotic water or large lakes where stratification and wind action allow for water movement. The results from our field experiment do not fully support the conclusions of Spens et al. (2016) or Deiner et al. (2015), both of whom conclude that filtration outperforms precipitation. In our experiment we observed no difference between the sampling methods when they are applied to pond water. However, these two studies utilised lake and river water respectively and so may not have faced the same limitations found with ponds.

Deiner et al. (2015) show that different combinations of sampling and extraction protocols are appropriate when targeting different taxa, and conclude that it is imperative to pick the combination best suited to the specific study, advice also advocated by Minamoto et al. (2016). Our results suggest that this approach should be extended to environment type as well as to taxon, given that the difference in recovery between the sample types, which we observed in tank experiments, disappears with pond water.

Within the laboratory tank experiment there was a significant relationship between the level of dilution and eDNA extract concentration, with more diluted samples showing a reduced extract concentration. However, some samples exhibited greater extract concentration than more heavily diluted samples collected from the same tank. Although this may result from sampling error, it was apparent with both collection

methods, but was more prominent in ethanol precipitation samples. This finding suggests that even though concentration of eDNA extracts are related to the amount of DNA within the water sampled, extraction efficiency between samples may not be consistent, or the amount of eDNA within a sample may be heavily influenced by the form that the eDNA takes (extracellular, single cells or aggregations of cells). Assuming that the majority of eDNA collected is intracellular (Rees et al., 2014b; Deiner et al., 2015), concentration may be influenced by aggregations of cells within samples, with larger water volumes used with the filtration samples helping to mitigate for this.

To detect statistically meaningful changes in pond occupancy by amphibians on a regional or national scale using traditional visual based survey methods, the number of sites needed to be visited and the survey effort required may be prohibitive (Biggs et al., 2014b; Wilkinson & Arnell, 2013). This would be the same for citizen scientists or professional surveyors. New survey methods, such as the targeting of eDNA, require fewer visits to a pond, less time on site and more sociable working hours. This may allow surveys to be undertaken on a larger scale, thereby improving estimates of occupancy and population change, which have been goals of citizen science led national monitoring projects such as the Amphibian and Reptile Conservations Trust (ARC) NARRS project (Wilkinson & Arnell, 2013). However, there is a financial cost associated with processing eDNA samples and the laboratory analysis would need to be budgeted from the outset. The use of the eDNA technique by citizen scientists within national or regional distribution assessment projects has been demonstrated within the ARC Great Crested Newt Detectives project in Scotland (Minting, 2016) and the Freshwater Habitats Trust PondNet project (Biggs et al., 2015). These projects are managed centrally by nature conservation charities working closely with a commercial laboratory. The laboratory provides eDNA sampling kits to the volunteer surveyors and undertakes the genetic analysis. The availability of eDNA survey

methods for projects utilizing samples collected by citizen scientists has the potential to be utilised for a wide range of taxa of conservation concern.

Citizen scientists clearly have options for eDNA sample collection. However, the collection of filtered samples from natural ponds with filtration was challenging as filters can easily become blocked and this may not lend itself to participant engagement and retention. Citizen scientists are likely to vary in the level of perseverance when trying to pass water across a filter. This may cause differences in the amount of water collected between individuals under the same conditions, impacting the consistency of the results and reducing the efficiency of the filtration method. Although filtration outperformed ethanol precipitation under experimental conditions, citizen scientists would be sampling natural ponds. Given that no difference in performance was observed between the methodologies in the field, either methodology would seem to be equally applicable under the conditions encountered here. However, different methods may recover different amounts of eDNA in different situations. We recommend pilot studies are undertaken to identify the most appropriate method for individual studies; with decisions on the most appropriate method taking into account practical considerations relating to the two methods, and the specific study needs.

2.6. Acknowledgements

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Chapter 3 – Is the detection of aquatic environmental DNA influenced by substrate type?

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3.1. Abstract

The use of environmental DNA (eDNA) to assess the presence-absence of rare, cryptic or invasive species is hindered by a poor understanding of the factors that can remove DNA from the system. In aquatic systems, eDNA can be transported out either horizontally in water flows or vertically by incorporation into the sediment. Equally, eDNA may be broken down by various biotic and abiotic processes if the target organism leaves the system. We use occupancy modelling and a replicated mesocosm experiment to examine how detection probability of eDNA changes once the target species is no longer present. We hypothesise that detection probability falls faster in a sediment which has a large number of DNA binding sites such as topsoil or clay, over lower DNA binding capacity substrates such as sand. Water removed from ponds containing the target species (the great crested newt) initially showed high detection probabilities, but these fell to between 40% and 60% over the first 10 days and to between 10% and 22% by day 15: eDNA remained detectable at very low levels until day 22. Very little difference in detection was observed between the control group (no substrate) and the sand substrate. A small reduction in detection probability was observed between the control and clay substrates, but this was not significant. However, a highly significant reduction in detection probability was observed with a topsoil substrate. This result is likely to have stemmed from increased levels of PCR inhibition, suggesting that incorporation of DNA into the sediment is of only limited importance. Surveys of aquatic species using eDNA clearly need to take account of substrate type as well as other environmental factors when collecting samples, analysing data and interpreting the results.

3.2. Introduction

Environmental DNA (eDNA) is a rapidly expanding method for the detection and survey of aquatic organisms. Targeted species detection from samples of water using qPCR is increasingly being used in local and regional assessments of invasive (Jerde et al., 2011), rare (Santas et al., 2013) or protected species (Biggs et al., 2015). The method is also being used to assess changes in site occupancy over time (Biggs et al., 2014b, 2015), where the use of traditional methodologies would be logistically onerous. For both national assessments and localised presence-absence surveys of target species it is important that limitations surrounding the technique and sampling strategy are understood. Indeed, where eDNA fails to detect a species that is known to have been recently present, understanding the persistence of eDNA is crucial for reliable interpretation of results.

Three processes contribute to the removal of eDNA from the aquatic environment, influencing the length of time a target organism can be detected. Firstly, transport in water flows in lotic systems (Pilliod et al., 2014) or currents in the marine environment (Thomsen et al., 2012a). However, this is unlikely in small lentic waterbodies such as ponds. Secondly, eDNA becomes unavailable for survey as the DNA is degraded through a wide variety of processes (Laramie et al., 2015; Turner et al., 2014a; Barnes et al., 2014; Piaggio et al., 2014; Strickler et al., 2015). Thirdly, eDNA can be transported vertically out of suspension by binding to particulate matter, settling and becoming incorporated into substrates such as clay (Turner et al., 2014a). The number of binding sites and binding mechanisms within the substrate play a role in its capacity to bind with DNA, with sand having a lower capacity than clay due to particle size (Levy-Booth et al., 2007). This difference in capacity means that substrate type can potentially alter the amount of DNA available in eDNA surveys. The persistence of aquatic eDNA is highly variable, with reports suggesting anything from a few hours (Thomsen et al., 2012a) to two months (Strickler et al., 2015)

depending on environmental conditions. However, when incorporated into soil sediments, eDNA persistence may be in excess of months (Turner et al., 2015) or even thousands of years (Anderson-Carpenter et al., 2011; Haile et al., 2009).

Where decreases in eDNA concentration are observed following the removal of the target organism, a pattern similar to a negative exponential decline has been documented (Thomsen et al., 2012b; Pilliod et al., 2014; Lance et al., 2017). In mesocosm experiments with Idaho giant salamanders (*Dicamptodon aterimus*), Pilliod et al. (2014), show eDNA degradation of between 94% and 98% over the first two days, with the last positive samples found after 11 days. Also using mesocosms, Thomsen et al. (2012b) monitored eDNA persistence of the common spadefoot toad (*Pelobates fuscus*) and the great crested newt (*Triturus cristatus*), with detection persisting between 2 and 9 days. Neither study attempted to look at qPCR limits of detection or quantification (Tréguier et al., 2014). It is therefore unclear whether the negative exponential decline is real or the studies merely reached their limits of quantification at the point where concentration decline appeared to slow.

Simply using the length of time during which eDNA remains detectable after the target species is removed does not show how the probability of detecting the species declines over time. Imperfect detection is commonplace within ecological studies causing errors within monitoring programs (MacKenzie et al., 2002; Pollock et al., 2002; Tyre et al., 2003; Schmidt, 2003; Kéry & Schmid, 2004; Schmidt, 2005; Field et al., 2005), and this is true for eDNA as well as conventional monitoring methods. Errors may arise during collection of the water sample, extraction of the DNA or amplification of the DNA. A false negative result (i.e., not detecting a species when in fact it is present) could result from non-uniform eDNA distribution within a waterbody, low concentration within the water body, degradation during sample transport and storage, PCR inhibition or poor affinity of the genetic assay with the target DNA (Sigsgaard et al., 2015; Jane et al., 2015).

PCR inhibition is common in environmental samples, with high concentrations of eDNA often being undetectable due to inhibitors (Jane et al., 2015). There are many sources of PCR inhibition (Alaeddini, 2012; Schrader et al., 2012), and the cause of inhibition is not always apparent. However, humic substances from the breakdown of organic material (Jane et al., 2015) or derived from soils (Alaeddini, 2012; Albers et al., 2013), are known to be PCR inhibitors. Humic acids cause uncompetitive inhibition, binding to the polymerase active sites preventing the PCR reaction from occurring (Alaeddini, 2012), reducing the efficiency of the PCR process and increasing the chance for false negative results.

The potential for false negative results therefore needs to be understood when using eDNA as a survey tool. Consequently, detection probability of eDNA – and how it changes over time – are important considerations. A number of studies report naïve detection rates based on the number of positives identified from a range of independent samples. In one well-studied species, the great crested newt, these naïve detection rates have been shown to vary widely from 60% to up to 99% (Thomsen et al., 2012b; Rees et al., 2014a; Biggs et al., 2015, 2014b), and this can lead to inconsistent – or even misleading – interpretation of the results.

Site occupancy detection models account for occasions when the sampling method may ‘miss’ the species (detectability) so that the proportion of sites in which the target species occurs (occupancy) can be reliably estimated (MacKenzie et al., 2002, 2003; Tyre et al., 2003). When replicated samples are taken, the same principle can be applied to estimating the actual ‘occupancy’ of eDNA at a site. Indeed, occupancy models have been utilised for eDNA with a variety of taxa with the probability of detection ranging from 0.74 to 0.95 (Schmidt et al., 2013; Hunter et al., 2015; Schmelzle & Kinziger, 2016; Vörös et al., 2017; Ficetola et al., 2015; Lahoz-Monfort et al., 2016; Guillera-Arroita et al., 2017; Ficetola et al., 2016).

In this study we utilise great crested newts, a semi-aquatic amphibian protected under UK and European legislation, as our study species. The species has been the subject of several eDNA studies (Thomsen et al., 2012b; Biggs et al., 2014b, 2015; Rees et al., 2014a), and eDNA surveys are now accepted practice in surveys of the species carried out as part of commercial development mitigation (Natural England, 2017). Using a mesocosm experiment with different pond substrates, we show how detection probability falls following the removal of the target species. We hypothesize that detection probability will reduce over time as eDNA becomes unavailable for the survey. We further predict this drop in detection probability will occur faster in water containing organic sediments or small particle size sediments than in water where no sediment is present or with large particle size inorganic sediments. Although changes in eDNA concentration and the proportion of amplifying replicates have been previously studied under semi-natural conditions (Thomsen et al., 2012b; Pilliod et al., 2014), we believe that this is the first time occupancy models have been utilised in relation to mesocosm experiments to reliably determine changes in detection over time. Equally, we show for the first time how eDNA detectability varies in relation to sediment type.

3.3. Methods

3.3.1. Experimental set up

Twenty opaque PVC plastic boxes with a maximum volume of 20 L (width 36 cm x depth 28 cm x height 20 cm) were set up in a 5 x 4 grid in an outdoor field, with tanks separated from one another by approximately 30 cm. Each tank was randomly assigned one of four treatments, with five replicates of each treatment.

The four treatment groups comprised clay, sand, topsoil and a no substrate control group. Smooth terracotta potter's clay was chosen to represent a substrate commonly used for pond lining, the substrate is 100% clay with impurities removed. Commercially available children's play sand was used to emulate ponds with a sandy

inorganic substrate. Commercially available garden centre topsoil was used to represent ponds with a high organic input. The topsoil consisted of 40% sand, 33.3% silt and 26.7% clay, identified using LaMottle Company soil texture test kit (following the manufacturer's instructions; LaMotte Company, 2017). This is a similar composition to that found in ponds with a high leaf litter content (unpublished data). No substrate was added to the control treatment groups.

The commercially available substrates were tested for great crested newt DNA using a modified QIAamp Stool DNA Mini Kit (Qiagen®) extraction protocol and qPCR conditions described later (n = 8 replicates). Each of the plastic treatment boxes (except the control group) had 2.5 kg of the substrate added to it. Thirty litres of water were collected from each of eight high density great crested newt ponds (Lewis, 2012), at the end of the breeding season on the 23rd of May 2016 and mixed together in a large fiberglass tank to ensure a homogenous starting concentration. Five eDNA samples were collected at this stage to represent a baseline starting detectability and concentration. Ten litres of water were then transferred from the large fiberglass tank to each of the 20 treatment tanks. Opaque plastic lids were added to each treatment tank to prevent rainfall having a dilution effect or the effect of UV on eDNA breakdown. This was considered appropriate in the case of these mesocosms, because of the shallow nature of the water in each tank, UV would have penetrated the majority of the water and had a disproportionate influence compared to a natural pond. eDNA samples were collected from each of the 20 tanks 1, 2, 3, 4, 7, 9, 11, 14, 18 and 22 days after the water had been removed from the ponds.

3.3.2. Environmental covariates

Various environmental covariates were collected during the course of the study. pH, total dissolved solids and electro-conductivity were measured in each tank at the end of the study using electronic "pen type" meters (Hanna® Instruments HI-98312 and AZ® Instrument, 8685 pH Pen) following manufacturer's instructions (Hanna

Instruments, n.d.; AZ Instruments, 2014). It was believed that these would not change considerably over the course of the study and the benefit of monitoring these daily was outweighed by the risk of contamination of target DNA between tanks. Air temperature was logged hourly at the site using Tinitag® Plus2 – TGP-4017 (Gemini Data Loggers, Chichester, UK).

3.3.3. eDNA sample collection protocol

eDNA samples were collected using the precipitation in ethanol approach as developed by Biggs et al. (2015). Six 50 mL centrifuge tubes (Corning, Centristar™ Cap, 430828) containing 33 mL of absolute ethanol and 1.5 mL of 3 M sodium acetate solutions, made up one sample. Using a sterilised disposable plastic pipette, each of the six centrifuge tubes was filled to the 50 mL gradation with water directly from the middle of the water column of the tank without stirring. This provided a total sample volume of approximately 90 mL. Samples were immediately stored at -20 ºC until extraction, this both aided sample preservation as well as the precipitation of DNA out of solution.

3.3.4. Laboratory protocol

eDNA sample extraction was undertaken using a modified Qiagen® DNeasy® Blood and Tissue kit protocol. A sample was removed from the freezer and centrifuged at 11000 RPM (14069g) for 30 minutes at 6 ºC. The supernatant was poured off leaving a pellet containing DNA and other matter that had precipitated out of solution on the side of each tube. The pellet from the first tube was suspended in 360 µL ATL buffer from the Qiagen® DNeasy® Blood and Tissue kit by vortexing for several minutes, the buffer solution containing the re-suspended pellet was then transferred to the second tube and the process repeated until each tube had been sequentially vortexed, and all six pellets suspended in the same solution. The ATL buffer solution was then transferred to a 2 mL microcentrifuge tube, 25 µL of ProK added and

samples incubated at 56 ºC overnight. Extraction continued as per extraction kit manufacturer's protocol, with spin columns eluted twice with 100 µL of warm AE buffer. Periodic extraction negative control samples were run through the course of the project.

Each sample was tested for PCR inhibition using TaqMan® Exogenous Internal Positive Control (IPC) Reagents (Applied Biosystems™), following manufacturer's instructions, with TaqMan® Environmental Master Mix 2.0 (Applied Biosystems™). Samples were identified as inhibited if the IPC failed to amplify or late amplification (amplification outside 1 qPCR cycle from the template negative control samples) was observed within the internal positive control.

qPCR was undertaken on all samples whether inhibited or not following the assay and conditions from Biggs et al. (2015), using *Triturus cristatus* PCR primers TCCBL, TCCBR and hydrolysis probe TCCB developed by Thomsen et al. (2012b). qPCR was conducted using a BioRad Laboratories, CFX Connect™ Real-Time PCR Detection System. qPCR was repeated on each sample eight times. Each qPCR plate contained three standards for quantification, each repeated three times, as acting as positive controls, and three PCR negative controls. qPCR standards were made up of a dilution from great crested newt tissue extract and were quantified using a Qubit® 2.0 with the Qubit® dsDNA high sensitivity assay (Life Technologies™) at concentrations of 12.500 ngµL^{-1}, 1.140 ngµL^{-1} and 0.120 ngµL^{-1}, qPCR R-squared values ranged between 0.994 and 0.999, with a mean efficiency of 85.5%. A replicate was deemed to be positive if an exponential growth phase was observed during qPCR. The median concentration of the eight qPCR replicates was utilized as the concentration for a sample in analysis. During qPCR all negative control samples showed no deviation from the baseline, and were therefore clear negatives. Limits of detection and quantification are presented in Chapter 2.

Due to high levels of inhibition within the topsoil treatment group (see results), all topsoil samples were treated as potentially inhibited. A 1 in 10 dilution using ddH_2O was then undertaken on inhibited samples, to attempt to remove inhibitors and improve detection (Jane et al., 2015; Al-Soud et al., 2000; Volkmann et al., 2004; Sigsgaard et al., 2015; Thornton & Passen, 2004; Palomares et al., 2002; Alaeddini, 2012). The diluted samples were then re-run using the internal positive control and qPCR protocol outlined above (Goldberg et al., 2011; Biggs et al., 2014b; Pilliod et al., 2013). Trials using Bovine Serum Albumin (BSA) and lower dilution levels were undertaken but failed to remove inhibitors sufficiently (data not presented).

3.3.5. Analysis

As eDNA concentrations often fall below the limit of quantification achieved by qPCR, the use of eDNA concentration within the analysis would only be of limited value. However, occupancy modelling can be used to generate the probability of detection, independently of the concentration within a sample. Single season occupancy models were constructed based on single eDNA samples (representing 'sites' in traditional occupancy modelling) and repeated qPCR runs (representing observations). Models were constructed using R version 3.3.2 (R Development Core Team, 2016) with package Unmarked version 0.11-0 (Fiske & Chandler, 2011), to observe the influences on detection probability across the study. Models were constructed using the *occu* function, with variable detection but constant occupancy. Site covariates, included in the analysis were substrate type, days since removal of target species and tank pH. Model selection was undertaken utilising the inbuilt model selection option within the *Unmarked* package. Models were ranked using the Akaike Information Criterion (AIC) and were weighted to indicate relative support of a model. Models with ΔAIC < 2 had strong support while models with a ΔAIC of >2 were considered to have less support (Burnham & Anderson, 2003). Detection probabilities

were then generated, using the predict function within the unmarked package and the model containing day and substrate variable detection.

We observed the rate at which the detection probability changed each day (Δp/day) by taking the difference between predicted detection probabilities from one day to the next, for each of the sediment types. We examined whether maximum, minimum and mean external temperature influenced detection probability or Δp/day, with generalised linear models (GLM) using R version 3.3.2 (R Development Core Team, 2016).

3.3.6. Ethical assessment

The experimental procedure was approved by the University of Kent, School of Anthropology and Conservation, Research and Ethics Committee. All sampling was undertaken from water and no animals were used as part of this work. Positive control samples within PCR were set up from DNA extracts from a long deceased great crested newt held under licence from Natural England licence number 2015-7591-SCI-SCI-1.

3.4. Results

3.4.1. Degradation

The commercially available sediments all tested negative for great crested newt DNA. In the clay, sand and control treatments eDNA concentration fell from 0.00108 ngµL^{-1}, the mean concentration found on day 0, to the limit of quantification of 0.00005 ngµL^{-1} by day 4, a decrease of over 95%. In the topsoil treatment, eDNA concentration fell faster, reaching the limit of quantification between days 2 and 3. Beyond day 4 most samples fell below the limit of quantification for qPCR and so no accurate analysis can be undertaken with regard to eDNA concentrations. Samples were first observed as negative in the topsoil treatment group on day 7, in clay on day 14, and in sand and the control on day 18.

3.4.2. Detection probability

Models were included to predict what was influencing detection probability. The model with most support included detection based on number of days since the species was present (estimate = -0.320; z = -20.56; SE = 0.0155; p < 0.0001), pH (estimate = -0.171; z = -1.66; SE = 0.1030; p = 0.0974) and substrate type with constant occupancy (Table 3.1). Although pH was included in the top model, it was not found to be significant. There was a significant reduction in detection in the topsoil treatment (estimate = -0.850; z = -3.85; SE = 0.2207; p = 0.000116), compared to the control group. However, no significant difference in detectability was found between the control group and both the clay treatment (estimate = -0.374; z = -1.83; SE = 0.2045; p = 0.0673) and the sand treatment (estimate = -0.003; z = -0.014; SE = 0.2053; p = 0.989). All covariates included within all models with a ΔAIC of < 2 with the exception of substrate type and day were found not to be significant.

Table 3.1 - Influences on detection probability model selection

Model	nPars	AIC	ΔAIC	AIC weight	Cumulative weight
Constant occupancy, detection variable by day, substrate and pH	7	1244.73	0.00	0.59	0.59
Constant occupancy, detection variable by day and substrate	6	1245.48	0.75	0.41	1.00
Constant occupancy, detection variable by day	3	1259.15	14.43	<0.01	1.00

The models with most support based on AIC criterion and AIC model selection. Top three models and all models with a ΔAIC of <2 presented. All models contain variable detection rates but constant occupancy. Days since the target species was in contact with the water, pH within each mesocosm and substrate treatment group were the only covariates found to be in the three models with most AIC support. nPars represents the number of parameters in the model.

The model with the second highest support included constant occupancy, but variable detection based on substrate type and day was used to predict detection probability in the different substrate types and across the study (Figure 3.1). The model with the most support was not used because the pH covariate was insignificant and would therefore have only confused the predictions. Detection probability (*p*), based on replicated PCR runs, was initially very high with sand and control treatment groups with $p > 0.96$, and the clay treatment group $p = 0.94$. The topsoil treatment group showed a reduced starting detection probability at $p = 0.91$ (Figure 3.1). Detection fell slowly for the first few days, and by day five detection probability had fallen to $p = 0.87$ in the control and sand treatments, $p = 0.83$ for clay and $p = 0.75$ for topsoil treatment. Detection rate then fell more sharply from $p = 0.58$ for the control and sand treatments by day 10 and $p = 0.22$ by day 15: this was more pronounced in the clay and topsoil treatments where detection fell more rapidly to $p = 0.49$ and $p = 0.37$ respectively by day 10 and $p = 0.16$ and $p = 0.10$ respectively by day 15. By day 20 detection probability had fallen to 0.05 or below in all treatments (Figure 3.1).

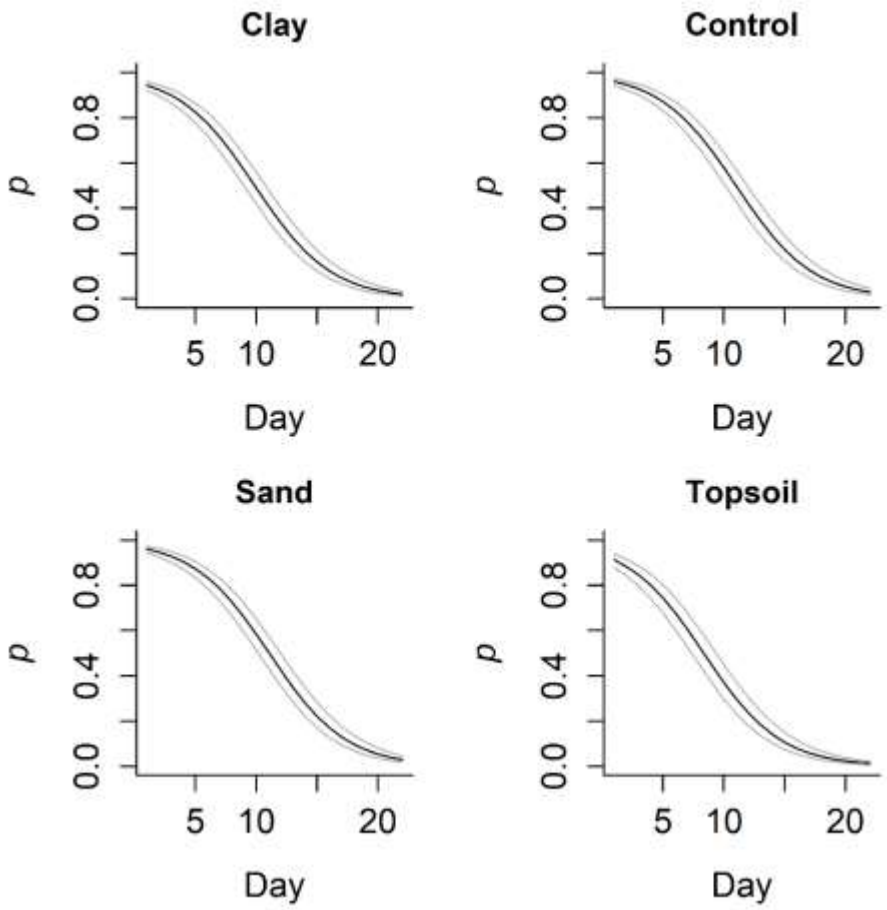

Figure 3.1 – Decline in detection probability over time. Decline in eDNA detection probability (*p*) over time, using eight qPCR runs per sampling occasion, following the removal of the target species from the water, with different substrate types. Light grey lines show 95% confidence limits.

Samples collected from tanks with a topsoil substrate were more likely to contain PCR inhibitors with 70% (35/50) of the samples showing signs of inhibition, compared to 2% (1/50) in the clay treatment group and no samples from the sand or control groups. Samples treated for the removal of inhibitors were found to all be free from inhibitors; however, a drastic reduction in detection probability was observed, from $p = 0.91$ in the original samples to $p = 0.39$ in the same samples when diluted to remove inhibitors. As a result the diluted data were discarded and analysis undertaken on the inhibited but undiluted data.

The change in detection probability per day (Δ*p/day;* Figure 3.2), is initially low for all treatment groups, increasing towards the middle of the study before reducing in the latter stages. No difference was observed between sand and the control treatment groups, initially at approximately 0.015 Δ*p/*day, increasing to a peak of 0.08 Δ*p/*day by day 11, before falling again until the end of the study. Rate of detection initially decreased more in the clay and topsoil treatments (0.02 and 0.03 Δ*p/*day respectively) than in the control treatment group. Both reached a peak rate of change of 0.08 Δ*p/*day on days 9 (clay) and 8 (topsoil). The rate of change for sand and topsoil then started to reduce earlier in the study than the control or clay treatment groups, and continued to reduce through the rest of the experiment.

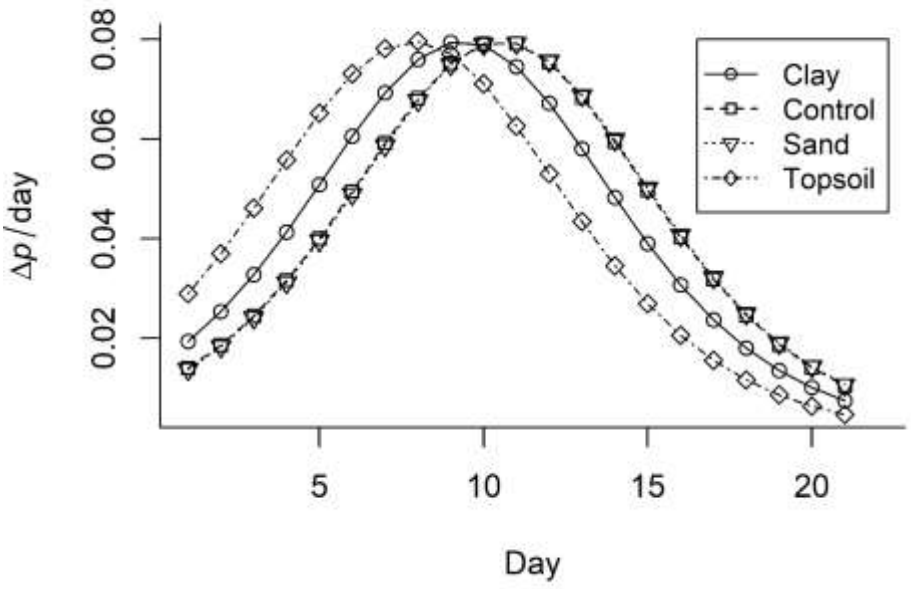

Figure 3.2 - Change in detection probability per day. The rate of change in eDNA detection probability (p) each day in mesocosms with four sediment types.

A general linear model was used to assess whether the external temperature influenced the rate of change in detection. A negative relationship between maximum

air temperature and change in detection probability was identified, with greater rate of change at lower temperatures (Figure 3.S1). This was found to be significant for all four sediment types (control: estimate = -0.0024, SE = 0.0007, t-value = -3.325, p-value = 0.0038; sand: estimate = -0.0024, SE = 0.0007, t-value = -3.309, p-value = 0.0039; clay: estimate = -0.0023, SE = 0.0007, t-value = -3.433, p-value = 0.0030; topsoil: estimate = -0.0018, SE = 0.0006, t-value = -3.862, p-value = 0.0104). Mean daily temperature was not found to be significant for any of the treatment groups, and minimum temperature was only found to have a significant (positive) influence on the topsoil treatment group (estimate = 0.0049, SE = 0.0020, t-value = 2.411, p-value = 0.0268). This is surprising given that increases in temperature are linked with increases in DNA degradation rate and this is likely to be a coincidental artefact of the weather during the course of the experiment rather than an overriding influence on the change in detection probability.

3.5. Discussion

If a species vacates a waterbody, detection of that species remains possible using eDNA. Detection when a waterbody is no longer occupied is a distinct advantage over traditional survey methods. We have shown that the probability of detecting a species decreases with time, following its removal; however, the rate at which the probability of detecting the species decreases is not constant. We have not only shown that detection probability of eDNA is dependent on time since the organism was present, but we also show that the type of sediment influences detectability and the rate at which detectability decreases.

The initial detection probability for eDNA was very high - between 91% and 95% - the exception being where samples had been diluted to remove inhibitors. This high detection probability may have been because the water was sourced from small ponds with a very high target species density, and collected at the end of the breeding season when eDNA concentration is high (see Chapter 5). As a result, the amount of

DNA within the water was likely to be higher than that found in more typical, natural ponds or at other times of year (see Chapter 5). There is a discrepancy between the results of our study and those of Thomsen et al. (2012b) in which eDNA was only detected for nine days - compared to 22 days in our study. Both studies targeted the same sequence in the cytochrome b gene and used the same target species. This discrepancy may be down to differences in both initial concentration of target DNA and collection methods used, with our study collecting a sample volume six times greater than Thomsen et al. (2012b).

The reduction in detection over time is likely due to the removal of target DNA through both degradation (Barnes et al., 2014) and vertical transport and incorporation into the sediment (Turner et al., 2014a). The rate of change in detection was initially slow, an increase in the middle part of the study was observed peaking at approximately 0.08 Δp/day in all treatment groups, with a reduced rate at the end. This pattern was observed in all sediment types. Reduced rate of change towards the end of the study may represent a slowing in the rate of degradation at lower eDNA concentrations, as at lower concentrations the chance of DNA molecules being broken down by DNase enzymes is reduced (Levy-Booth et al., 2007).

Detectability and the rate of change in detectability varied between the sediment types. It is likely that the type and quantity of PCR inhibitors released into the water differs between sediment types (Albers et al., 2013; Schrader et al., 2012). Soil structure may also influence the capacity for DNA to become incorporated into the different sediments (Levy-Booth et al., 2007). No differences were observed in detectability or rate of change in detectability between the sand and control groups. However, we observed both a lower starting probability of detection and an increase in the initial rate of fall in detectability within the topsoil compared to the control treatment group. There was a tendency for a reduction in probability of detection in

the clay treatment group compared to the control and sand treatment groups, although this was not significant.

PCR inhibitors interact either with DNA or DNA polymerase and can result in an increase in the number of cycles required to observe amplification of the target DNA over uninhibited samples; additionally reductions in the number of qPCR repeats which amplify, inconsistent amplification of the qPCR repeats or complete failure to amplify any target DNA may be observed if PCR inhibitors are present (Alaeddini, 2012; Schrader et al., 2012). PCR inhibitors are common within environmental samples and strong seasonality in inhibition has been observed and linked with the accumulation and degradation of leaf litter (Jane et al., 2015), consistent with high organic content of topsoil. PCR inhibition led to a drop in detection probability for the topsoil treatment group relative to the other sample groups, and lower extract concentration was observed in qPCR due to late amplification.

Initially, total dissolved solids (TDS) within each tank was included in in the analysis. However, TDS value was found to be significantly dependent on substrate type (Figure 3.S2) and so the two factors are not independent. TDS may therefore increase in suspended solids within the water column, rather than within the sediment itself. This may be responsible for the difference in PCR inhibition seen between the treatment groups: 0% of samples inhibited in control and sand, 2% of samples inhibited in the clay group and over 70% of samples inhibited in the topsoil treatment group. TDS within the topsoil treatment group was by far the highest and it is likely that PCR inhibitors within the dissolved solids such as humic acid (Wilson, 1997; Alaeddini, 2012; Schrader et al., 2012) were the cause of the reduction in detection probability over the other treatment groups.

The dilution of the topsoil samples to remove inhibitors led to a 52% reduction in detection probability and therefore an increase in false negative results, as the DNA was diluted to undetectable levels (Juen & Traugott, 2006; Alaeddini, 2012; Jane et

al., 2015). We therefore argue that dilution approaches for the removal of inhibitors from eDNA samples compromises detection probability: other methods which do not result in a dilution of target DNA should therefore be explored.

In addition to inhibition, the rate at which DNA is incorporated into the sediment may cause the availability of eDNA within the water column to vary (Turner et al., 2014a). eDNA can become incorporated into substrates and absorbed onto minerals, binding to both humic compounds and soil minerals (Levy-Booth et al., 2007). Little difference was identified in detection between the control group and the sand treatment group. Sand has a very large particle size, which results in a lower surface area and fewer binding sites than substrates with smaller particle size such as clay (Levy-Booth et al., 2007). As a result, more DNA would be expected to remain detectable in water with a sandy substrate, than with a clay or topsoil substrate. Humic substances likely to be found within the organic topsoil also provide key binding sites for DNA (Levy-Booth et al., 2007); this may reduce the availability of the target DNA for survey. However, it is likely that the greatly reduced detection probability in the topsoil treatment group results from a combination of an increase in PCR inhibitors (Alaeddini, 2012), as well as removal of available target DNA from the water column.

eDNA research is still an evolving discipline. Unlike more widely recognised survey methods for freshwater species, the influences on and limitations of detection are still being identified. Our findings have important implications for how eDNA results are analysed and interpreted. Although detection of eDNA does not necessarily correspond to the concurrent presence of the species, the chance of detecting the species after it has vacated a pond reduces rapidly, and after three weeks can be as low as just 3.9%, as observed in our control group. To maximise the chance of detection, it is therefore advisable to collect samples when the target species is likely to be present, to minimise the chance of false absences. Pond specific characteristics such as the sediment also influence the probability of detecting the target organism,

either by increasing PCR inhibition or through other mechanisms. It is therefore important to recognise when planning or interpreting the results from an eDNA study, that sediment has an influence of the efficacy of the survey method, and ponds with organic sediment types - or sediments that become suspended easily - can be a source of false negative results.

3.6. Acknowledgments

We would like to thank Galatea Swart and Debbie Fogell for assistance within the laboratory. We would also like to thank Robert Ward for assistance with occupancy modelling and Izabela Barata for assistance with occupancy modelling and comments on an early draft of the text. We would also like to thank Jim Foster and John Wilkinson from Amphibian and Reptile Conservation and Jeremy Biggs from Freshwater Habitats Trust for advice on the project. We would also like to thank two anonymous reviews for their comments on an earlier version of the manuscript.

3.7. Chapter 3 – Supplementary Information

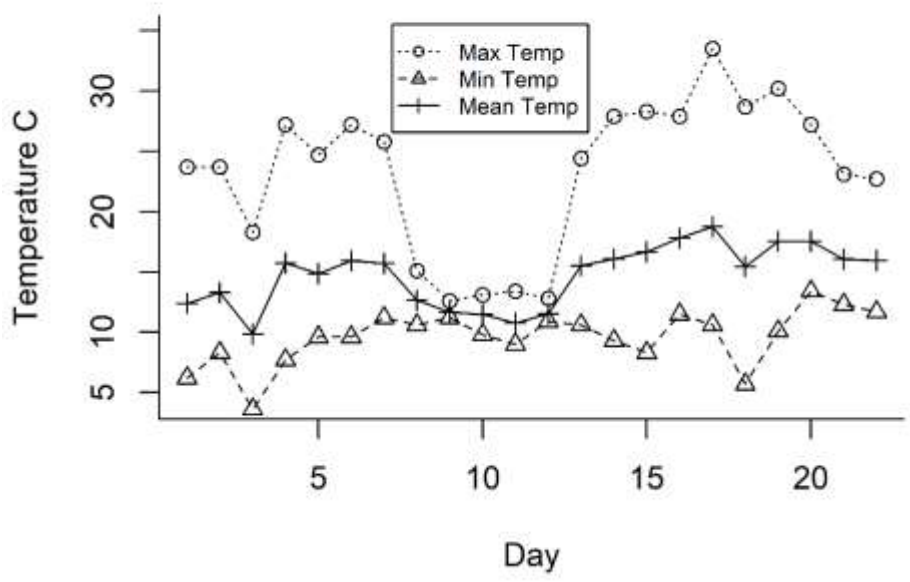

Figure 3.S1 – Mean daily air temperatures. Mean, maximum and minimum air temperature over the course of the study. Day one temperatures relate to the 24 hours leading up to the sampling time on the 24th of May 2016.

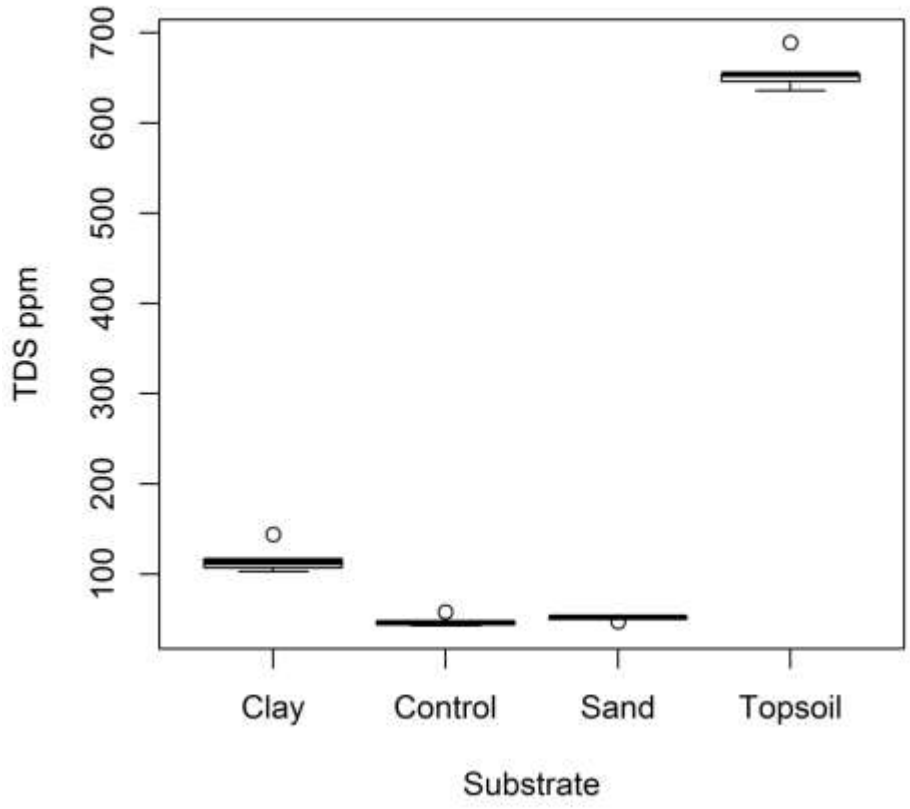

Figure 3.S2 – Total dissolved solids. Difference in total dissolved solid (TDS) levels within the mesocosms of different substrate types. Showing the median values with interquartile ranges. An analysis of variance yielded significant variation between sediment type and the TDS loading ($F(3,16)=2464$; $p<0.0001$). A post-hoc Tukey test showed no significant difference between the control group and sand ($p=0.98$) but all other pairs had highly significant differences ($p<0.0001$).

Chapter 4 – Seasonal variation in environmental DNA detection in sediment and water samples

This chapter has been published in the peer reviewed journal *PLoS ONE*:

Buxton, A.S., Groombridge, J.J. & Griffiths, R.A. (2018). Seasonal variation in environmental DNA detection in sediment and water samples PLoS ONE. 13. e019173

4.1. Abstract

The use of aquatic environmental DNA (eDNA) to detect the presence of species depends on the seasonal activity of the species in the sampled habitat. eDNA may persist in sediments for longer than it does in water, and analysing sediment could potentially extend the seasonal window for species assessment. Using the great crested newt as a model, we compare how detection probability changes across the seasons in eDNA samples collected from both pond water and pond sediments. Detection of both aquatic and sedimentary eDNA varied through the year, peaking in the summer (July), with its lowest point in the winter (January): in all seasons, detection probability of eDNA from water exceeded that from sediment. Detection probability of eDNA also varied between study areas, and according to great crested newt habitat suitability and sediment type. As aquatic and sedimentary eDNA show the same seasonal fluctuations, the patterns observed in both sample types likely reflect current or recent presence of the target species. However, given the low detection probabilities found in the autumn and winter we would not recommend using either aquatic or sedimentary eDNA for year-round sampling without further refinement and testing of the methods.

4.2. Introduction

The advent of aquatic environmental DNA (eDNA) protocols for surveying aquatic organisms has revolutionised the assessment of both protected and invasive species. Extra-organismal DNA is collected as part of a sample of environmental material and isolated in a laboratory to identify the recent presence of a species (Lodge et al., 2012; Jane et al., 2015; Taberlet et al., 2012; Barnes et al., 2014). However, as with all survey methods, sampling aquatic eDNA is limited to time periods when the species is active and in its aquatic phase. DNA bound to sediments has been found to persist much longer (Turner et al., 2015), and therefore may be an appropriate source of DNA to allow the detection of a species outside its active period.

Animals constantly shed DNA into their environment through the expulsion of waste products, skin secretions, sloughing of skin cells, release of reproductive cells (eggs and sperm), through the decay of dead individuals and through many other processes (Lydolph et al., 2005; Haile et al., 2009; Waits & Paetkau, 2005). This organic material becomes suspended in the water column (Jane et al., 2015). The persistence of aquatic eDNA depends on a range of factors and is highly variable (Thomsen et al., 2012a; Strickler et al., 2015; Chapter 3). eDNA is broken down through both biotic and abiotic processes (Pilliod et al., 2014; Levy-Booth et al., 2007; Barnes et al., 2014; Ravanat et al., 2001; Piaggio et al., 2014; Strickler et al., 2015; Turner et al., 2014a). eDNA in marine or lotic environments can be transported out of the system it was released in and diluted to undetectable levels (Pilliod et al., 2014; Thomsen et al., 2012a). Additionally, eDNA becomes undetectable by settling out of the suspension through vertical transport and incorporation into sediment (Turner et al., 2015). This process may result in progressive accumulation of eDNA in the sediment (Corinaldesi et al., 2011). The rate at which particles settle out and therefore the amount of eDNA suspended within the water column is related to particle size (Maggi, 2013). Turner et al., (2014a) found that although the highest amounts of total eDNA

pass through 0.2 µm filters, 71% of targeted carp eDNA was trapped by 1 µm filter membranes. Particles greater than 1 µm therefore settle out of natural waters (Isao et al., 1990) and accumulate in the sediment (Turner et al., 2014a). Consequently, sediment may be a valuable but as yet largely untested source of eDNA.

Within the sediment, extracellular DNA can bind to the mineral particles and humic compounds (Greaves & Wilson, 1969; Lorenz & Wackernagel, 1987; Crecchio & Stotzky, 1998), with the capacity varying with sediment characteristics (Levy-Booth et al., 2007; Saeki & Kunito, 2010). Long-term persistence of the DNA molecules is therefore predominantly due to bound DNA molecules being protected from degradation (Crecchio & Stotzky, 1998; Cai et al., 2006; Romanowski et al., 1991; Paget et al., 1992; Recorbet et al., 1993). Consequently, DNA has the potential to persist in the sediment for a short time or for thousands of years (Hofreiter et al., 2003; Haile et al., 2007, 2009; Boessenkool et al., 2014; Anderson-Carpenter et al., 2011; Matisoo-Smith et al., 2008; Parducci et al., 2013; Jørgensen et al., 2012a, 2012b) depending on these conditions.

The isolation of DNA from sediment was developed with microbial DNA (Steffan et al., 1988). The field of ancient DNA has subsequently emerged using the same principles to isolate DNA from terrestrial and aquatic sediments (Matisoo-Smith et al., 2008; Anderson-Carpenter et al., 2011; Thomsen & Willerslev, 2015; Hofreiter et al., 2003). However, techniques to isolate contemporary DNA from soils or aquatic sediments have emerged only relatively recently. The potentially extensive persistence of DNA bound to sediments is very valuable for analysis of ancient DNA, but it may be difficult to identify when the target species was last present. In experimental conditions, big headed Asian carp (*Hypophthalmichthys* spp.) eDNA has been found to persist in sediments for longer than four months and to be more concentrated in the sediment than the water column (Turner et al., 2015).

With either direct field observation or aquatic eDNA surveys, the short survey season available for semi-aquatic species such as amphibians can reduce the application of the method. For protected species, missing the effective survey window can lead to false negatives and poorly informed conservation decision-making, which has potential economic implications. Reliable year-round detection methods that can detect the recent presence of a species therefore have great benefits. Year-round detection using aquatic eDNA has been proposed with great crested newts (*Triturus cristatus;* Rees et al., 2017), a semi-aquatic amphibian with which eDNA has become rapidly adopted as a survey protocol (Biggs et al., 2015; Rees et al., 2014a, 2017). Simple positive or negative results from a single eDNA sample are being used for distribution assessments of the species and to inform mitigation of development impacts on newt habitat (Bormpoudakis et al., 2016). However, the reliability of this in different seasons has not been assessed. We use great crested newts as a model species to examine the reliability of eDNA sampling in different seasons. In addition we develop a method to extract eDNA from pond sediments and assess how the probability of detection changes seasonally and how it compares to aquatic eDNA samples. We discuss whether eDNA from pond sediment could be used to allow year-round detection for a semi-aquatic species.

4.3. Methods

4.3.1. Study areas

Eighteen ponds in three study areas in south and south-east England all known to support great crested newts were chosen. These comprised eight ponds at Little Wittenham in Oxfordshire, a designated Special Area of Conservation (SAC) for great crested newts; and two study areas in Essex, both created as mitigation habitat for local development projects containing translocated individuals, one at Wickford (six ponds) and one at Stanford-le-Hope (four ponds). An additional pond located in an isolated position inaccessible to great crested newt colonisation on an island in the

centre of Canterbury City, was used as a negative control and a second negative control pond which had yet to establish vegetation and had no record of great crested newts was located near the Stanford-le-Hope population.

4.3.2. Visual surveys and habitat suitability index

A combination of torch-light surveys, aquatic funnel traps and visual searches for eggs were used to confirm the presence of great crested newts in each pond (English Nature, 2001). A well-established Habitat Suitability Index (HSI) assessment exists for great crested newts and was calculated for each pond (Oldham et al., 2000). The HSI is a measure of the suitability of a pond and associated habitat for the target species (Oldham et al., 2000). Ten habitat variables are recorded in the field, comprising geographic location (SI_1), pond area (SI_2), frequency of pond drying (SI_3), water quality (SI_4), pond shading (SI_5), waterfowl presence (SI_6), fish presence (SI_7), pond density in the immediate landscape (SI_8), terrestrial habitat quality (SI_9) and macrophyte cover (SI_{10}), and are each scored between 0.01 and 1.0. The final HSI index is calculated as the geometric mean of the variables using the equation (Oldham et al., 2000):

$$HSI = (SI_1 * SI_2 * SI_3 * SI_4 * SI_5 * SI_6 * SI_7 * SI_8 * SI_9 * SI_{10})^{1/10}$$

The index gives a broad indication of the quality of the habitat for great crested newts on a numerical scale of 0 (unsuitable habitat) to 1 (optimal habitat; Unglaub et al., 2015).

4.3.3. Sample collection

All equipment was sterilised using a 10% bleach solution and/or UV light. Before sampling the sediment, an aquatic eDNA sample was collected from the undisturbed water column. The aquatic eDNA sampling followed a precipitation in ethanol method described in Biggs et al. (2015), and replicated the commonly used protocol for commercial great crested newt eDNA sampling in the UK. To allow a single

representative sample of sediment to be collected from a pond, ten subsamples were collected from the accessible pond perimeter at evenly spaced intervals and combined. Using shoulder length disposable gloves to avoid contamination, a 60 mL scoop of the surface of the pond sediment was collected in a polypropylene collection pot from the ten sampling locations. Any pond water collected as part of the sampling process was then drained off and the sediment transferred to a 1000 mL wide-mouth plastic bottle. 250 mL of double distilled water was then added to the sample, and the bottle shaken vigorously for 60 seconds to suspend the sediment within the distilled water. Fifteen mL of this solution was immediately subsampled and added to a 50 mL centrifuge tube containing 33 mL of absolute ethanol and 1.5 mL of 3 M sodium acetate solution to preserve the sample. The remainder of the distilled water sediment suspension was retained for sediment texture analysis.

Both aquatic and sedimentary eDNA samples were transported on the day of collection to the laboratory at the University of Kent and stored at -20 ˚C until extraction. Samples were collected from ponds at approximately three-monthly intervals in April, July and October 2016 and January 2017 to cover the four seasons. If ponds were dry and an aquatic eDNA sample could not be collected then aquatic eDNA was considered to be negative in the analysis.

4.3.4. eDNA extraction

Extraction of the aquatic eDNA sample followed the same modified Qiagen® DNeasy® blood and tissue extraction kit protocol used by Biggs et al. (2015). Extraction of sedimentary eDNA samples followed modified Qiagen® QIAamp® DNA Stool Mini Kit protocol (Chaves et al., 2010). The 50 mL centrifuge tube containing the ethanol preservative with the suspension of pond sediment and distilled water was removed from the freezer and shaken vigorously to homogenise the sample. The sample was then centrifuged at 8500 rpm for 30 minutes to separate the sediment from the preservative, the supernatant was carefully poured off and discarded.

Sediment was removed from the centrifuge tube, placed on a sterile Petri-dish, and then stirred to mix once again. Half of one milliliter of sediment was then transferred to a 2 mL micro-centrifuge tube. Extraction continued as per Chaves et al. (2010) and is outlined in supporting information S4.1 methods. Both aquatic and sedimentary eDNA extracts were stored at -20 ˚C until qPCR could be undertaken.

4.3.5. eDNA qPCR and IPC

Quantitative real-time PCR was undertaken on all samples following the assay and PCR conditions in Biggs et al. (2015), with PCR primers TCCBL and TCCBR as well as hydrolysis probe TCCB from Thomsen et al. (2012b). Each sample was repeated eight times and run in parallel with both positive and negative controls. All samples were checked for PCR inhibition using TaqMan® Exogenous Internal Positive Control Reagents (Applied Biosystems™), following manufacturer's instructions, with TaqMan® Environmental Master Mix 2.0 (Applied Biosystems™). Samples were identified as inhibited if the IPC failed to amplify or late amplification (amplification outside 1 qPCR cycle from the qPCR negative control samples) was observed within the internal positive control.

4.3.6. Sediment texture analysis

Sediment texture can be categorised through the proportion of sand, silt and clay found within it. Following the collection of the sediment eDNA sample, the remaining homogenised mixture of distilled water and collected sediment was saved. This mixture was allowed to dry completely before the proportions of sand, silt and clay were analysed using a LaMottle Company soil texture test kit following the manufacturer's instructions (LaMotte Company, 2017). This procedure produced a percentage of each of the components within the sediment for each of the four visits, of which the mean was taken for the analysis. In addition, this allowed the sediment texture to be categorised using the United States Department of Agriculture (USDA) soil texture calculator (United States Department of Agriculture, n.d.).

4.3.7. Statistical analysis

The concentration of DNA recovered was consistently below the limit of quantification (Tréguier et al., 2014; Chapter 2), and so could not be accurately measured. However, single season occupancy models use repeated observations with detection and non-detection data to estimate the probability of detecting a species (Mackenzie & Kendall, 2002; MacKenzie et al., 2003; Tyre et al., 2003). Occupancy modelling has been widely used with eDNA (Schmidt et al., 2013; Hunter et al., 2015; Schmelzle & Kinziger, 2016; Vörös et al., 2017) to estimate detection probability, with repeated observations represented by replication of qPCR runs. This process allowed detection probability to be estimated, with each sample representing a "site" and each qPCR run considered an independent observation as in a traditional occupancy analysis. Models were fitted in R version 3.4.1 (R Development Core Team, 2016) with package Unmarked version 0.12-2 (Fiske & Chandler, 2011), to identify differences in detection probability. Models were fitted using the `occu` function, with covariates of detection, but with a constant occupancy (i.e., no covariates fitted for occupancy, only for detectability). Site covariates included in the models were the time of year, the type of samples (aquatic or sediment), study area, the pond sediment texture and the HSI score. The default model selection option within package Unmarked was utilised, ranking models based on Akaike Information Criterion (AIC) and weighted to indicate relative model support. AIC model selection was corroborated using package AICcmodavg version 2.1.1 (Mazerolle, 2017) to generate Bayesian Information Criterion (BIC) to confirm relative model support. Models with strong support were identified having a ΔAIC or ΔBIC ≤ 2 with models with a ΔAIC or ΔBIC of >2 but ≤ 7 were considered to have some support (Burnham & Anderson, 2003; Marchetti et al., 2004). AIC and BIC importance weights for the covariates were generated as measures of covariate importance, by summing the respective weights for each model that contains that covariate (Marchetti et al., 2004; Johnson et al.,

2011). Covariates were classed as strongly supported by our models if they were significant in all strongly supported models (ΔAIC ≤ 2) and had a cumulative AIC or BIC weight of > 0.75 (Marchetti et al., 2004). Covariates were considered to be somewhat supported if they were significant in any of the strongly supported models regardless of cumulative AIC or BIC importance weight (Marchetti et al., 2004). Goodness of fit, using the chi-square statistic and c-hat was performed using package AICcmodavg version 2.1.1 (Mazerolle, 2017), and the `mb.gof.test` function, with a bootstrap value of 1000, for all somewhat or strongly supported models. The model with greatest support was used with the `predict` function within the Unmarked package to generate predicted detection probabilities under different covariate combinations.

4.3.8. Ethical assessment

The experimental procedure was approved by the University of Kent, School of Anthropology and Conservation, Research and Ethics Committee. Surveys for great crested newts using traditional methodologies were undertaken following best practice guidelines by experienced surveyors and under licence from Natural England (Licence number 2015-16607-CLS-CLS). All eDNA sampling was undertaken from water or sediment and no animals were disturbed. PCR Positive control samples were set up from DNA extracts from a long deceased great crested newt held under licence from Natural England (licence number 2015-7591-SCI-SCI-1).

4.4. Results

Using the visual survey methods great crested newts were confirmed from all ponds except the two negative control ponds. This result was corroborated with eDNA samples, with no samples from the two negative control ponds found to be positive.

With the exception of the control ponds, each pond was positive using either sediment or water eDNA samples on at least one occasion. The mean number of qPCR

replicates amplifying out of a possible eight for water in spring was 5.67 (standard deviation (SD) = 3.24), which compared to 1.83 (SD = 2.60) for sediment; this increased in the summer to 6.22 (SD = 3.42) for water and 3.28 (SD=3.34) for sediment. A reduction was seen in autumn, 2.11(SD = 2.70) for water and 1.00 (SD = 1.75) for sediment, reducing further into the winter 0.33 (SD = 0.59) for water and 0.78 (SD = 1.06) for sediment. Only one sample from each sediment and water samples showed signs of inhibition. We constructed models to identify what was influencing the differences in detection probability.

The model with the greatest AIC and BIC support (ΔAIC to the second model = 4.95; ΔBIC to the second model = 2.77; Table 4.1), for the influences on detection probability included detection based on the season, study area, sediment texture and HSI score, as well as whether the sample was water or sediment. No other models were found to have substantial support (ΔAIC or ΔBIC $\leq$ 2), although three additional models were shown to have some support (ΔAIC or ΔBIC $\leq$ 7; Table 4.1).

Table 4.1 - Influences on detection probability model selection

Model		nPars	AIC	ΔAIC	AIC weight	AIC Cumulative weight	BIC	ΔBIC	BIC weight	BIC Cumulative weight	GOF - χ^2	GOF – p-value	GOF-c-hat
Occupancy	**Detection**												
Constant	Season, Sample Type, Study Area, Texture, HSI Score	13	827.19	0.00	0.90	0.90	841.37	0.00	0.75	0.75	183.9754	1	0.71
Constant	Season, Texture, Sample Type, HSI Score	11	832.14	4.95	0.075	0.97	844.14	2.77	0.19	0.93	188.8944	1	0.73
Constant	Season, Sample Type, Study Area, Texture	12	834.96	7.77	0.018	0.99	848.05	6.68	0.03	1.00	185.9396	1	0.72
Constant	Season, Texture, Sample Type	10	836.23	9.05	0.0097	1.00	847.14	5.77	0.04	0.97	188.4366	0.998	0.73

Occupancy models with most support based on AIC and BIC criteria and ordered with AIC model selection. The six most supported models through both AIC and BIC as well as all models with a ΔAIC or ΔBIC of < 10 presented. All models contain variable detection rates but constant occupancy. Goodness of fit (GOF) χ^2, p-value and c-hat also shown. nPars represents the number of parameters in the model.

Within the model of greatest support, samples from water were found to have a significantly greater detection probability of eDNA than samples from sediment (SE = 0.228; z = 7.59; p < 0.0001). Detection of eDNA was significantly increased in samples collected in summer compared to those taken in the spring (SE = 0.264; z = 3.00; p = 0.003), but a significant reduction was seen between spring and autumn (SE = 0.314; z = -5.19; p < 0.0001) as well as between spring and winter (SE = 0.359; z = -8.07; p < 0.0001; Figure 4.1). Significant differences were also identified between the study areas with Little Wittenham having greater detection probability of eDNA than the two study areas in Essex, Stanford-le-Hope (SE = 0.300; z = -2.83; p = 0.005) and Wickford (SE = 0.327; z = -2.04; p = 0.041). Detection probability was also positively related to the HSI (SE = 1.026; z = 3.09; p = 0.002; Figure 4.2). eDNA in clay was found to have a significantly greater detection probability than in clay loam (SE = 0.618; z = -5.02; p < 0.0001) and sandy clay loam (SE = 0.341; z = -2.97; p = 0.003). However, eDNA in clay was found to have a lower detection probability than in sandy clay (SE = 0.483; z = 3.93; p < 0.0001), and no significant difference was found between clay and sandy loam substrates (SE = 0.471; z = -0.22; p = 0.828; Figure 4.3).

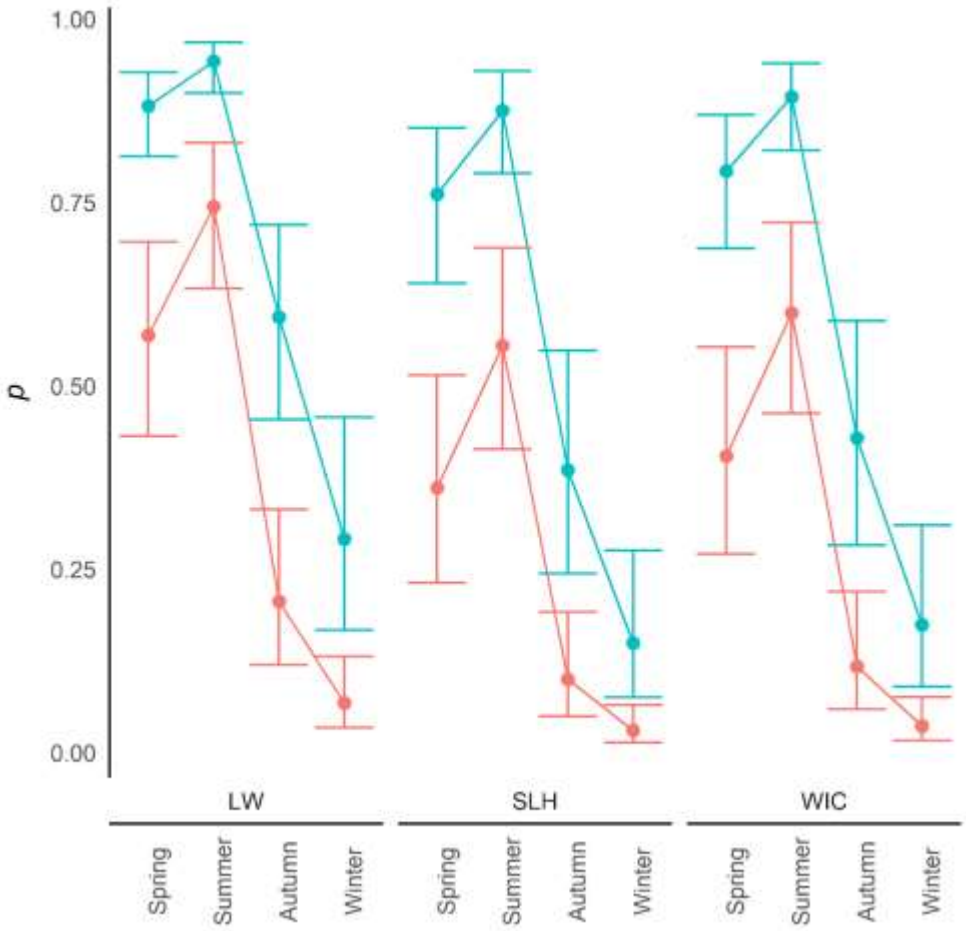

Figure 4.1 - Seasonal variation in detection probability (*p*) between water samples (Blue) and sediment samples (Red) across the seasons, in the different study areas (LW – Little Wittenham; SLH – Stanford-le-Hope; WIC – Wickford), with 95% confidence intervals. These results are based on a clay substrate and an HSI of 0.65 (a score considered mid-range for great crested newt occupancy). Comparisons with ponds in other HSI categories are shown in Figure 4.S1.

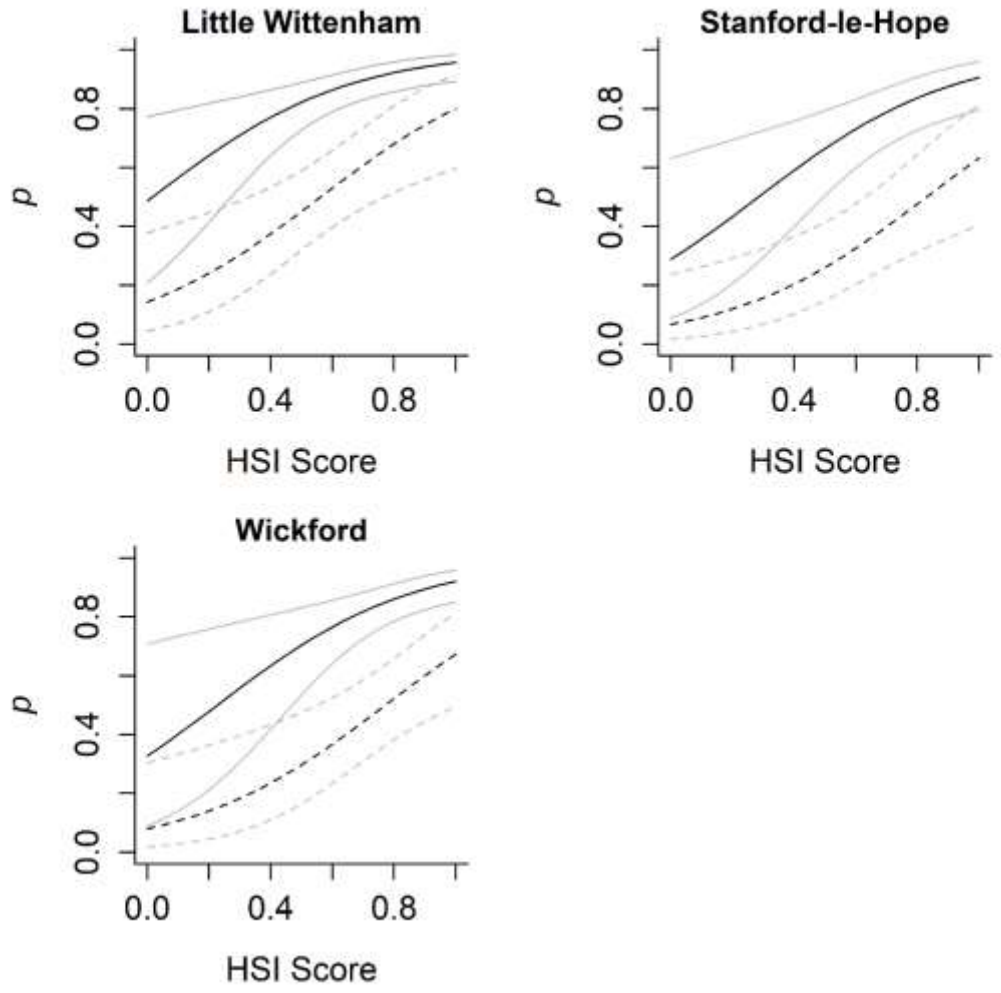

Figure 4.2 - Habitat suitability and detection probability. Variation in detection probability (*p*) between water samples (solid line) and sediment samples (dotted line) in relation to HSI score at three study areas. 95% confidence intervals in light colours. These results are based on a clay substrate and samples collected in spring. Comparisons across the seasons are shown in Figure 4.S2.

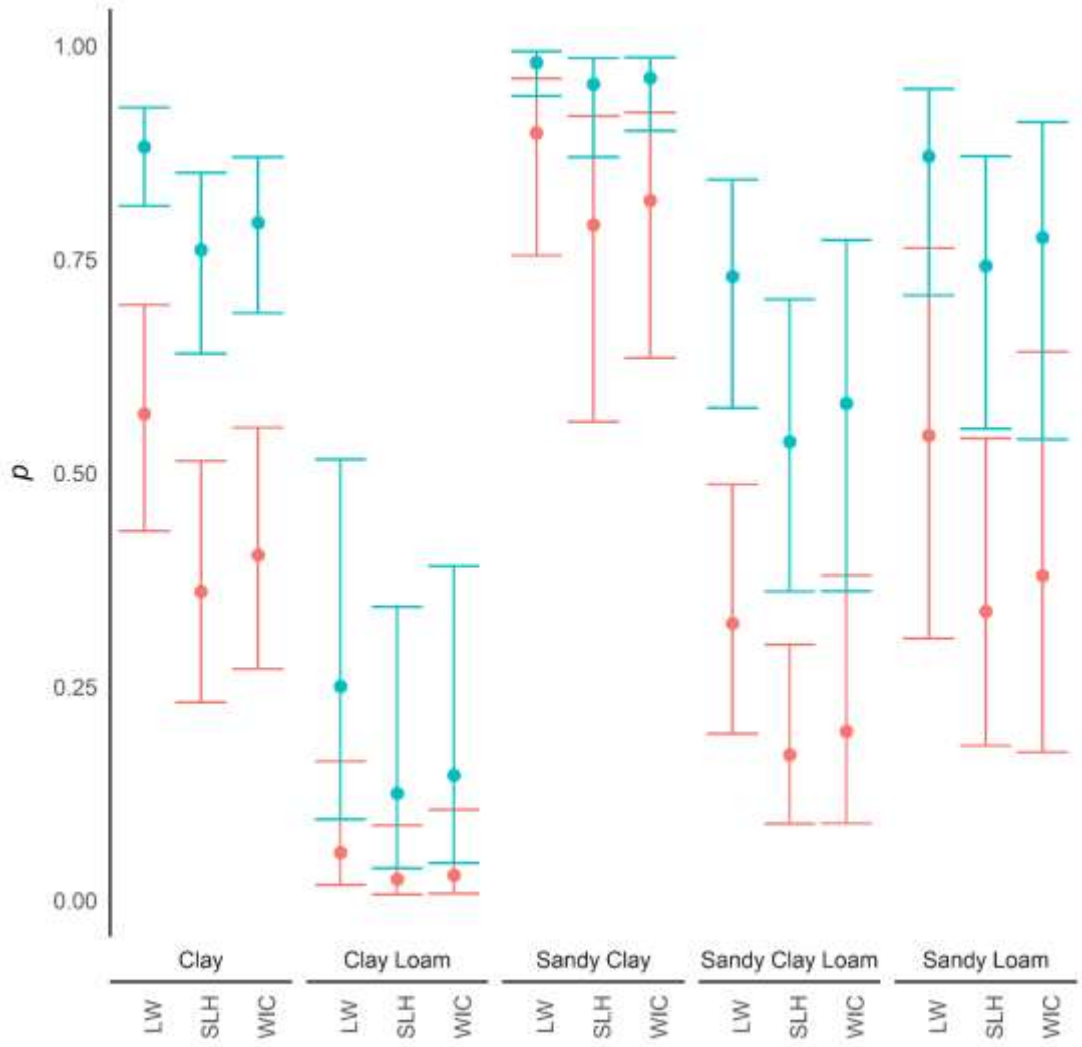

Figure 4.3 - Sediment type and detection probability. Variation in detection probability (*p*) between water samples (Blue) and sediment samples (Red) with various sediment types, and study area (LW – Little Wittenham; SLH – Stanford-le-Hope; WIC – Wickford), with 95% confidence intervals. These are based on sample collection in spring and an HSI of 0.65 (a score considered mid-range for great crested newt occupancy). Comparisons with ponds in other seasons are shown in Figure 4.S3.

Further analysis was undertaken on AIC and BIC importance weights for individual covariates with season (cumulative AIC weight = 1.00; cumulative BIC weight = 1.00), sample type (cumulative AIC weight = 1.00; cumulative BIC weight = 1.00), study

area (cumulative AIC weight = 0.92; cumulative BIC weight = 0.78), sediment texture (cumulative AIC weight = 1.00; cumulative BIC weight = 1.00), and HSI score (cumulative AIC weight = 0.98; cumulative BIC weight = 0.94) all strongly supported by the analysis and therefore considered to be important.

4.5. Discussion

The probability of detecting eDNA varies with its concentration (Chapter 3) and the ability to recover it. We have shown it is possible to detect great crested newts from samples of both pond water and sediment through all seasons, supporting previous work (Rees et al., 2017). However, the probability of detecting the target DNA varies through the year in eDNA samples from both water and sediment. We also found that eDNA detection from sediment was lower than from water samples in all seasons. We show that the influences on detection probability vary according to the sediment texture, pond HSI score and the study area.

We demonstrate that detection probability from aquatic samples varies over the year with detection increasing between spring and summer in each of the three study areas, and declining through the autumn to lowest levels in the winter. The trend in the detection probability through the seasons was similar in sediment and aquatic eDNA samples. This finding suggests that contemporary eDNA has a strong influence on detection probability in sediment eDNA samples as seasonal changes in detection exist. However, some detection may be from longer-term DNA deposits within the sediment, as the seasonal changes are not as pronounced as in the water samples. The seasonally variable detection probability, with rates much lower in the winter than spring or summer, suggests a low level of confidence in a negative result outside the core aquatic activity season for the species.

DNA bound to sediment is protected from processes which break it down (Levy-Booth et al., 2007). During sample collection we were only targeting the very surface of the

sediment, which we assume to contain the most recent deposits. Suspended material within the water column including whole cells and extracellular DNA settle out of the suspensions and progressively accumulate within the sediment (Turner et al., 2015; Corinaldesi et al., 2011), but do not necessarily bind to it. Unbound DNA within sediments has been found to be broken down more quickly than DNA bound to sediments (Levy-Booth et al., 2007). DNA that has been incorporated into sediments through the settling of cellular material, but remains unbound may explain why our samples did not show a constant level of detection all year. This would suggest unbound target DNA building up through the spring and summer, when the target species is present. However, the amount of target DNA in the sediment is reduced when there are fewer inputs in the autumn and winter.

We also identified that HSI score – a measure of how suitable the habitat is for great crested newts – is positively related to detection. Although all ponds used within the study contained great crested newts, HSI scores ranged from 0.34 to 0.91, with the majority between 0.65 and 0.80. Our data suggests that ponds with higher HSI scores have greater detection probabilities. This may be because the HSI values of ponds in this study were biased towards higher scores. Equally, higher HSI scores and better habitats may mean higher population densities (Oldham et al., 2000; Unglaub et al., 2015), and thereby more DNA being released. However, some studies have reported no relationship between HSI value and newt abundance (Lewis et al., 2007) and abundance is not the only influence on eDNA concentration within a pond (Chapter 5).

Detection of eDNA also varied according to sediment texture. Ponds with clay loam and sandy clay loam had lower detection probability than clay or the other substrate textures. The pattern of lower detectability in clay loam and sandy clay loam was apparent in all four seasons (Figure 4.S3), but more pronounced in spring and autumn. Fourteen of the nineteen ponds were found to have a clay texture substrate,

whereas only two ponds had a sandy clay loam texture, and one of each had sandy loam, sandy clay and clay loam. Due to the unbalanced distribution of the pond substrates between different ponds, other factors that vary between ponds may have exaggerated or masked any influence pond sediment texture had on detection probability. Substrate texture may therefore not be as important as these results suggest although the influence of texture was found to be strong with both the AIC and BIC cumulative weight analysis. As eDNA is often released in particles of sizes large enough to settle into the sediment, which may be within whole cells or aggregations of whole cells, these then accumulate within the sediment but do not necessarily bind to it (Turner et al., 2014a). The mechanism and capacity for DNA binding would therefore be less important between the sediment textures, and differences between the textures would not be observed.

The sample collection and DNA extraction method allowed for a single homogenous sample to be collected from a pond, rather than multiple independent samples. We chose this method as it allowed for a simple kit-based extraction method with inbuilt steps to remove inhibition. However, most kits designed for extraction of DNA from soil require mechanical cell lysis which have been shown to generate lower yields of eukaryotic eDNA than kits with chemical cell lysis (Eland et al., 2012; Deiner et al., 2015; Hinlo et al., 2017). The DNA extraction kit chosen was developed and tested on stool samples which we assumed would have greater efficiency extracting DNA from eukaryotic cells. However, the small volume of sediment used within the analysis may have resulted in low yields and a different extraction process may have recovered more target DNA.

As aquatic eDNA is usually broken down within weeks, detection of great crested newts in water using eDNA indicates current or recent presence of the species (Thomsen et al., 2012b; Chapter 3). Positive detections in the winter therefore suggest some adults or larvae are present in the ponds over this period. Likewise,

the seasonal fluctuation of eDNA in sediments also indicates the current or recent presence of the species. Nevertheless, some eDNA within sediment samples may originate from longer-term DNA deposits. However, the lower probability of detection of eDNA extracted from sediments indicates that sediment analysis should not be used to attempt year-round detection of a seasonally aquatic species, at least using the current methods. Refinement of the sample collection protocol, collection of multiple samples from a pond or alterations to the DNA extraction process used may increase DNA recovery rate, detection probability, and ultimately the use of the method for year-round detection of species from sediments.

4.6. Acknowledgments

We would like to thank the Earth Trust for granting us access to their site at Little Wittenham in Oxfordshire, UK for sample collection, similarly we would like to thank Herpetologic for access to two sites they manage (Wickford and Stanford-le-Hope) both in Essex, UK. We would also like to thank Herpetologic for providing great crested newt presence results using traditional survey techniques, for the 11 ponds managed by them. We would like to thank Stephanie Green for help with field work and Galatea Swart for assistance in the laboratory. We would also like to thank Jim Foster and Dr John Wilkinson from Amphibian and Reptile Conservation, and Dr Jeremy Biggs from Freshwater Habitats Trust for their advice. Finally, we would like to thank Isabela Barata and two anonymous reviewers for their comments on the manuscript.

4.7. Chapter 4 - Supplementary Information

4.7.1. Methods

Detailed eDNA from sediment extraction protocol.

Extraction of sedimentary eDNA samples followed modified Qiagen® QIAamp® DNA Stool Mini Kit protocol (Chaves et al., 2010).

i. The 50 mL centrifuge tube containing the ethanol preservative with the suspension of pond sediment and distilled water, was removed from the freezer and shaken vigorously to homogenise the sample. The sample was then centrifuged at 8500 rpm for 30 minutes to separate the sediment from the preservative.
ii. The supernatant was carefully poured off and discarded, making sure to leave all sediment within the tube.
iii. Sediment was removed from the centrifuge tube and placed on a sterile petri-dish, the sediment was then mixed by hand and 0.5 mL transferred to a 2 mL micro-centrifuge tube. 1.5 mL of ASL buffer from the QIAamp® DNA Stool Mini Kit was added to the micro-centrifuge and mixed by vortexing for 15 seconds.
iv. Samples were incubated at 55˚C overnight on a rotating block to separate DNA from the sediment.
v. After incubation the samples were centrifuged at 13,300 rpm for 3 minutes to pellet the unwanted sediment.
vi. The supernatant was transferred to a new 2 mL tube and 1 InhibitEX® tablet from the extraction kit added per 1.5mL of sample, the remaining sediment was discarded.
vii. The samples with InhibitEX® tables were vortexed for 1 minute and then incubated at room temperature for 1 minute, to allow inhibitors to adsorb onto the InhibitEX® matrix.

viii. The samples were then centrifuged at 13,300 rpm for 12 minutes to pellet the tablet and inhibitors. The supernatant was split between two new 2 mL micro-centrifuge tubes. 25 µL of proteinase K was added to each tube, and an equal volume of AL buffer to the supernatant added to each tube.

ix. The samples were vortexed for 15 seconds and incubated at 70˚C for 15 minutes.

x. Equal volumes of 100% ice cold ethanol, to the volume of AL buffer added in step eight was then added to each tube and mixed by vortexing.

xi. 600 µL of sample was then transferred to the QIAamp® spin column provided. This was centrifuged at 13,300 rpm for 1 minute, trapping the DNA on the spin column, and the flow through discarded.

xii. This process was repeated until all of the sample had passed through the spin column.

xiii. Each spin column was then washed with 500 µL of AW1 buffer and centrifuged for 1 minute at 13,300 rpm.

xiv. A second wash step using 500 µL of AW2 buffer was undertaken, centrifuging for 3 minutes at 13,300 rpm both times. This step was repeated to aid in sample cleaning.

xv. The spin column was then transferred to a new 1.5 mL microcentrifuge tube for the elution step and 100 µL of hot AE buffer pipetted directly onto the spin column membrane, this was then incubated at room temperature for 45 minutes before centrifuging at 8,000rpm for 1 minute.

xvi. A second elution step was undertaken; a further 100 µL of hot AE buffer was added directly to the spin column membrane, and incubated at room temperature for 15 minutes before centrifuging at 8,000rpm for 1 minute.

xvii. eDNA extracts were stored at -20 ˚C until the qPCR analysis.

4.7.2. Figures

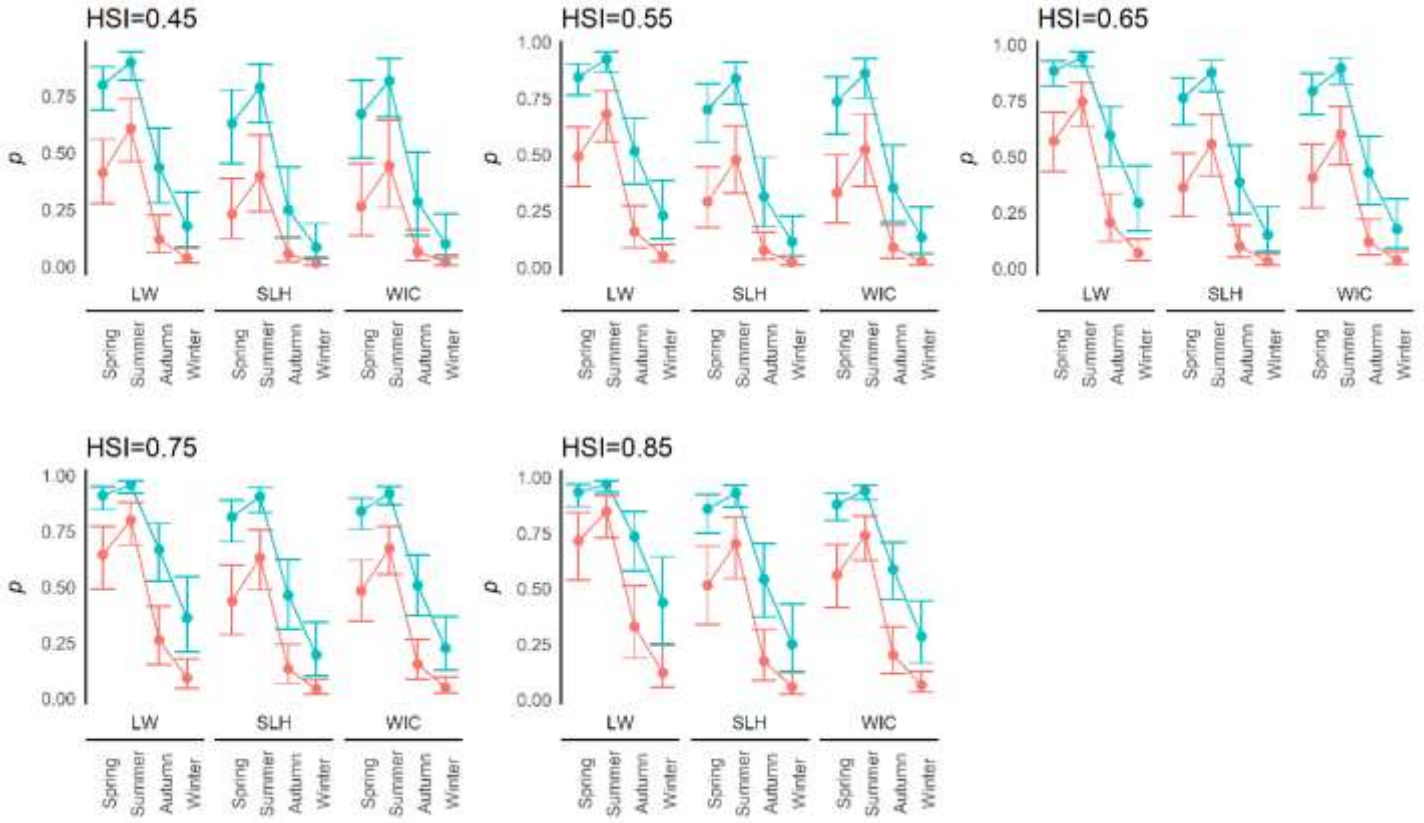

Figure 4.S1. Seasonal detection probability. – Variation in detection probability (*p*) between water samples (Blue) and sediment samples (Red) across the seasons, in the different study areas, with 95% confidence intervals. Predictions shown assume a clay substrate.

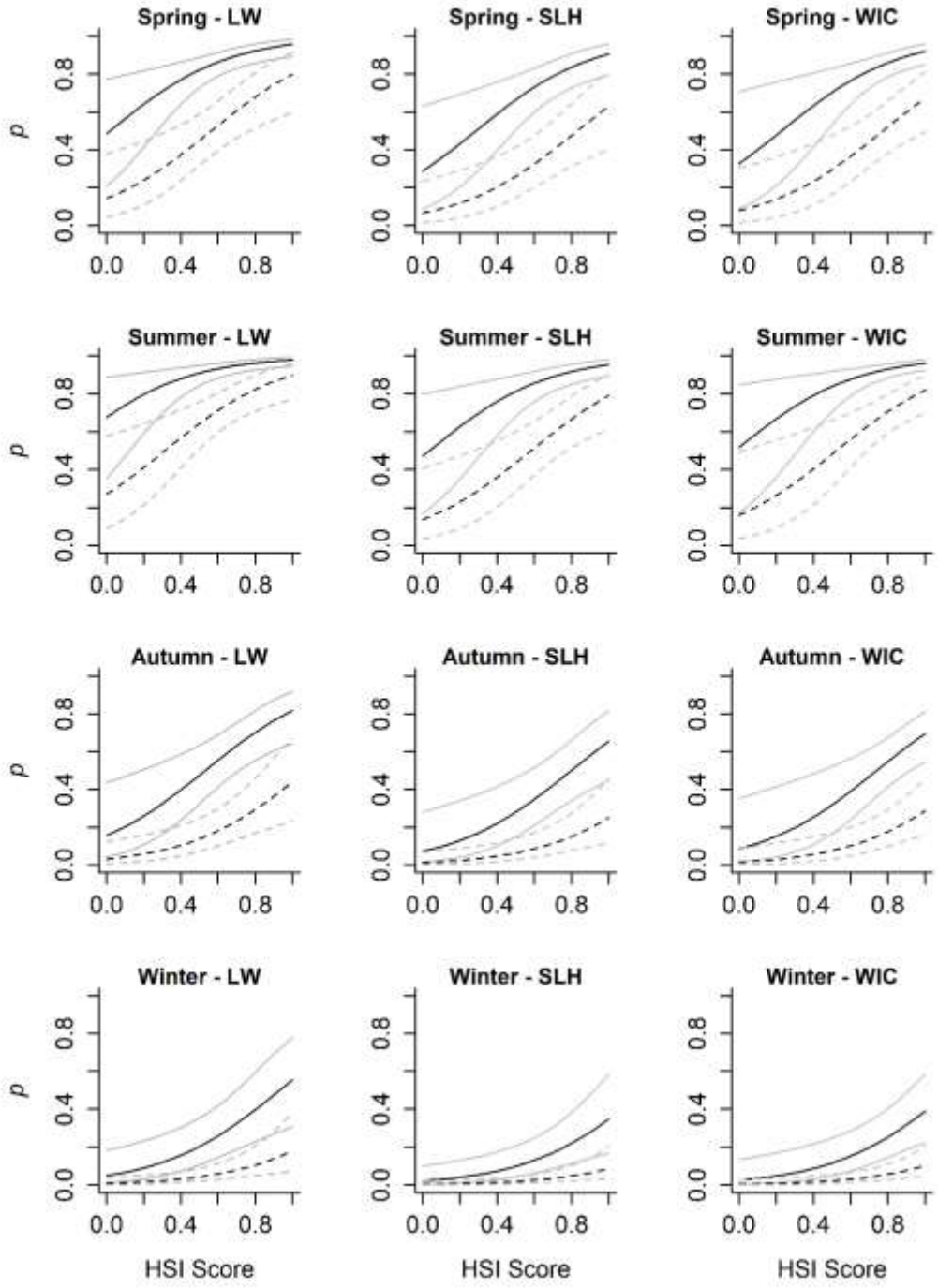

Figure 4.S2. Habitat suitability and detection probability. – Variation in detection probability (*p*) between water samples (solid) and sediment samples (dotted) in relation to HSI score, in all seasons. Little Wittenham (LW), Stanford-le-Hope (SLH), and Wickford (WIC), with 95% confidence intervals. These predictions assume a clay substrate.

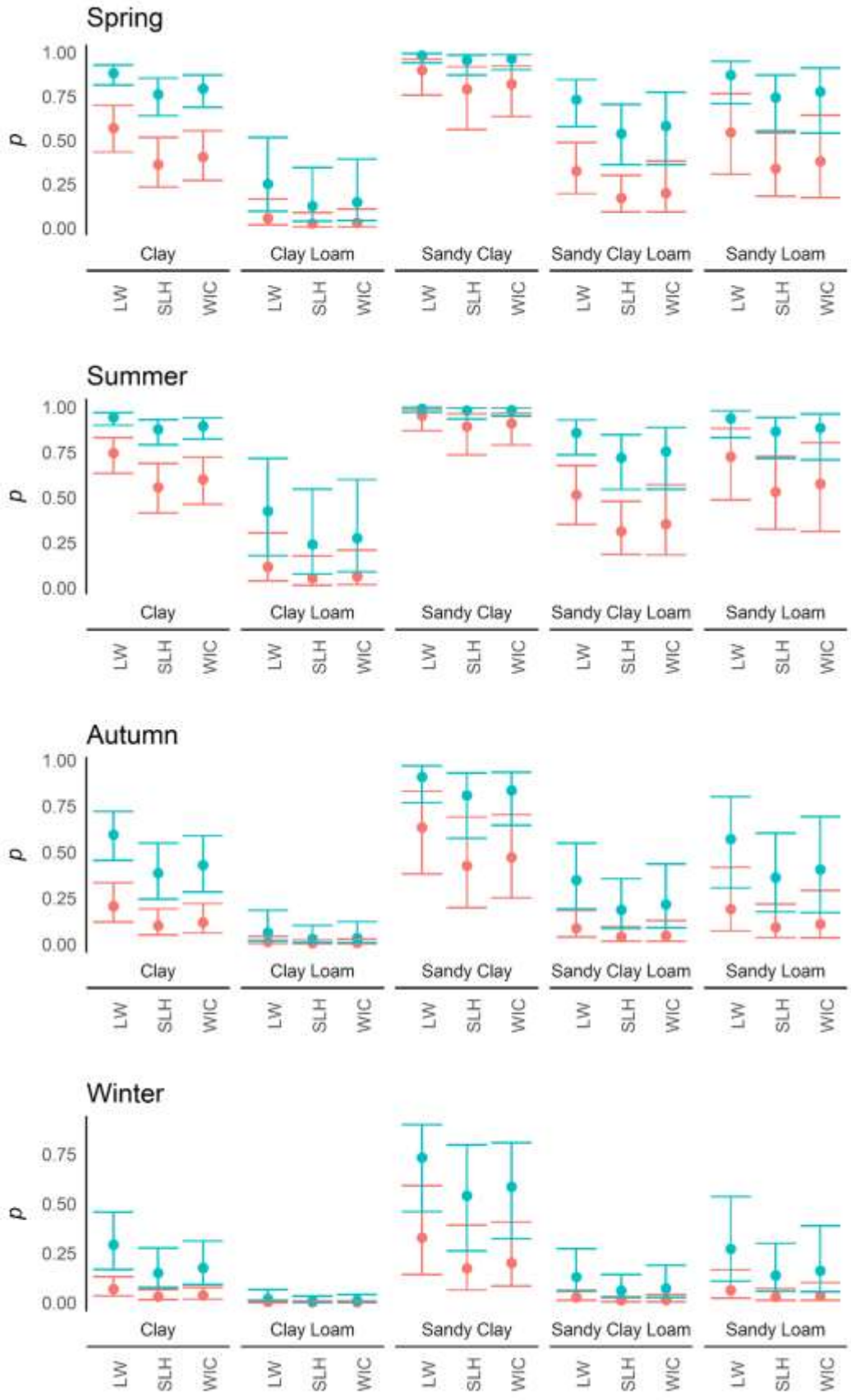
Spring
Summer
Autumn
Winter
p
1.00
0.75
0.50
0.25
0.00
Clay
Clay Loam
Sandy Clay
Sandy Clay Loam
Sandy Loam
LW
SLH
WIC

Figure 4.S3. Sediment type and detection probability. – Variation in detection probability (*p*) between water samples (Blue) and sediment samples (Red) in relation to sediment types, in the different study areas, and the different seasons, with 95% confidence intervals. All based on an HSI of 0.65 (a score considered mid-range for great crested newt occupancy)

Chapter 5 – Seasonal variation in environmental DNA in relation to population size and environmental factors

This chapter has been published in the peer reviewed journal *Scientific Reports*:

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

5.1. Abstract

Analysing DNA that organisms release into the environment (environmental DNA, or eDNA) has enormous potential for assessing rare and cryptic species. At present the method is only reliably used to assess the presence-absence of species in natural environments, as seasonal influences on eDNA in relation to presence, abundance, life stages and seasonal behaviours are poorly understood. A naturally colonised, replicated pond system was used to show how seasonal changes in eDNA were influenced by abundance of adults and larvae of great crested newts (*Triturus cristatus*). Peaks in eDNA were observed in early June when adult breeding was coming to an end, and between mid-July and mid-August corresponding to a peak in newt larval abundance. Changes in adult body condition associated with reproduction also influenced eDNA concentrations, as did temperature (but not rainfall or UV). eDNA concentration fell rapidly as larvae metamorphosed and left the ponds. eDNA concentration may therefore reflect relative abundance in different ponds, although environmental factors can affect the concentrations observed. Nevertheless, eDNA surveys may still represent an improvement over unadjusted counts which are widely used in population assessments but have unreliable relationships with population size.

5.2. Introduction

All living organisms continually expel DNA into the environment via faeces, urine, skin secretions, skin cells and gametes (Lydolph et al., 2005; Haile et al., 2009; Waits & Paetkau, 2005). The emergence of techniques that are able to detect low levels of such environmental DNA (eDNA) has enormous potential to break new ground in areas such as invasive species research (Dejean et al., 2012; Jerde et al., 2013; Takahara et al., 2012), pathogen detection (Walker et al., 2007), palaeoecology (Anderson-Carpenter et al., 2011), and forensics and law enforcement (Mahon et al., 2014). The use of eDNA to survey rare and cryptic species that are difficult to detect using traditional methods also has wide implications for biodiversity assessment and the protection of species (Bohmann et al., 2014; Ikeda et al., 2016). A relationship between the amount of eDNA present and measures of abundance has been demonstrated in both natural and mesocosm systems (Thomsen et al., 2012b; Pilliod et al., 2013; Eichmiller et al., 2014; Doi et al., 2015b, 2017; Lacoursière-Roussel et al., 2016a; Matsuhashi et al., 2016; Takahara et al., 2012). Although some studies suggest peaks in eDNA are associated with breeding (Spear et al., 2015; Fukumoto et al., 2015; Doi et al., 2017), the seasonal dynamics of eDNA in relation to population size are poorly understood. Consequently, eDNA is currently largely limited to surveys of presence and absence. Measures of abundance are more useful than presence-absence, but are often based on count data that are not adjusted for detection probability which can be misleading (Schmidt, 2003). As such, producing reliable population, biomass or relative abundance estimates would be much more informative for conservation practitioners (Bohmann et al., 2014). Before predicting abundance, the factors that influence eDNA concentration in relation to changes in population size and environmental factors need to be understood.

The concentration of eDNA at any point in time will depend on (1) the rate of production of eDNA by the species; and (2) how long eDNA persists in the

environment (Dejean et al., 2011). eDNA release and accumulation rates depend on a number of factors including the density of individuals, their physiology, metabolism and temperature (Klymus et al., 2015). However, eDNA can be broken down by biotic and abiotic factors such as extracellular enzymes, high temperatures, UV, and chemicals (Tréguier et al., 2014; Barnes et al., 2014; Piaggio et al., 2014; Levy-Booth et al., 2007; Pilliod et al., 2014; Strickler et al., 2015; Dejean et al., 2011). In aquatic environments, eDNA can also become incorporated into sediment (Turner et al., 2015). Persistence of eDNA in water after organisms are removed can range from less than one day (Thomsen et al., 2012a), to over three weeks (Dejean et al., 2011) depending on environmental conditions, whereas persistence in soil or sediment is likely to be much longer (Turner et al., 2015). Despite this knowledge base, and the fact that eDNA concentration can vary seasonally (Spear et al., 2015; Fukumoto et al., 2015), to our knowledge no studies have identified how seasonal population dynamics impact eDNA concentration in relation to other factors that influence DNA release and degradation. Therefore, whilst eDNA surveys promises to redefine how biodiversity is monitored in the future, there is considerable uncertainty about the relationship between eDNA concentrations and seasonal changes in population size, because of the influence of other environmental factors.

In this study we examined the relationship between eDNA and the seasonal population dynamics of great crested newts (*Triturus cristatus*) using a replicated but naturally colonised system of eight ponds. Adult great crested newts migrate into ponds to breed in the spring, with most returning to land in early summer. Breeding occurs in water with females laying eggs that hatch into aquatic larvae that metamorphose and emerge in the late summer or, occasionally, overwinter (Beebee & Griffiths, 2000). All of these stages may release eDNA into the water. As a European Protected Species, great crested newt eDNA surveys are currently being used to assess the presence-absence of species, but how eDNA fluctuates over this

aquatic phase is unknown (Biggs et al., 2014a). To fill this knowledge gap, adult and larval abundance, adult body condition and environmental factors including temperature, rainfall and UV, were used to evaluate their influences on eDNA concentrations throughout the aquatic period.

5.3. Methods

5.3.1. Study site

The study site was located at the University of Kent campus in Canterbury, UK. The site consists of eight identical ponds measuring 1 m x 2 m x 0.6 m deep constructed using PVC liner and a water volume when full of 600 L. The eight ponds are arranged in a grid pattern with approximately 3 m between each pond. All eight ponds can be considered to experience the same environmental conditions. All eight ponds had been in place for a minimum of six years at the time of the study and were allowed to be colonised naturally by the three species of newts in the area (Lewis, 2012). All species could freely move from one pond to another and to immigrate or emigrate. Over the winter prior to the study, all eight ponds were drained, liners replaced and filled with tap water so that all ponds were identical at the start of the study.

5.3.2. eDNA sampling

eDNA samples were collected from the eight ponds every 14 days from 26 February through to 22 October 2015. To avoid contamination, on each occasion eDNA samples were collected prior to the population monitoring. Two eDNA collection methods were used: (1) filtration of 1 L of sample water using a 0.7 µm glass-microfiber syringe filter (Sterlitech Corporation®, Kent Washington State, USA); and (2) precipitation of DNA from a 0.09 L sample volume in an ethanol, sodium acetate solution (Biggs et al., 2015). All field equipment was sterilised using 10% bleach, UV-Crosslinker or autoclave and sealed prior to transport to the study site, and a separate set of nitrile disposable gloves were used for each sample. Due to the small dimensions of each pond, a single 1 L surface sample, collected using a

polypropylene wide mouth bottle, was deemed sufficient to provide a representative sample from each pond. The bottle was rinsed with pond water and used to stir the pond as suggested by Biggs et al. (2014a) prior to being filled.

Filtered samples were collected using a 100 mL syringe. The sample was removed from the collection bottle, and then drawn through a 0.7 µm glass microfiber syringe filter. The process was repeated, with the sample homogenised before filling each syringe, until 1 L had been filtered or two filter units had become blocked. Residual water was removed from the filter unit by passing two syringes of air through each unit. Both filter units were then sealed in bags prior to transport to the laboratory where they were stored at -20 ˚C until extraction.

Samples collected using precipitation in ethanol consisted of six, sterile 50 mL centrifuge tubes containing 33 mL of absolute ethanol and 1.5 mL of 3 M sodium acetate solution. All six tubes were filled from the collection bottle to make the volume in each up to 50 mL, using a sterile disposable plastic pipette. This equates to a total volume per sample of approximately 90 mL. Each sample was placed in a sealable bag for transport to the laboratory, where they were stored at -20 °C until extraction.

5.3.3. Population assessments

The population in each pond was assessed using aquatic funnel traps (Griffiths, 1985). Trapping commenced in the last week of February 2015 and continued weekly until the end of October 2015, encompassing the period adult and larval great crested newts are active (Langton et al., 2001). Traps were left in place for between 11 and 12 hours overnight depending on the season. Ventral patterns of all adults caught were photographed and used for individual identification to allow for capture-mark-recapture analysis to provide weekly detection probabilities (White & Burnham, 1999). Each adult was weighed on each capture event to the nearest 0.1 g, and snout-vent and tail length measured to the nearest 1 mm to assess body condition. To avoid contamination between ponds, surveyors wore disposable nitrile gloves that were

changed between ponds. Additionally all bottle trapping equipment was sterilised at the start of the season with 10% bleach and dedicated equipment was used for sampling each of the eight ponds.

Torchlight counts of larvae were also conducted from the beginning of July onwards. This allowed calibration of the counts of larvae captured in the bottle traps at the same time. Torchlight counts involve shining a 1 million candle power torch through the surface of the water after dark. The light was moved systematically from one end of the pond to the other, counting all of individuals that could be seen within the water column. Due to the size of each of the study ponds and absence of vegetation, counts could be undertaken across the entire surface area and water column of each of the ponds.

5.3.4. Laboratory protocol

DNA extractions were conducted in a UV sterilisable work station in a laboratory with dedicated equipment. All extractions were based on the DNeasy Blood & Tissue Extraction kit (Qiagen®, Hilden, Germany) with amended protocols as outlined in the supplementary information. Periodic extraction blanks for both methods were undertake through the laboratory phase of the project to check for equipment contamination, and were all negative.

Real-Time qPCR was performed on all samples in a separate lab from DNA extraction and in a dedicated UV-sterilisable work station. qPCR was performed using previously published primers and hydrolysis probe (Thomsen et al., 2012b) and qPCR assay and cycle condition (Biggs et al., 2015) using a CFX Connect Real-Time PCR detection system (BIO-RAD® Hercules, California, USA). Eight qPCR replicates were performed per sample. qPCR standards were created from a serial dilution of a great crested newt tissue extract, quantified using a Qubit® 2.0 flurometer (Life Technologies™, Carlsbad, California, USA) with Double Stranded DNA High Sensitivity Kit following manufacturers' instructions, qPCR negative controls were

also included in each run. The median value for the eight qPCR replicates was taken forward into the analysis for each sample. eDNA was found in all ponds, but not in each calendar week, with concentration varying between zero and 0.00845 ngµL^{-1}. The mean R-squared value of all qPCR standard curves was 0.99 and the efficiency was 90.3%.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated through qPCR from a serial dilution of a tissue extract from a great crested newt. The LOD related to the minimum concentration amplification was observed, while the LOQ was assigned to the minimum level that exhibited a high degree of conformity between qPCR replicates (Tréguier et al., 2014). The LOD was found to be less than 10^{-7} ngµL^{-1}, with an LOQ of 10^{-5} ngµL^{-1} (Chapter 2).

Great crested newt eDNA was detected in some or all ponds on each survey occasion. Eleven out of 200 eDNA samples analysed returned as negative. Negative results were split between both survey methods and were only found when eDNA concentrations were low either towards the start or end of the study.

5.3.5. Environmental data

Mean temperature as well as UV levels for the 14 days between sampling were generated for the study site as a whole. Air temperature was recorded from the site hourly using a Tinitag® Plus 2 – TGP-4017 (Gemini Data Loggers, Chichester, UK) commencing on the 30 January. UV was recorded on a TR-74Ui – Illuminance UV Recorder (T&D Corporation®, Nagano, Japan) at hourly intervals, from 17 February. An indication of the level of rainfall that occurred between each survey period was collected using a standard rain gauge, emptied at the time of the visual surveys.

5.3.6. Analysis

Losses of body mass during the breeding season are associated with egg deposition (females), spermatophore production (males) and utilization of fat reserves for

breeding activity. Body condition estimates were generated using the Scaled Mass Index (SMI; Peig & Green, 2009). The mean of the SMI values for all individuals caught each week were taken to produce each weekly value. SMI values could only be generated until the middle of July due to low adult numbers caught beyond that point. This was done for males and females separately as well as both sexes combined.

The Cormack-Jolly-Seber model and Program MARK (White & Burnham, 1999) were used to generate a detection probability each week for adults captured in traps. The best fitting model was phi(.)p(t), or constant survival with variable detection probability. Detectability varied each week and ranged from 0 to 1 with the majority of results falling between 0.3 and 0.6, with outliers from this range only found in weeks when few individuals were caught. A single detection probability was generated for the larvae, using torchlight counts, as using capture-mark-recapture was not a viable option for larvae. Using ponds with high visibility, which allowed the entire pond to be observed, the number of larvae captured in traps was divided by the number of larvae counted in the torchlight surveys. This approach is appropriate in the case of this study due to the small size of the ponds allowing the entire pond to be searched by torchlight. A fixed detection probability of 0.39 was used in all weeks for two reasons. Firstly, the low number of individuals in the last few weeks of the study skewed detectability estimates. Secondly, torchlight counts only started on 9 June, after the first larvae were caught in traps, therefore no detection probability could be generated for the weeks before the introduction of torchlight counts. The population size for each pond in each week was estimated by multiplying the number of newts caught in traps by the reciprocal of the detection probability (Schmidt, 2003). Population estimates and body condition scores are only included in the analysis for the weeks eDNA was collected.

5.3.7. Statistical analysis

eDNA concentrations were transformed prior to analysis using y = log10 (x+0.0001) to ensure normality. All statistics were conducted using linear mixed effect models (LMM; Crawley, 2007) using R version 3.1.3 (R Development Core Team, 2016) and package nlme (Pinheiro et al., 2016), LMM were chosen to account for the repeated measures on the same ponds through the season (treated as a random effect). Akaike's Information Criterion (AIC) was used to assess support for different models using package MuMIn (Bartoń, 2016). Models with a ΔAIC of $\leq$ 2 were considered to have substantial support, while models with a ΔAIC of $\leq$ 7 were considered to have some support (Marchetti et al., 2004). Using the full set of models, Akaike importance weights for predictors were calculated as measures of parameter importance, by summing the Akaike weights for each model containing that variable (Johnson et al., 2011; Marchetti et al., 2004). Parameters were classed as strongly supported by our models if they were significant in all strongly supported models (ΔAIC of $\leq$ 2) and had a cumulative Akaike weight of > 0.75 (Marchetti et al., 2004). Parameters were considered somewhat supported if they were significant in any of the strongly supported models (ΔAIC of $\leq$ 2) regardless of Akaike weight (Marchetti et al., 2004).

Two models were run, because different factors potentially influence eDNA concentration at different times of year: (1) a model encompassing the core adult aquatic period (26 February to 18 June); and (2) a model encompassing the post-breeding season when most adults will be on land (18 June to 22 October). A single model would be inappropriate because estimates for body condition were only available for those weeks when adults were in the ponds in high numbers, and would lead to a high degree of non-random missing data biasing the output. The first set of models therefore incorporated the breeding season (i.e. 26 February to 18 June), and comprised nine eDNA sampling occasions across 18 weeks. These models were constructed with "Pond" as the random variable to account for repeated sampling,

and combinations of adult abundance, larval abundance, male body condition, female body condition, combined body condition, calendar week, collection method, air temperature, water temperature, rainfall and UV included as covariates. All variables were treated as continuous co-variates with the exception of collection method which was nominal. Correlation coefficients were examined for covariates included in all strongly supported models (ΔAIC of $\leq$ 2), a pair of covariates were considered to be highly correlated with a correlation coefficient of > 0.7 (Dormann et al., 2013). This was found to be the case for UV and female body condition (r = 0.868 in the top model), and as a result UV was excluded from the analysis. No other pairs of covariates were found to be above this threshold.

The second set of models explored variation in eDNA concentration outside the breeding season (i.e. 18 June to the 22 October), including ten eDNA sampling occasions across 19 weeks, with “Pond” again used as the random variable to account for repeated sampling. Adult abundance, larval abundance, eDNA collection method, air temperature, rainfall, UV, and calendar week, were all included as covariates. All variables were treated as continuous co-variates with the exception of collection method which was nominal. Correlation coefficients were examined for covariates included in all strongly supported models (ΔAIC of $\leq$ 2), a pair of covariates were considered to be highly correlated with a correlation coefficient of > 0.7 (Dormann et al., 2013).This was found to be the case for UV and calendar week (r = 0.960 in the top model), and as a result UV was excluded from the analysis, no other pairs of covariates were found to be above this threshold.

Collection method (i.e. ethanol precipitation versus glass-microfiber syringe filtration) was included as a variable in all of the models to check that there was no method-related bias. This was subsequently corroborated, with paired sample analysis showing no difference in eDNA extract concentration between the two methods (Chapter 2).

5.3.8. Ethics statement

The experimental procedure was approved by the School of Anthropology and Conservation (University of Kent) Research and Ethics Committee, with disturbance and handling of live animals undertaken under EPS Licence 2014-5025-CLS-CLS issued by Natural England, in accordance with the conditions of the licence.

5.4. Results

Between 26 February 2015 and 29 October 2015, a total of 389 captures of 49 individuals were made across the eight ponds, with capture-mark-recapture models yielding an overall population size of between 53 and 60 individuals with a most likely population size of 57, although the numbers varied between ponds. Likewise, 408 larvae were captured between 28 May 2015 and 29 October 2015, with an estimated bottle trapping detectability of 0.39.

Two distinct peaks were seen in eDNA concentration (Figure 5.1). The first peak corresponded to the end of the adult breeding season in early June. The second peak was observed from mid-July to mid-August and corresponded with the peak in larval numbers. The influences on eDNA concentration over the breeding season (26 February to 18 June) were identified using the first set of models. The change in body condition measured by the Scaled Mass Index (SMI; Peig & Green, 2009) fell from a peak on 6 March through the breeding season and continued to fall into the post breeding season, with most of the decline occurring from 9 April through to 4 June. Both sexes showed declines in SMI score with females showing a slightly greater decrease than males (Figure 5.1). The sharpest decline in body condition for both males and females occurred in the key breeding months of April and May. During the same core period of April and May the mean eDNA concentration rose considerably but adult population changed very little, and larvae were first identified in the ponds at the beginning of June. As would be expected, temperature and UV both increased as the breeding season progressed, from early spring into early summer. This

resulted in the model with the greatest AIC support (ΔAIC to second model = 0.5) comprising adult abundance, larval abundance, temperature, and male and female body condition as predictors of eDNA concentration (Table 5.1). Three other models were shown to have strong support ($\Delta AIC \leq 2$) also detailed in Table 5.1.

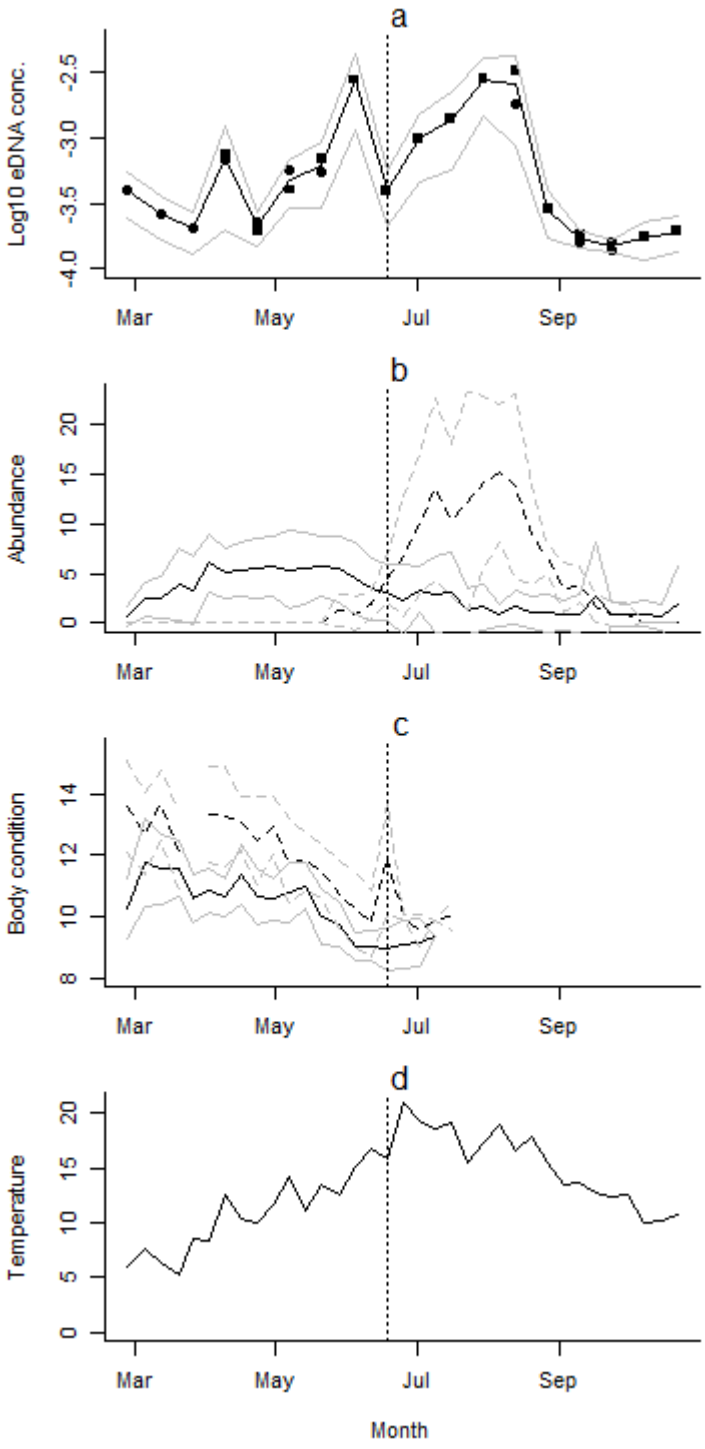

Figure 5.1 - Seasonal variations in eDNA concentration. Seasonal variations in eDNA concentration, in relation to adult and larval population size, adult body condition and temperature. a - shows Log10(x+0.0001) of the mean eDNA concentration (ngµL^{-1}), per pond (black line, solid circles collected using glass-microfiber filters, solid squares collected using

precipitation in ethanol) with 95% confidence intervals (grey) across the eight ponds. b - shows the mean estimated population size per pond in black (adults - solid line, larvae - broken line) with 95% confidence intervals (grey). c - shows mean body condition (males – solid line, females – dashed line) using the scaled mass index of adults caught each week throughout a survey season with 95% confidence intervals (grey). d - shows mean weekly temperatures in degrees Celsius through the study period. The vertical dotted line represents the end of the breeding season and the start of the post-breeding season, as related to the models described in Tables 5.1-5.2.

Table 5.1 - Breeding season model selection

Predictor	Random	Value	SE	DF	t-value	p-value	AIC	AICc	ΔAIC	Weights
Adult Abundance	Pond	0.029	0.008	83	3.68	0.0004	97.91	99.6	0.00	0.263
Larval Abundance		0.043	0.021	83	2.01	0.0481				
Male Body Condition		-0.189	0.072	83	-2.61	0.0108				
Female Body Condition		-0.328	0.065	83	-5.02	<0.0001				
Temperature		-0.117	0.022	83	-5.32	<0.0001				
Larval Abundance	Pond	0.054	0.020	85	2.65	0.0095	99.13	100.1	0.50	0.204
Female Body Condition		-0.398	0.063	85	-6.31	<0.0001				
Temperature		-0.101	0.023	85	-4.33	<0.0001				
Adult Abundance	Pond	0.026	0.008	84	-4.99	<0.0001	99.13	100.4	0.83	0.174
Larval Abundance		0.066	0.020	84	3.32	0.0013				
Female Body Condition		-0.404	0.061	84	-6.66	<0.0001				
Temperature		-0.113	0.0223	84	3.223	0.0018				
Female Body Condition	Pond	-0.176	0.039	87	4.53	<0.0001	100.79	101.2	1.66	0.115

Linear mixed effect models showing influences on eDNA concentration in the breeding season (26 February to 18 June). All models showing substantial support based on ΔAIC shown.

Further analysis was undertaken on AIC importance weights for individual predictors over the breeding season, with female body condition (cumulative AIC weight = 0.99), larval abundance (cumulative AIC weight = 0.80) and air temperature (cumulative AIC weight = 0.79) strongly supported by the analysis, while male body condition (cumulative AIC weight = 0.43) and adult abundance (cumulative AIC weight = 0.44) were only somewhat supported by the analysis.

Influences on eDNA concentration after adult newts had finished breeding were examined through the second set of models, which included potential predictors from 18 June to 22 October. eDNA concentration increased dramatically between 18 June and 30 July, corresponding with an increase in mean larval abundance. During the same period adult abundance nearly halved, indicating that the increase in eDNA was more likely due to larval than adult influences. Temperature also increased through this period from a mean weekly temperature of 15.9 ºC to over 19 ºC for all of July. eDNA concentration remained high until the middle of August when it fell by over 90% between 13 August and 27 August, and continued to fall into the autumn. Metamorphosis of larvae from the ponds resulted in larval abundance falling over the same period. Temperature remained above 15 ºC through August but then fell to below 10 ºC in October. The model with the greatest AIC support (ΔAIC = 4.82) included larval abundance and air temperature (Table 5.2) as predictors of eDNA concentration. No other models were shown to have strong support (ΔAIC $\leq$ 2), but one was shown to have limited support (ΔAIC $\leq$ 7) also detailed in Table 5.2.

Table 5.2 - Post-breeding season model selection

Predictor	Random	Value	SE	DF	t-value	p-value	AIC	AICc	ΔAIC	Weights
Larval Abundance	Pond	0.013	0.002	94	5.36	<0.0001	100.74	101.4	0.00	0.898
Temperature		0.056	0.014	94	6.13	<0.0001				
Collection Method	Pond	0.065	0.079	93	0.82	0.4166	105.31	106.2	4.82	0.081
Larval Abundance		0.012	0.002	93	5.29	<0.0001				
Temperature		0.088	0.014	93	6.16	<0.0001				

Linear mixed effect models showing influences on eDNA concentration post-breeding season (18 June to 22 October). All models showing substantial or some support based on ΔAIC shown.

Further analysis was undertaken on AIC importance weights for individual predictors for the post-breeding season, with larval abundance (cumulative AIC weight = 0.998), and temperature (cumulative AIC weight = 1.0) strongly supported by the analysis; no other variables were found to be strongly supported by the analysis. Sample collection method was not found to be a significant predictor of eDNA in any of the models.

5.5. Discussion

Both laboratory and field studies have shown that an increase in abundance or density of target species can lead to an increase in either eDNA concentration (Goldberg et al., 2013; Thomsen et al., 2012b; Takahara et al., 2012; Pilliod et al., 2013; Klymus et al., 2015; Lacoursière-Roussel et al., 2016a) or eDNA detectability (Mahon et al., 2013). Our results take this further by showing that the eDNA contribution from different life stages of a semi-aquatic species varies seasonally. Although it was artificially created, our replicated pond system was ideal for this work, as it allowed for truly replicated samples to be taken, with robust population estimates of naturally colonising newts obtained. eDNA concentration within the breeding season increases as females lose body condition through reproductive behaviour and laying eggs. Male body condition and adult abundance also have some influence on eDNA concentration during the breeding season but not to the same extent as other variables. After adult breeding activity has finished, eDNA increases again as larval abundance increases, but with temperature also having an influence at this time.

The amount of eDNA in the environment depends on both DNA release from organisms and eDNA degradation rate (Dejean et al., 2011). These rates are likely to vary seasonally in response to environmental changes and the ecology of the species (Goldberg et al., 2011; Lacoursière-Roussel et al., 2016a; Barnes et al., 2014). Strong temporal increases in eDNA during months associated with breeding have been observed in the Eastern hellbender (*Cryptobranchus alleganiensis alleghaniensis*;

Spear et al., 2015) and Chinese and Japanese giant salamanders (*Andrias davidianus* and *A. japonicus* respectively; Fukumoto et al., 2015). Doi et al. (2017) found that seasonal variations in eDNA concentration were related to total biomass, rather than abundance or behaviour, in stream dwelling fish (Doi et al., 2017). Our data support this with an increase in eDNA concentration associated with both peak breeding and peak larval abundance.

Current eDNA survey protocols for great crested newts focus on the period adults are present in ponds (Biggs et al., 2014a). In the past, positive great crested newt eDNA samples have been identified outside the breeding season (Rees et al., 2014a). We find a second period with high eDNA concentration at a time of year when adults are moving out of ponds into their terrestrial phase (Jehle et al., 2011). This post-breeding season spike can be attributed to other life stages, predominantly larvae, and the late August fall in eDNA, corresponds to the period larvae are metamorphosing and leaving the ponds (Jehle et al., 2011). Seasonal changes in eDNA therefore have implications for survey strategy. If the eDNA surveys are focused on assessing breeding rates, it may be more appropriate to attempt to target larvae by sampling over the post-breeding months. On the other hand, if surveys are aimed at determining occupancy by adults, this approach may be inappropriate. As with many other amphibians, great crested newts live in a metapopulations, where some ponds hold reservoirs of adults that are not breeding each year (Griffiths et al., 2010). We have shown that one of the key influences on eDNA concentration after adults have finished breeding is larval abundance. Samples taken outside the core adult aquatic period may be useful in identifying successful breeding, due to the presence of larvae. However, in the cases of occupied but non-breeding ponds, samples in this period would likely return negative results, potentially missing important non-breeding sites for the species.

The relationship between eDNA water concentration and population size varies by season. For example, an increase in temperature is likely to influence both eDNA release, through higher activity levels (Takahara et al., 2012), and breakdown rates, with an increase in DNA degradation (Barnes et al., 2014; Pilliod et al., 2014). We found that temperature had a significant influence on eDNA concentration during both breeding and non-breeding periods. During the breeding season, temperature increased as did eDNA concentration, while during the post-breeding season (late summer and autumn) both eDNA and temperature decreased. This suggests that the seasonal activity of newts outweighs any influence temperature has on DNA degradation. High levels of rainfall would potentially dilute ponds thereby reducing eDNA concentration. However, we found that rainfall had no influence on eDNA concentration in our system. Although UV has been found to influence DNA (Ravanat et al., 2001), its impact on degradation rates appears to be variable (Dick et al., 2010; Green et al., 2011; Bae & Wuertz, 2009, 2012; Waits & Paetkau, 2005; Pilliod et al., 2014). In the present study the correlation of UV with other potential environmental predictors means that separating its precise effects is confounded.

During the breeding season newts expend energy in courtship and reproduction, releasing pheromones (Janssenswillen & Bossuyt, 2016; Treer et al., 2013), spermatophores and eggs into the environment, all potentially directly or indirectly releasing DNA with them. The release of these products into the environment will not only lead to an increase in eDNA but it will reduce the mass of an individual and lead to a reduction in body condition. We observed a fall in both male and female body condition through the breeding season both of which were shown to be a significant influence on eDNA concentration. Reductions in male body condition were not as pronounced as for females and are likely to come from the release of spermatophores and expenditure of energy during courtship. The greater decline in female body

condition and influence on eDNA over that from males is likely to be related to the greater loss of body mass due to egg production and laying.

Great crested newt females lay between 200 and 400 eggs per year (Griffiths, 1996), which take between 15 and 20 days to develop (Griffiths et al., 1994). However, this species suffers from a development arrest syndrome, with a chromosomal abnormality causing 50% of eggs to abort during the first two weeks of development (Horner & Macgregor, 1985). As a result, this mortality is likely to release a large amount of eDNA into the water as eggs decompose. As egg production, egg abortion and hatching would be difficult to measure without destructive sampling, we believe that female body condition was a proxy measure for egg laying.

Can eDNA concentration be used as an index of relative abundance of target organisms rather than just presence or absence? Our analyses – which provide a more accurate estimate of adult and larval numbers than widely used visual count or trap-based survey methods – demonstrate that factors other than newt abundance influence the amount of eDNA present seasonally. Using eDNA to map population trends would therefore be problematical, although a relative abundance estimate between similar ponds, sampled concurrently under the same environmental conditions may be possible. Current traditional count-based population assessments from visual or trapping surveys for amphibians or other aquatic organisms suffer from the same issues, as detection rates may have poorly understood relationships to total population sizes and vary according to environmental conditions (Griffiths et al., 2015). For stream fish, predictive models incorporating eDNA concentration are developing to identify detection probabilities, abundance, as well as eDNA production and discharge (Wilcox et al., 2016). To apply this to population assessments of lentic, semi-aquatic amphibians, and models would need to include seasonally variable DNA release and degradation rates, as well as taking into account multiple life stages. As these relationships become clearer, the role of eDNA in assessing populations is

likely to become an increasingly valuable and cost-effective tool in assessing and mitigating the challenging problem of global amphibian declines.

5.6. Acknowledgements

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5.7. Chapter 5 - Supplementary Information

Extraction protocols

The glass-microfiber filters were extracted in a fume hood, sterilised with a 10% bleach solution and UV-light the filter paper was removed from the sealed syringe filter holder using sterilised wire cutters and sterilised forceps. Once removed the filters were cut into strips approximately 3 mm in width with each filter placed length ways into a separate 1.5 mL microcentrifuge tube. As a result for the digestion step each sample consisted of two microcentrifuge tubes, one for each of the two filters. 675 µL of the ATL buffer from the DNeasy® Blood & Tissue kit was added to each tube; it was then vortexed for 15 seconds to mix before 20 µL of Pro K was added and again vortexed. The samples were then incubated on a rotating block, for 3 hours at 56 °C or overnight at 37 °C. Following incubation the liquid was then transferred to a fresh microcentrifuge tube; the two digestion reactions for a sample were combined at this stage. 200 µL of AL buffer and 200 µL of ice cold absolute ethanol was added to each tube and vortexed for 15 seconds to facilitate DNA precipitation. 650 µL of the extraction solution was transferred into a DNeasy® Blood and Tissue kit Mini spin column and centrifuged at 8000rpm for one minute with the flow through discarded. This was then repeated using the same mini spin column until the entire sample had been passed through. DNA extraction continued as per the DNeasy® Blood and Tissue kit manufacturers' protocol, eluting into 200 µL of the elution buffer.

Extraction from ethanol precipitation samples was undertaken using a modified protocol from Biggs et al. (2015). The mixture was centrifuged at 10,020 g, (8500 rpm) for 35 minutes and the supernatant discarded. The remainder of the extraction protocol was conducted as per Biggs et al. (2015).

Chapter 6 - How many newts are there? Towards assessing abundance from environmental DNA samples

This Chapter is In Prep for submission to the scientific literature and was conducted at the NERC Biomolecular Analysis Facility at the University of Sheffield.

Running title: Newt population size estimates from aquatic eDNA

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Key words –Population size estimates; Environmental DNA (eDNA); Microsatellite; Allele frequencies; *Triturus cristatus;* Great crested newt; Pond water sampling

6.1. Abstract

Ecological surveys frequently focus on the presence/absence of a species. However, a measure of population size can increase the value of data, allowing the monitoring of species trends. Most rapid methods for monitoring the presence of amphibians involve visual surveys, and simple counts, but the numbers observed often have little relationship with population size. Environmental DNA (eDNA) is an emerging survey method that can be used to determine species presence from an environmental sample, such as water or sediment. However, the method has not yet been developed to measure population sizes. We explore the use of microsatellite allele frequencies and the probability of an observed array of alleles occurring, to predict the number of individuals contributing to a mixed sample of DNA. We use a real population of great crested newts (*Triturus cristatus*) to simulate mixed samples of different numbers of individuals. We observed that the population estimate produced a mean overestimation of less than 2%. However, attempts to test the technical feasibility of this by amplifying great crested newt microsatellite alleles from eDNA samples were met with limited success, due to low concentrations of genomic DNA within environmental samples. This extension is widely applicable to circumstances where a non-invasive mixed genetic sample can be collected, but where the individuals that contributed to it cannot be separated. We conclude eDNA techniques have the potential to accurately identify the number of individuals present. However, to be effectively applied, additional development of methodologies is required in order to increase the reliability of the extraction of microsatellites from environmental samples.

6.2. Introduction

Identifying DNA in samples collected from the environment is an increasingly popular non-invasive sampling strategy. Most studies using environmental DNA (eDNA) seek to determine the presence of a particular species, with few attempting to quantify the abundance of the target species. However, some studies have drawn relationships between eDNA concentration and population size in natural environments (Takahara et al., 2012; Doi et al., 2017; Lacoursière-Roussel et al., 2016a; Stoeckle et al., 2017a). Consequently, the method is widely used to assess distribution (Deiner & Altermatt, 2014; Eichmiller et al., 2014; Foote et al., 2012; Fukumoto et al., 2015; Jerde et al., 2011; Olson et al., 2012; Takahara et al., 2013), but needs to advance from species detection to analysis of abundance to reach its full potential (Sigsgaard et al., 2016; Corse et al., 2017).

Although occupancy data is valuable to ecologists, and can be used for setting or monitoring conservation goals, a measure of population size can give additional information about the status of a species (Bohmann et al., 2014). At present most rapid methods for assessing population size of amphibians are based on simple counts that bear little relationship to population size (Griffiths et al., 2015). To generate a reliable estimate of population size, extensive and labour-intensive capture-mark-recapture studies are required, which are often only practical on a small scale. Owing to the utility of eDNA methods with large scale monitoring projects (Biggs et al., 2015), the ability to generate a reliable estimate of the number of individuals which contribute to a sample of DNA (mixed sample of DNA), collected from the environment, would be a considerable benefit to biodiversity assessment, while remaining logistically feasible. This may allow quantification of increases and decreases in population size of protected or rare taxa, or allow the quantification of removal effort in relation to introduced or invasive species.

Relationships between aquatic eDNA concentration and the number of individuals of a species present have been identified (Takahara et al., 2012). However; Chapter 5 shows that although the concentration of eDNA is influenced by population size, other environmental factors have an overriding effect. As a result, a comparison of relative abundance between two similar water bodies (i.e. waterbody 'A' has greater abundance than water body 'B') sampled at the same time using eDNA concentration may be possible. However, this in itself would not allow accurate comparisons to be made over time or between waterbodies that vary in their characteristics (Chapter 5; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016). For the full utility of eDNA to be reached with an assessment of population size, the estimate needs to be independent of the environmental conditions, time of year and waterbody characteristics.

Mitochondrial DNA (mtDNA) is usually targeted within eDNA studies because it has a much higher copy number in each cell compared to genomic DNA (Alberts et al., 2002; Minamoto et al., 2017). However, mtDNA is maternally inherited with males and females possessing the same sequence as their mother (Rubinoff, 2006). The regions selected for amplification also typically target coding regions that are conserved, consequently mitochondrial DNA sequences are likely to show little variation between individuals within a species. This feature makes mtDNA ideal for taxonomic assessment (Rubinoff, 2006), but is limited in its utility for identifying individuals, although this was attempted by Corse et al. (2017) and Sigsgaard et al. (2016). Genomic DNA, is predominantly used for population genetics studies at the individual level using either single nucleotide polymorphisms (SNPs; Hajibabaei et al., 2007) or short tandem repeat (STR) variation identified through microsatellite genotyping (Aziz et al., 2017). Microsatellites are regions of genomic DNA occurring in non-coding regions, which contain short nucleotide motifs tandemly repeated. For each microsatellite locus the number of repeats in each individual is hypervariable within and between individuals in a population. The number of repeat motifs making

up these repeat regions differ, resulting in alleles of different lengths (Russell, 2006). Diploid organisms contain two copies of DNA at each locus, one inherited from each parent, and these may be the same (homozygote) or different (heterozygote) in length (Russell, 2006). The combination of alleles at different loci found within an individual (genotype) forms a core component of population genetics (Russell, 2006). Allele frequencies are the relative frequency of a specific allele at a particular locus within a population or species (Russell, 2006).

Microsatellites are routinely used for population assessments where genotypes are used to identify individuals and allow mark-recapture analysis to be undertaken. DNA is often collected directly from individuals through tissue samples (Dufresnes et al., 2016; Jehle et al., 2001) or swabs (Broquet et al., 2007; Dufresnes et al., 2016). However, non-invasive sampling is becoming more common with isolation and amplification of microsatellites from DNA that has been extracted from scat samples (Aziz et al., 2017), shed hair (Sawaya et al., 2012), and other sources (Waits & Paetkau, 2005). With all of these methods DNA can be isolated from single individuals, whereas many environmental samples contain DNA from a mixture of individuals. It is therefore not possible to separate which alleles originate from which individual and so individual genotypes cannot be constructed, rendering a conventional approach to population genetics impossible.

Although the most common technology used for microsatellite genotyping is capillary electrophoresis, there are drawbacks to this and new technologies such as high-throughput sequencing may offer advantages (Gan et al., 2015; Salipante et al., 2014). High-throughput sequencing produces the exact sequences, allowing unambiguous allele identification, and the parallel sequencing abilities of the technique offer advantages in processing capacity (Salipante et al., 2014; De Barba et al., 2017; Suez et al., 2016; Vartia et al., 2016). Additionally high-throughput sequencing has the potential to increase accuracy, sensitivity, efficiency and

standardization of microsatellite genotyping (Salipante et al., 2014; De Barba et al., 2017; Vartia et al., 2016; Zavodna et al., 2014). High-throughput sequencing has been demonstrated with ecology, evolution and conservation applications for the assignment of genotyping of brown bear (*Ursus arctos;* De Barba et al., 2017), warbling finches (*Poospiza* spp.; Raposo do Amaral et al., 2015), fruit flies (*Drosophila melanogaster;* Suez et al., 2016), red deer (*Cervus elaphus;* Suez et al., 2016), Atlantic cod (*Gadus morhua;* Vartia et al., 2016), and guppy (*Poecilia reticulata;* Zhan et al., 2016). The use of high-throughput sequencing will improve the reliability of genotyping from low quality or degraded DNA often relied upon within ecology, when samples are collected from the environment or from samples of faecal material (De Barba et al., 2017). Programs have started to be constructed to allow for the processing of high-throughput sequencing reads for genotyping such as MEGASAT (Zhan et al., 2016).

Studies analysing mixed samples of genomic DNA from multiple individuals have used microsatellites and allele frequencies within a population to observe polyandry within insects (Bretman & Tregenza, 2005; Demont et al., 2011; Bussière et al., 2010). Analysis of sperm stores produces an estimate of the number of males a female has mated with. This approach uses the probability of the observed array of alleles, found within a sample of mixed DNA, occurring based on a known allele frequency from the wider population of the target species. This approach allows the most likely number of individuals to be estimated. The use of mixed sample analysis with eDNA would require the isolation of genomic DNA and amplification of microsatellite loci from eDNA samples; which has been attempted, but so far with only limited success (Barnes & Turner, 2016).

As far as the authors are aware mixed sample analysis has only been demonstrated using a single locus at a time (Bretman & Tregenza, 2005; Bussière et al., 2010; Demont et al., 2011). This is appropriate if the expected number of individuals is small

or the microsatellite locus is highly variable within a population. However, where populations are larger or the microsatellite loci have low variation, the use of a single marker would limit the accuracy of estimating population size and could indicate an infinite population size with a relatively small number of individuals. Prior to any attempts at isolating microsatellite loci from eDNA samples we assessed the applicability of the mixed sample analysis approach with larger numbers of individuals that may represent the individuals contributing to an eDNA sample. We initially amend the previously used approach (Bretman & Tregenza, 2005; Bussière et al., 2010; Demont et al., 2011) to allow multiple microsatellite loci to be included within the analysis. We then use simulation models to predict population size with a degree of accuracy acceptable for most ecological assessment. We use allele frequencies from a real population of great crested newts (*Triturus cristatus*), a species widely used in the development and testing of eDNA (Thomsen et al., 2012b; Rees et al., 2014a; Biggs et al., 2015; Harper et al., 2018), to generate the predictions. Finally we make attempts to amplify microsatellite loci from eDNA samples.

6.3. Methods

6.3.1. Study population

The study population of great crested newts inhabit a network of eight identical artificial ponds arranged in a grid pattern, located within the campus of the University of Kent, Canterbury in south-eastern United Kingdom. The ponds are lined with PVC, 2m by 1m and 0.6m deep at one end in a wedge shape giving a total volume of approximately 600 L each. The distance between neighbouring ponds is 2 - 3 m. The ponds had been in place for a minimum of six years at the time the project was undertaken (Lewis, 2012; Matechou & Caron, 2017). However the PVC liners for all eight ponds were replaced in December 2014, and refilled with tap water, prior to the start of the eDNA project. The ponds are naturally colonised and no species have been deliberately introduced, all individuals are free to enter and leave the population

and to move between the eight ponds. However, it is thought that there is relatively low dispersal of adults between this population and others in the area, due to anthropogenic barriers. The colonisation, population and seasonal dynamics of great crested newts that utilise this pond network has been subject to a long-term capture-mark-recapture study with a very high individual capture rate each year (Matechou & Caron, 2017). Each year the site is surveyed weekly from March to July using a bottle trapping capture methodology (Chapter 5; Griffiths, 1985). In the year of the study (2015), 49 separate individuals were caught, this compares to 57 (95% confidence – 53 - 60 individuals) as the most likely total population generated by Program MARK analysis Chapter 5. The small size of the ponds means they exhibit much higher population densities of great crested newts than would be expected with more typical ponds occupied by the species, and therefore the eDNA concentration within the water body is likely to be high.

6.3.2. Individual genotypes and population allele frequency

During the 2015 survey season a cloacal swab was collected from every individual the first time it was captured using Thermo Scientific™ Sterilin™ Plain Swabs. The newt was held either upside down in the left hand or by a second surveyor and the swab was rolled gently into the cloacal opening. This method was simple for males due to large size of the cloacal opening, but was not always possible for small females due to small cloacal size; in these cases the body surface of the individual was also swabbed to attempt to pick up additional skin cells.

Swabs were transported to the lab and frozen at -20 ºC within 2 hours of being taken, and frozen for up to eight months before extraction. DNA extraction was undertaken using Qiagen® DNeasy® Blood and Tissue Kits. The swab tips were incubated over night at 55 ºC in 360 µL of ATL buffer and 40 µL of Proteinase K on a rotating block prior to extraction continuing as per manufacturer's instructions.

Initially 19 microsatellite markers were identified from the literature; *Tcri13, Tcri27, Tcri29, Tcri35, Tcri36, Tcri42, Tcri46* (Krupa et al., 2002a, 2002b), *TC50, TC52, TC58, TC66, TC68b, TC69, TC70, TC71, TC74, TC81* and *TC85* (Drechsler et al., 2013). For *Tcri32* two different reverse primers were quoted and we therefore tested for amplification with the two different reverse primers (Jehle et al., 2005; Krupa et al., 2002b). Fluoro-labels were added to the forward primers as per Table 6.S1. These loci were initially tested as singleplex reactions with tissue extracts from great crested newt and the other newt species native to the United Kingdom: the palmate newt (*Lissotriton helveticus*) and the smooth newt (*L. vulgaris*), as well as alpine newt (*Ichthyosaura alpestris*), the most common non-native newt within the UK.

The PCR assay consisted of 1 µL of extracted DNA, dried in the reaction vessel, 1 µL of 0.2 µM labelled PCR primers and, 1 µL of QIAGEN Multiplex PCR Master Mix, with a total reaction volume of 2 µL (following Kenta et al. (2008)). Samples were amplified using the PCR protocol 95 ºC for 15 minutes, 35 cycles of 94 ºC of 30 seconds, 60 ºC for 1 minute 30 seconds, 72 ºC for 1 minute 30 seconds and a final elongation step of 60 ºC for 30 minutes. PCR product was diluted with 20 µL of ddH2O (ultrapure), and DNA fragment analysis conducted using capillary electrophoresis on an ABI 3730 (Applied Biosystems, MA, USA), with 1 µL of diluted PCR product and 9 µL of Formamide with LIZ size standard. The DNA fragment analysis data was analysed using GENEMAPPER Version 3.7 (Applied Biosystems, MA, USA).

Each of the swab samples were analysed using the 19 loci split into five multiplex reactions (Table 6.S1), following identical PCR and DNA fragment analysis protocols to above. The genotype of each individual was assigned using GENEMAPPER Version 3.7. The frequency of each allele within the population for each microsatellite locus was generated using CERVUS version 3.0.7 (Kalinowski et al., 2007).

6.3.3. Simulation of mixed sample approach

To ascertain whether mixed sample analysis would produce reasonable estimates of population size, simulations were used to predict the alleles present if different combinations of individuals were present within a hypothetical sample. Individuals were picked at random from the known population to be included in each simulated allele array; this was done with a random number generator in R, for between one and 30 individuals, and was repeated 70 times.

The probabilistic method of mixed sample analysis first used by Bretman & Tregenza (2005) but also used by Demont et al. (2011) was followed with some modifications. Initially the probability of not observing an allele when a given number of individuals were within the sample was calculated using the equation $P_{not\ observed} = [1\text{-}f(a)^t]$, with f(a) representing allele frequency in the wider population and 't' being the number of attempts at observing the allele, which is twice the number of individuals (as great crested newts are diploid). The probability of observing an allele was then calculated using the equation $P_{observed} = 1\text{-}P_{not\ observed.}$ We then calculated the probability of observing the array of alleles within each simulated sample by multiplying together $P_{obseqrved}$ for each allele within the sample at the loci and $P_{not\ observed}$ for each allele not in the sample but found within the population. The probability of observing the observed array of alleles was calculated with 0.5 to 100 individuals, representing t=1 to 200. The value for 't' with the highest probability, indicates two times the most likely population size. This was carried out for each microsatellite locus where four or more alleles were found within the population; this led to the exclusion of loci *Tcri13, Tcri29, TC58, TC66, TC69, TC70,* and *TC81* due to low allelic diversity at these markers within the study population. Single loci analysis is limited in its effectiveness at estimating population size to populations comprising only a small number of individuals. When the number of alleles is low, the chance of identifying them all when only a small number of individuals are present is high; which restricts population size

estimation to when only small numbers of individuals are present. To allow the method to work with larger populations a high number of alleles need to be incorporated in the analysis. We multiplied the probability of observing the observed array of alleles at each locus together, before identifying the value for 't' with greatest probability, to generate the final population estimate, advancing the mixed sample analysis to operate across multiple loci and with larger numbers of individuals.

6.3.4. Amplification of microsatellites from eDNA

We identified the limit of reliable detection for each of the microsatellite loci. A 1 in 10 serial dilution of a tissue extract was then performed to identify the point at which allelic dropout began to be observed using a single PCR and the ABI3730. The analysis was undertaken using 2 µL of DNA template and the PCR protocol described above, with multiplex reactions. The concentration of extracted DNA was measured on a Life Technologies® Qubit® 2.0 Fluorometer, before the 1 in 10 dilution series was created.

We then attempted to demonstrate the utility of this by amplifying microsatellite loci from eDNA samples. Remaining eDNA sample extracts, positive for great crested newt mitochondrial DNA using qPCR, previously reported in Chapter 5, were tested with a number of different protocols. These included increasing the volume of DNA template used to 5 µL, multiple PCRs, whole genome amplification kits, biotinylated microsatellite probe magnetic bead separation, and microsatellite amplification through high-throughput sequencing on an Illumina® MiSeq® platform (supplementary information 6.1). An allele was deemed to have been amplified if a scoreable peak was identified within the expected size range for the locus in either GENEMAPPER or MEGASAT (Zhan et al., 2016). An analysis of variance (ANOVA) was conducted in R version 3.4.1 (R development core team) to assess whether the minimum and maximum allele lengths at each locus as well as the combined lengths of the PCR primers and combined flank lengths (Table 6.1) influenced isolation of

microsatellite from eDNA samples. Akaike's Information Criterion (AIC) was used to assess support for different models using package MuMIn (Bartoń, 2016). Models with a ΔAIC of ≤ 2 were considered to have substantial support (Marchetti et al., 2004).

6.3.5. Ethics

The experimental procedure was approved by the University of Kent, School of Anthropology and Conservation, Research and Ethics Committee. All disturbance to great crested newts was undertaken following standard methodologies under licence from Natural England, licence number 2014-5025-CLS-CLS and newt tissue stored under licence 2015-7591-SCI-SCI-1.

6.4. Results

6.4.1. Allele frequencies

All 19 of the microsatellites tested were found to amplify with the great crested newt DNA from tissue samples collected from four individuals. Some amplification was observed with the other species; however, this was always below the threshold for selection as an allele and outside the size range expected for the markers based on the allele sizes observed in great crested newts (Drechsler et al., 2013; Krupa et al., 2002b, 2002a; Jehle et al., 2005). Useable genotypes were recovered from 47 of the 49 great crested newt individuals within the population, one juvenile and one young female were ruled out from the analysis due to insufficient DNA recovered from the swab samples. Ninety-four alleles were identified from 19 microsatellite loci, with allele frequencies ranging from 0.01 for allele 194bp at the *Tc52* locus to 0.92 for allele 228bp at the *Tc70* locus (Table 6.S2).

6.4.2. Simulation of mixed sample approach

The simulated number of individuals compared to the expected can be seen in Figure 6.1. A simple linear regression was calculated to predict "Predicted Population Size"

based on “Expected Populaion Size”. A significant regression was found ($F(1,68)=129.6$; $p<0.0001$), with an R^2 of 0.558. The mean predicted number of individuals across all simulations was found to be only a 1.89% overestimation of the expected number. However, smaller expected values tended towards underestimation of the number of individuals, with larger expected populations yielding an overestimation of the number of individuals, demonstrated by a regression intercept of -2.791 and slope of 1.423. When five individuals were expected, the mean predicted number of individuals was an 8.0% underestimation. However, the predicted figures were still close to what was expected. When the number of individuals was below 10, the predicted number was a maximum of +/- 3.5 individuals either side of the expected, a mean underestimation of 7.3%. This ranged from a 50% underestimation when 1 or 2 individuals were expected to a 37% overestimation when 11 individuals were predicted when eight were expected. As the expected population size increased the conformity of the predicted population size reduced. When 30 individuals were expected the predicted results a range from 30 to 100 individuals, a mean overestimation of 60.3% (0% to 233% overestimation; Figure 6.1), consequently overestimation increasedwith higher expected number of individuals.

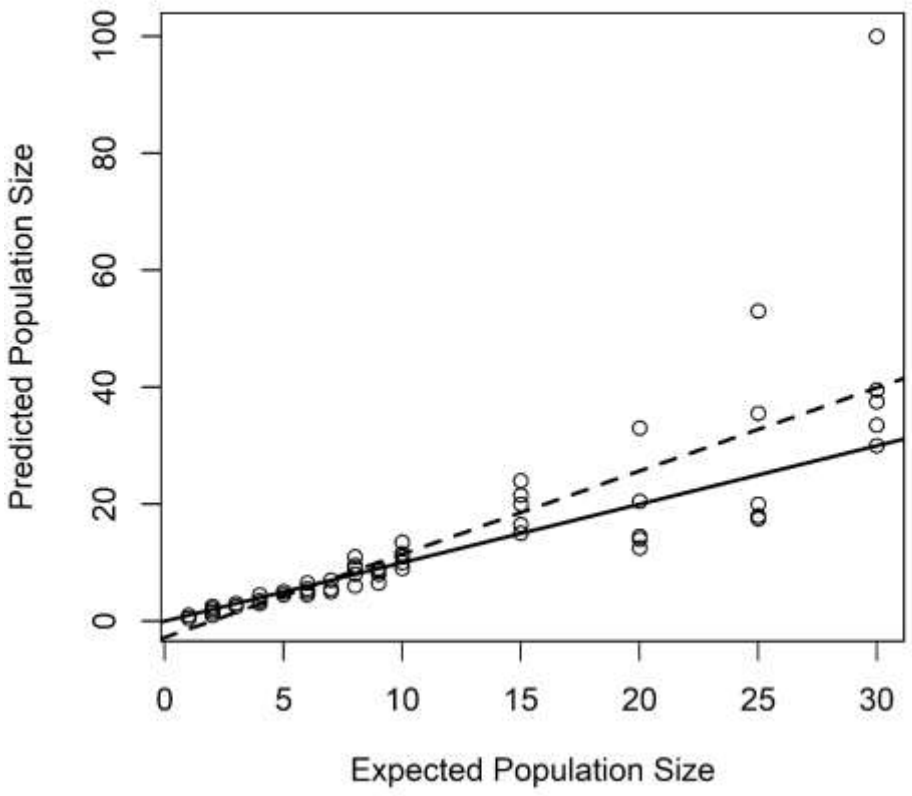

Figure 6.1: Mixed sample analysis simulations: the predicted population size (Predicted Population Size) against the number of individuals included in a simulation (Expected Population Size), based on the genotypes of individuals included within simulated samples and allele frequencies within the populaion as a whole. The solid line represents expected abundance, with the dashed line a linear regression of predicted population size based on expected population size (intercept = -2.791; slope = 1.423; R^2=0.558; F(1,68)=129.6; p<0.0001).

6.4.3. Amplification of microsatellites from eDNA

The concentration of the undiluted tissue extract was found to be 71.5 $nguL^{-1}$. The lowest point detection without dropout was achieved, varied between loci and ranged from a 1 in 100 dilution (0.715 $nguL^{-1}$) to a 1 in 10,000 dilution (0.00715 $nguL^{-1}$) with

detection at lower concentrations showing allelic dropout (Table 6.S3). The maximum concentration of DNA recovered from eDNA samples tested within this study, based on mitochondrial qPCR analysis was 0.00845 $nguL^{-1}$ (Chapter 5), which is up to two orders of magnitude lower than was detectable in the serial dilution, and so below levels identifiable with conventional PCR and DNA fragment analysis.

Due to the low concentration of eDNA, amplification of microsatellite loci was attempted using a range of techniques aiming to increase detectability (supplementary information 6.1). Using increased volumes (5µL) of template DNA and minimal post PCR dilution, followed by a second round of PCR under the same protocol, small levels of inconsistent amplification at some loci were observed. Small amounts of amplification were observed after a second PCR in loci *TC58* (1 allele observed in 1 of 2 eDNA samples)*, TC66* (1 allele observed in 1 of 2 eDNA samples)*, TC68b* (4 alleles observed in the first eDNA sample, and 3 in the second sample)*, TC71* (1 allele observed in 2 of 2 eDNA samples) and *Tcri46* (1 allele observed in 1 of 2 eDNA samples), with a third PCR adding nothing additional. Positive PCR product was tested on an Agilent Technologies 2100 BioAnalyzer Instrument following manufacturer's instructions, with high quantities of product found within the expected size ranges.

Some amplification was also observed when using Biotinylated Microsatellite Probes (BMP), using the CTTT probe at loci *Tcri13* (1 allele observed in 1 of 4 eDNA samples)*, Tcir29* (1 allele observed in 1 of 4 eDNA samples)*, Tcri35* (1 allele observed in 1 of 4 eDNA samples) and *Tcri36* (1 allele observed in 1 of 4 eDNA samples). When amplification was observed with either multiple rounds of PCR or with the use of the BMP, it was inconsistent and few alleles amplified. Inconsistent detection of single alleles is insufficient to conduct the mixed sample analysis.

We additionally attempted to amplify microsatellites from eDNA samples using high-throughput sequencing. Increased, but inconsistent, amplification was observed

when eDNA samples were analysed in this way, with low levels of amplification observed at ten loci, but, this was not sufficient to run the mixed sample analysis. The loci with most success tended to be those with smaller product sizes (Table 6.1). All other methods used to attempt to identify microsatellite alleles from eDNA yielded no results (Table 6.1).

Table 6.1 – Microsatellite amplification from eDNA using different laboratory protocols. Microsatellite loci where inconsistent levels of positive amplification have been observed are indicated (the number of eDNA samples with amplification / the number tested). Positive amplification was classed as any scoreable peak occurring within the expected size range using GENEMAPPER or MEGASAT (Zhan et al., 2016).

Locus	Allele size range (bp)	Forward primer length (bp)	Reverse primer length (bp)	5'flank (bp)	3'flank (bp)	Increased template volume (5 µL template) – ABI3730	Second PCR – ABI3730	Whole genome amplification – ABI3730	Biotinylated microsatellite probe magnetic bead separation – ABI3730	High-throughput sequencing
Tcri13	125-129	16	23	13	20	0/4	0/2	0/2	1/4	1/1
Tcri27	256-283	29	25	19	98	0/4	0/2	0/2	0/4	0/1
Tcri29	315-323	17	17	62	136	0/4	0/2	0/2	1/4	0/1
Tcri32	464-480	20	20	52	0	0/4	0/2	0/2	0/4	0/1
Tcri35	207-226	18	25	2	36	0/4	0/2	0/2	1/4	1/1
Tcri36	262-300	19	19	29	40	0/4	0/2	0/2	1/4	0/1
Tcri43	264-280	25	19	30	105	0/4	0/2	0/2	0/4	0/1
Tcri46	272-298	20	22	15	127	0/4	1/2	0/2	0/4	0/1
TC50	182-248	20	24	66	4	0/4	0/2	0/2	Not tested	0/1
TC52	190-202	20	20	68	20	0/4	0/2	0/2	Not tested	1/1
TC58	201-205	18	20	27	107	0/4	1/2	0/2	Not tested	0/1
TC66	231-239	21	22	8	110	0/4	1/2	0/2	Not tested	1/1
TC68b	179-203	23	20	33	21	0/4	2/2	0/2	Not tested	1/1
TC69	176-184	20	20	40	48	0/4	0/2	0/2	0/4	0/1
TC70	216-228	20	20	10	124	0/4	0/2	0/2	Not tested	1/1
TC71	177-187	20	20	78	24	0/4	2/2	0/2	Not tested	1/1
TC74	185-205	24	20	66	28	0/4	0/2	0/2	Not tested	1/1
TC81	131-149	21	20	50	11	0/4	0/2	0/2	Not tested	1/1
TC85	161-169	21	20	0	80	0/4	0/2	0/2	Not tested	1/1

We examined whether any loci characteristics influenced in which loci amplification was observed from eDNA samples using an ANOVA. No significant effects were found when examining the data produced with multiple rounds of PCR or BMP. However, within the eDNA samples analysed with high-throughput sequencing, the model with greatest AIC support (Table 6.2) suggested that maximum allele size had a significant negative influence on amplification ($F(1,17)=8.226$; $p=0.00468$). In the models ranked second ($\Delta AICc = 1.70$; $F(1,17)=8.226$; $p=0.0107$) and third ($\Delta AICc = 1.92$; $F(1,16)=9.081$; $p=0.00824$) minimum allele size was also found to have a significant influence on which loci amplified; however in the model ranked third maximum allele size was not significant ($F(1,16)=2.766$; $p=0.11572$; Table 6.2). All other models were found to have a $\Delta AICc > 2$.

Table 6.2 – Amplification prediction ANOVA model selection. All models showing substantial support based on ΔAIC for eDNA microsatellite amplification with high throughput sequencing.

Model parameter	Intercept	DF	Sums of Squares	Mean Sums of Squares	F-value	p-value	AICc	ΔAICc	AICc Weight
Maximum Allele Size (bp)	1.488	1,17	1.817	1.817	10.58	0.00468	25.9	0	0.504
Minimum Allele Size (bp)	1.367	1,17	1.545	1.5447	8.226	0.0107	27.6	1.7	0.216
Minimum Allele Size (bp)	1.546	1,16	1.5447	1.5447	9.081	0.00824	27.9	1.92	0.193
Maximum Allele Size (bp)		1,16	0.4706	0.4706	2.766	0.11572			
Minimum Allele Size (bp)	2.385	1,15	1.5447	1.5447	9.177	0.00845	30.2	4.25	0.06
Maximum Allele Size (bp)		1,15	0.4706	0.4706	2.796	0.11524			
Minimum Allele Size (bp) : Maximum Allele Size (bp)		1,15	0.1969	0.1969	1.17	0.29655			

6.5. Discussion

Changes in population size are often needed to inform conservation. This need is particularly true for monitoring eradication programs of invasive species or for arresting decline or increasing the population of rare or threatened species. Simple visually based counts for assessing populations are notoriously unreliable, with

detection probabilities dependent on a large number of factors (Schmidt, 2003; Griffiths et al., 2015), and varying between sites and between survey visits (Sewell et al., 2010).

In previous studies the number of individuals contained within a mixed sample of DNA has been predicted with a reasonable degree of accuracy, to a maximum number of approximately ten individuals (Demont et al., 2011; Bretman & Tregenza, 2005). By combining multiple loci within the analysis, we demonstrate that mixed sample analysis can be used to predict the number of individuals contributing to a mixed DNA sample when the number contributing is much higher. Simulated sampling of mixed samples of DNA showed a relationship with the numbers of newts included within a mixed sample, although on average an overestimation of 1.89% was observed relative to the actual number. However, the overestimation was non-uniform with a mean overestimation with larger simulated sample sizes, and a mean underestimation with smaller simulated sample sizes. Other forms of population size modelling such as capture-mark-recapture have wide confidence intervals (Ward et al., 2017), and within amphibians simple counts with unknown confidence intervals are often used (Lewis et al., 2017; Griffiths et al., 2015). The method presented here may therefore be more accurate in showing general changes in a population, and prove more informative for conservation decision making, than currently used counting methods. The deviation of the predicted population size from the actual population size is likely to be of little consequence to practitioners, given the constraints of the methods widely used at present.

Our simulations indicate a general trend towards an overestimate of population size, particularly when over 50% of the population used to generate the allele frequencies was included in the simulated sample. Our simulation is likely to have been restricted by the study population chosen being relatively small, comprising genotypes from only 47 individuals. Compared to previously published data for these microsatellite

markers, allelic diversity in this study was relatively low, with the exception of *TC85* locus, where five alleles were identified compared to three reported previously (Table 6.S1; Drechsler et al., 2013). The reduced diversity observed over that previously published is not unexpected given that all individuals sampled were from one relatively isolated population. If the allele frequencies were generated from a larger number of individuals with a lower level of relatedness, and if the allele arrays used to simulate the mixed samples came from individuals not included in the allele frequency analysis, the overestimations of predicted number of individuals may have been reduced.

Although the amplification of genomic DNA is not yet reliable, from the serial dilution of tissue we demonstrate that successful amplification of microsatellites from eDNA samples requires DNA extract concentrations over 100 times higher than the concentrations at which eDNA samples are routinely encountered. It is therefore not surprising that we were unable to achieve consistent microsatellite amplification from eDNA using any of the measures put in place to improve amplification. Although we did demonstrate some amplification of microsatellites from eDNA, we were unable to get consistently reliable amplification: this has also been found elsewhere (Barnes & Turner, 2016). However, as we were conducting tests on pre-existing eDNA samples we were making no attempts to alter extraction methods to boost the concentration of DNA present, and this may be the stage which needs to be improved to retain more high quality DNA. eDNA collection, concentration and extraction methods from water are under constant review (Renshaw et al., 2015; Turner et al., 2014b; Deiner et al., 2015; Spens et al., 2016). With further development of the methodologies, the amplification of genomic DNA from eDNA will become possible in the near future. We encourage work on methodologies to facilitate improved eDNA recovery, this could be done through the use of different eDNA collection methods as methods such as syringe filtration may offer advantages over ethanol precipitation (Spens et al., 2016). Additionally, the data recovered from the high-throughput sequencing suggests that

shorter markers have greater success in amplifying than longer markers, this is likely to relate to the PCR amplification process as marker length can influence amplification efficiency (Toouli et al., 2000). We recommend that primer sequences are redesigned or new loci identified to allow the shortest amplicon size possible. We also recommend that high-throughput sequencing is explored further as it seems to offer advantages over capillary electrophoresis in terms of sensitivity.

For mixed sample analysis to work with aquatic eDNA sampling, amplification of microsatellites from eDNA will need to be reliable enough to be undertaken with only low levels of allelic dropout. The mixed sample analysis makes the assumption that there is no allelic dropout, a situation which is highly unlikely with eDNA. Allelic dropout would bias results towards an underestimation of population size. However, as our simulations suggest a general trend towards an overestimation of population size, particularly when the numbers were approaching a higher proportion of the total population used to generate the allele frequencies, low levels of allelic dropout may not constitute a major problem.

One drawback to the method presented here is that knowledge of allele frequencies is required for the target species or preferably for the population. The preparation of these data can be time consuming and costly; however, it could be made more cost-effective and rapid through the analysis of pooled DNA samples. Genotyping of pooled DNA samples has been used to find allele frequencies through quantitative microsatellite genotyping (Khatib et al., 1994; LeDuc et al., 1995; Schnack et al., 2004). This approach has considerable cost savings compared to genotyping individuals at thousands of genetic markers (Daniels et al., 1998; Breen et al., 1999; Collins et al., 2000; Schnack et al., 2004) and would be applicable in identifying baseline allele frequencies within a species or population. Quantitative microsatellite genotyping can be achieved with a variety of methods including quantitative estimation of relative band areas through densitometry of autoradiographs of the gel

electrophoresis gels (Khatib et al., 1994) or measurement of the peak height following genotyping (LeDuc et al., 1995; Schnack et al., 2004; Skalski et al., 2006), but may also be possible with high-throughput sequencing read counts when used with microsatellites. The method has been found to have a 92-97% accuracy in assessing allele frequencies with protists (Minter et al., 2015). However, it would still be a necessary to collect DNA from many individuals and pool it at the same concentration from each individual, to ascertain allele frequency, rather than attempt this from environmental samples.

Although our attempts at mixed sample analysis have focused on the emerging field of aquatic eDNA, the field of non-invasive DNA sampling promises wider scope for using mixed DNA samples to generate population size estimates. Although reliable amplification of aquatic eDNA from microsatellites was not possible in this study, advances in sample collection and extraction technologies mean this will become possible in the future. The simulations presented here provide a valuable framework to achieve this.

6.6. Acknowledgements

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6.7. Chapter 6 - Supplementary Information

6.7.1. Methods

Allele frequency

Allele information from GENEMAPPER was analysed using CERVUS (Kalinowski et al., 2007), individuals with over four loci failing to amplify were excluded from the analysis as it was considered likely they would have high levels of allelic dropout and appear to have more homozygous alleles that actually present. CERVUS was used to look for null alleles as well as homozygote and heterozygote excess among the markers. GENEPOP (Rousset, 2008) was then used to identify whether each of the markers was in Hardy-Weinberg equilibrium. Markers out of Hardy-Weinberg equilibrium were at this point excluded as were markers that failed to amplify or were found to be monomorphic within the population. The individual genotypes were analysed for relatedness, however it was considered that related individuals would exist within any population so no individuals were excluded on these grounds.

eDNA extraction

eDNA was collected from each of the eight study ponds at 2 weekly intervals through the 2015 survey season to coincide with traditional survey events. Samples collected from the end of March to the end of June were included in this analysis, which is the time period adults are predominantly in their aquatic phase.

eDNA samples were collected using precipitation in ethanol as per Biggs et al. (2015) with a number of alterations to the collection protocol due to the small nature of the ponds. A sterile 1 L plastic bottle was placed in the centre of the pond using a gloved hand, rinsed twice with pond water and the water mixed using the bottle, the bottle was then filled with surface water. Six 50 mL centrifuge tubes containing 33 mL of absolute ethanol and 1.5 mL of 3M sodium acetate solution, were topped up to the 50 mL gradation using a sterile pipette. The bottle was shaken repeatedly through the

pipetting process to ensure full homogenisation of DNA in the bottle. Samples were then stored at -20 ºC until extraction.

Samples were extracted following the protocol in Biggs et al. (2015) using Qiagen® DNeasy® Blood and Tissue Kits and amendments to the manufacturers' protocols. The protocol was followed as per Biggs et al. (2015) with the exception of initial centrifuge speed being limited to 8500 rpm in an unrefrigerated centrifuge due to availability of laboratory equipment. Extraction was conducted in a UV sterilisable work station, which was cleaned with bleach between samples to avoid contamination (Chapter 5).

All samples were initially tested for the presence of great crested newt DNA using qPCR and primer, hydrolysis probe combination targeting an 81 base pair section of the CytB gene on the mitochondrial genome, developed by Thomsen et al. (2012) and used by Biggs et al. (2015) and Rees et al. (2014). qPCR setup was conducted in a separate laboratory again in a UV sterilisable work station as represented in Chapter 5.

Amplification of microsatellites from eDNA

Amplification was attempted on known positive eDNA samples following a similar protocol to the singleplex reaction outlined for tissue extracts. An increased volume of template DNA was initially used, drying 5 µL of DNA rather than one; increasing the primer concentration from 0.2 to 0.6 µM was tried, however all failed to produce any amplification.

Multiple PCRs

PCR was performed on eDNA samples collected via ethanol precipitation, following a protocol adapted from Kenta et al. (2008), 5 µL of extracted eDNA sample was dried in a well and 2 µL of singleplex PCR primer and QIAGEN Multiplex PCR Master Mix added as before. A separate reaction was performed for each of the 19 microsatellite

loci. PCR was performed using the same protocol as before. After the completion of the first round of PCR the product was diluted with 15 µL of ddH2O and 2 µL transferred to a second PCR plate and PCR was repeated a second then third time. Alleles were detected using an Applied Biosystems ABI3730, with 1 µL of PCR product diluted with 15 µL of ddH2O, on 9 µL of Formamide and LIZ size standard. No detection was achieved with the product of the first PCR, however some product was visible in 5 of the markers of the second PCR, and no additional markers amplified in the 3rd PCR however detection was stronger.

Whole Genome Amplification kit

eDNA extracts were concentrated in a vacuum concentrator, decreasing their volume to approximately 40 µL. LGC Genomics Whole Genome Amplification Kits (WGA; KBS-1003-001) were used in an attempt to enhance the starting concentration of DNA as per manufacturer's instructions. Amplification was conducted in a 0.2 mL PCR tube containing 2.2 µL of Buffer B (concentrated) solution, 0.6 µL of 50mM $MgCl_2$, 1.3 µL dNTP Mix, 1.1 µL primer Mix and 1.1 µL KlearTaq Polymerase. The volume was made up to 25 µL with the eDNA extract that had been concentrated in the vacuum concentrator. The tube was sealed to prevent evaporation. The samples were then placed in a PCR thermocycler under the following conditions: 94 ºC for 15 minutes, 10 cycles of 94 ºC for 1 minute, 37 ºC for 2 minutes, and 55 ºC for 4 minutes, then 36 cycles of 94 ºC for 1 minute, then 37 ºC to 55 ºC increasing from 37ºC by 0.5ºC each cycle for 2 to 11 minutes increasing from 2 minutes by 15 seconds each cycle and 4 minutes at 55 ºC, with a final step at 94ºC for 30 minutes[1]. Following WGA, a double

[1] This PCR protocol takes approximately 10 hours to complete.

PCR was conducted under the same protocols as for the multiple PCR's, visualising microsatellite products on the ABI 3730.

Biotinylated Microsatellite Probe magnetic bead separation

Biotinylated Microsatellite Probe (BMP) magnetic bead separation isolates just sections of DNA with the repeat motif of interest, other DNA and inhibitors within a sample can then be washed and removed from the target DNA. This process involves attaching target DNA to magnetic beads to be able to isolate only repeat motifs of interest. Biotinylated bead separation was undertaken on eDNA samples collected via ethanol precipitation following a protocol outlined in Zimmer & Roalson (2005).

Hybridization

Initially within a 0.2 mL PCR tube 15 µL of target eDNA sample was placed with 10 µL of biotinylated microsatellite probe at 1 uM concentration (initially CTTT and GATA repeats were trialled given their availability in the lab), and 25 µL of 2 x Hyb solution (consisting of 12 x SCC, 0.2% SDS). This was placed in a thermal cycler under conditions of 95ºC for 5 minutes, then 99 cycles ramping down from 70 ºC to 50.2 ºC at 5 second intervals. 50 ºC was held for 10 minutes then 20 cycles ramping down from 50 ºC to 40 ºC every 5 seconds. The temperature was then dropped to 15 ºC and held for 30 minutes. This process attached the target DNA to the biotinylated microsatellite probes with the complementary motif. This was done with both eDNA extract and eDNA extract that had been reduced in volume by 50% in the vacuum concentrator.

Enrichment capture

Dynabeads M280 (Invitrogen) were repeatedly washed in TE buffer, then twice with 1x Hyb Solution and were re-suspended in 150 uL of 1x Hyb Solution. The DNA probe

mix was added to the Dynabeads and incubated on a sideways orbital shaker for over 30 minutes to allow the target DNA loaded probes to attach to the magnetic beads. The beads loaded with DNA containing the target repeat motifs were captured using a magnetic particle concentration. The supernatant containing non-target DNA and non-magnetic inhibitors was discarded and the beads suspended in a 2 x SSC, 0.1% SDS solution. Washing of the beads was conducted once more using "2 x SSC, 0.1% SDS, twice with cool and twice with warm (40ºC, approximately 5-10ºC lower than the Tm for the Oligo mix used) 1 x SSC, 0.1 SDS, each time with the supernatant discarded. Following this the magnetic beads were incubated at 95 ºC for 5 minutes in 200 µL of TLE buffer, the TLE buffer steps the DNA from the magnetic beads suspending it in a solution, the heated tubes were placed in magnetic particle concentration and supernatant containing the target DNA was removed and retained. The DNA within the TLE buffer solution was precipitated out of solution using NaOAc/EDTA solution [2] with 95% ethanol, incubated at -80 ºC for 10 minutes. The DNA was pelleted by centrifuging at full speed for 10 minutes, the supernatant was then discarded and pellet washed with 70% ethanol. The supernatant was removed and the pellet air dried before being suspended in 25 µL of TLE. Following biotinylated probe microsatellite magnetic bead separation, a double PCR was conducted under the same protocols as for the multiple PCR's, visualising microsatellite products via capillary electrophoresis.

High-throughput sequencing

eDNA samples were vacuum concentrated by two, in an attempt to increase the concentration of target DNA within the sample, one of the samples was then treated

[2] NaOAc/EDTA Solution: To a 50 mL conical, make 20 ml of 3M NaOAc from the dry chemical stock. Do not adjust the pH. Add 20 mL of 500 mM EDTA, pH 8.0. This makes a solution that is 1.5 M NaOAc and 250 mM EDTA (Zimmer & Roalson, 2005).

with the WGA kit as described above. In addition DNA from 5 of the swab extracts were combined to be taken forward for analysis on the Illumina® MiSeq with DNA from one great crested newt tissue extract. Samples were amplified as before using unlabelled primers and then prepared for sequencing using the NEBNext library prep kit for Illumina, before being run on the Illumina® MiSeq. To identify microsatellite alleles from the high-throughput sequencing data, the program MEGASAT (Zhan et al., 2016) was used to analyse the sequence data.

6.7.2. Tables

Table 6.S1. - Characterisation of the full set of 19 microsatellite loci for *Triturus cristatus* used within this study. Table adapted from Drechsler et al. (2013). *Reverse primer for *Tcri32* taken from Krupa et al. (2002a, 2002b) as reverse primer reported in Jehle et al. (2005) failed to produce amplification.

Locus	Primer sequence (5'-3')	Repeat motif	Observed allele sizes (bp)	Number of alleles reported by others	Number of alleles found in the study population (n=47)	Fluoro label	Multiplex	Marker reference	GenBank sequence accession number
Tcri13	F: GTGATGGTTGCCAAGC R:GATCCAAGACACAGAATATTTAG	(GT)36 Interrupted	125-129	10 (Drechsler et al., 2013)	3	6FAM	TcMP1	Krupa et al. (2002b)	AJ292500
Tcri27	F:GATCCACTATAGTGAAAATAAATAATAAG R:CAAGTTAGTATATGATATGCCTTTG	(GAAA)27	256-283	18 (Drechsler et al., 2013)	7	NED	TcMP1	Krupa et al. (2002b)	AJ292517
Tcri29	F: CGAGTTGCCCAGACAAG R: GATCACATGCCCATGGA	(TTTC)22 (CA)11	315-323	11 (Drechsler et al., 2013)	2	NED	TcMP1	Krupa et al. (2002b)	AJ292505
Tcri32	F: GAAACTCGTAATCCAGCCCTAA R: CAAGCCTCTTGCCTTTGAGT*	(TTTC)28 Interrupted	464-480	7 (Krupa et al., 2002b)	5	6FAM	TcMP1	Krupa et al. (2002b)	AJ292487
Tcri35	F: CCAACTGGTATGGCATTG R:GATCACAGAAACTCTGAATATAAGC	(GAAA)32 Interrupted	207-226	10 (Drechsler et al., 2013)	6	NED	TcMP4	Krupa et al. (2002b)	AJ292490
Tcri36	F: GATCATCTGAATCCCTCTG R: ATACATTCATGACGTTTGG	(GAAA)36 Interrupted	262-300	24 (Drechsler et al., 2013)	6	VIC	TcMP1	Krupa et al. (2002b)	AJ292491
Tcri43[3]	F:GAAGTAACTGAAAGATAACATGTAG R: GTTTC-TATTCATTTTTGTTACGCAC	(GAAA)30	264-280	9 (Krupa et al., 2002b)	5	6FAM	TcMP4	Krupa et al. (2002b)	AJ292511
Tcri46[4]	F: CAAGTTTCCTCTGAAGCCAG R:GTTTC-TTGCCTGACAAAGTAATGCTTC	(TTTC)23	272-298	15 (Drechsler et al., 2013)	9	PET	TcMP4	Krupa et al. (2002b)	AJ292494

[3] Pigtails were added following Brownstein, Carpten, & Smith (1996)
[4] Pigtails were added following Brownstein et al. (1996)

Locus	Primer sequence (5'-3')	Repeat motif	Observed allele sizes (bp)	Number of alleles reported by others	Number of alleles found in the study population (n=47)	Fluoro label	Multiplex	Marker reference	GenBank sequence accession number
TC50	F: GCGGATACATGGTCTTCGTT R:TTCAGTTAAAAGTGTCCTCTGTGG	(ACTC)18	182-248	26 (Drechsler et al., 2013)	10	PET	TcMP3	Drechsler et al. (2013)	KF442195
TC52	F: GGCTCTTCGACTGAATGGAG R: CGGTCAATTGGTTGTAGCAG	(ATTG)17	190-202	6 (Drechsler et al., 2013)	4	VIC	TcMP4	Drechsler et al. (2013)	KF442196
TC58	F: ACAGGCAGTGCGAAAGAAAG R: CTGACCCAAGACCACCTCTC	(AATC)7	201-205	2 (Drechsler et al., 2013)	2	NED	TcMP2	Drechsler et al. (2013)	KF442201
TC66	F: CCTTTGTACACCACTGGCAAA R: TGGTCCTATAAAGCCATCTTGG	(ATCC)18	231-239	8 (Drechsler et al., 2013)	3	6FAM	TcMP5	Drechsler et al. (2013)	KF442197
TC68b	F: AAAGTGCACTCTTTCTCTGAAGC R: TGCAAAGTGCATGTGTGACT	(ATCC)24	179-203	13 (Drechsler et al., 2013)	10	6FAM	TcMP2	Drechsler et al. (2013)	KF442198
TC69	F: AGGTAGCCTTCCGCCACTAT R: GCTTGATCCTGGCATGAAAT	(AGAT)13	176-184	6 (Drechsler et al., 2013)	3	NED	TcMP3	Drechsler et al. (2013)	KF442202
TC70	F: GGGTTGCAAAGCACCTTAAT R: TACCTGGGTCCTCCTCCAAG	(ACAT)14	216-228	6 (Drechsler et al., 2013)	2	VIC	TcMP3	Drechsler et al. (2013)	KF442199
TC71	F: CCGCCAATCAGCAATATTTA R: AGTGGAAGCACCTGCTGAAG	(ACAT)11	177-187	5 (Drechsler et al., 2013)	4	PET	TcMP1	Drechsler et al. (2013)	KF442203
TC74	F:TCTGTGACATGTCCTGATAGTGAA R: TAGCACCATGAGACCCTCAC	(AATC)13	185-205	9 (Drechsler et al., 2013)	5	6FAM	TcMP3	Drechsler et al. (2013)	KF442204
TC81	F: TTTAGTCTCTCCGCTCTGCAA R: AGCGGAATCTGCCTTATGGT	(AATC)13	131-149	10 (Drechsler et al., 2013)	3	VIC	TcMP5	Drechsler et al. (2013)	KF442200
TC85	F: GTTAGACCTCGCATCTGTTGG R: CCTCAAGACCTGGCTCTACG	(AATC)11	161-169	3 (Drechsler et al., 2013)	5	VIC	TcMP2	Drechsler et al. (2013)	KF442205

Table 6.S2. – Great crested newt allele frequencies from capillary electrophoresis found within the study population of 47 individuals

Microsatellite locus	Allele size (bp)	Allele frequency
Tcri13	125	0.80
	127	0.18
	129	0.02
Tcri27	256	0.06
	260	0.29
	264	0.03
	268	0.05
	275	0.45
	279	0.06
	283	0.06
Tcri29	315	0.12
	323	0.88
Tcri32	464	0.18
	467	0.05
	471	0.63
	476	0.07
	480	0.07
Tcri35	207	0.25
	211	0.24
	214	0.05
	218	0.06
	222	0.21
	226	0.19
Tcri36	262	0.17
	285	0.11
	289	0.37
	293	0.11
	297	0.22

Microsatellite locus	Allele size (bp)	Allele frequency
	300	0.01
Tcri43	264	0.42
	268	0.08
	272	0.26
	276	0.17
	280	0.07
Tcri46	272	0.07
	278	0.09
	282	0.10
	286	0.10
	290	0.31
	292	0.15
	294	0.15
	296	0.01
	298	0.01
TC50	182	0.11
	198	0.02
	201	0.15
	205	0.01
	213	0.12
	217	0.19
	221	0.14
	228	0.06
	240	0.13
	248	0.06
TC52	190	0.03
	194	0.01
	198	0.82
	202	0.14
TC58	201	0.51
	205	0.49

Microsatellite locus	Allele size (bp)	Allele frequency
TC66	231	0.01
	235	0.52
	239	0.47
TC68b	179	0.10
	181	0.01
	183	0.13
	185	0.02
	187	0.10
	189	0.01
	191	0.27
	199	0.12
	201	0.02
	203	0.21
TC69	176	0.08
	180	0.59
	184	0.34
TC70	216	0.08
	228	0.92
TC71	177	0.02
	179	0.08
	183	0.76
	187	0.14
TC74	185	0.27
	189	0.30
	193	0.15
	201	0.08
	205	0.18
TC81	131	0.34
	135	0.60
	149	0.07

Microsatellite locus	Allele size (bp)	Allele frequency
TC85	161	0.33
	163	0.01
	165	0.82
	167	0.01
	169	0.13

Table 6.S3 – Minimum concentration at which alleles were amplified for each microsatellite loci used within this study without allelic dropout (DNA was extracted from great crested newt tissue samples). The total undiluted concentration of the original sample based on Qubit 2.0 analysis using the High Sensitivity DNA kit was 71.5 ngµL^{-1}.

Locus	Observed allele sizes (bp)	Minimum dilution at which the locus amplified without any allelic dropout
Tcri13	125-129	1/10000
TC81	131-149	1/100
TC85	161-169	1/100
TC69	176-184	1/100
TC71	177-187	1/1000
TC68b	179-203	Failed
TC50	182-248	1/100
TC74	185-205	1/100
TC52	190-202	1/1000
TC58	201-205	1/1000
Tcri35	207-226	1/1000
TC70	216-228	1/10000
TC66	231-239	1/1000
Tcri27	256-283	Failed
Tcri36	262-300	1/1000
Tcri43	264-280	1/1000
Tcri46	272-298	1/100
Tcri29	315-323	1/10000
Tcri32	464-480	Failed

Chapter 7 - Discussion

Since the commencement of this project the volume of research into eDNA has expanded dramatically. A Google Scholar search for the phrase “environmental DNA” in the title of articles increased from just 12 results in 2005 to 40 in 2014 the year this project started, increasing to 157 results for the year 2017 (Figure 7.1). This may not necessarily only capture studies on animals and plants and not all articles on eDNA include the exact phrase in the title. However, the increase in number of publications annually gives an indication of the uptake and development of the method, since the proof of concept (Martellini et al., 2005) and over the course of this project. As a result this PhD has been adapted as new research has been undertaken by other research groups.

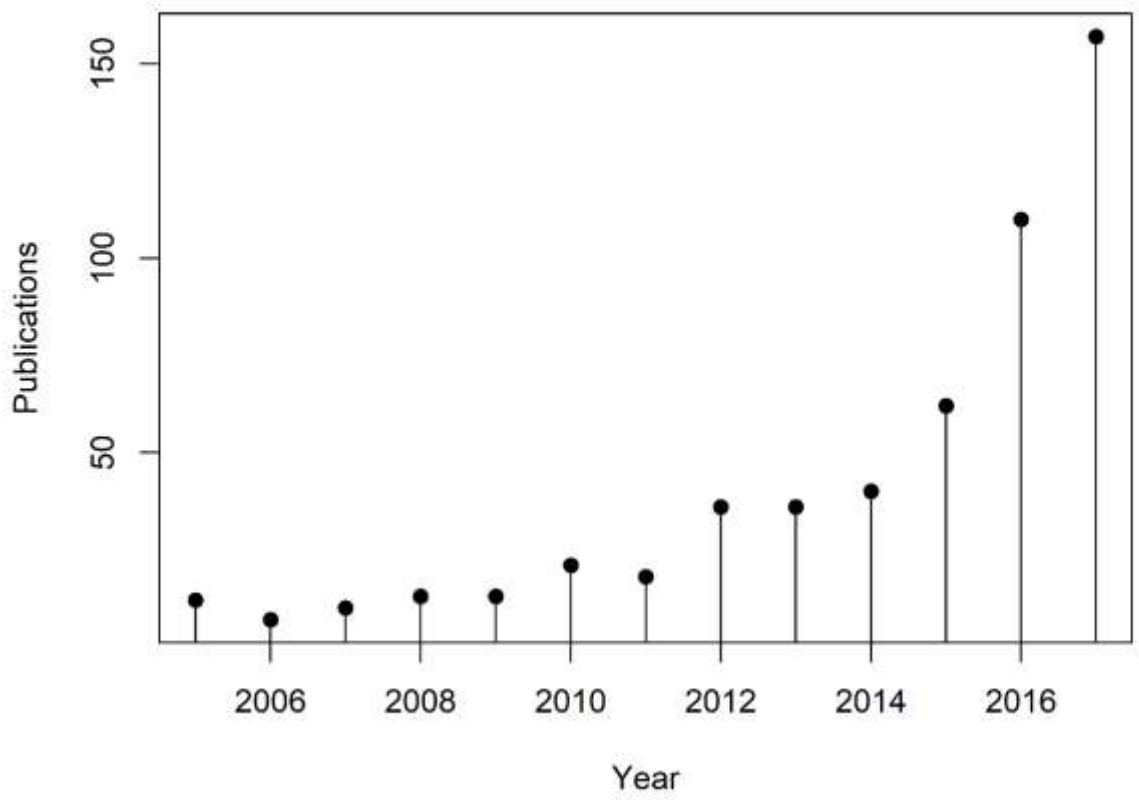

Figure 7.1 - Publications with environmental DNA in the title by year. Google Scholar search results for the term “Environmental DNA” in the title of publications from 2005 to 2017, demonstrating the increase in research into eDNA. Note that not all publications with environmental DNA as the subject contain the full phrase in the title.

The objectives outlined in the introduction aimed to advance the field of eDNA, specifically linking back to advances in the methodology sought by Natural England. These objectives included improvements of detection of small populations, estimation of population size, extending the survey window, identification of true detection rates and their covariates, and developing proficiency testing for great crested newt eDNA. All of these were also key concerns raised by practitioners at the Herpetofauna Workers Meeting workshop in February 2017 (Appendix II). We have attempted to

address each of the aims laid out in the introduction through the work we have undertaken as part of this project. The aims will be discussed individually, incorporating relevant work that has been published in parallel to this project. The exception to this is the laboratory proficiency testing where Natural England has taken a leading role; however, an initial test was completed in September 2017, involving seven commercial labs (Rees, 2017).

7.1. Assess the currently accepted commercial eDNA collection protocol for great crested newt eDNA within the UK and evaluate protocols that might recover greater amounts of eDNA.

Within this project we concentrated on methodologies that would be practical for large scale sample collection, and which would likely be collected by numerous surveyors with minimal training. For example, studies using a volunteer base workforce (Biggs et al., 2015) such as the Freshwater Habitats Trust *Pond Net* project (Freshwater Habitats Trust, 2017a) or the Amphibian and Reptile Conservation *Great Crested Newt Detectives* project (Minting, 2016). Additionally, these methods would be applicable to commercial eDNA sampling, where ecological consultants are collecting samples and sending them to laboratories for analysis (Henson, 2016), again potentially with many individuals each collecting a small number of samples. Studies conducted by universities or other research organisations, where sampling is undertaken by one team with a high degree of training often involve expensive and bulky electronic pumping equipment or the transport of large volumes of sample water to a central location for filtration. However, the provision of such equipment to citizen scientists would be prohibitively expensive, and the coordination of transport of time-sensitive samples would be logistically impossible for large scale projects with high numbers of surveyors (Biggs et al., 2014b).

A large number of studies have undertaken some form of methodological comparison of eDNA collection methods (Minamoto et al., 2016; Spens et al., 2016; Turner et al., 2014a; Djurhuus et al., 2017; Eichmiller et al., 2016b; Lacoursière-Roussel et al., 2016b). However, few studies have compared filtration methods to precipitation in ethanol sample collection (Deiner et al., 2015; Eichmiller et al., 2016b; Spens et al., 2016; Minamoto et al., 2016), with the majority of these using electronic pumping equipment for filtration. In general, these studies have concluded that filtration yields more total and target DNA than precipitation in ethanol. However, one study found precipitation in ethanol to return more copies of DNA than filtration (Minamoto et al., 2016).

In Chapter 2 we collected paired eDNA samples from both laboratory based mesocosm experiments and natural ponds. We observed differences in post-extraction concentration, from two eDNA sample collection methods which would be applicable to distribution assessments of great crested newts with eDNA for both volunteer and commercial surveys. We compared an ethanol precipitation based methodology, which is commonly used with great crested newts in the UK following a protocol similar to Biggs et al. (2015), and also a syringe based filtration methodology, using 0.7 µm glass-microfiber syringe filters. We found that in a mesocosm setting where the water was 'clean', syringe filtration of nearly 1 L of water was straightforward but using ethanol precipitation there was little other material to aid in the capture of DNA. This approach resulted in syringe filters consistently recovering greater concentrations of target eDNA than ethanol precipitation from mesocosms. However, the results from the field were less clear. With water from more natural ponds, filters often became blocked relatively quickly, subsequently requiring considerable physical exertion to pass only small volumes across them. However, algae and other suspended solids within the ethanol precipitation samples aid in the formation of the pellet during the centrifugation step, which may help trap target DNA.

As a result no significant difference in eDNA concentration was found between ethanol precipitation and syringe filtration samples from natural ponds.

Neither method we tried can therefore be recommended over the other in terms of eDNA extract concentration, and therefore likely detection rate. A decision as to which method to use may depend on practicalities or cost - ethanol precipitation is much quicker and simpler in the field, but filtration has benefits in terms of sample batch size and ease of laboratory work. Since undertaking this work new products such as Sterivex® filter capsules have started to be used with eDNA. Although Sterivex® filter capsules still block easily, these filters have been found to recover more DNA than ethanol precipitation from lake water (Spens et al., 2016). Undertaking direct comparisons between new products as they become available and the currently used methodologies, with samples from the environments that they are going to be used in, needs to be a priority. It is unlikely that one universal method for eDNA capture and extraction will be appropriate for all studies; methodologies best suited to specific studies should be identified and applied (Minamoto et al., 2016; Deiner et al., 2015). For turbid waters precipitation in ethanol may be optimal as filters will block easily (Williams et al., 2017), while in cleaner waters filter-based approaches may yield better results.

We have not focused on the eDNA preservation, extraction and amplification stages within this study as work in these areas has been advanced by others. Preservation of DNA generally relates to filtered samples, which may be frozen, or preserved in ethanol or lysis buffer (Stein et al., 2013; Renshaw et al., 2015; Wegleitner et al., 2015; Minamoto et al., 2016; Williams et al., 2016), but some have examined other preservatives such as cationic surfactants (Yamanaka et al., 2017).

Again we have not focused on DNA extraction or amplification methods, as mentioned in the introduction a wide variety of protocols are used with eDNA, each with advantages and disadvantages (Minamoto et al., 2016; Renshaw et al., 2015; Deiner

et al., 2015; Schiebelhut et al., 2017; Djurhuus et al., 2017; Eichmiller et al., 2016b; Turner et al., 2014b). Samples with certain characteristics may favour certain extraction protocols. One example of this is within the Qiagen® DNeasy® blood and tissue kit, used within this study, which utilize spin columns that aid in the removal of PCR inhibitors. Additionally the Mo Bio® PowerWater® extraction kit targets eubacteria and contains a mechanical cell lysis step, whereas the Qiagen® DNeasy® blood and tissue kit as well as phenol based extractions use chemical cell lysis, leading to reduced DNA fragmentation and greater detection from eukaryotic biodiversity (Deiner et al., 2015). Extraction methods with chemical cell lysis have been found to outperform those with mechanical cell lysis for eukaryotic targets, but it was less clear when eubacteria were targeted (Deiner et al., 2015).

At present qPCR is overwhelmingly recommended over conventional PCR, for single species analysis with qPCR one or two orders of magnitude more sensitive (Turner et al., 2014b; Qu & Stewart, 2017). However, new technologies are becoming available and being tested with droplet digital PCR (Doi et al., 2015a) and loop-mediated isothermal amplification (LAMP) technology starting to be used (Lee, 2017), these may offer advantages in assay sensitivity and in reducing PCR inhibition. As with sample collection, multiple protocols are available at the extraction and amplification stages of eDNA analysis, it is unlikely that a single method will be the most appropriate in all studies.

7.2. Identify the probability of detection of great crested newts using the currently accepted environmental DNA survey protocol.

The chance of detecting a species is likely to be a function of the amount of DNA that is present within the sample, and the methods used to collect the sample. This is no different from observational detection which can depend on the abundance of a species at a site (Guillera-Arroita et al., 2014) and the detection methods used (Sewell et al., 2010). The amount of DNA present is related to the shedding and decay rates

of eDNA from an organism in a particular environment (Klymus et al., 2015; Sassoubre et al., 2016; Sansom & Sassoubre, 2017). As a result detection probability will be different when targeting different organisms in different environments.

When great crested newts are present in a pond, naïve detection rates for eDNA vary between 60% and 99% (Thomsen et al., 2012b; Rees et al., 2014a; Biggs et al., 2014b, 2015). However, these estimates are not true detection probabilities and only relate to the percentage of samples, expected to be positive, which returned a positive result. Site occupancy detection models use repeated observations from many sites to estimate the probability that the target species is being missed (detection). The models can then be used to provide more reliable estimates of the proportion of sites actually occupied (occupancy; Mackenzie & Kendall, 2002; Tyre et al., 2003; MacKenzie et al., 2003; Lahoz-Monfort et al., 2016; Ficetola et al., 2016; Guillera-Arroita et al., 2017). This type of analysis has been used with eDNA on a wide variety of taxa generating detection probabilities which range from 0.74 to 0.95 (Schmidt et al., 2013; Hunter et al., 2015; Schmelzle & Kinziger, 2016; Vörös et al., 2017). We also use single season site occupancy detection models within Chapter 3, to estimate how the probability of detection decreases once great crested newts have left the water body. Additionally in Chapter 4, we use occupancy models to estimate the detection probability in ponds known to be occupied by great crested newts, at different times of the year, for both aquatic and sedimentary eDNA samples, and a Bayesian variant on occupancy modelling in Appendix III for estimating false positive and negative error rates.

We showed that the location of the study area influenced detection probability as did time of year and the suitability of the habitat for great crested newts. Using the current commercial survey protocol, spring-time detection levels using samples collected during the accepted survey window from ponds of average habitat suitability (HSI = 0.65) were found to be p = 0.88 (95% CI = 0.81-0.93) in one survey area, p = 0.76

(95%CI = 0.64-0.85) in a second and p = 0.79 (95% CI = 0.69-0.87) in the third. These results suggest that false negative results may be as high as 31% from an 'average pond' in England when sampled in April. However, when this approach was repeated for samples collected later in the summer (July) detection probability was higher, p = 0.94 (95% CI = 0.90-0.97) in one survey area, p = 0.88 (95% CI = 0.79-0.93) in a second and p = 0.89 (95% CI = 0.82-0.94) in the third. This result suggests that at this time of year the chance of false negative results is reduced to a maximum of 21%. Great crested newt courtship and egg-laying activity is highest through May and June (Langton et al., 2001); however, in Chapter 4 samples were not collected during this time period. It is likely that greater amounts of eDNA are released into the water with the increased activity, particularly surrounding egg laying (Chapter 5), and so detection probability between April and July may be higher than found in either our spring or summer samples. Detection probabilities were considerably lower in the autumn and winter with maximum values of p = 0.59 and p = 0.29 found respectively, and surveys at these times would not be recommended.

We have additionally used a Bayesian occupancy modelling approach to generate false positive and false negative error rates from a commercially collected eDNA data set. Occupancy within the study was identified as 0.21 (0.11-0.36). We identified a false negative error of between 12% (8-19%) and additionally a false positive error of between 6% (4 - 9%; Appendix III). This more in-depth analysis shows false negative rates similar to those we have presented within Chapter 4, adding confidence to a false negative error rate of between 10 and 20%, for commercial eDNA samples.

A detection probability of p > 0.75 using eDNA for a single visit during the spring and summer, is greater than or equivalent to a single survey using a combination of traditional visual based survey methods (in 2007: edge species of range p = 0.38, SE = 0.094; core species range p = 0.68, SE = 0.081; Sewell et al., 2010). However, the standard commercial survey protocols for great crested newts require four visits using

a combination of a minimum of three methods during the great crested newt breeding season. For traditional methods this level of survey effort has been shown to relate to an 80% confidence that a non-detection indicates absence of the species on the edge of the species range, and 95% confidence that a non-detection indicates absence of the species in the core of the species range (Sewell et al., 2010). This suggests the currently accepted practice of using a single eDNA sample to determine presence and absence from a pond will be generating a greater number of false absence results than the conventional observational methods, over multiple visits. As yet no analysis has been undertaken to identify the optimum number of eDNA samples to collect for great crested newts. However, this could be easily achieved, by collecting multiple samples and the use of power analysis (Barata et al., 2017). Research has suggested that just adding a second eDNA sample greatly increases the probability of detecting platypus (*Ornithorhynchus anatinus*) (Lugg et al., 2017).

To inform a planning application, if a pond is found to be positive using eDNA there is a requirement that traditional surveys are used to estimate the population 'size class' of great crested newts within a pond, despite the fact that such size class estimates are highly inaccurate (Griffiths et al., 2015). This requirement is leading commercial ecologists to collect eDNA samples at the earliest possible opportunity in the 15th of April to the 30th of June survey window (Appendix II). We have shown that the early part of the accepted survey window has comparatively low amounts of eDNA within the water (Chapter 5) and lower detection probability than the peak later in the summer (Chapter 4). This practice could be exaggerating the number of false negative results, leading to populations being missed, mitigation on development projects not being applied, and the risk of expensive delays if great crested newts are subsequently discovered once construction has started. The use of a single eDNA sample will also lead to an under-estimate of actual occupancy when eDNA is used for distribution assessments (Freshwater Habitats Trust, 2017b). Improved sampling

protocols or collecting more samples on multiple occasions from the same pond will increase detection probability. However, increasing the number of samples and the number of visits to a pond will increase the cost, making eDNA less attractive to commercial ecologists, developers and volunteer groups (Appendix II).

7.3. Evaluate the appropriateness of the commercial sample collection window in relation to newt phenology.

The recommended survey window for great crested newts using eDNA in the UK, when results are to be used to inform planning decisions, is between the 15th of April and the 30th of June. This recommended survey window was put in place because it was assumed that the highest levels of target eDNA would be present while peak numbers of adults were present in the water to breed, and coincided with the timing of the original pilot study (Biggs et al., 2014b; Natural England, 2014). However, year round detection of great crested newts using aquatic eDNA has recently been suggested by Rees et al. (2017).

In Chapter 5 we observed how the concentration of target eDNA changed across the active period from March through to October, and how this related to the number of adults, larvae, adult body condition and environmental variables. We found that the amount of target eDNA was highest in early June coinciding with peak breeding as had been expected. Although eDNA was detectable during the early part of the recommended survey window, the amount of DNA present between mid- and late April was very low when compared to the amount present at the end of May or early June. From this finding we can assume that samples collected in the early part of the survey window are more likely to produce false negatives due to lower concentrations of target DNA, than samples collected towards the middle or end of the window.

The potential for samples collected early in the survey window to be less reliable than those collected later is corroborated by the data presented in Chapter 4. Detection

probability from aquatic samples collected from typical ponds was found to be lower in samples collected in spring (April) than it was in the summer (July). However, a number of the ponds had completely dried by July and no sample could be collected. These ponds had been found to be occupied in the early part of the year; for this reason dry ponds should not be treated as negative and mid-summer sample collection may be inadvisable.

Although in Chapter 5 a peak in eDNA concentration was observed in early June associated with peak breeding, a second longer peak was also identified between mid-July and mid-August, a time when few adults were in the ponds, but larval numbers were at their peak. eDNA concentration then fell abruptly when larvae metamorphosed and emerged from the ponds. This peak in eDNA concentration observed out of season opens opportunities for the use of eDNA to distinguish between ponds with breeding and non-breeding populations of newts through the targeting of eDNA from larvae.

We also developed a method for collecting and extracting eDNA from pond sediments in an attempt to achieve more reliable year-round detection (Chapter 4). Although we did achieve year-round detection from both samples collected from sediment and samples collected from water, detection probability was low in both aquatic and sediment samples in the autumn (October) and winter (January) samples, compared to the spring and the summer. Therefore we concluded that a negative result could not be relied upon in samples collected in the autumn and the winter.

The optimal timing of eDNA sampling for great crested newts varies depending on the aims of the study, and whether pond occupancy or an indication of breeding is required. The level of confidence that is acceptable in estimating non-detection will also be influenced by the timing of the sampling. We found that the end of the breeding season (early June) returned the highest concentration of eDNA and is therefore likely to have the highest detection probability and provide the most reliable

occupancy data. However, detection earlier in the spring is possible with a reduced detection probability. Consequently, moving the start of the currently accepted survey window for great crested newts to the beginning of May would reduce false negative results. Although high detection probabilities and eDNA concentration were found after June, they may only be reliable in detecting breeding ponds and not occupied but non-breeding ponds. This is shown by the analysis conducted in Chapter 5 indicating that peaks in eDNA concentration in the summer months were highly reliant on larval presence, but less so on the presence of adults. As detection probability was considerably reduced in autumn and winter (often below 50%), we would not recommend sample collection at these times. Optimal timing is likely to vary year on year depending on weather conditions as well as location, as both of these can alter great crested newt activity and timing. The duration of the acceptable survey window needs to be linked to the confidence in results deemed acceptable by the licencing authorities or conservation practitioners.

7.4. Determine whether an estimate of abundance of great crested newts can be made from eDNA samples.

Although species occupancy has considerable value for ecologists and conservation managers, a measure of abundance can give additional information on the status, and trends of the species, and inform management decisions (Joseph et al., 2006; Bohmann et al., 2014). With the exception of capture-mark-recapture methodologies which are logistically demanding, most methods used for the assessment of abundance of amphibians are based on traditional quasi-quantitative practices with little scientific justification (Griffiths et al., 2015). To date eDNA has largely been limited to presence and absence of a species; the value of eDNA will increase considerably if a reliable estimate of abundance can be drawn from it (Goldberg et al., 2016; Kelly, 2016). However, a weak relationship has been found between the

proportion of qPCR replicates to amplify and great crested newt counts (Biggs et al., 2014b, 2015).

We have shown that both the number of adults and the number of larvae influence the concentration of DNA within a sample (Chapter 5). eDNA may therefore allow comparisons of abundance between similar water bodies, experiencing similar environmental conditions, which are sampled simultaneously. However, we also showed that both survey timing and body condition of adults were of far greater influence over eDNA concentration than the number of individuals. As a result, eDNA concentration or changes in eDNA concentration alone would not comprise a reliable estimate of abundance or change in abundance (Chapter 5); although models are being developed (Chambert et al., 2018). Nevertheless, simple counts of individuals by bottle trapping or torch light also vary between waterbodies, with environmental conditions and the time of year, but this is largely ignored by most practitioners when undertaking an assessment of abundance. Before eDNA concentration can be used for abundance estimates, eDNA shedding and decay rates need to be quantified (Klymus et al., 2015; Sassoubre et al., 2016; Sansom & Sassoubre, 2017) and corrections applied for seasonal variability and non-adult life stages.

eDNA concentration from qPCR and sequence reads from metabarcoding analysis of mitochondrial DNA will vary between species and between the cell types released into the water. This variation will depend on the abundance of mitochondria within a cell and so the number of copies of target DNA present is likely to bear little relationship to the number of individuals of a species present. Nevertheless, the amount of eDNA present has been found to have a relationship with the abundance or biomass of a single targeted species in both mesocosms (Thomsen et al., 2012b; Klymus et al., 2015; Sassoubre et al., 2016; Matsuhashi et al., 2016; Lacoursière-Roussel et al., 2016b) and natural environments (Takahara et al., 2012; Pilliod et al., 2013; Lacoursière-Roussel et al., 2016a; Erickson et al., 2016; Baldigo et al., 2017;

Tillotson et al., 2018). Although not strictly eDNA, metabarcoding read abundance has been shown to correlate with specimen abundance or biomass, when DNA has been extracted from mixed specimen samples (Elbrecht & Leese, 2015; Thomas et al., 2016; Elbrecht et al., 2017). This approach has been repeated with aquatic eDNA samples with similar results, with the number of sequence reads correlating with either species abundance or biomass within both mesocosms (Kelly et al., 2014; Evans et al., 2016) and natural environments (Stoeckle et al., 2017b; O'Donnell et al., 2017; Hänfling et al., 2016; Thomsen et al., 2016). The majority of these studies allow an estimate of relative abundance or biomass between samples to be generated, but do not allow for a direct estimate of population size to be drawn. As we have shown in Chapter 5, environmental factors and time of year will influence shedding rates and the amount of DNA present; eDNA concentration and number of sequence reads recovered are therefore poor indicators of abundance. We have, however, theorised a different approach which may allow for the production of an estimate of population abundance (Chapter 6).

In Chapter 6 we take a mixed sample analysis approach, by analysing microsatellite allele arrays from genomic DNA and using this to predict abundance. We demonstrate this through the simulation of mixed samples from multiple individuals, and predict abundance, based on the alleles present and the known allele frequency within the study population. Although this allowed the prediction of the number of individuals within simulated data to within an acceptable degree of confidence, we were unable to achieve reliable amplification of genomic DNA from eDNA samples. We found reliable detection of genomic DNA from tissue extracts to be lost at concentrations at least two orders of magnitude higher than eDNA sample extracts are routinely found to be. However, the abundance estimation theory we present, along with the speed of technological development within eDNA detection, provides great future potential and a very valuable addition to the field.

7.5. Identify environmental influences on great crested newt environmental DNA and its detection.

The chance of detecting eDNA is related to the amount of DNA that is present within a waterbody. The amount of DNA present within a sample is in turn related to the rate of production of eDNA by the species and how long it persists in the environment (Dejean et al., 2011). Influences on detection may be acting on the newts and the amount of DNA released into the water or acting on the eDNA itself and influencing its degradation rate and persistence time.

Factors which influence detection that are acting on the newts themselves include habitat variables which will influence the density of individuals present within a pond. As discussed earlier, the density of individuals has been shown to influence the amount of eDNA within a sample (Takahara et al., 2012; Thomsen et al., 2012b; Pilliod et al., 2014; Eichmiller et al., 2014; Doi et al., 2015b; Klymus et al., 2015; Doi et al., 2017; Lacoursière-Roussel et al., 2016b; Matsuhashi et al., 2016; Chapter 5). The analysis in the replicated pond system (Chapter 5) supports larval abundance influencing the amount of eDNA present, both in and outside the breeding season, with adult abundance having a lesser effect outside the breeding season. We show in Chapter 4 that detection probability increases with habitat suitability for great crested newts, based on the great crested newt Habitat Suitability Index (HIS; Oldham et al., 2000). Improved habitat suitability may be allowing great crested newts to persist in higher abundance, or may allow for higher levels of activity or breeding, therefore increasing the release of DNA. Within Appendix III we use a Bayesian occupancy modelling approach to generate false positive and false negative error rates. In addition to this we attempt to identify influences on detection based on the parameters measured within the HSI metric. Within this we identify that as water depth increases, a decrease in false negative error rates are observed, whereas higher pond density and better surrounding terrestrial habitat increase false negative

error rates. However, the presence of fish was found to reduce the instances of false positive error.

We have shown in Chapter 5 that changes in adult body condition associated with breeding behaviour influence the amount of DNA within the water. Although significant influences were found for both males and females, the impact of female body condition on eDNA concentration was much greater than that of males. This is likely to relate to the decrease in body mass associated with the production and laying of eggs, and the additional DNA released through laying eggs and through non-viable eggs decomposing (Horner & Macgregor, 1985) which leads to release of DNA into the water. Other studies have observed seasonal peaks in eDNA associated with breeding (Spear et al., 2015), or habitat use varying in time and space (Erickson et al., 2016; Pfleger et al., 2016; Stewart et al., 2017). Consequently habitat variables which influence the behaviour of a species in a location can affect the amount of DNA present within a sample.

Influences on the accumulation and loss of eDNA within an environment and how eDNA remains detectable after a species has left a waterbody are constantly being identified. In contrast to others (Barnes et al., 2014; Klymus et al., 2015), we did not find an influence of temperature on the rate of change in detection, when eDNA was repeatedly sampled from mesocosms (Chapter 3). Temperature was, however, found to influence the amount of eDNA present across the year (Chapter 5). During the breeding season temperature rose as did the amount of eDNA, with both falling in the late season. This positive relationship between temperature and amount of eDNA is likely to be caused by the activity of newts following seasonal patterns which are in turn influenced by temperature.

Detectability of eDNA was also found to decline after great crested newts were removed from a waterbody, and so the time since great crested newts vacated the water body influences detection (Chapter 3). Detection probability began in excess of

p = 0.96 falling to p < 0.6, 10 days after newts were removed. We did demonstrate, however, that detection was possible beyond 20 days albeit at very low levels (p < 0.05). We also found that substrate type had an influence on the rate of change in detection probability. Samples of eDNA from water with topsoil or clay substrates decrease in detection probability faster than those with sand substrates or control samples (Chapter 3). This result was supported by our analysis of natural ponds (Chapter 4), where ponds with a sandy clay substrate were found to have a greater eDNA detection probability than pure clay substrate or sandy loam substrates, which in turn had greater detection probability than clay loam and sandy clay loam substrates. We have also demonstrated that the timing of sample collection, and the study area can influence the detection of eDNA (Chapter 4).

PCR inhibition has the ability to reduce detection probability dramatically (Alaeddini, 2012; Schrader et al., 2012). In Chapter 3, we found 70% of samples from water with the topsoil substrate showed signs of inhibition, this compared to just 2% for clay and 0% for sand or control groups. PCR inhibitors come in many forms, but the samples from the topsoil treatments had substantially higher levels of total suspended solids in the water column than the other treatment groups, which are likely to have introduced inhibitors such as humic acids (Wilson, 1997; Alaeddini, 2012; Schrader et al., 2012) into the samples. We demonstrated that dilution of samples, one of the commonly recommended methods for removing the effects of PCR inhibitors (McKee et al., 2015; Biggs et al., 2014b), was not appropriate with eDNA. Although a 1 in 10 dilution factor applied to the eDNA extract was sufficient to remove the effects of inhibition, it reduced detection probability from p > 0.96 in the control group and p = 0.91 in the inhibited data set to just p = 0.39 in the diluted data set. This reduction in detection probability was caused by diluting target DNA (Juen & Traugott, 2006; Alaeddini, 2012; Jane et al., 2015) and could have misleading implications for the interpretation of any eDNA sampling.

Variable detection rates and PCR inhibition need to be taken into consideration by practitioners when applying eDNA results to decisions concerning great crested newt presence-absence in planning applications. It is essential to ensure sample collection is at an optimal time of year to detect the target species. Additionally when interpreting results it needs to be recognised that the chance of detecting the species is greater in more optimal habitat than less suitable habitat, even if both are occupied. To allow for appropriate interpretation of eDNA results in a commercial context and greatest confidence in results, measures to identify the presences of PCR inhibitors need to be applied as recommended in Appendix I. Appropriate measures to reduce inhibition should also be sought, for example droplet digital PCR is more resistant to inhibitors and has been shown to be more sensitive and consistent at lower DNA concentrations than qPCR (Doi et al., 2015b, 2015a).

7.6. Conclusions

This PhD adds to the general field of eDNA research demonstrating that there are options available for sample collection methodology. We have been one of the first groups of researchers to explain seasonality within detection using eDNA. We show that for a semi-aquatic species the optimal timing for sample collection is around or just after peak egg laying when the highest amount of genetic material is entering the water. We have identified influences on detection probability for eDNA samples and have demonstrated how the chance of detecting a target species changes after they are no longer present. We have also shown that it is difficult to identify direct relationships between the number of individuals and the amount of eDNA present within a sample, but we have, we believe, in the context of eDNA been the first to attempt the use of genomic DNA and allele frequencies in the prediction of population abundance.

Although the initial aims of the project marry well with those of the great crested newt commercial eDNA sector within the UK, the work we have done contributes to the

advancement of and understanding of the limitations of environmental DNA survey methodology more generally. When tested in a field environment the commercially-used great crested newt eDNA collection protocol recovers a similar amount of DNA to other methods which do not require mechanical pumps to be used in the field or the transport of large volumes of water to a central location for filtering. However, the use of different filter types or alterations to extraction protocols used may still prove beneficial.

We have demonstrated that the detection probability of eDNA targeting great crested newts is high, but not as high as some of the naïve estimates originally quoted in the scientific literature (Biggs et al., 2015). The detection probability estimates generated as part of this project suggest that a single eDNA sample was equivalent to a single visit using a suite of traditional methods, in the core of the species range; however, no analysis has been undertaken on eDNA detection probabilities at the edge of the species range. To increase the confidence in non-detections, multiple samples may be necessary, as are required when using traditional survey methodologies. Increased sample numbers need to be combined with power analysis to ascertain the optimal number of samples to collect (Sewell et al., 2010; Barata et al., 2017). However, this may reduce the appeal and cost-effectiveness of eDNA surveys to commercial surveyors. We have also demonstrated that detection probability is influenced by survey timing, the suitability of the habitat for the species, the time since great crested newts were present, as well as other environmental factors.

The ability to estimate abundance from eDNA is progressing. At present, under certain conditions, an indication of relative abundance appears to be possible from either eDNA concentration in qPCR or sequence reads from metabarcoding data. These approaches seem unlikely to generate estimates that would have the capacity to monitor the change in abundance within a pond or compare ponds experiencing different environmental conditions. We demonstrate mixed sample analysis, which is

more likely to generate realistic abundance estimates that would allow robust spatial and temporal inferences to be drawn about changes in population abundance. However, technology and eDNA methods have not quite progressed sufficiently to make this approach a reality at this stage. This is a likely direction for future research.

The most appropriate survey window depends on the detection probability tolerance that is acceptable to surveyors. We attempted to extend the survey window for great crested newts with samples collected from pond sediments. However, this leads to a reduced detection probability when compared to water samples and would not be an appropriate methodological advancement. We did find that detection in the early part of the currently accepted window (the middle of April to the end of June) was low, with low concentration of DNA within the water, whereas throughout parts of the summer, detection probability and amount of DNA present was significantly higher. It may therefore be appropriate to recommend a shift in the recommended survey window to later in the year to enable more reliable detection.

We have not only demonstrated advancements to, and limitations of, the currently utilised method of eDNA assessment of great crested newts in the UK; we have also gone some way to addressing the priorities for advancing surveys using eDNA outlined by Natural England in 2014. With the interest in and use of eDNA growing (Figure 7.1), there are still advancements to be made within the eDNA field. At present metabarcoding options show a slight reduction in species specific sensitivity over qPCR (Harper et al., 2018), but the costs are reducing and it offers much greater ecological information than just the presence of a single species. As the cost decreases and multi-taxa primers are developed and tested for metabarcoding, the scope of species community analysis will flourish. Additionally technology such as droplet digital PCR will reduce the impact of PCR inhibitors and increase the limits of detection for single species analysis (Doi et al., 2015b, 2015a). As technologies such as LAMP (Lee, 2017) and MinION nanopore sequencing (Laszlo et al., 2014; Brown

et al., 2017) become more accurate and cost effective, these will allow analysis of eDNA to be conducted in the field. It is clear that eDNA is taking us one step closer to making technology only realised in science fiction a reality; however, there is still some way to go before all an ecologist needs to carry is a Tricorder from Gene Roddenberry's television series *Star Trek*.

Legislation

- Convention on the Conservation of European Wildlife and Natural Habitats (19 September 1979; The 'Bern Convention')
- Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. *Official Journal* **L206**, 22/07/1992 0007-0050 (The 'Habitats Directive')
- The Conservation (Natural Habitats, &c.) Regulations 1994 (Statutory Instrument 1994 No. 2716; The 'Habitats Regulations') www.opsi.gov.uk/si/si1994/Uksi_19942716_en_1.htm
- The Conservation of Habitats and Species Regulations 2010 (Statutory Instrument 2017 No. 1012) http://www.legislation.gov.uk/uksi/2010/490/contents/made
- Wildlife and Countryside Act 1981 (1981 Chapter 69)
    - www.naturenet.net/law/wcagen.html
- Natural Environment and Rural Communities Act 2006 (Statutory Instrument 2006 No. 16) https://www.legislation.gov.uk/ukpga/2006/16/contents
- The Nature Conservation (Scotland) Act 2004 (2004 ASP6) www.opsi.gov.uk/legislation/scotland/acts2004/20040006.htm

References

Abrego, N., Norros, V., Halme, P., Somervuo, P., Ali-Kovero, H. & Ovaskainen, O. (2018). Give me a sample of air and I will tell which species are found from your region – molecular identification of fungi from airborne spore samples. *Molecular Ecology Resources*. In Press.

Al-Soud, W.A., Jönsson, L.J. & Rådström, P. (2000). Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *Journal of Clinical Microbiology*. 38. 345–350.

Alaeddini, R. (2012). Forensic implications of PCR inhibition - A review. *Forensic Science International: Genetics*. 6. 297–305.

Albers, C.N., Jensen, A., Bælum, J. & Jacobsen, C.S. (2013). Inhibition of DNA Polymerases used in q-PCR by structurally different soil-derived humic substances. *Geomicrobiology Journal*. 30. 675–681.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. (2002). *Molecular Biology of the Cell*. 4th editio. New York: Garland Science.

Andersen, K., Bird, K.L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kjær, K.H., Orlando, L., Gilbert, M.T.P. & Willerslev, E. (2012). Meta-barcoding of 'dirt' DNA from soil reflects vertebrate biodiversity. *Molecular Ecology*. 21. 1966–1979.

Anderson-Carpenter, L.L., McLachlan, J.S., Jackson, S.T., Kuch, M., Lumibao, C.Y. & Poinar, H.N. (2011). Ancient DNA from lake sediments: bridging the gap between paleoecology and genetics. *BMC evolutionary biology*. 11. 30.

Ando, A., Camm, J., Polasky, S. & Solow, A. (1998). Species distributions, land values, and efficient conservation. *Science*. 279. 2126–2128.

Andújar, C., Arribas, P., Gray, C., Bruce, K., Woodward, G., Yu, D.W. & Vogler, A.P. (2017). Metabarcoding of freshwater invertebrates to detect the effects of a

pesticide spill. *Molecular Ecology*. 00. 1–21.

Arntzen, J. and Teunis, S. (1993). A six years study on the population dynamics of the crested newt (*Triturus cristatus*) following the Colonization of a newly created pond. *Herpetological Journal*. 3. 99–110.

Arntzen, J.W., Jehle, R., Bardakci, F., Burke, T. & Wallis, G.P. (2009). Asymmetric viability of reciprocal-cross hybrids between crested and marbled newts (*Triturus cristatus* and *T. marmoratus*). *Evolution*. 63. 1191–1202.

Austen, G.E., Bindemann, M., Griffiths, R.A. & Roberts, D.L. (2016). Species identification by experts and non-experts: Comparing images from field guides. *Scientific Reports*. 6. 33634.

AZ Instruments (2014). *Opperation manual pen type pH meter*. [Online]. 2014. Available from: http://www.az-instrument.com.tw/az-instrument/upload/product_pdf/ODY4NQ==-1414549904-YSkeQ.pdf. [Accessed: 4 July 2017].

Aziz, M.A., Tollington, S., Barlow, A., Greenwood, C., Goodrich, J., Smith, O., Shamsuddoha, M., Islam, M.A. & Groombridge, J. (2017). Using non-invasively collected genetic data to estimate density and population size of tigers in the Bangladesh Sundarbans. *Global Ecology and Conservation*. 12. 272–282.

Bae, S. & Wuertz, S. (2009). Rapid decay of host-specific fecal Bacteroidales cells in seawater as measured by quantitative PCR with propidium monoazide. *Water Research*. 43. 4850–4859.

Bae, S. & Wuertz, S. (2012). Survival of host-associated bacteroidales cells and their relationship with *Enterococcus spp.*, *Campylobacter jejuni*, *Salmonella enterica serovar*, *Typhimurium*, and adenovirus in freshwater microcosms as measured by propidium mon. *Applied and Environmental Microbiology*. 78. 922–932.

Baker, J., Beebee, T., Buckley, J., Gent, T., Orchard, D., Bennett, A., Bernhard, T., Brady, L., Coward, D., Driver, B., Foster, J., Gleed-Owen, C., Herder, J., Holmes, F., King, P., Mcgrath, A., Reynolds, A., Shaw, B. & Woodhead, D. (2011). *Amphibian habitat management handbook*. Bournemouth, UK: Amphibian and Reptile Conservation.

Baldigo, B.P., Sporn, L.A., George, S.D. & Ball, J.A. (2017). Efficacy of environmental DNA to detect and quantify brook trout populations in headwater streams of the Adirondack Mountains, New York. *Transactions of the American Fisheries Society*. 146. 99–111.

Barata, I.M., Griffiths, R.A. & Ridout, M.S. (2017). The power of monitoring: optimizing survey designs to detect occupancy changes in a rare amphibian population. *Scientific Reports*. 7. 16491.

De Barba, M., Miquel, C., Lobréaux, S., Quenette, P.Y., Swenson, J.E. & Taberlet, P. (2017). High-throughput microsatellite genotyping in ecology: improved accuracy, efficiency, standardization and success with low-quantity and degraded DNA. *Molecular Ecology Resources*. 17. 492–507.

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

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

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C., Mersey, B. & Ferrer, E. a (2011). Has the Earth's sixth mass extinction already arrived? *Nature*. 471. 51–57.

Bartoń, K. (2016). *MuMIn: Multi-Model Inference*.

Beebee, T.J.C. (1997). Changes in dewpond numbers and amphibian diversity over 20 years on chalk downland in Sussex, England. *Biological Conservation*. 81. 215–219.

Beebee, T.J.C. (1981). Habitats of the British amphibians (4): Agricultural lowlands and a general discussion of requirements. *Biological Conservation*. 21. 127–139.

Beebee, T.J.C. & Griffiths, R. (2000). *Amphibians and Reptiles: A Natural History of the British Herpetofauna*. HarperCollins UK.

Beebee, T.J.C. & Griffiths, R.A. (2005). The amphibian decline crisis: A watershed for conservation biology? *Biological Conservation*. 125. 271–285.

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

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R., Foster, J., Wilkinson, J., Arnett, A., Williams, P. & Dunn, F. (2014a). *Analytical and methodological development for improved surveillance of the great crested newt Appendix 5. Technical advice note for field and laboratory sampling of great crested newt (Triturus cristatus) environmental DNA*.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R., Foster, J., Wilkinson, J., Arnett, A., Williams, P. & F, D. (2014b). *Analytical and methodological development for improved surveillance of the great crested newt. Defra Project WC1067.* Oxford.

Blaustein, A.R. & Kiesecker, J.M. (2002). Complexity in conservation: Lessons from the global decline of amphibian populations. *Ecology Letters*. 5. 597–608.

Blaustein, A.R., Wake, D.B. & Sousa, W.P. (1994). Amphibian declines: Judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology*. 8. 60–71.

Boehrer, B. & Schultze, M. (2008). Stratification of lakes. *Reviews of Geophysics*. 46. 1–27.

Boessenkool, S., Mcglynn, G., Epp, L.S., Taylor, D., Pimentel, M., Gizaw, A., Nemomissa, S., Brochmann, C. & Popp, M. (2014). Use of ancient sedimentary DNA as a novel conservation tool for high-altitude tropical biodiversity. *Conservation Biology*. 28. 446–455.

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

Bonney, R., Cooper, C.B., Dickinson, J., Kelling, S., Phillips, T., Rosenberg, K. V. & Shirk, J. (2009). Citizen science: A developing tool for expanding science knowledge and scientific literacy. *BioScience*. 59. 977–984.

Bormpoudakis, D., Foster, J., Gent, T., Griffiths, R.A., Russell, L., Starnes, T., Tzanopoulos, J. & Wilkinson, J. (2016). *Developing models to estimate the occurrence in the English countryside of great crested newts, a protected species under the Habitats Directive. Defra Project WC1108*. London, UK.

Breen, G., Sham, P., Li, T., Shaw, D., Collier, D.A. & St Clair, D. (1999). Accuracy and sensitivity of DNA pooling with microsatellite repeats using capillary electrophoresis. *Molecular and cellular probes*. 13. 359–65.

Bretman, A. & Tregenza, T. (2005). Measuring polyandry in wild populations: A case study using promiscuous crickets. *Molecular Ecology*. 14. 2169–2179.

Broquet, T., Berset-Braendli, L., Emaresi, G. & Fumagalli, L. (2007). Buccal swabs

allow efficient and reliable microsatellite genotyping in amphibians. *Conservation Genetics*. 8. 509–511.

Brown, B.L., Watson, M., Minot, S.S., Rivera, M.C. & Franklin, R.B. (2017). MinION nanopore sequencing of environmental metagenomes: A synthetic approach. *GigaScience*. 6. 1–10.

Brownstein, M.J., Carpten, J.D. & Smith, J.R. (1996). Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques*. 20. 1004–1010.

Brys, R. (2017). How far can we get: Detection and quantification limits of fish species in space and time. In: *5th UK DNA Working Group Meeting; 2017 Dec 5-6*. 2017, Salford, UK.

Burnham, K.P. & Anderson, D.R. (2003). *Model selection and multimodel inference: a practical information-theoretic approach*.

Bussière, L.F., Demont, M., Pemberton, A.J., Hall, M.D. & Ward, P.I. (2010). The assessment of insemination success in yellow dung flies using competitive PCR. *Molecular Ecology Resources*. 10. 292–303.

Cai, P., Huang, Q.Y. & Zhang, X.W. (2006). Interactions of DNA with clay minerals and soil colloidal particles and protection against degradation by DNase. *Environmental Science and Technology*. 40. 2971–2976.

Chambert, T., Pilliod, D.S., Goldberg, C.S., Doi, H. & Takahara, T. (2018). Estimating species density and abundance from environmental DNA. *Ecology and Evolution*. In Press.

Champlot, S., Berthelot, C., Pruvost, M., Andrew Bennett, E., Grange, T. & Geigl, E.M. (2010). An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. *PLoS ONE*. 5. e13042.

Chaves, L., Dias, I. & Pomilla, C. (2010). *Extraction of genomic DNA from carnivore fecal samples using QIAamp DNA Stool Mini kit*. [Online]. 2010. Sackler Institute for Comparative Genomics. Available from: https://research.amnh.org/genomics/Resources/Extraction-genomic-DNA. [Accessed: 20 January 2016].

Collins, H.E., Li, H., Inda, S.E., Anderson, J., Laiho, K., Tuomilehto, J. & Seldin, M.F. (2000). A simple and accurate method for determination of microsatellite total allele content differences between DNA pools. *Human Genetics*. 106. 218–226.

Corinaldesi, C., Barucca, M., Luna, G.M. & Dell'Anno, A. (2011). Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments. *Molecular Ecology*. 20. 642–654.

Corse, E., Meglécz, E., Archambaud, G., Ardisson, M., Martin, J.F., Tougard, C., Chappaz, R. & Dubut, V. (2017). A from-benchtop-to-desktop workflow for validating HTS data and for taxonomic identification in diet metabarcoding studies. *Molecular Ecology Resources*. 17. e146–e159.

Crawley, M.J. (2007). *The R Book*. Chichester, UK: John Wiley and Sons, Ltd.

Crecchio, C. & Stotzky, G. (1998). Binding of DNA on humic acids: Effect on transformation of *Bacillus subtilis* and resistance to DNase. *Soil Biology and Biochemistry*. 30 (8–9). 1061–1067.

Cresswell, W.J. & Whitworth, R. (2004). *An assessment of the efficiency of capture techniques and the value of different habitats for the great crested newt Triturus cristatus*. Peterborough, UK.

D'Amen, M. & Bombi, P. (2009). Global warming and biodiversity: Evidence of climate-linked amphibian declines in Italy. *Biological Conservation*. 142. 3060–3067.

Daniels, J., Holmans, P., Williams, N., Turic, D., McGuffin, P., Plomin, R. & Owen, M.J. (1998). A simple method for analyzing microsatellite allele image patterns generated from DNA pools and its application to allelic association studies. *The American Journal of Human Genetics*. 62. 1189–1197.

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

Deiner, K. & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PLoS ONE*. 9. e88786.

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

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P. & Miaud, C. (2011). Persistence of environmental DNA in freshwater ecosystems. *PLoS ONE*. 6. e23398.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E. & Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology*. 49. 953–959.

Demont, M., Buser, C.C., Martin, O.Y. & Bussière, L.F. (2011). Natural levels of polyandry: Differential sperm storage and temporal changes in sperm competition intensity in wild yellow dung flies. *Functional Ecology*. 25. 1079–1090.

Denoël, M. (2012). Newt decline in Western Europe: Highlights from relative distribution changes within guilds. *Biodiversity and Conservation*. 21. 2887–

2898.

Denoël, M. & Ficetola, G.F. (2008). Conservation of newt guilds in an agricultural landscape of Belgium: the importance of aquatic and terrestrial habitats. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 18. 714–728.

Díaz-Ferguson, E. (2014). Development of molecular markers for eDNA detection of the invasive African jewelfish (*Hemichromis letourneuxi*): a new tool for monitoring aquatic invasive species in National Wildlife Refuges. *Management of Biological Invasions*. 5. 121–131.

Dick, L.K., Stelzer, E.A., Bertke, E.E., Fong, D.L. & Stoeckel, D.M. (2010). Relative decay of Bacteroidales microbial source tracking markers and cultivated *Escherichia coli* in freshwater microcosms. *Applied and Environmental Microbiology*. 76. 3255–3262.

Dickinson, J.L., Zuckerberg, B. & Bonter, D.N. (2010). Citizen science as an ecological research tool: challenges and benefits. *Annual Review of Ecology, Evolution, and Systematics*. 41. 149–172.

Djurhuus, A., Port, J., Closek, C.J., Yamahara, K.M., Romero-Maraccini, O., Walz, K.R., Goldsmith, D.B., Michisaki, R., Boehm, A.B., Breitbart, M. & Chavez, F.P. (2017). Evaluation of filtration and DNA extraction methods for environmental DNA biodiversity assessments across multiple trophic levels. *Frontiers in Marine Science*. 4. 314.

Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T. & Minamoto, T. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshwater Biology*. 62. 30–39.

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K. & Yamanaka, H. (2015a). Droplet digital polymerase chain reaction (PCR) outperforms real-time

PCR in the detection of environmental DNA from an invasive fish species. *Environmental science & technology*. 49. 5601–5608.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H. & Minamoto, T. (2015b). Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS ONE*. 10. e0122763.

Dormann, C.F., Elith, J., Bacher, S., Buchmann, C., Carl, G., Carré, G., Marquéz, J.R.G., Gruber, B., Lafourcade, B., Leitão, P.J., Münkemüller, T., Mcclean, C., Osborne, P.E., Reineking, B., Schröder, B., Skidmore, A.K., Zurell, D. & Lautenbach, S. (2013). Collinearity: A review of methods to deal with it and a simulation study evaluating their performance. *Ecography*. 36. 027–046.

Drechsler, A., Geller, D., Freund, K., Schmeller, D.S., Künzel, S., Rupp, O., Loyau, A., Denoël, M., Valbuena-Ureña, E. & Steinfartz, S. (2013). What remains from a 454 run: Estimation of success rates of microsatellite loci development in selected newt species (*Calotriton asper*, *Lissotriton helveticus*, and *Triturus cristatus*) and comparison with Illumina-based approaches. *Ecology and Evolution*. 3. 3947–3957.

Dufresnes, C., Pellet, J., Bettinelli-Riccardi, S., Thiébaud, J., Perrin, N. & Fumagalli, L. (2016). Massive genetic introgression in threatened northern crested newts (*Triturus cristatus*) by an invasive congener (*T. carnifex*) in Western Switzerland. *Conservation Genetics*. 17. 839–846.

Edgar, P. & Bird, D.R. (2006). *Action Plan for the conservation of the crested newt Triturus cristatus species complex in Europe*. Bournemouth, UK.

Edgar, P.W., Griffiths, R.A. & Foster, J.P. (2005). Evaluation of translocation as a tool for mitigating development threats to great crested newts (*Triturus cristatus*) in England, 1990-2001. *Biological Conservation*. 122. 45–52.

Eichmiller, J.J., Bajer, P.G. & Sorensen, P.W. (2014). The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS ONE*. 9. e112611.

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

Eichmiller, J.J., Miller, L.M. & Sorensen, P.W. (2016b). Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular Ecology Resources*. 16. 56–68.

Eland, L.E., Davenport, R. & Mota, C.R. (2012). Evaluation of DNA extraction methods for freshwater eukaryotic microalgae. *Water Research*. 46. 5355–5364.

Elbrecht, V. & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. *PLoS ONE*. 10. e0130324.

Elbrecht, V., Peinert, B. & Leese, F. (2017). Sorting things out: Assessing effects of unequal specimen biomass on DNA metabarcoding. *Ecology and Evolution*. 7. 6918–6926.

Elith, J. & Leathwick, J.R. (2009). Species distribution models: ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution, and Systematics*. 40. 677–697.

English Nature (2001). *Great crested newt mitigation guidelines*. Peterborough, UK: English Nature.

Erickson, R.A., Rees, C.B., Coulter, A.A., Merkes, C.M., McCalla, S.G., Touzinsky, K.F., Walleser, L., Goforth, R.R. & Amberg, J.J. (2016). Detecting the movement and spawning activity of bigheaded carps with environmental DNA. *Molecular*

Ecology Resources. 16. 957–965.

Evans, N.T., Olds, B.P., Renshaw, M.A., Turner, C.R., Li, Y., Jerde, C.L., Mahon, A.R., Pfrender, M.E., Lamberti, G.A. & Lodge, D.M. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular Ecology Resources*. 16. 29–41.

Ficetola, G.F., Cagnetta, M., Padoa-Schioppa, E., Quas, A., Razzetti, E., Sindaco, R. & Bonardi, A. (2014). Sampling bias inverts ecogeographical relationships in island reptiles. *Global Ecology and Biogeography*. 23. 1303–1313.

Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C.M., Boyer, F., Pompanon, F., Rayé, G. & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*. 15. 543–556.

Ficetola, G.F., Taberlet, P. & Coissac, E. (2016). How to limit false positives in environmental DNA and metabarcoding? *Molecular Ecology Resources*. 16. 604–607.

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

Field, S.A., Tyre, A.J., Thorn, K.H., O'Connor, P.J. & Possingham, H.P. (2005). Improving the efficiency of wildlife monitoring by estimating detectability: A case study of foxes (*Vulpes vulpes*) on the Eyre Peninsula, South Australia. *Wildlife Research*. 32. 253–258.

Fiske, I. & Chandler, R. (2011). unmarked : An R package for fitting hierarchical models of wildlife occurrence and abundance. *Journal of Statistical Software*. 43. 1–23.

Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A.,

Salling, A.B., Galatius, A., Orlando, L. & Gilbert, M.T.P. (2012). Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS ONE*. 7. e41781.

Freshwater Habitats Trust (2017a). *eDNA great crested newt survey data - 2016 eDNA results*. [Online]. 2017. Available from: https://freshwaterhabitats.org.uk/edna-great-crested-newt-survey-data/. [Accessed: 22 November 2017].

Freshwater Habitats Trust (2017b). *Great crested newt conservation*. [Online]. 2017. Available from: https://freshwaterhabitats.org.uk/projects/environment-bank/. [Accessed: 27 November 2017].

Fukumoto, S., Ushimaru, A. & Minamoto, T. (2015). A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: A case study of giant salamanders in Japan. *Journal of Applied Ecology*. 52. 358–365.

Gan, C., Love, C., Beshay, V., Macrae, F., Fox, S., Waring, P. & Taylor, G. (2015). Applicability of next generation sequencing technology in microsatellite instability testing. *Genes*. 6. 46–59.

Gibson, J.F., Shokralla, S., Curry, C., Baird, D.J., Monk, W.A., King, I. & Hajibabaei, M. (2015). Large-scale biomonitoring of remote and threatened ecosystems via high-throughput sequencing. *PLoS ONE*. 10. e0138432.

Goldberg, C.S., Pilliod, D.S., Arkle, R.S. & Waits, L.P. (2011). Molecular detection of vertebrates in stream water: A demonstration using rocky mountain tailed frogs and Idaho giant salamanders. *PLoS ONE*. 6. e22746.

Goldberg, C.S., Sepulveda, A., Ray, A., Baumgardt, J. & Waits, L.P. (2013). Environmental DNA as a new method for early detection of New Zealand

mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science*. 32. 792–800.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P. & Gilbert, M. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*. 7. 1299–1307.

Greaves, M.P. & Wilson, M.J. (1969). The adsorption of nucleic acids by montmorillonite. *Soil Biology and Biochemistry*. 1. 317–323.

Green, H.C., Shanks, O.C., Sivaganesan, M., Haugland, R.A. & Field, K.G. (2011). Differential decay of human faecal Bacteroides in marine and freshwater. *Environmental Microbiology*. 13. 3235–3249.

Griffiths, R.A. (1985). A simple funnel trap for studying newt populations and an evaluation of trap behaviour in smooth and palmate newts, *Triturus vulgaris* and *Triturus helveticus*. *British Journal of Herpetology*. 1. 5–10.

Griffiths, R.A. (1996). *Newts and Salamanders of Europe*. London: Poyser.

Griffiths, R.A., Foster, J., Wilkinson, J.W. & Sewell, D. (2015). Science, statistics and surveys: a herpetological perspective. *Journal of Applied Ecology*. 52. 1413–1417.

Griffiths, R.A. & Inns, H. (1998). Surveying. In: T. Gent & S. Gibson (eds.). *Herpetofauna Workers' Manual*. Peterborough, UK: Joint Nature Conservation Committee.

Griffiths, R.A., Raper, S.J. & Brady, L.. (1996). Evaluation of a standard method for surveying common frogs (*Rana temporaria*) and newts (*Triturus cristatus*, *T. helveticus* and *T. vulgaris*). *JNCC Report No. 259*.

Griffiths, R.A., Sewell, D. & McCrea, R.S. (2010). Dynamics of a declining amphibian metapopulation: Survival, dispersal and the impact of climate. *Biological Conservation*. 143. 485–491.

Griffiths, R.A., de Wijer, P., May, R.T., Griffiths, A.R.A., Wijer, P. De, May, R.T., Griffiths, R.A., Wijer, P. De & May, R.T. (1994). Predation and competition within an assemblage of larval newts (*Triturus*). *Ecography*. 17. 176–181.

Gu, W. & Swihart, R.K. (2004). Absent or undetected? Effects of non-detection of species occurrence on wildlife-habitat models. *Biological Conservation*. 116. 195–203.

Guillera-Arroita, G., Lahoz-Monfort, J.J., MacKenzie, D.I., Wintle, B.A. & McCarthy, M.A. (2014). Ignoring imperfect detection in biological surveys is dangerous: A response to 'fitting and interpreting occupancy models'. *PLoS ONE*. 9. e99571.

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

Haile, J., Froese, D.G., Macphee, R.D.E., Roberts, R.G., Arnold, L.J., Reyes, A. V, Rasmussen, M., Nielsen, R., Brook, B.W., Robinson, S., Demuro, M., Gilbert, M.T.P., Munch, K., Austin, J.J., Cooper, A., Barnes, I., Möller, P. & Willerslev, E. (2009). Ancient DNA reveals late survival of mammoth and horse in interior Alaska. *Proceedings of the National Academy of Sciences of the United States of America*. 106. 22352–22357.

Haile, J., Holdaway, R., Oliver, K., Bunce, M., Gilbert, M.T.P., Nielsen, R., Munch, K., Ho, S.Y.W., Shapiro, B. & Willerslev, E. (2007). Ancient DNA chronology within sediment deposits: Are paleobiological reconstructions possible and is DNA leaching a factor? *Molecular Biology and Evolution*. 24. 982–989.

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. & Hickey, D.A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*. 23. 167–172.

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

Hanna Instruments (n.d.). *Instruction manual HI98311-IH98312*. [Online]. Available from: http://www.hannacan.com/PDF/manHI98311.pdf. [Accessed: 4 July 2017].

Harper, L.R., Handley, L.L., Hahn, C., Boonham, N., Rees, H.C., Gough, K.C., Lewis, E., Adams, I.P., Brotherton, P., Phillips, S. & Haenfling, B. (2018). Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of great crested newt (*Triturus cristatus*). *Ecology and Evolution*. [Online]. In Press. Available from: https://www.biorxiv.org/content/early/2017/11/15/215897.

Harvey, C.T., Qureshi, S.A. & MacIsaac, H.J. (2009). Detection of a colonizing, aquatic, non-indigenous species. *Diversity and Distributions*. 15. 429–437.

Haubrock, P.J., Altrichter, J.A.N., Romana, V., Haubrock, P.J. & Altrichter, J. (2016). Northern crested newt (*Triturus cristatus*) migration in a nature reserve : multiple incidents of breeding season displacements exceeding 1km. *The Herpetological Bulletin*. 5. 31–33.

Henson, N. (2016). Technical appendices chapter 9 terrestrial and avian ecology. In: *North west coast connections preliminary environmental information (PEI)*. Leeds: National Gird.

Herder, J., Valentini, A., Bellemain, E., Dejean, T., van Delft, J., Thomsen, P.F. &

Taberlet, P. (2014). *Environmental DNA - a review of the possible applications for the detection of (invasive) species*. p.Report 2013-104.

Hinlo, R., Gleeson, D., Lintermans, M., Furlan, E., Klaveness, D. & Jussila, J. (2017). Methods to maximise recovery of environmental DNA from water samples. *Plos One*. 12. e0179251.

Hofreiter, M., Mead, J.I., Martin, P. & Poinar, H.N. (2003). Molecular caving. *Current Biology*. 13. 693–695.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Fach, P. & Wagner, M. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of clinical microbiology*. 42. 1863–8.

Horner, H.A. & Macgregor, H.C. (1985). Normal development in newts (*Triturus*) and its arrest as a consequence of an unusual chromosomal situation. *Journal of Herpetology*. 19. 261–270.

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J.A., Smith, B.J., Hunter, C.T., Reed, R.N. & Hart, K.M. (2015). Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS ONE*. 10. e0121655.

Ikeda, K., Doi, H., Tanaka, K., Kawai, T. & Negishi, J.N. (2016). Using environmental DNA to detect an endangered crayfish *Cambaroides japonicus* in streams. *Conservation Genetics Resources*. 8. 231–234.

Innis, S.A., Naiman, R.J. & Elliott, S.R. (2000). Indicators and assessment methods for measuring the ecological integrity of semi-aquatic terrestrial environments. *Hydrobiologia*. 422/423. 111–131.

Inns, H. (2009). *Britain's reptiles and amphibians: a guide to the reptiles and amphibians of Great Britain, Ireland and the Channel Islands*. WILDGuides.

Isao, K., Hara, S., Terauchi, K. & Kogure, K. (1990). Role of sub-micrometre particles in the ocean. *Nature*. 345. 242–244.

IUCN (2018a). *Amphibians | IUCN*. [Online]. 2018. Available from: https://www.iucn.org/theme/species/our-work/amphibians. [Accessed: 6 February 2018].

IUCN (2018b). *The IUCN red list of threatened species*. [Online]. 2018. Available from: http://www.iucnredlist.org/search. [Accessed: 2 January 2018].

Iversen, L.L., Kielgast, J. & Sand-Jensen, K. (2015). Monitoring of animal abundance by environmental DNA - An increasingly obscure perspective: A reply to Klymus et al., 2015. *Biological Conservation*. 192. 479–480.

Jackson, R.B., Carpenter, S.R., Dahm, C.N., McKnight, D.M., Naiman, R.J., Postel, S.L. & Running, S.W. (2001). Water in a changing world. *Ecological Applications*. 11. 1027–1045.

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

Janssenswillen, S. & Bossuyt, F. (2016). Male courtship pheromones induce cloacal gaping in female newts (Salamandridae). *PLoS ONE*. 11. e0144985.

Jarvis, L.E. (2016). Terrestrial ecology of juvenile great crested newts (*Triturus cristatus*) in a woodland area. *Herpetological Journal*. 26. 287–296.

Jehle, R. & Arntzen, J.W. (2000). Post-breeding migrations of newts (*Triturus cristatus* and *T. marmoratus*) with contrasting ecological requirements. *Journal of Zoology*. 251. 297–306.

Jehle, R., Arntzen, J.W., Burke, T., Krupa, A.P. & Hödl, W. (2001). The annual

number of breeding adults and the effective population size of syntopic newts (*Triturus cristatus, T. marmoratus*). *Molecular Ecology*. 10. 839–850.

Jehle, R., Thiesmeier, B. & Foster, J. (2011). *The crested newt*. Bielefield: Laurenti-Verlag.

Jehle, R., Wilson, G.A., Arntzen, J.W. & Burke, T. (2005). Contemporary gene flow and the spatio-temporal genetic structure of subdivided newt populations (*Triturus cristatus, T. marmoratus*). *Journal of Evolutionary Biology*. 18. 619–628.

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

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

Johnson, P.T.J., Mckenzie, V.J., Peterson, A.C., Kerby, J.L., Brown, J., Blaustein, A.R. & Jackson, T. (2011). Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. *Conservation Biology*. 25. 556–566.

Jørgensen, T., Haile, J., Möller, P., Andreev, A., Boessenkool, S., Rasmussen, M., Kienast, F., Coissac, E., Taberlet, P., Brochmann, C., Bigelow, N.H., Andersen, K., Orlando, L., Gilbert, M.T.P. & Willerslev, E. (2012a). A comparative study of ancient sedimentary DNA, pollen and macrofossils from permafrost sediments of northern Siberia reveals long-term vegetational stability. *Molecular Ecology*. 21. 1989–2003.

Jørgensen, T., Kjaer, K.H., Haile, J., Rasmussen, M., Boessenkool, S., Andersen, K., Coissac, E., Taberlet, P., Brochmann, C., Orlando, L., Gilbert, M.T.P. & Willerslev, E. (2012b). Islands in the ice: Detecting past vegetation on Greenlandic nunataks using historical records and sedimentary ancient DNA Meta-barcoding. *Molecular Ecology*. 21. 1980–1988.

Joseph, L.N., Field, S.A., Wilcox, C. & Possingham, H.P. (2006). Presence-absence versus abundance data for monitoring threatened species. *Conservation Biology*. 20. 1679–1687.

Juen, A. & Traugott, M. (2006). Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biology and Biochemistry*. 38. 1872–1879.

Kalinowski, S.T., Taper, M.L. & Marshall, T.C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*. 16. 1099–1106.

Kelly, R.P. (2016). Making environmental DNA count. *Molecular Ecology Resources*. 16. 10–12.

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

Kenta, T., Gratten, J., Haigh, N.S., Hinten, G.N., Slate, J., Butlin, R.K. & Burke, T. (2008). Multiplex SNP-SCALE: A cost-effective medium-throughput single nucleotide polymorphism genotyping method. *Molecular Ecology Resources*. 8. 1230–1238.

Kéry, M. & Schmid, H. (2004). Monitoring programs need to take into account imperfect species detectability. *Basic and Applied Ecology*. 5. 65–73.

Khatib, H., Darvasi, A. & Plotski, Y. (1994). Determining relative micorsatellite allele

frequencies in pooled DNA samples. *PCR Methods and Applications*. 4. 13–18.

Klymus, K., Richter, C., Thompson, N. & Hinck, J. (2017a). Metabarcoding of environmental DNA samples to explore the use of uranium mine containment ponds as a water source for wildlife. *Diversity*. 9. 54.

Klymus, K.E., Marshall, N.T. & Stepien, C.A. (2017b). Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. *PLoS ONE*. 12. e0177643.

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

Kowalchuk, G.A., Austin, J.J., Gooding, P.S. & Stephen, J.R. (2007). Valid recovery of nucleic acid sequence information from high contamination risk samples - ancient DNA and environmental DNA. In: *Perspectives in Bioanalysis*. 357–371.

Kraaijeveld, K., de Weger, L.A., Ventayol García, M., Buermans, H., Frank, J., Hiemstra, P.S. & den Dunnen, J.T. (2015). Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. *Molecular Ecology Resources*. 15. 8–16.

Kröpfli, M., Heer, P. & Pellet, J. (2010). Cost-effectiveness of two monitoring strategies for the great crested newt (*Triturus cristatus*). *Amphibia-Reptilia*. [Online]. 31. 403–410. Available from: http://booksandjournals.brillonline.com/content/journals/10.1163/156853810791769374.

Krupa, A.P., Jehle, R., Dawson, D.A., Gentle, L.K., Gibbs, M., Arntzen, J.W. & Burke, T. (2002a). Erratum - Microsatellite loci in the crested newt (*Triturus cristatus*)

and their utility in other newt taxa. *Conservation Genetics*. 3. 87–89.

Krupa, A.P., Jehle, R., Dawson, D.A., Gentle, L.K., Gibbs, M., Arntzen, J.W. & Burke, T. (2002b). Microsatellite loci in the crested newt (*Triturus cristatus*) and their utility in other newt taxa. *Conservation Genetics*. 3. 87–89.

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

Lacoursière-Roussel, A., Rosabal, M. & Bernatchez, L. (2016b). Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. *Molecular Ecology Resources*. 16. 1401–1414.

Lahoz-Monfort, J.J., Guillera-Arroita, G. & Tingley, R. (2016). Statistical approaches to account for false-positive errors in environmental DNA samples. *Molecular Ecology Resources*. 16. 673–685.

LaMotte Company (2017). *Soil texture test - 1067*. [Online]. 2017. Available from: http://www.lamotte.com/en/education/soil-testing/1067.html. [Accessed: 24 May 2017].

Lance, R.F., Klymus, K.E., Richter, C.A., Guan, X., Farrington, H.L., Carr, M.R., Thompson, N., Chapman, D. & Baerwaldt, K.L. (2017). Experimental observations on the decay of environmental DNA from bighead and silver carps. *Management of Biological Invasions*. 8. 343–359.

Langton, T.E.. T., Beckett, C.L.C. & Foster, J.J.P. (2001). *Great crested newt conservation handbook*. Halesworth: Froglife.

Laramie, M.B., Pilliod, D.S. & Goldberg, C.S. (2015). Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological*

Conservation. 183. 29–37.

Laroche, O., Wood, S.A., Tremblay, L.A., Ellis, J.I., Lejzerowicz, F., Pawlowski, J., Lear, G., Atalah, J. & Pochon, X. (2016). First evaluation of foraminiferal metabarcoding for monitoring environmental impact from an offshore oil drilling site. *Marine Environmental Research*. 120. 225–235.

Laroche, O., Wood, S.A., Tremblay, L.A., Lear, G., Ellis, J.I. & Pochon, X. (2017). Metabarcoding monitoring analysis: the pros and cons of using co-extracted environmental DNA and RNA data to assess offshore oil production impacts on benthic communities. *PeerJ*. 5. e3347.

Laszlo, A.H., Derrington, I.M., Ross, B.C., Brinkerhoff, H., Adey, A., Nova, I.C., Craig, J.M., Langford, K.W., Samson, J.M., Daza, R., Doering, K., Shendure, J. & Gundlach, J.H. (2014). Decoding long nanopore sequencing reads of natural DNA. *Nature Biotechnology*. 32. 829–833.

LeDuc, C., Miller, P., Lichter, J. & Parry, P. (1995). Batched analysis of genotypes. *Genome Research*. 4. 331–336.

Lee, P. (2017). DNA amplification in the field: move over PCR, here comes LAMP. *Molecular Ecology Resources*. 17. 138–141.

Levy-Booth, D.J., Campbell, R.G., Gulden, R.H., Hart, M.M., Powell, J.R., Klironomos, J.N., Pauls, K.P., Swanton, C.J., Trevors, J.T. & Dunfield, K.E. (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology and Biochemistry*. 39. 2977–2991.

Lewis, B. (2012). *An evaluation of mitigation actions for great crested newts at development sites, PhD Thesis*. Durrell Institute of Conservation and Ecology, University of Kent, UK.

Lewis, B., Griffiths, R.A. & Barrios, Y. (2007). *Field assessment of great crested newt*

Triturus cristatus mitigation projects in England. Natural England research report NERR001. Peterborough, UK.

Lewis, B., Griffiths, R.A. & Wilkinson, J.W. (2017). Population status of great crested newts (*Triturus cristatus*) at sites subjected to development mitigation. *Herpetological Journal*. 27. 133–142.

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

Lodge, D.M., Williams, S., Macisaac, H.J., Hayes, K.R., Leung, B., Mack, R.N., Moyle, P.B., Smith, M., Andow, D. a, Carlton, J.T., Mcmichael, A. & Reichard, S. (2006). Biological invasions: Recommendations for U.S. policy and management. *Ecological Applications*. 16. 2035–2054.

Lorenz, M.G. & Wackernagel, W. (1987). Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Applied and environmental microbiology*. 53. 2948–52.

Lugg, W.H., Griffiths, J., van Rooyen, A.R., Weeks, A.R. & Tingley, R. (2017). Optimal survey designs for environmental DNA sampling. *Methods in Ecology and Evolution*. In Press.

Lydolph, M.C., Jacobsen, J., Arctander, P., Gilbert, M.T.P., Gilichinsky, D.A., Hansen, A.J., Willerslev, E. & Lange, L. (2005). Beringian paleoecology inferred from permafrost-preserved fungal DNA. *Applied and Environmental Microbiology*. 71. 1012–1017.

MacKenzie, D.D., Nichols, J.D., Hines, J.E., Knutson, M.G. & Franklin, A.B. (2003). Estimating site occupancy, colonization, and local extinction when a species is

detected imperfectly. *Ecology*. 84. 2200–2207.

Mackenzie, D.I. & Kendall, W.L. (2002). How should detection probability be incorporated into estimates of relative abundance? *Ecology*. 83. 2387–2393.

MacKenzie, D.I., Nichols, J.D., Lachman, G.B., Droege, S., Royle, A.A. & Langtimm, C.A. (2002). Estimating site occupancy rates when detection probabilities are less than one. *Ecology*. 83. 2248–2255.

Maggi, F. (2013). The settling velocity of mineral, biomineral, and biological particles and aggregates in water. *Journal of Geophysical Research: Oceans*. 118. 2118–2132.

Magurran, A. (2004). *Measuring biological diversity*. Oxford; UK: Blackwell Science Ltd.

Mahon, A.R., Jerde, C.L., Galaska, M., Bergner, J.L., Chadderton, W.L., Lodge, D.M., Hunter, M.E. & Nico, L.G. (2013). Validation of eDNA surveillance sensitivity for detection of Asian carps in controlled and field experiments. *PLoS ONE*. 8. e58316.

Mahon, A.R., Nathan, L.R. & Jerde, C.L. (2014). Meta-genomic surveillance of invasive species in the bait trade. *Conservation Genetics Resources*. 6. 563–567.

Majeneva, M., Diserud, O.H., Eagle, S.H.C., Boström, E., Hajibabaei, M. & Ekrem, T. (2018). Environmental DNA filtration techniques affect recovered biodiversity. *Scientific Reports*. In Press.

Marchetti, M.P., Light, T., Moyle, P.B. & Viers, J.H. (2004). Fish invasions in California watersheds : testing hypotheses using landscape patterns. *Ecological Applications*. 14. 1507–1525.

Martellini, A., Payment, P. & Villemur, R. (2005). Use of eukaryotic mitochondrial DNA

to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Research*. 39. 541–548.

Matechou, E. & Caron, F. (2017). Modelling individual migration patterns using a Bayesian nonparametric approach for capture–recapture data. *Annals of Applied Statistics*. 11. 21–40.

Matheson, C.D., Gurney, C., Esau, N. & Lehto, R. (2010). Assessing PCR inhibition from humic substances. *The Open Enzyme Inhibition Journal*. 3. 38–45.

Matisoo-Smith, E., Roberts, K., Welikala, N., Tannock, G., Chester, P., Feek, D. & Flenley, J. (2008). Recovery of DNA and pollen from New Zealand lake sediments. *Quaternary International*. 184. 139–149.

Matsuhashi, S., Doi, H., Fujiwara, A., Watanabe, S. & Minamoto, T. (2016). Evaluation of the environmental DNA method for estimating distribution and biomass of submerged aquatic plants. *PLoS ONE*. 11. e0156217.

Matsui, K., Honjo, M. & Kawabata, Z. (2001). Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. *Aquatic Microbial Ecology*. 26. 95–102.

Mazerolle, M.J. (2017). *AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). R package version 2.1-1*. [Online]. Available from: https://cran.r-project.org/package=AICcmodavg.

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

Minamoto, T., Naka, T., Moji, K. & Maruyama, A. (2016). Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction.

Limnology. 17. 23–32.

Minamoto, T., Uchii, K., Takahara, T., Kitayoshi, T., Tsuji, S., Yamanaka, H. & Doi, H. (2017). Nuclear internal transcribed spacer-1 as a sensitive genetic marker for environmental DNA studies in common carp *Cyprinus carpio*. *Molecular Ecology Resources*. 17. 324–333.

Minter, E.J.A., Lowe, C.D., Brockhurst, M.A. & Watts, P.C. (2015). A rapid and cost-effective quantitative microsatellite genotyping protocol to estimate intraspecific competition in protist microcosm experiments. *Methods in Ecology and Evolution*. 6. 315–323.

Minting, P. (2016). *Great crested newt detectives*. Bornmouth, UK: Amphibian and Reptile Conservation.

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

Natural England (2014). *Environmental DNA (eDNA) detects the presence of great crested newts in water*. [Online]. 2014. Available from: http://webarchive.nationalarchives.gov.uk/20140605105717/http://www.naturalengland.org.uk/ourwork/regulation/wildlife/gcn-eDNA-feature.aspx. [Accessed: 12 November 2014].

Natural England (2017). *Wildlife licensing newsletter March 2017*. Peterborough, UK.

Newton, J., Sepulveda, A., Sylvester, K. & Thum, R. (2015). Potential utility of environmental DNA for early detection of Eurasian watermilfoil (*Myriophyllum spicatum*). *Journal of Aquatic Plant Management*. 54. 46–49.

O'Brien, C.D., Hall, J.E., Orchard, D., Barratt, C.D., Arntzen, J.W. & Jehle, R. (2015).

Extending the natural range of a declining species: genetic evidence for native great crested newt (*Triturus cristatus*) populations in the Scottish Highlands. *European Journal of Wildlife Research*. 61. 27–33.

O'Brien, D., Hall, J., Miró, A. & Wilkinson, J. (2017). Testing the validity of a commonly-used habitat suitability index at the edge of a species' range: great crested newt *Triturus cristatus* in Scotland. *Amphibia-Reptilia*. 38. 265–273.

O'Donnell, J.L., Kelly, R.P. & Lowell, N.C. (2017). Spatial distribution of environmental DNA in a nearshore marine habitat. *PeerJ*. 5. e3044.

Oldham, R.S., Keeble, J., Swan, M.J.S. & Jeffcote, M. (2000). Evaluating the suitability of habitat for the great crested newt (*Triturus cristatus*). *Herpetological Journal*. 10. 143–155.

Oliver, I., Pik, A., Britton, D., Dangerfield, J.M., Colwell, R.K. & Beattie, A.J. (2000). Virtual biodiversity assessment systems. *Computers in Biology*. 50. 441–450.

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

Paget, E., Monrozier, J.. M. & Simonet, P. (1992). Adsorption of DNA on clay minerals: protection against DNaseI and influence on gene transfer. *FEMS Microbiology Letters*. 97. 31–39.

Palomares, F., Godoy, J.A., Piriz, A., O'Brien, S.J. & Johnson, W.E. (2002). Faecal genetic analysis to determine the presence and distribution of elusive carnivores: Design and feasibility for the Iberian lynx. *Molecular Ecology*. 11. 2171–2182.

Parducci, L., Matetovici, I., Fontana, S.L., Bennett, K.D., Suyama, Y., Haile, J., Kjær, K.H., Larsen, N.K., Drouzas, A.D. & Willerslev, E. (2013). Molecular- and pollen-based vegetation analysis in lake sediments from central Scandinavia. *Molecular*

Ecology. 22. 3511–3524.

Peig, J. & Green, A.J. (2009). New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos*. 118. 1883–1891.

Pfleger, M.O., Rider, S.J., Johnston, C.E. & Janosik, A.M. (2016). Saving the doomed: Using eDNA to aid in detection of rare sturgeon for conservation (*Acipenseridae*). *Global Ecology and Conservation*. 8. 99–107.

Piaggio, A.J., Engeman, R.M., Hopken, M.W., Humphrey, J.S., Keacher, K.L., Bruce, W.E. & Avery, M.L. (2014). Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Molecular Ecology Resources*. 14. 374–380.

Piggott, M.P. (2016). Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and Evolution*. 6. 2739–50.

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

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

Pimentel, D., Zuniga, R. & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics*. 52. 273–288.

Pimm, S.L., Russell, G.J., Gittleman, J.L. & Brooks, T.M. (1995). The future of

biodiversity. *Science*. 269. 347–350.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R-Core Team (2016). *nlme: Linear and Nonlinear Mixed Effects Models.*

Pollock, K.H., Nichols, J.D., Simons, T.R., Farnsworth, G.L., Bailey, L.L. & Sauer, J.R. (2002). Large scale wildlife monitoring studies: Statistical methods for design and analysis. *Environmetrics*. 13. 105–119.

Qu, C. & Stewart, K.A. (2017). Comparing conservation monitoring approaches: traditional and environmental DNA tools for a critically endangered mammal. *PeerJ Preprints*. 5. e2828v1.

R Development Core Team (2016). *R: language and environment for statistical computing*.

Raposo do Amaral, F., Neves, L.G., Resende, M.F.R., Mobili, F., Miyaki, C.Y., Pellegrino, K.C.M. & Biondo, C. (2015). Ultraconserved elements sequencing as a low-cost source of complete mitochondrial genomes and microsatellite markers in non-model amniotes. *PLoS ONE*. 10. 1–9.

Ravanat, J.-L., Douki, T. & Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. *Journal of photochemistry and photobiology. B, Biology*. 63. 88–102.

Recorbet, G., Picard, C., Normand, P. & Simonet, P. (1993). Kinetics of the persistence of chromosomal DNA from genetically engineered *Escherichia coli* introduced into soil. *Applied and environmental microbiology*. 59. 4289–94.

Rees, H. (2017). *eDNA proficiency testing*. [Online]. 2017. ADAS. Available from: http://www.adas.uk/News/edna-proficiency-testing. [Accessed: 21 September 2017].

Rees, H.C., Baker, C.A., Gardner, D.S., Maddison, B.C. & Gough, K.C. (2017). The

detection of great crested newts year round via environmental DNA analysis. *BMC Research Notes*. 10. 327.

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

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

Renshaw, M.A., Olds, B.P., Jerde, C.L., Mcveigh, M.M. & Lodge, D.M. (2015). The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. *Molecular Ecology Resources*. 15. 168–176.

Romanowski, G., Lorenz, M.G. & Wackernagel, W. (1991). Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. *Applied and environmental microbiology*. 57. 1057–1061.

Roose-Amsaleg, C.L., Garnier-Sillam, E. & Harry, M. (2001). Extraction and purification of microbial DNA from soil and sediment samples. *Applied Soil Ecology*. 18. 47–60.

Rousset, F. (2008). GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*. 8. 103–106.

Rubinoff, D. (2006). Utility of mitochondrial DNA barcodes in species conservation. *Conservation Biology*. 20. 1026–1033.

Russell, P.J. (2006). *iGenetics a molecular approach*. Second edi. San Francisco, USA: Pearson Education Inc.

Saeki, K. & Kunito, T. (2010). Adsorptions of DNA molecules by soils and variable-charged soil constituents. In: A. Méndez-Vilas (ed.). *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. Badajoz, Spain: Formatex Research Center, 188–195.

Salipante, S.J., Scroggins, S.M., Hampel, H.L., Turner, E.H. & Pritchard, C.C. (2014). Microsatellite instability detection by next generation sequencing. *Clinical Chemistry*. 60. 1192–1199.

Sansom, B.J. & Sassoubre, L.M. (2017). Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. *Environmental Science & Technology*. 51. 14244–14253.

Santas, A.J., Persaud, T., Wolfe, B.A., Bauman, J.M., Santas, A.J., Persaud, T., Wolfe, B.A. & Bauman, J.M. (2013). Noninvasive method for a statewide survey of eastern hellbenders *Cryptobranchus alleganiensis* using environmental DNA. *International Journal of Zoology*. 2013. 174056.

Sassoubre, L.M., Yamahara, K.M., Gardner, L.D., Block, B.A. & Boehm, A.B. (2016). Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environmental Science and Technology*. 50. 10456–10464.

Sawaya, M.A., Stetz, J.B., Clevenger, A.P., Gibeau, M.L. & Kalinowski, S.T. (2012). Estimating grizzly and black bear population abundance and trend in Banff National Park using noninvasive genetic sampling. *PLoS ONE*. 7. e34777.

Schiebelhut, L.M., Abboud, S.S., Gómez Daglio, L.E., Swift, H.F. & Dawson, M.N. (2017). A comparison of DNA extraction methods for high-throughput DNA analyses. *Molecular Ecology Resources*. 17. 721–729.

Schmelzle, M.C. & Kinziger, A.P. (2016). Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of

an endangered aquatic species. *Molecular Ecology Resources*. 16. 895–908.

Schmidt, B.R. (2003). Count data, detection probabilities, and the demography, dynamics, distribution, and decline of amphibians. *Comptes rendus biologies*. 326. S119–S124.

Schmidt, B.R. (2005). Monitoring the distribution of pond-breeding amphibians when species are detected imperfectly. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 15. 681–692.

Schmidt, B.R., Kéry, M., Ursenbacher, S., Hyman, O.J. & Collins, J.P. (2013). Site occupancy models in the analysis of environmental DNA presence/absence surveys: A case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*. 4. 646–653.

Schnack, H.G., Bakker, S.C., van't Slot, R., Groot, B.M., Sinke, R.J., Kahn, R.S., Pearson, P.L., van 't Slot, R., Groot, B.M., Sinke, R.J., Kahn, R.S. & Pearson, P.L. (2004). Accurate determination of microsatellite allele frequencies in pooled DNA samples. *European Journal of Human Genetics*. 12. 925–934.

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

Schrader, C., Schielke, A., Ellerbroek, L. & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology*. 113. 1014–1026.

Sewell, D., Beebee, T.J.C. & Griffiths, R.A. (2010). Optimising biodiversity assessments by volunteers: The application of occupancy modelling to large-scale amphibian surveys. *Biological Conservation*. 143. 2102–2110.

Sewell, D., Griffiths, R.A., Beebee, T.J.C., Foster, J. & Wilkinson, J.W. (2013). *Survey*

protocols for the British herpetofauna Version 1.0. [Online]. Available from: http://www.narrs.org.uk/documents/Survey_protocols_for_the_British_herpetofauna.pdf.

Seymour, M., Durance, I., Cosby, B.J., Deiner, K., Ormerod, S.J., Colbourne, J.K., Wilgar, G., Carvalho, G.R., Bruyn, M. De, Edwards, F., Emmett, B.A. & Creer, S. (2018). Acidity promotes degradation of multi-species eDNA in lotic mesocosms. *Communications Biology*. 1. 4.

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

Sigsgaard, E.E., Nielsen, I.B., Bach, S.S., Lorenzen, E.D., Robinson, D.P., Knudsen, S.W., Pedersen, M.W., Jaidah, M. Al, Orlando, L., Willerslev, E., Møller, P.R. & Thomsen, P.F. (2016). Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology & Evolution*. 1. 0004.

Skalski, G.T., Couch, C.R., Garber, A.F., Weir, B.S. & Sullivan, C. V. (2006). Evaluation of DNA pooling for the estimation of microsatellite allele frequencies: A case study using striped bass (*Morone saxatilis*). *Genetics*. 173. 863–875.

Skei, J., Dolmen, D., Rønning, L. & Ringsby, T. (2006). Habitat use during the aquatic phase of the newts *Triturus vulgaris* (L.) and *T. cristatus* (Laurenti) in central Norway: proposition for a conservation and monitoring area. *Amphibia-Reptilia*. 27. 309–324.

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

Smith, K.F., Wood, S.A., Mountfort, D.O. & Cary, S.C. (2012). Development of a real-time PCR assay for the detection of the invasive clam, *Corbula amurensis*, in environmental samples. *Journal of Experimental Marine Biology and Ecology*. 412. 52–57.

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

Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E. & Hellström, M. (2016). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*. 8. 635–645.

Steffan, R.J., Goksoyr, J., Bej, A.K. & Atlas, R.M. (1988). Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology*. 54. 2908–2915.

Stein, E.D., White, B.P., Mazor, R.D., Miller, P.E. & Pilgrim, E.M. (2013). Evaluating ethanol-based sample preservation to facilitate use of DNA barcoding in routine freshwater biomonitoring programs using benthic macroinvertebrates. *PLoS ONE*. 8. e51273.

Stewart, K., Ma, H., Zheng, J. & Zhao, J. (2017). Using environmental DNA to assess population-wide spatiotemporal reserve use. *Conservation Biology*. 31. 1173–1182.

Stoeckle, B.C., Beggel, S., Cerwenka, A.F., Motivans, E., Kuehn, R. & Geist, J. (2017a). A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. *Plos One*. 12. e0189119.

Stoeckle, M.Y., Soboleva, L. & Charlop-powers, Z. (2017b). Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. *PLoS ONE*. 12. e0175186.

Strand, D.A., Holst-Jensen, A., Viljugrein, H., Edvardsen, B., Klaveness, D., Jussila, J. & Vrålstad, T. (2011). Detection and quantification of the crayfish plague agent in natural waters: Direct monitoring approach for aquatic environments. *Diseases of Aquatic Organisms*. 95. 9–17.

Strand, D.A., Jussila, J., Johnsen, S.I., Viljamaa-Dirks, S., Edsman, L., Wiik-Nielsen, J., Viljugrein, H., Engdahl, F. & Vrålstad, T. (2014). Detection of crayfish plague spores in large freshwater systems. *Journal of Applied Ecology*. 51. 544–553.

Strand, D.A., Jussila, J., Viljamaa-Dirks, S., Kokko, H., Makkonen, J., Holst-Jensen, A., Viljugrein, H. & Vrålstad, T. (2012). Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Veterinary Microbiology*. 160. 99–107.

Strayer, D.L. & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society*. 29. 344–358.

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

Van Strien, A.J., Van Swaay, C.A.M. & Termaat, T. (2013). Opportunistic citizen science data of animal species produce reliable estimates of distribution trends if analysed with occupancy models. *Journal of Applied Ecology*. 50. 1450–1458.

Stuart, S.N., Chanson, J.S., Cox, N. a, Young, B.E., Rodrigues, A.S.L., Fischman, D.L. & Waller, R.W. (2004). Status and trends of amphibian declines and

extinctions worldwide. *Science*. 306. 1783–1786.

Suez, M., Behdenna, A., Brouillet, S., Graça, P., Higuet, D. & Achaz, G. (2016). MicNeSs: Genotyping microsatellite loci from a collection of (NGS) reads. *Molecular Ecology Resources*. 16. 524–533.

Taberlet, P., Coissac, E., Hajibabaei, M. & Rieseberg, L.H. (2012). Environmental DNA. *Molecular Ecology*. 21. 1789–1793.

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

Takahara, T., Minamoto, T. & Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS ONE*. 8. e56584.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. & Kawabata, Z. (2012). Estimation of fish biomass using environmental DNA. *PLoS ONE*. 7. e35868.

Tanadini, L.G. & Schmidt, B.R. (2011). Population size influences amphibian detection probability: Implications for biodiversity monitoring programs. *PLoS ONE*. 6. e28244.

Tebbe, C.C. & Vahjen, W. (1993). Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Applied and Environmental Microbiology*. 59. 2657–2665.

Thomas, A.C., Deagle, B.E., Eveson, J.P., Harsch, C.H. & Trites, A.W. (2016). Quantitative DNA metabarcoding: Improved estimates of species proportional biomass using correction factors derived from control material. *Molecular Ecology Resources*. 16. 714–726.

Thomsen, P.F., Kielgast, J., Iversen, L., Møller, P.R., Rasmussen, M. & Willerslev, E. (2012a). Detection of a diverse marine fish fauna using environmental DNA from

seawater samples. *PLoS ONE*. 7. e41732.

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

Thomsen, P.F., Møller, P.R., Sigsgaard, E.E., Knudsen, S.W., Jørgensen, O.A. & Willerslev, E. (2016). Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. *PLoS ONE*. 11. e0165252.

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

Thornton, C.G. & Passen, S. (2004). Inhibition of PCR amplification by phytic acid, and treatment of bovine fecal specimens with phytase to reduce inhibition. *Journal of Microbiological Methods*. 59. 43–52.

Tillotson, M.D., Kelly, R.P., Duda, J.J., Hoy, M., Kralj, J. & Quinn, T.P. (2018). Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. *Biological Conservation*. 220. 1–11.

Toouli, C.D., Turner, D.R., Grist, S.A. & Morley, A.A. (2000). The effect of cycle number and target size on polymerase chain reaction amplification of polymorphic repetitive sequences. *Analytical Biochemistry*. 280. 324–326.

Treer, D., Van Bocxlaer, I., Matthijs, S., Du Four, D., Janssenswillen, S., Willaert, B. & Bossuyt, F. (2013). Love is blind: Indiscriminate female mating responses to male courtship pheromones in newts (Salamandridae). *PLoS ONE*. 8. e56538.

Tréguier, A., Paillisson, J.-M.M., Dejean, T., Valentini, A., Schlaepfer, M.A.A. & Roussel, J.-M.M. (2014). Environmental DNA surveillance for invertebrate

species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*. 51. 871–879.

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

Turner, C.R., Miller, D.J., Coyne, K.J. & Corush, J. (2014b). Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (Hypophthalmichthys spp.). *PLoS ONE*. 9. e114329.

Turner, C.R., Uy, K.L. & Everhart, R.C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*. 183. 93–102.

Tyre, A.J., Tenhumberg, B., Field, S.A., Niejalke, D. & Possingham, H.P. (2003). Improving precision and reducing bias in biological surveys : estimating false-negative error rates. *Ecological Applications*. 13. 1790–1801.

Unglaub, B., Steinfartz, S., Drechsler, A. & Schmidt, B.R. (2015). Linking habitat suitability to demography in a pond-breeding amphibian. *Frontiers in Zoology*. 12. 9.

United States Department of Agriculture (n.d.). *Soil texture calculator*. [Online]. Available from: https://www.nrcs.usda.gov/wps/portal/nrcs/detail/?cid=nrcs142p2_054167. [Accessed: 30 June 2017].

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G.H., Geniez, P., Pont, D., Argillier, C., Baudoin,

J.M., Peroux, T., Crivelli, A.J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P.R., Willerslev, E. & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*. 25. 929–942.

Vartia, S., Villanueva-Cañas, J.L., Finarelli, J., Farrell, E.D., Collins, P.C., Hughes, G.M., Carlsson, J.E.L., Gauthier, D.T., McGinnity, P., Cross, T.F., FitzGerald, R.D., Mirimin, L., Crispie, F., Cotter, P.D. & Carlsson, J. (2016). A novel method of microsatellite genotyping-by-sequencing using individual combinatorial barcoding. *Royal Society Open Science*. 3. 150565.

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S. & Obst, U. (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *Journal of Microbiological Methods*. 56. 277–286.

Vörös, J., Márton, O., Schmidt, B.R., Gál, J.T. & Jelić, D. (2017). Surveying Europe's only cave-dwelling chordate species (*Proteus anguinus*) using environmental DNA. *Plos One*. 12. e0170945.

Waits, L.P. & Paetkau, D. (2005). Noninvasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection. *Journal of Wildlife Management*. 69. 1419–1433.

Walker, S.F.F., Salas, M.B.B., Jenkins, D., Garner, T.W.J.W.J., Cunningham, A.A.A., Hyatt, A.D.D., Bosch, J. & Fisher, M.C.C. (2007). Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms*. 77. 105–112.

Ward, R.J., Griffiths, R.A., Wilkinson, J.W. & Cornish, N. (2017). Optimising monitoring efforts for secretive snakes: a comparison of occupancy and N-mixture models for assessment of population status. *Scientific Reports*. 7. 18074.

Wegleitner, B.J., Jerde, C.L., Tucker, A., Chadderton, W.L. & Mahon, A.R. (2015). Long duration, room temperature preservation of filtered eDNA samples. *Conservation Genetics Resources*. 7. 789–791.

White, G.C. & Burnham, K.P. (1999). Program MARK: survival estimation from populations of marked animals. *Bird study*. 46 (S1). S120–S139.

Wielstra, B. & Arntzen, J.W. (2016). Description of a new species of crested newt, previously subsumed in *Triturus ivanbureschi* (Amphibia: Caudata: Salamandridae). *Zootaxa*. 4109. 73–80.

Wielstra, B. & Arntzen, J.W. (2011). Unraveling the rapid radiation of crested newts (*Triturus cristatus* superspecies) using complete mitogenomic sequences. *BMC Evolutionary Biology*. 11. 162.

Wielstra, B., Crnobrnja-Isailović, J., Litvinchuk, S.N., Reijnen, B.T., Skidmore, A.K., Sotiropoulos, K., Toxopeus, A.G., Tzankov, N., Vukov, T. & Arntzen, J.W. (2013). Tracing glacial refugia of *Triturus* newts based on mitochondrial DNA phylogeography and species distribution modeling. *Frontiers in Zoology*. 10. 1–14.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R. & Schwartz, M.K. (2013). Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS ONE*. 8. e59520.

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

Wilkinson, J.. W. & Arnell, A.P. (2013). *NARRS report 2007-2012*. Bournemouth, UK.

Williams, K.E., Huyvaert, K.P. & Piaggio, A.J. (2016). No filters, no fridges: a method

for preservation of water samples for eDNA analysis. *BMC Research Notes*. 9. 298.

Williams, K.E., Huyvaert, K.P., Piaggio, A.J., Duncan, R., Luo, C. & Poretsky, R. (2017). Clearing muddied waters: Capture of environmental DNA from turbid waters. *Plos One*. 12. e0179282.

Wilson, C., Wright, E., Bronnenhuber, J., Macdonald, F., Belore, M. & Locke, B. (2014). Tracking ghosts: combined electrofishing and environmental DNA surveillance efforts for Asian carps in Ontario waters of Lake Erie. *Management of Biological Invasions*. 5. 225–231.

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

Xia, Z., Zhan, A., Gao, Y., Zhang, L., Haffner, G.D. & MacIsaac, H.J. (2017). Early detection of a highly invasive bivalve based on environmental DNA (eDNA). *Biological Invasions*. 17. 1–11.

Yamanaka, H., Minamoto, T., Matsuura, J., Sakurai, S., Tsuji, S., Motozawa, H., Hongo, M., Sogo, Y., Kakimi, N., Teramura, I., Sugita, M., Baba, M. & Kondo, A. (2017). A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. *Limnology*. 18. 233–241.

Zavodna, M., Bagshaw, A., Brauning, R. & Gemmell, N.J. (2014). The accuracy, feasibility and challenges of sequencing short tandem repeats using next-generation sequencing platforms. *PLoS ONE*. 9. 1–14.

Zhan, L., Paterson, I.G., Fraser, B.A., Watson, B., Bradbury, I.R., Nadukkalam Ravindran, P., Reznick, D., Beiko, R.G. & Bentzen, P. (2016). MEGASAT: automated inference of microsatellite genotypes from sequence data. *Molecular Ecology Resources*. 17. 247–256.

Zimmer, E.A. & Roalson, E.H. (2005). *Molecular evolution : producing the biochemical data. Part B*. San Diego, USA: Elsevier Achademic Press.

Zobel, M., Davison, J., Edwards, M.E., Brochmann, C., Coissac, E., Taberlet, P., Willerslev, E. & Moora, M. (2018). Ancient environmental DNA reveals shifts in dominant mutualisms during the late Quaternary. *Nature Communications*. 9. 139.

Appendix I – Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds

This appendix has been submitted to the peer reviewed journal Methods in Ecology and Evolution and is under review. Its production was a joint project between members of the eDNA in Ponds working group, a subgroup of the UK DNA Working group. A.S. Buxton is the second author and has been heavily involved with the production of this review, with Lynsey Harper from The University of Hull. Lynsey Harper and A.S. Buxton conceived the review paper, and between them produced the first draft of text, which has been commented on and edited, under their coordination, by the other authors.

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Summary

1. Environmental DNA (eDNA) analysis is a relatively rapid, non-invasive, cost-efficient method of species detection and identification, which may complement or interchange with conventional biodiversity monitoring. eDNA analysis is currently acknowledged as a tool to inform aquatic conservation and management. Development is ongoing, with strong commercial interest, and new applications are continually being discovered. However, formal recognition of this monitoring tool by governing authorities is uncommon. Although the potential applications of eDNA have already been reviewed, and several eDNA research guidelines are established, eDNA applications in ponds and methodological constraints specific to these environments remain unaddressed.
2. Despite the proliferation of eDNA surveys in research and professional practice, there is no consensus on standardisation of sampling or analytical procedures. This can lead to misleading and even erroneous interpretation of results, and loss of credibility. Following a stakeholder workshop in 2017, researchers combined knowledge and expertise to review aspects of eDNA ecology that differ between ponds and other freshwater ecosystems. In this review, we discuss current and prospective applications of eDNA analysis, and challenges that need to be addressed for the future and consistency of biodiversity monitoring in ponds.
3. The greatest challenges for eDNA surveys of ponds are the problems of potential PCR inhibition, alongside ensuring representative sampling and optimal method of eDNA capture. We provide recommendations for sampling, eDNA capture, inhibition testing, and ideal laboratory practice, which should aid those beginning eDNA projects or currently using this tool in ponds.
4. If implemented, these recommendations will contribute towards an eventual broad standardisation of eDNA research and practice, with room to tailor workflows for optimal analysis and different applications. Such standardisation will provide more

robust, comparable, and ecologically meaningful data to enable effective management and conservation of pond biodiversity.

Key-words: biodiversity, environmental DNA (eDNA), metabarcoding, monitoring, ponds, quantitative PCR (qPCR), survey

Introduction

Globally, there are an estimated 64 million to 3 billion ponds or small lakes (Downing *et al.* 2006; Biggs, von Fumetti & Kelly-Quinn 2016), with ponds outnumbering larger lentic freshwater systems approximately 100:1 (Downing *et al.* 2006; Céréghino *et al.* 2008). Ponds represent a high proportion of global freshwater habitat despite their limited size, comprising up to 30% of standing freshwater by area (Downing *et al.* 2006). These small water bodies occur in all land-use types at high frequency (Céréghino *et al.* 2008) and possess ecological, aesthetic, and recreational value (Biggs *et al.* 2016). Ponds are species-rich, containing many rare, protected, and unique species not found in other freshwater habitats (Wood, Greenwood & Agnew 2003). Moreover, pond networks support more species at landscape-scale than lakes or rivers (Davies *et al.* 2008).

Ponds have enormous scientific value as small and abundant ecosystems along broad ecological gradients, enabling experimental validation and hypothesis testing in ecology and conservation (De Meester *et al.* 2005). However, until recently, pondscapes – a pond, its immediate catchment, and the terrestrial matrix of land between ponds – were poorly understood (Wood *et al.* 2003). Ponds were not mentioned or included in the Water Framework Directive (Davies *et al.* 2008) and have been neglected in research, scientific monitoring, and policy (De Meester *et al.* 2005; Céréghino *et al.* 2008; Oertli *et al.* 2009), despite being threatened by anthropogenic activity and environmental change, and having greater vulnerability to environmental stressors than larger water bodies with larger catchments (Biggs *et al.* 2016). Poor study of these important ecosystems may be due in part to a lack of appropriate monitoring tools and sheer abundance. Pond biodiversity assessment can be costly, time-consuming, and dependent on taxonomic expertise (Briers & Biggs 2005). Often data is at the genus- or family-level when species-level knowledge is required for effective conservation.

In this context, molecular tools offer a solution through rapid, sensitive, cost-effective, non-invasive monitoring and promise to enhance our understanding of global biodiversity. One tool, environmental DNA (eDNA) analysis (see Supplemntary Information: Box. 1), is particularly relevant for aquatic biodiversity monitoring (Rees *et al.* 2014b; Thomsen & Willerslev 2015; Lawson Handley 2015). Ponds were the first natural habitats screened for eDNA by Ficetola *et al*. (2008), who demonstrated reliable detection of invasive American bullfrogs *Lithobates catesbeianus*, even at low densities. Since this initial publication, a large and growing number of studies have utilised eDNA in a range of environments (reviewed for example by Rees *et al*. 2014b; Lawson Handley 2015; Thomsen & Willerslev 2015; Deiner *et al.* 2017). eDNA approaches are often more affordable and logistically feasible than conventional counterparts, and have enormous potential to enable ecological study at greater temporal and spatial scales (Deiner *et al.* 2017). However, there are unique challenges associated with using eDNA in ponds that are not faced in other aquatic environments, largely due to the physical and chemical properties of ponds that influence eDNA capture and detection, which are not taken into account by current methodologies.

Although eDNA and its applications have been reviewed extensively (Rees *et al.* 2014b; Thomsen & Willerslev 2015; Lawson Handley 2015; Barnes & Turner 2015; Goldberg *et al.* 2016; Deiner *et al.* 2017), examinations of eDNA in relation to specific environments are distinctly lacking. In this review, we evaluate eDNA analysis as a tool for biodiversity monitoring in ponds. We first discuss the prospects of eDNA monitoring in these ecosystems. We then identify how ponds differ from other freshwater habitats, and examine the implications this has for eDNA detection. We outline the challenges associated with eDNA analysis in ponds and use the existing literature and combined experience of all authors to provide recommendations that will help standardise eDNA workflows for passive or targeted monitoring of pond

biodiversity. Finally, we look into the future of eDNA monitoring in ponds and explore avenues of research that would enhance our understanding of these ecosystems.

Prospects of eDNA monitoring in ponds

eDNA analysis continues to gain popularity with numerous studies on lentic ecosystems, including ponds (Table A1.S1), but we are only beginning to realise the potential of ponds for eDNA monitoring. The most obvious potential is biodiversity assessment, but they also offer endless experimental opportunities for ecological hypothesis testing and heightened understanding of eDNA dynamics due to the vast physical and chemical heterogeneity of ponds.

Ponds are important biodiversity hotspots in fragmented landscapes; however, eDNA analysis may revolutionise how we record and measure this biodiversity (Biggs *et al.* 2016). The sensitivity of this tool over conventional methods of monitoring pond biodiversity has been repeatedly demonstrated. The work of Thomsen *et al*. (2012) was pivotal to the development of eDNA surveillance for many rare and endangered species across the globe (Torresdal, Farrell & Goldberg 2017; Bylemans *et al.* 2017; Weltz *et al.* 2017; Doi *et al.* 2017). eDNA analysis has since shown potential for estimation of relative abundance and biomass (Thomsen *et al.* 2012; Takahara *et al.* 2012; Buxton *et al.* 2017b), and has begun to outperform conventional counterparts, for example, large-scale sampling and distribution modelling of the protected great crested newt *Triturus cristatus* (Biggs *et al.* 2015), and may deepen our understanding of species distribution patterns and activity.

eDNA analysis can drastically enhance the scope and scale of wildlife surveys, enabling research that would be impractical with conventional tools. Indeed, eDNA metabarcoding was recently used to identify abiotic and biotic determinants of *T. cristatus* at the pondscape (Harper *et al.* 2018). Ponds are often considered to be

closed systems, but may receive inputs from inflow and land surface run-off (especially during high rainfall and flood events), mobile species (e.g. birds, dragonflies, amphibians, water beetles), and other sources. Ponds are impacted both directly and indirectly, through large aquatic-terrestrial contact zones, by anthropogenic and environmental stressors. They can therefore act as natural samples of biodiversity in the wider environment, and provide information on entire ecosystems (De Meester *et al.* 2005). For example, eDNA metabarcoding revealed wildlife using uranium mine containment ponds as water sources, and supplemented conventional assessment of ecotoxicological effects of uranium mining on local biodiversity (Klymus *et al.* 2017b).

Pond water is comparatively stagnant, and the lack of flow and relatively small water volumes in ponds allows eDNA to accumulate over time to concentrations not attainable in most other water bodies. This benefits the amount of target DNA present, and subsequent detection probability (Buxton, Groombridge & Griffiths 2017a). However, eDNA accumulation can reduce ability to distinguish contemporary from recent or historic presence (Rees *et al.* 2014b). Under stagnant conditions, eDNA can settle out of suspension, but become incorporated into the water column again following sediment disturbance (Turner, Uy & Everhart 2015; Buxton, Groombridge & Griffiths 2018). eDNA may remain detectable in ponds for several weeks under 'optimal' conditions (Buxton *et al.* 2017a), but can also degrade rapidly with complete disappearance of target eDNA within one week (Brys, R. & Halfmaerten, D., unpublished results). Ponds are further influenced by the activity of domestic and wild animals which can increase suspended solids within the water column and change the properties of an eDNA sample. These external influences may also transfer eDNA between water bodies and potentially cause false positive detections (Klymus *et al.* 2017b).

The small and shallow nature of ponds subjects these systems to more extreme conditions than deeper water bodies, including larger fluctuations in temperature range and potentially greater exposure to ultraviolet light (UV); although, higher turbidity and dense vegetation in some ponds will limit UV penetration (Kazanjian *et al.* 2018). Temperature, UV, and pH all influence eDNA shedding and degradation rates, and can affect the amount of eDNA present within a waterbody (Strickler, Fremier & Goldberg 2015; Robson *et al.* 2016; Buxton *et al.* 2017b). Many ponds are successional in nature and often support an abundant emergent and semi-terrestrial vegetation with substantial (relative to waterbody size) shallow marginal drawdown zones in some cases, creating ideal habitat for multiple amphibian species. As water volume decreases over time, ponds become increasingly ephemeral or seasonal (Wood *et al.* 2003). Accessing these waters via wet, vegetated margins may make cross-contamination between sites hard to avoid, while high levels of organic debris in late succession ponds and duckweed (*Lemna* spp.) dominated ponds can exacerbate difficulties in collecting clean, debris-free samples.

Crucially, ponds can be highly anoxic due to poor wind-mixing and mass decomposition of terrestrial, submerged, and emergent vegetation, resulting in extremely low oxygen content at the bottom of the water column (Sayer *et al.* 2013; Kazanjian *et al.* 2018). Anoxic conditions were shown to slow marine eDNA decay (Weltz *et al.* 2017) but impacts of anoxia on pond eDNA have not been investigated. Slow decay may affect inferences made from eDNA regarding contemporary species presence; however, anoxic conditions dramatically enhance preservation of pond sediments and the communities that live there, providing information on historical pond biodiversity (Alderton *et al.* 2017; Emson *et al.* 2017).

Challenges, considerations and recommendations for eDNA monitoring in ponds

A universal methodology for eDNA analysis may not be appropriate across habitat types as water bodies vary considerably in their biological, physical and chemical properties (Goldberg *et al.* 2016). These fundamental differences can affect eDNA behaviour, including origin, state, fate and transport (Barnes & Turner 2015), and may ultimately have repercussions for eDNA detection. However, no reviews to date examine eDNA in the context of a single freshwater habitat and the challenges specific to this environment. The characteristics of ponds that make them ideal systems for eDNA monitoring and research are the very characteristics that challenge eDNA analysis. It is likely no one standard workflow will be appropriate in all circumstances. Practitioners must instead determine the most appropriate workflow options on a study-by-study basis. Figure A1.1 outlines these options and other considerations that must be taken into account throughout the eDNA workflow.

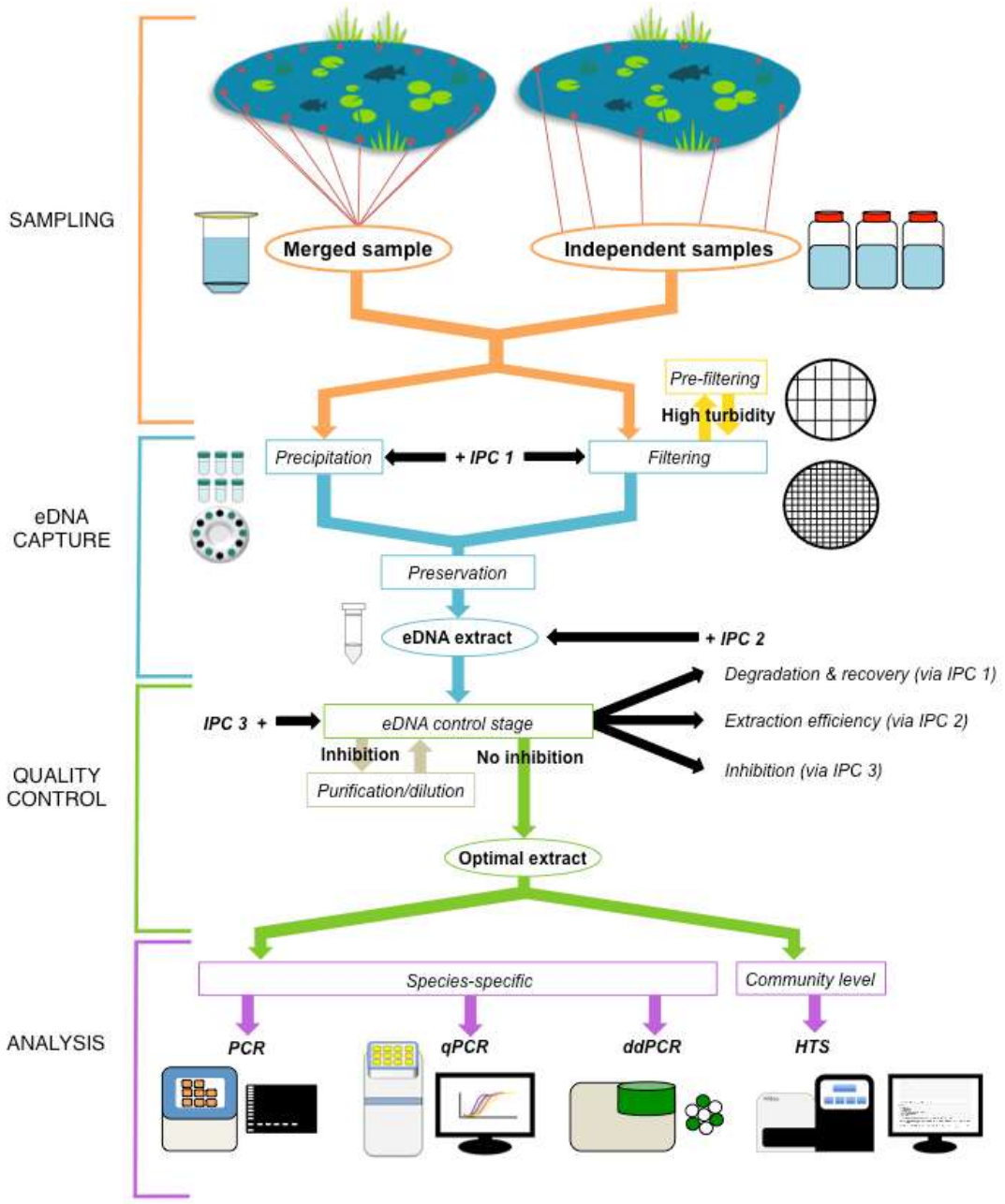

Figure A1.1. Schematic of eDNA workflow for samples collected from ponds. Three different Internal Positive Controls (IPCs) are recommended for inclusion during the stages of eDNA capture and quality control to identify substandard samples which require reanalysis or resampling. Pre-filtering is recommended if water samples are turbid.

SAMPLING

The distribution and dispersion of eDNA in ponds complicates design of sampling strategies. In contrast to lotic systems, eDNA has patchy distribution in lentic systems due to uneven distribution of organisms (Takahara *et al.* 2012; Eichmiller, Bajer & Sorensen 2014), possibly resulting from available microhabitats (Nicolet *et al.* 2004). eDNA distribution and dispersion in ponds is limited both horizontally by the presence of barriers to water movement (e.g. fallen trees and dense stands of aquatic vegetation; Biggs *et al.* 2015), and vertically by chemical stratification of the water column due to minimal wind-mixing (Sayer *et al.* 2013). This large variation in eDNA on fine spatial scales has severe consequences for species detection. Eichmiller *et al*. (2014) detected common carp *Cyprinus carpio* eDNA at points within tens of metres where it went undetected in a small lake. More recent caging experiments of fish and amphibians in ponds revealed a strong decrease in eDNA detection probability with distance from the cage, with most species almost undetectable after a few metres (Brys, R. & Halfmaerten, D., unpublished data; Li, J. *et al.*, unpublished data). We recommend water is collected underneath or around barriers to eDNA dispersion, and at different depths in ponds to maximise species detection.

The patchy distribution of pond eDNA means one sample of surface water will not sufficiently represent true biodiversity. Representation can be achieved with a timely, thought-out sampling strategy that accounts for location, number and volume of samples, and method of collection. Crucially, ecology of target species should be taken into consideration when choosing sampling time frame and methodology. Comprehensive sampling, at many different locations on fine spatial scales, will be required for pond eDNA surveys. There are two main options: collection of stratified or random subsamples around a pond, or sampling/subsampling locations known to be suitable for target species. Samples may be combined for sample preservation, DNA capture, and analysis, or processed independently as biological replicates

(Figure A1.1). The chosen strategy will be context-dependent as surveyors must ensure their targeted or merged sample(s) are representative of their focal species. For example, *T. cristatus* detection may be best achieved through collection of 20 x 30 mL samples which are combined and homogenised before 6 x 15 mL subsamples are taken for subsequent DNA extraction and qPCR analysis (Biggs *et al.* 2015). Volume and number of samples are standardised with this protocol, but whether all or any aspects would be effective for other species or different applications is unclear. Indeed, Harper *et al*. (in press) observed lower *T. cristatus* detection with eDNA metabarcoding than qPCR using this protocol. For information on entire communities, it may be better to take stratified samples around a pond and process these as biological replicates (Evans *et al.* 2017). Independent sample processing is also necessary to investigate species distribution and habitat use in ponds. We advocate that eDNA studies include sample-based rarefaction to evaluate sample number required to fully represent pond biodiversity.

Limited accessibility to a waterbody can hamper optimisation of sampling strategies for aquatic environments, particularly ponds. Typically, the full pond perimeter may be inaccessible due to distance from the shoreline, areas of dense vegetation, high steep banks, or other risks to health and safety. Sampling poles, boats or drones (aerial or aquatic) can enable water sample collection beyond the shoreline, but routine use is prevented by expense of purchase and operation (Barnes & Turner 2015) and they may potentially transfer contaminants between ponds. Therefore, surveyors are often unable to systematically sample the full pond perimeter or areas most suitable for focal species, and instead can only collect samples where access can be gained. This may influence detection rates but as yet, there is no evidence to support or refute this. Better insights to the confidence and resolution of eDNA detection in ponds could be obtained if surveyors report the total size of the pond

perimeter and proportion that was inaccessible, the number of samples and distance at which these were taken, and volume of water collected per sample.

It is not uncommon for ponds to undergo summer drying, causing a reduction in water volume (Nicolet *et al.* 2004) which may complicate sample collection. In some extremes, ponds completely dry in summer months, reducing suitability for fully aquatic species and preventing any sample collection. However, ponds may still be used by semi-aquatic species earlier in the season (Nicolet *et al.* 2004) thus dry ponds should not be automatically deemed negative for a target species when no sample can be collected (Buxton *et al.* 2018). In these circumstances, eDNA samples from sediment may provide better insight as to which species utilise a pond, provided method of eDNA capture is appropriate and cautious inferences are made regarding species detection (Turner *et al.* 2015; Buxton *et al.* 2018).

eDNA CAPTURE

Two broad methods are used in the capture of eDNA: filtration or ethanol precipitation. Comparative studies have generally shown that filtration approaches have higher sample throughput and can process greater water volumes, thereby increasing potential to recover greater amounts of DNA (Spens *et al.* 2016; Hinlo *et al.* 2017; Klymus *et al.* 2017b). However, studies tend to exclude ponds and make comparisons for water from rivers, lakes and experimental aquaria.

As ponds can contain high levels of suspended solids and algae, as well as high levels of organic debris from detached, degrading aquatic and terrestrial vegetation, filters tend to become blocked when sampling comparatively small water volumes (Klymus *et al.* 2017b). Where water is turbid, centrifugation, increased pore size, or pre-filtering will be necessary (Figure A1.1; Takahara *et al.* 2012; Robson *et al.* 2016; Klymus *et al.* 2017b). However, pre-filters increase cost and potential for cross-

contamination, and larger pore sizes trade capture of smaller particle sizes for greater proportions of target DNA, reducing total eDNA yield (Turner *et al.* 2014). These issues make it difficult to standardise the exact filtration method or volume of water processed. Nonetheless, a recent study comparing different filter sizes in ponds found filter size did not impede metabarcoding detection probability of fish, despite differences in filtration time and eDNA recovery (Li, J. *et al*. unpublished data). In contrast, water volumes are consistent with ethanol precipitation and species recovery may be the same or higher (Klymus *et al.* 2017b). However, water volume is usually limited to ~90 mL per sample due to logistical and financial constraints on the number of tubes of ethanol that can be taken into the field (Biggs *et al.* 2015). Moreover, ethanol is not always easy to obtain and is subject to dangerous goods regulations for transportation.

Where possible, we advise filtration is performed on-site using enclosed capsule or syringe filters to minimise risk of contamination (Spens *et al.* 2016). If on-site filtration is unfeasible, samples should be kept cool and processed in the laboratory within 24 hours, or preservative added if this time frame cannot be met, to maximise DNA recovery (Hinlo *et al.* 2017). Filters should be placed in preservative solution or frozen to prevent eDNA degradation prior to extraction (Hinlo *et al.* 2017). Ethanol precipitation remains an effective method of eDNA capture where field sites are remote, filtration facilities are not available, or surveyors are relatively inexperienced (i.e. volunteers).

INHIBITION

PCR inhibition can affect eDNA samples from any environment (Jane *et al.* 2015), but the stagnant nature of ponds means they are particularly prone to inhibitor build-up. Ponds have high organic inputs due to dense vegetation, lack of water flow, and

soil run-off, which encourages the build-up of algae, supports dense planktonic communities, and leads to high levels of natural turbidity. Turbid water with high suspended particulate matter not only clogs filters, but blocks extraction spin columns reducing DNA recovery. DNA extracts produced from turbid water often contain humic acid and tannin compounds, created through non-enzymatic decay of the organic material. These compounds can inactivate DNA polymerase and inhibit the PCR amplification process, reducing its efficiency or causing complete failure (Alaeddini 2012; Albers *et al.* 2013; McKee, Spear & Pierson 2015).

PCR inhibition can cause false negatives, thus it is imperative that eDNA practitioners test for it (Goldberg *et al.* 2016) using qPCR amplification of Internal Positive Controls (IPCs, see Supplementary Information: Box. 1), such as Applied Biosystems™ TaqMan Exogenous Internal Positive Control Reagents (Figure A1.1, IPC3), or by spiking reactions with control DNA that will not be found in the sample. The impact of inhibition can be minimised through optimisation of reagents, protocols, and thermocycling conditions (Alaeddini 2012; McKee *et al.* 2015; Jane *et al.* 2015). Some DNA extraction kits contain specific inhibitor removal steps that can be adapted for use with difficult (e.g. turbid, high algal content) pond eDNA samples (Buxton *et al.* 2018; Sellers *et al*. 2018), while stand-alone clean-up kits (e.g. Zymo® or Qiagen®) can be effective when applied to inhibited samples after DNA extraction (McKee *et al.* 2015). Alternatively, addition of protein to PCR reactions (e.g. Bovine-serum albumin, BSA) can reduce inhibition (Albers *et al.* 2013).

Diluting eDNA extracts (Biggs *et al.* 2015; McKee *et al.* 2015) or reducing PCR template (Takahara, Minamoto & Doi 2015) were previously recommended to overcome inhibition; however, we would not advise either approach. eDNA samples are characterised by low target DNA concentrations and dilution may ultimately reduce target DNA concentration below the limit of detection, causing false negatives despite diluting out inhibiting compounds (Buxton *et al.* 2017a). Use of droplet digital

PCR (ddPCR, see Supplementary Information: Box. 1) may overcome the aforementioned limitations for detection and quantification, particularly in turbid waters containing high concentrations of PCR inhibitors. In ponds, ddPCR outperformed qPCR, especially at very low eDNA concentrations (Doi *et al*. 2015a), and may be more accurate for abundance or biomass estimation due to lower variability (Nathan *et al.* 2014; Doi *et al*. 2015b).

Finally, in addition to running equipment, extraction and amplification blanks, and identification of inhibition using IPCs (Rees *et al.* 2014b; Goldberg *et al.* 2016), we recommend that quality control measures are taken to identify sample degradation and extraction efficiency (Figure A1.1, IPC1 and 2). A known amount of non-target DNA can be introduced as IPC1 into ethanol precipitation sample kits before they are taken into the field, or non-target DNA can be introduced into a preservative solution for filtered samples. IPC2 can be added before or during the first step of DNA extraction. In similar fashion to IPC3, this sequence would be targeted during qPCR and failure to amplify, or amplification after more cycles than expected, would indicate sample degradation or low extraction efficiency. This will help improve confidence in negative results.

Future perspectives

RARE AND INVASIVE SPECIES

Use of eDNA for presence-absence assessment of rare, threatened or invasive species has been widely investigated since it was first identified as a major challenge in previous eDNA reviews (Rees *et al.* 2014b; Thomsen & Willerslev 2015). eDNA analysis can complement conventional methods, act as an early warning system for invasive species (Goldberg *et al.* 2013; Piaggio *et al.* 2014; Smart *et al.* 2015; Blackman *et al.* 2017) and improve distribution mapping for rare species (Thomsen

et al. 2012; Biggs *et al.* 2015; Torresdal *et al.* 2017; Doi *et al.* 2017). This tool will continue to scale-up rare and invasive species monitoring by enabling rapid and cost-efficient screening of multitudes of sites. However, substantial variation exists in design, validation, and application of species-specific assays, even for the same target species e.g. invasive signal crayfish *Pacifastacus leniusculus* (Dunn *et al.* 2017; Larson *et al.* 2017; Agersnap *et al.* 2017; Harper *et al.* 2018). False positives and negatives remain pertinent issues in eDNA monitoring and intuitive counter-strategies are required for their mitigation. For purposes of eventual standardisation and consistency of eDNA research independent of target species or environment, researchers must ensure they familiarise themselves with existing guidelines for assay development, such as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009) and the eDNA minimum reporting guidelines established by Goldberg *et al*. (2016).

COMMUNITY COMPOSITION AND MONITIORING AT THE PONDSCAPE

Despite their biodiversity value, monitoring of ponds is problematic due to abundance and available sampling tools which may not be representative of all biodiversity (Biggs *et al.* 2016). eDNA metabarcoding holds enormous potential for conservation and management at the pondscape by providing species-level distribution data for entire communities (Harper *et al*. 2018). eDNA metabarcoding has been successfully used in ponds to survey temperate and tropical amphibian communities (Valentini *et al.* 2016; Bálint *et al.* 2017), fish assemblages (Valentini *et al.* 2016; Evans *et al.* 2017), and has strong capacity to detect semi-aquatic and terrestrial species (Ushio *et al.* 2017a; c; Klymus *et al.* 2017b; Harper *et al.* 2018). Issues with the metabarcoding approach remain and have been reviewed (Deiner *et al.* 2017) but it holds great promise for community study.

In contrast to vertebrates, published eDNA metabarcoding studies on pond invertebrates are distinctly lacking despite strong interest in this sector. A small number of studies successfully detected a range of macroinvertebrate taxa from running water (Deiner *et al.* 2016; Blackman *et al.* 2017; Klymus, Marshall & Stepien 2017a) and lakes (Bista *et al.* 2017), but these taxa often comprise a low proportion of total sequence reads if generic primers are used (Deiner *et al.* 2016). The standard barcode gene, cytochrome c oxidase subunit I (*COI*, see Hebert *et al*. 2003), for which the most extensive reference databases exist, appears to be problematic for invertebrate metabarcoding. Often *COI* metabarcoding primers do not recover all taxa or show substantial amplification bias toward non-metazoan taxa (e.g. bacteria, fungi, algae), even when carefully designed to be specific to a particular metazoan group (Elbrecht & Leese 2017). This bias may be more pronounced in ponds containing high densities of phyto- and zooplankton.

Metabarcoding has yet to be routinely implemented for pond biodiversity monitoring but has a number of applications which could improve our knowledge and understanding of pond biodiversity, such as multi-species distribution, individual pond occupancy, species associations, ecological networks, and biomonitoring (Deiner *et al.* 2017; Klymus *et al.* 2017b; Harper *et al*. 2018).

ESTIMATION OF ABUNDNACE OR BIOMASS

Estimation of abundance or biomass of target species was previously identified as a major challenge in eDNA research (Rees *et al.* 2014b; Thomsen & Willerslev 2015; Lawson Handley 2015). Accurate estimation may be most feasible in ponds as their small size may allow well-represented sampling versus large lakes or lotic environments. Some studies have achieved estimates of abundance/biomass from eDNA in ponds (Thomsen *et al.* 2012; Takahara *et al.* 2012; Biggs *et al.* 2015; Buxton

et al. 2017b), but others observed no link (Rees *et al.* 2014a; Doi *et al.* 2017). Similarly, semi-quantitative estimates have been made from metabarcoding for vertebrate eDNA (Evans *et al.* 2016; Hänfling *et al.* 2016; Ushio *et al.* 2017b) and invertebrate DNA (Elbrecht & Leese 2015), but whether these approaches can be applied in ponds and to invertebrate eDNA remain untested. Fully quantitative estimates may also be unrealistic due to potential species masking and amplification bias that occurs when degenerate primers are applied to highly diverse systems (Deiner *et al.* 2017; Klymus *et al.* 2017b).

The relationship between eDNA concentration and abundance/biomass is highly variable in natural systems due to the influence of biotic and abiotic factors on release, persistence and degradation of eDNA (Strickler *et al.* 2015; Buxton *et al.* 2017a). These factors may be especially influential in ponds, due to their physicochemical heterogeneity and use by semi-aquatic and terrestrial wildlife. Life stage, behaviour and seasonality of *T. cristatus* substantially affected eDNA concentration in ponds (Buxton *et al.* 2017b). Relationships between biomass and eDNA concentration may only be observed during certain life cycle phases e.g. egg production and spawning (Dunn *et al.* 2017; Bylemans *et al.* 2017). Abiotic factors alter rates of organismal eDNA degradation and release, and their effects may be exaggerated in ponds where environmental extremes are observed e.g. hydroperiod, nutrient loading, pH (De Meester *et al.* 2005). Temperature (Takahara *et al.* 2012; Robson *et al.* 2016; Buxton *et al.* 2017b) and sediment type (Buxton *et al.* 2017a) were found to influence eDNA concentration of target species in ponds. Consequently, care must be taken when estimating abundance/biomass of pond species to ensure estimates are not confounded by under-representative sampling, inhibition, abiotic or biotic variables. Pond eDNA monitoring will continue to benefit from further investigation into the role of organisms and environmental variables (e.g. UV, temperature, pH, anoxia) on eDNA release, persistence, degradation and detection.

DISEASE MANAGEMENT

Detection and management of disease in freshwater environments is crucial to preventing spread and further infection. Crayfish plague *Aphanomyces astaci* and chytrid fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans* pose major threats to pond biodiversity. Chytrid fungi has decimated amphibian populations and contributed to global decline and extinction risk of species (Walker *et al.* 2007). Microscopy or molecular techniques were once used to detect zoosporangium in host individuals but swabs were required from the host's skin or mouth. eDNA presented an alternative avenue of diagnosis: water is sampled and filtered, followed by detection of chytrid zoospores using qPCR (Walker *et al.* 2007; Schmidt *et al.* 2013). A similar procedure was developed to detect crayfish plague spores, carried by invasive North American crayfish but lethal to European crayfish species (Strand *et al.* 2014). eDNA metabarcoding may be the next logical step to screen for multiple freshwater diseases that threaten biodiversity, or monitor host, threatened species, and pathogens simultaneously. Microbiome research is another field that has been pivotal to understanding chytrid fungus resistance and immunity in amphibian species, and cure development. Obtaining microbiome data has been dependent on whole body or ventral swabbing but eDNA metabarcoding of bacterial communities may be an option where tissue samples are not available.

FROM RESEARCH INTO PRACTICE

A broad group of stakeholders are invested in eDNA and ponds outside of academia. This group includes: commercial ventures, who provide ecological and laboratory services to developers and the building industry; industries (e.g. utility companies) who manage large amounts of land and are responsible for its management/exploitation; government departments and agencies who are

responsible for monitoring environmental quality (e.g. Environment Agency, Natural England, United States Fish and Wildlife Service); and end users, whether conservation organisations, the development industry, government departments, or quangos. These end users have identified immediate and long-term priorities for DNA-based environmental monitoring and assessment (DNA End User Group 2017). They seek methodological advances within eDNA that will allow assessment of ecosystem predictors and/or stressors, and feed into routine biodiversity assessment, monitoring, and other statutory responsibilities. Beyond determining current range, distribution and response of species to conservation interventions, these advances may include ecological responses to eutrophication and other chemical inputs, spread of invasive species, and range pressures such as climate change and environmental impact assessment. All of these goals are pertinent to pond conservation and management (*pers. comm.* UK DNA Working Group).

In the UK, ponds are now a Biodiversity Action Plan (BAP) priority habitat which may increase incentive for their routine monitoring. Here, eDNA surveys are being adopted to aid pond conservation and steadily incorporated into policy, for example, *T. cristatus* (Biggs *et al.* 2014). eDNA results are being used to model *T. cristatus* distribution and inform new Natural England policies that will provide district-level species protection, as opposed to site-by-site survey and mitigation which has done little to improve *T. cristatus* conservation status (Lewis, Griffiths & Wilkinson 2016). This policy shift offers a more unified approach to *T. cristatus* conservation, and pilot projects testing these reforms are underway (see Woking Borough Council report, 2016). eDNA surveys underpinning district-level policy would provide critical baseline distribution data for *T. cristatus* throughout England, and radically improve our understanding of the conservation status of this species.

Conclusions

eDNA analysis is starting to change the way we design and implement biodiversity monitoring programs and has opened up new possibilities for the future. This tool holds particular promise in ponds for monitoring biodiversity, testing hypotheses and understanding eDNA, but there are a number of challenges specific to these environments, in conjunction with those faced by all freshwater habitats. These challenges must be overcome to achieve accurate, standardised tools that can be routinely and reproducibly implemented. At present, there is no consensus on how much water, and how many samples should be taken from an individual pond to achieve representative samples from water that is patchy horizontally, vertically and temporally. Further investigation is required to determine the number of samples needed to achieve a set detection probability for a target species, or representative community composition. Similarly, methods of eDNA capture diverge widely in ponds between filtration (various pore sizes and filter types) and ethanol precipitation. Evidence suggests that pond water samples should be processed by filtration, but intuitive strategies are needed to prevent clogging. All captured and extracted DNA requires PCR amplification, whether PCR, qPCR, or ddPCR, but PCR inhibition remains a pressing issue in pond eDNA monitoring. It is therefore crucial that researchers and practitioners test for and report steps taken to prevent inhibition of the amplification process. A broad standardisation of eDNA workflows (with flexibility depending on sample type and downstream application), will ensure more robust, comparable, and ecologically meaningful data to guide effective management and conservation of pond biodiversity without stifling innovation or development.

Author contributions

L.R.H. and A.S.B. conceived the review and organised the stakeholder workshop held in 2017 to discuss opportunities and challenges of using eDNA monitoring in ponds. L.R.H., A.S.B., H.C.R., K.B., D.H., H.V.W. and G.B. attended the workshop which was hosted by D.S.R. at Centre for Ecology and Hydrology, Wallingford. L.R.H. and A.S.B. wrote the manuscript, which all authors revised.

References

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

Alaeddini, R. (2012) Forensic implications of PCR inhibition-A review. *Forensic Science International: Genetics*, **6**, 297–305.

Albers, C.N., Jensen, A., Bælum, J. & Jacobsen, C.S. (2013) Inhibition of DNA polymerases used in q-PCR by structurally different soil-derived humic substances. *Geomicrobiology Journal*, **30**, 675–681.

Alderton, E., Sayer, C.D., Davies, R., Lambert, S.J. & Axmacher, J.C. (2017) Buried alive: Aquatic plants survive in “ghost ponds” under agricultural fields. *Biological Conservation*, **212**, 105–110.

Bálint, M., Nowak, C., Márton, O., Pauls, S., Wittwer, C., Aramayo, J.L., Schulze, A., Chambert, T., Cocchiararo, B. & Jansen, M. (2017) Twenty-five species of frogs in a liter of water: eDNA survey for exploring tropical frog diversity. *bioRxiv*, 176065.

Barnes, M.A. & Turner, C.R. (2015) The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, **17**, 1–17.

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

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C. & Griffiths, R.A. (2014) Analytical and methodological development for improved surveillance of the great crested newt. *Defra Project WC1067.*

Biggs, J., von Fumetti, S. & Kelly-Quinn, M. (2016) The importance of small waterbodies for biodiversity and ecosystem services: implications for policy makers. *Hydrobiologia*, **793**, 3-39.

Bista, I., Carvalho, G.R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., Christmas, M. & Creer, S. (2017) Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nature Communications*, **8**, 14087.

Blackman, R.C., Constable, D., Hahn, C., Sheard, A.M., Durkota, J., Hänfling, B. & Handley, L.L. (2017) Detection of a new non-native freshwater species by DNA metabarcoding of environmental samples—first record of *Gammarus fossarum* in the UK. *Aquatic Invasions*, **12**, 177-189.

Briers, R.A. & Biggs, J. (2005) Spatial patterns in pond invertebrate communities: separating environmental and distance effects. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **15**, 549–557.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. & Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, **55**, 611–622.

Buxton, A.S., Groombridge, J.J. & Griffiths, R.A. (2017a) Is the detection of aquatic environmental DNA influenced by substrate type? *PLoS One*, **12**, e0183371.

Buxton, A.S., Groombridge, J.J. & Griffiths, R.A. (2018) Seasonal variation in environmental DNA detection in sediment and water samples. *PLoS One*, **13**, e0191737.

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

Bylemans, J., Furlan, E.M., Hardy, C.M., McGuffie, P., Lintermans, M. & Gleeson, D.M. (2017) An environmental DNA-based method for monitoring spawning activity: a case study, using the endangered Macquarie perch (*Macquaria australasica*). *Methods in Ecology and Evolution*, **8**, 646–655.

Céréghino, R., Biggs, J., Oertli, B. & Declerck, S. (2008) The ecology of European ponds: defining the characteristics of a neglected freshwater habitat. *Hydrobiologia*, **597**, 1–6.

Davies, B., Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Sear, D., Bray, S. & Maund, S. (2008) Comparative biodiversity of aquatic habitats in the European agricultural landscape. *Agriculture, Ecosystems & Environment*, **125**, 1–8.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., de Vere, N., Pfrender, M.E. & Bernatchez, L. (2017) Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Molecular Ecology*, **26**, 5872-5895.

Deiner, K., Fronhofer, E.A., Mächler, E., Walser, J.-C. & Altermatt, F. (2016) Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature Communications*, **7**, 12544.

De Meester, L., Declerck, S., Stoks, R., Louette, G., Van De Meutter, F., De Bie, T., Michels, E. & Brendonck, L. (2005) Ponds and pools as model systems in conservation biology, ecology and evolutionary biology. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **15**, 715–725.

DNA End User Group (2017). DNA End User Group - DNA based method development interests. Accessed on 10th January 2018.

Doi, H., Katano, I., Sakata, Y., Souma, R., Kosuge, T., Nagano, M., Ikeda, K., Yano, K. & Tojo, K. (2017) Detection of an endangered aquatic heteropteran using environmental DNA in a wetland ecosystem. *Royal Society Open Science*, **4**, 170568.

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K. & Yamanaka, H. (2015a) Droplet digital polymerase chain reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. *Environmental Science & Technology*, **49**, 5601–5608.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H. & Minamoto, T. (2015b) Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS One*, **10**, e0122763.

Downing, J.A., Prairie, Y.T., Cole, J.J., Duarte, C.M., Tranvik, L.J., Striegl, R.G., McDowell, W.H., Kortelainen, P., Caraco, N.F., Melack, J.M. & Middelburg, J.J. (2006) The global abundance and size distribution of lakes, ponds, and impoundments. *Limnology and Oceanography*, **51**, 2388–2397.

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

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

Elbrecht, V. & Leese, F. (2015) Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. *PLoS One*, **10**, e0130324.

Elbrecht, V. & Leese, F. (2017) Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. *Frontiers in Environmental Science*, **5**, 1-11.

Emson, D., Sayer, C.D., Bennion, H., Patmore, I.R. & Rioual, P. (2017) Mission possible: diatoms can be used to infer past duckweed (lemnoid Araceae) dominance in ponds. *Journal of Paleolimnology*, 1–13.

Evans, D.N.T., Li, M.Y., Renshaw, M.M.A., Olds, D.B.P., Deiner, D.K., Turner, D.C.R., Jerde, D.C.L., Lodge, D.D.M., Lamberti, D.G.A. & Pfrender, D.M.E. (2017) Fish community assessment with eDNA metabarcoding: effects of sampling design and bioinformatic filtering. *Canadian Journal of Fisheries and Aquatic Sciences*, **74**, 1362-1374.

Evans, N.T., Olds, B.P., Renshaw, M.A., Turner, C.R., Li, Y., Jerde, C.L., Mahon, A.R., Pfrender, M.E., Lamberti, G.A. & Lodge, D.M. (2016) Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular Ecology Resources*, **16**, 29–41.

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

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

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E. & Taberlet, P. (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, **7**, 1299–1307.

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

Harper, L.R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H.C., Gough, K.C., Lewis, E., Adams, I.P., Brotherton, P., Phillips, S. & Hänfling, B. (in press) Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution*.

Harper, L.R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H.C., Lewis, E., Adams, I.P., Brotherton, P., Phillips, S. & Hänfling, B. (2018) Understanding biodiversity at the pondscape using environmental DNA: a focus on great crested newts. *bioRxiv*, 278309.

Harper, K.J., Anucha, N.P., Turnbull, J.F., Bean, C.W. & Leaver, M.J. (2018) Searching for a signal: Environmental DNA (eDNA) for the detection of invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). *Management of Biological Invasions*, **9**.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biological Ssciences*, **270**, 313–321.

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

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

Kazanjian, G., Flury, S., Attermeyer, K., Kalettka, T., Kleeberg, A., Premke, K., Köhler, J. & Hilt, S. (2018) Primary production in nutrient-rich kettle holes and consequences for nutrient and carbon cycling. *Hydrobiologia*, **806**, 77–93.

Klymus, K.E., Marshall, N.T. & Stepien, C.A. (2017a) Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. *PLoS One*, **12**, e0177643.

Klymus, K.E., Richter, C.A., Thompson, N. & Hinck, J.E. (2017b) Metabarcoding of environmental DNA samples to explore the use of uranium mine containment ponds as a water source for wildlife. *Diversity*, **9**, 54.

Larson, E.R., Renshaw, M.A., Gantz, C.A., Umek, J., Chandra, S., Lodge, D.M. & Egan, S.P. (2017) Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes of North America. *Hydrobiologia*, 1–13.

Lawson Handley, L. (2015) How will the “molecular revolution” contribute to biological recording? *Biological Journal of the Linnean Society*, **115**, 750–766.

Lewis, B., Griffiths, R.A. & Wilkinson, J.W. (2016) Population status of great crested newts (*Triturus cristatus*) at sites subjected to development mitigation. *The Herpetological Journal*, **27**, 133-142.

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

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

Nicolet, P., Biggs, J., Fox, G., Hodson, M.J., Reynolds, C., Whitfield, M. & Williams, P. (2004) The wetland plant and macroinvertebrate assemblages of temporary ponds in England and Wales. *Biological Conservation*, **120**, 261–278.

Oertli, B., Céréghino, R., Hull, A. & Miracle, R. (2009) Pond conservation: from science to practice. *Hydrobiologia*, **634**, 1–9.

Piaggio, A.J., Engeman, R.M., Hopken, M.W., Humphrey, J.S., Keacher, K.L., Bruce, W.E. & Avery, M.L. (2014) Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Molecular Ecology Resources*, **14**, 374–380.

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

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

Robson, H.L.A., Noble, T.H., Saunders, R.J., Robson, S.K.A., Burrows, D.W. & Jerry, D.R. (2016) Fine tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. *Molecular Ecology Resources*, **16**, 922-932.

Sayer, C., Shilland, E., Greaves, H., Dawson, B., Patmore, I., Emson, D., Alderton, E., Robinson, P., Andrews, K., Axmacher, J. & Others. (2013) Managing Britain's ponds – conservation lessons from a Norfolk farm. *British Wildlife*, **25**, 21–28.

Schmidt, B.R., Marc, K., Ursenbacher, S., Hyman, O.J. & Collins, J.P. (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, **4**, 646–653.

Sellers, G.S., Di Muri, C., Hänfling, B., Gómez, A. (2018) Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types. Available at http://dx.doi.org/10.17504/protocols.io.nbedaje. Accessed on 7th March 2018.

Smart, A.S., Tingley, R., Weeks, A.R., van Rooyen, A.R. & McCarthy, M.A. (2015) Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications*, **25**, 1944–1952.

Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E. & Hellström, M. (2016) Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*, **8**, 635-645.

Strand, D.A., Jussila, J., Johnsen, S.I., Viljamaa-Dirks, S., Edsman, L., Wiik-Nielsen, J., Viljugrein, H., Engdahl, F. & Vrålstad, T. (2014) Detection of crayfish plague spores in large freshwater systems. *Journal of Applied Ecology*, **51**, 544–553.

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

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

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. & Kawabata, Z. (2012) Estimation of fish biomass using environmental DNA. *PLoS One*, **7**, e35868.

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

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

Torresdal, J.D., Farrell, A.D. & Goldberg, C.S. (2017) Environmental DNA detection of the golden tree frog (*Phytotriades auratus*) in bromeliads. *PLoS One*, **12**, e0168787.

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

Turner, C.R., Uy, K.L. & Everhart, R.C. (2015) Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, **183**, 93–102.

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh,

M. & Miya, M. (2017a) Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources,* **17**, e63-e65.

Ushio, M., Murakami, H., Masuda, R., Sado, T., Miya, M., Sakurai, S., Yamanaka, H., Minamoto, T. & Kondoh, M. (2017b) Quantitative monitoring of multispecies fish environmental DNA using high-throughput sequencing. *bioRxiv*, 113472.

Ushio, M., Murata, K., Sado, T., Nishiumi, I., Takeshita, M., Iwasaki, W. & Miya, M. (2017c) Demonstration of the potential of environmental DNA as a tool for the detection of avian species. *bioRxiv*, 199208.

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

Walker, S.F., Salas, M.B., Jenkins, D., Garner, T.W.J., Cunningham, A.A., Hyatt, A.D., Bosch, J. & Fisher, M.C. (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms*, **77**, 105–112.

Weltz, K., Lyle, J.M., Ovenden, J., Morgan, J.A.T., Moreno, D.A. & Semmens, J.M. (2017) Application of environmental DNA to detect an endangered marine skate species in the wild. *PLoS One*, **12**, e0178124.

Wood, P.J., Greenwood, M.T. & Agnew, M.D. (2003) Pond biodiversity and habitat loss in the UK. *Area*, **35**, 206–216.

Woking Borough Council (2016) Natural Woking biodiversity and green infrastructure strategy supporting information, 118 – 131. Available at

https://www.woking.gov.uk/environment/greeninf/naturalwoking/nwsuppinfo. Accessed on 10th January 2018.

Appendix I – Supplementary Information

Box 1. Glossary of technical terms.

Environmental DNA (eDNA): intra- or extracellular DNA that has been shed from an organism (via skin cells, mucous, scales, urine, faeces, saliva, gametes, eggs or deceased remains) and suspended within an environmental matrix, such as water, soil, or air (Rees et al., 2014b; Thomsen & Willerslev 2015; Lawson Handley 2015; Deiner et al., 2017). This DNA can be captured, amplified, identified and assigned, allowing taxonomic composition and distribution to be inferred. Current eDNA approaches largely use PCR based methods where DNA is amplified using targeted approaches to detect single-species or non-targeted approaches to examine community composition (Lawson Handley 2015).

Polymerase Chain Reaction (PCR): process used to generate millions of copies (amplify) of a particular section of DNA.

Real-time quantitative PCR (qPCR): PCR and detection are combined in a process which allows users to monitor their amplification reaction as it happens. Fluorescent dyes bind to DNA as it amplifies and the fluorescent signal produced is measured by qPCR instruments. Dyes may be non-specific and bind to any DNA amplified (SYBR green) or designed to bind to DNA from a target species (hydrolysis probe). The fluorescent signal of eDNA samples is often quantified against the signal produced by a known amount of synthetic or purified DNA from the target species.

Droplet digital PCR (ddPCR): a new method of DNA detection, also known as "third-generation PCR", which performs PCR using water-oil emulsion droplet technology.

Thousands of nano-litre droplets are generated for each eDNA sample, thus some ideally contain only one or a few copies of target DNA. Within each of those droplets, an individual PCR reaction occurs and end-point PCR amplification is detected by the fluorescence intensity of PCR probes.

eDNA metabarcoding: a passive community sequencing approach, which enables taxonomic identification of multiple species simultaneously. eDNA samples are amplified with conserved (or universal) primers using PCR, and the PCR products sequenced on a High-Throughput platform.

High-Throughput Sequencing (HTS): massively parallel sequencing technologies, such as the Illumina, Roche, or IonTorrent series, which produce millions of sequences for analysis opposed to Sanger sequencing technologies which process one sequence at a time. HTS is also known as Next Generation Sequencing (NGS).

Internal Positive Control (IPC): PCR controls which allow detection of failed DNA extraction or PCR inhibition. Typically, artificial or synthetic DNA not found in biological samples is used, and detected using a different set of primers (and probe) from those used for the target species.

Table A1.S1 Overview of technical details from described case studies. Gene abbreviations denote the marker gene used in each study, including cytochrome c oxidase subunit I (*COI*) and cytochrome c oxidase subunit III (*COIII*), cytochrome-b (*cyt-b*), 12S and 16S ribosomal RNA (rRNA), internal transcribed spacer (*ITS*), and maturase K (*matK*). Abbreviations for filter types are as follows: polycarbonate track-etched (PCTE), glass fibre (GF), polyvinylidene difluoride (PVDF), cellulose acetate (CA) and cellulose nitrate (CN).

Organism(s)	Volume sampled	Capture method	Storage conditions	Extraction method	Technology	Marker(s)	Reference
American bullfrog *Lithobates catesbeianus*	3 x 15 mL	Ethanol precipitation	-20 °C	Qiagen® QIAamp Tissue Extraction Kit	PCR 3-5 replicates	*cyt-b*	Ficetola *et al*., (2008)
American bullfrog Siberian sturgeon *Acipenser baerii*	3 x 15 mL	Ethanol precipitation	-20 °C	Qiagen® QIAamp Blood and Tissue Extraction Kit	PCR 3 replicates	*cyt-b* 79 bp 98 bp	Dejean *et al*., (2012)
Common spadefoot toad *Pelobates fuscus* Great crested newt *Triturus cristatus* European weather loach *Misgurnus fossilis* Large white-faced darter *Leucorrhinia pectoralis* Tadpole shrimp *Lepidurus apus* Otter *Lutra lutra*	3 x 15 mL	Ethanol precipitation	-20 °C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan MGB qPCR 3 replicates Metabarcoding (PCR and 454 pyrosequencing) 6 replicates	*Cyt-b, COI* 72 - 130 bp	Thomsen *et al*., (2012)
Bluegill sunfish *Lepomis macrochirus*	1 L frozen at -30 °C	Filtration 3 µm CA filters	-25 °C in DNA-free aluminium foil	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 8 replicates	*cyt-b* 100 bp	Takahara *et al*., (2013)

Organism(s)	Volume sampled	Capture method	Storage conditions	Extraction method	Technology	Marker(s)	Reference
Chytrid fungus *Batrachochytrium dendrobatidis*	600 mL	Filtration Sterivex	Unknown	Gentra Systems Puregene kit	TaqMan qPCR SYBR green qPCR 2 replicates	*ITS-1* 97 bp 145 bp	Schmidt *et al*., (2013)
Red swamp crayfish *Procambarus clarkii*	20 x 40 mL pooled and subsampled for 6 x 15 mL	Ethanol precipitation	-20 ^{o}C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 12 replicates	*COI* 65 bp	Tréguier *et al*., (2014)
Great crested newt	3 x 50 mL subsampled for 3 x 15 mL	Ethanol precipitation	-20 ^{o}C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 12 replicates	*cyt-b* 81 bp	Rees *et al*., (2014a)
Common carp *Cyprinus carpio*	12 L subsampled for 300 mL	Filtration 0.2 - 180 µm Nylon net and PCTE filters	-20 ^{o}C in CTAB buffer	CTAB extraction	SYBR green qPCR 3 replicates	D-loop 146 bp	Turner *et al*., (2014a)
Bighead carp *Hypophthalmichthys nobilis* Silver carp *H. molitrix*	8 x 2 L	Filtration 1.5 µm GF filters 10 µm PCTE filters	-20 ^{o}C	MoBio® PowerWater DNA Isolation kit CTAB extraction	TaqMan qPCR 3 replicates	D-loop 100 bp	Turner *et al*., (2014b)
Great crested newt	20 x 30 mL pooled and subsampled for 6 x 15 mL	Ethanol precipitation	-20 ^{o}C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 12 replicates	*cyt-b* 81 bp	Biggs *et al*., (2015)

Organism(s)	Volume sampled	Capture method	Storage conditions	Extraction method	Technology	Marker(s)	Reference
Turtles	1 L	Filtration 1.2 µm GF filters	-20 °C	Qiagen® DNeasy Blood and Tissue Kit	PCR SYBR green qPCR	*COI* 155-299 bp	Davy *et al*. (2015)
Brazilian waterweed *Egeria densa*	5 mL in laboratory trial 1 L in field trial	Ethanol precipitation Filtration GF filters	None (samples processed rapidly after collection)	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 3 replicates (laboratory trial) 6 replicates (field trial)	*trnL–trnF* intergenetic spacer 373 bp	Fujiwara *et al.* (2016)
Waterthyme *Hydrilla verticillata*	0.6 – 1 L	Filtration ~0.7 µm GF filters	-20 °C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 3 replicates	*matK*	Matsuhashi *et al.* (2016)
Eurasian watermilfoil *Myriophyllum spicatum*	1 L	Filtration 0.45 µm PVDF filters	-20 °C in absolute ethanol	Qiagen® Plant Mini Kit	PCR 3 replicates	*ITS* 133 bp	Newton *et al*. (2016)
Amphibians Fish	20 x 30 mL pooled and subsampled for 6 x 15 mL	Ethanol precipitation	-20 °C	Qiagen® DNeasy Blood and Tissue Kit	Metabarcoding (PCR and Illumina MiSeq) 12 replicates	12S rRNA < 100 bp	Valentini *et al*. (2016)

Organism(s)	Volume sampled	Capture method	Storage conditions	Extraction method	Technology	Marker(s)	Reference
Pike *Esox lucius* Perch *Perca fluviatilis*	1 L	Ethanol precipitation Filtration 0.45 µm CN/CA 0.2 µm PCTE 0.6 µm GF 0.22 µm Sterivex	99% ethanol at room temperature, Longmire's buffer at room temperature, RNAlater at room temperature, no buffer frozen at 20 °C, no buffer refrigerated at 8–10 °C.	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 12 replicates	*cyt-b* 81 bp 89 bp	Spens *et al*. (2016)
Mozambique tilapia *Oreochromis mossambicus*	2 x 2 L	Filtration 0.7 µm GF syringe filter	-20 °C	Bioline® Isolate II Genomic DNA kit	SYBR green qPCR 5 replicates	16S rRNA 189 bp	Robson *et al*. (2016)
Great crested newt	1 L 6 x 15 mL	Filtration 20 µm, nylon filter Ethanol precipitation	-20 °C -20 °C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 8 replicates	*Cyt-b* 81 bp	Buxton *et al*. (2017b)

Organism(s)	Volume sampled	Capture method	Storage conditions	Extraction method	Technology	Marker(s)	Reference
Great crested newt	20 x 30 mL pooled and subsampled for 6 x 15 mL	Ethanol precipitation	-20 ^{o}C	Qiagen® DNeasy Blood and Tissue Kit	TaqMan qPCR 8 replicates	*Cyt-b* 81 bp	Buxton *et al*. (2017a)
Tropical frogs	1 L	Filtration 0.7 µm GF filters 0.2 µm nylon filters	Dried or CTAB preservation Dried	CTAB chloroform Qiagen® DNeasy Blood and Tissue Kit	Metabarcoding (PCR and Illumina NextSeq 500) 4 replicates	16S rRNA 150 bp	Bálint *et al.(2017)*
Fish	30 x 250 mL	Filtration 1.2 µm, PCTE filters	CTAB preservation at -20 ^{o}C	Chloroform-Isoamyl alcohol extraction and an isopropanol precipitation	Metabarcoding (PCR and Illumina MiSeq)	*Cyt-b* 12S rRNA 16S rRNA	Evans *et al.(2017)*
Mammals	500 mL	Filtration 0.7 µm, GF filters	-20 ^{o}C in aluminium foil	Qiagen® DNeasy Blood and Tissue Kit	Metabarcoding (PCR and Illumina MiSeq) 3 replicates	12S rRNA	Ushio *et al.(2017a)*
Birds	100 - 200 mL	Filtration 0.45 µm, Sterivex filters	-20 ^{o}C in RNAlater	Qiagen® DNeasy Blood and Tissue Kit	Metabarcoding (PCR and Illumina MiSeq) 3 replicates	12S rRNA	Ushio *et al.(2017b)*

Organism(s)	Volume sampled	Capture method	Storage conditions	Extraction method	Technology	Marker(s)	Reference
Vertebrates	27 - 100 mL	Filtration 0.45 µm CN filters	-20 °C in 1 mL 100% ethanol	Qiagen® Qiashredder and DNeasy Blood and Tissue Kit	Metabarcoding (PCR and Illumina MiSeq) 0 replicates	12S rRNA 16S rRNA	Klymus *et al*. (2017)

References

Bálint, M., Nowak, C., Márton, O., Pauls, S., Wittwer, C., Aramayo, J.L., Schulze, A., Chambert, T., Cocchiararo, B. & Jansen, M. (2017) Twenty-five species of frogs in a liter of water: eDNA survey for exploring tropical frog diversity. *bioRxiv*, 176065.

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

Buxton, A.S., Groombridge, J.J. & Griffiths, R.A. (2017a) Is the detection of aquatic environmental DNA influenced by substrate type? *PLoS One*, 12, e0183371.

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

Davy, C.M., Kidd, A.G. & Wilson, C.C. (2015) Development and Validation of Environmental DNA (eDNA) Markers for detection of freshwater turtles. *PLoS One*, 10, e0130965.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., de Vere, N., Pfrender, M.E. & Bernatchez, L. (2017) Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Molecular Ecology*, 26, 5872–5895.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E. & Miaud, C. (2012) Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology*, 49, 953–959.

Evans, D.N.T., Li, M.Y., Renshaw, M.M.A., Olds, D.B.P., Deiner, D.K., Turner, D.C.R., Jerde, D.C.L., Lodge, D.D.M., Lamberti, D.G.A. & Pfrender, D.M.E. (2017) Fish community assessment with eDNA metabarcoding: effects of sampling design and bioinformatic filtering. *Canadian Journal of Fisheries and Aquatic Sciences*, 74, 1362-1374.

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

Fujiwara, A., Matsuhashi, S., Doi, H., Yamamoto, S. & Minamoto, T. (2016) Use of environmental DNA to survey the distribution of an invasive submerged plant in ponds. *Freshwater Science*, 35, 748–754.

Klymus, K.E., Richter, C.A., Thompson, N. & Hinck, J.E. (2017) Metabarcoding of environmental DNA samples to explore the use of uranium mine containment ponds as a water source for wildlife. Diversity, 9, 54.

Lawson Handley, L. (2015) How will the “molecular revolution” contribute to biological recording? *Biological Journal of the Linnean Society*, 115, 750–766.

Matsuhashi, S., Doi, H., Fujiwara, A., Watanabe, S. & Minamoto, T. (2016) Evaluation of the environmental DNA method for estimating distribution and biomass of submerged aquatic plants. *PLoS One*, 11, e0156217.

Newton, J., Sepulveda, A., Sylvester, K. & Thum, R.A. (2016) Potential utility of environmental DNA for early detection of Eurasian watermilfoil (*Myriophyllum spicatum*). *Journal of Aquatic Plant Management*, 54, 46–49.

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

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

Robson, H.L.A., Noble, T.H., Saunders, R.J., Robson, S.K.A., Burrows, D.W. & Jerry, D.R. (2016) Fine tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. *Molecular Ecology Resources*, 16, 922-932.

Schmidt, B.R., Kéry, M., Ursenbacher, S., Hyman, O.J. & Collins, J.P. (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, 4, 646–653.

Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E. & Hellström, M. (2016) Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*, 8, 635–645.

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

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

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

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M.A. & Roussel, J.-M. (2014) Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, 51, 871–879.

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

Turner, C.R., Miller, D.J., Coyne, K.J. & Corush, J. (2014b) Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys spp*.). *PLoS One*, 9, e114329.

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh, M. & Miya, M. (2017a) Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, 17, e63-e65.

Ushio, M., Murata, K., Sado, T., Nishiumi, I., Takeshita, M., Iwasaki, W. & Miya, M. (2017b) Demonstration of the potential of environmental DNA as a tool for the detection of avian species. *bioRxiv*, 199208.

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

Appendix II – Advantages and disadvantages of eDNA surveys for great crested newts: perceptions from practitioners

This appendix is the output from workshops run by A.S. Buxton and others into the practitioners, perceptions of eDNA. A.S. Buxton conceived the workshops, designed and ran them with help from R.A. Griffiths, P. Edgar (Natural England), Gillian Benson (Natural England) and Katherine Bruce (Nature Metrics). A.S. Buxton analysed the data from the workshops and produced this document as an output with comments from R.A. Griffiths, Gillian Benson (Natural England) and Jim Foster (ARC). It has been published as an online advice note on the Amphibian and Reptile Conservation website with the citation:

Buxton A.S. & Griffiths R.A. (2017) Advantages and disadvantages of eDNA surveys for great crested newts: perceptions from practitioners. Note on a workshop held at Herpetofauna Workers Meeting, February 2017. Unpublished.

Background and methods

Environmental DNA (eDNA) has been used for the assessment of great crested newt presence in ponds in the UK since 2013 and for commercial surveys since 2014. Since then thousands of eDNA samples have been collected and analysed by a wide range of laboratories. Two workshops were held at the 2017 Herpetofauna Workers Meeting – attended by commercial ecologists, volunteer recorders, scientists and statutory agency staff – to discuss the merits and concerns about the use of eDNA for commercial and voluntary great crested newt surveys. Participants were split into 14 small groups of 4-8 participants. Each group was asked to explore two of four themes: 1) reliability of detection; 2) interpretation of results; 3) laboratory and field methods; and 4) potential methodological advances, and score the importance of each discussion points that emerged. In addition to open discussion, within the "potential methodological advances" topic participants were asked to consider such issues as (i) additional time needed for sampling at a pond; (ii) potential extra physical effort needed to push water through a filter rather than preserving in ethanol; and (iii) implications of an increase in the number of samples required. We recognise that hosting the workshops within a wider conservation conference may have restricted the sample of participants to those already familiar with the technique, potentially biasing the workshop outcomes.

Results and discussion

A wide variety of subjects was raised during the discussions and are outlined in Table A2.1. The key topics have been identified based on the frequency at which they were raised between the groups and the importance assigned to them by the participants.

Table A2.1: Discussion points raised by participants, under the different topic themes, points are listed in order from most to least important based on participant responses (+ Positive comments raised; * both positive and negative comments were raised)

Theme	Discussion points
Reliability of detection;	Chance of detecting newts if they are present; Follow-up surveys and survey timing; How do pond characteristics influence detection?; Variation between labs; Limits of detection of eDNA; Number of visits*; False positive results; Loss of field skills; Health and safety+; Limited detail in results; Surveyor inconsistencies; Lack of incorporation of wider ecology.
Interpretation of results;	Chance of detecting newts if they are present; Interpreting inconclusive results; Interpretation of qPCR replicates; Client and local planning authority understanding of the method; Variation between labs; Limits of detection of eDNA; Legal implications; Surveyor variation and; False positive results.
Laboratory and field methods;	Follow-up surveys and survey timing; Contamination; Variation between laboratories; Health and safety+; Inconclusive results; Animal welfare+; Sample storage and transport; Perimeter access; Sediment within samples; Protocol standardisation; Pond topography; Simplicity+; Cost*; Useful when addition to other methods+; Public engagement+ and; Logistics*
Potential methodological advances	Complexity of the kit; Non-recyclable waste produced; Protocol validation; Useful when addition to other methods+; Laboratory validation; Sediment within samples; Perimeter access; Cost and; Legislation.

Variation between the labs in both the quality of the results and the quality of the reporting was identified under all four themes. A laboratory proficiency testing scheme was announced in the March 2017 Wildlife Licencing Newsletter (Natural England, 2017), the scheme is underway with the first round having been completed in mid-2017 (Rees, 2017).

The probability of detecting the species, if present, was a key issue raised in both the detection and interpretation discussion groups. Some responses showed a belief that the method was 100% effective while others revealed distrust in negative results. It is important that further data on the reliability of eDNA in detecting the species when it is present is obtained, as well as environmental and sampling factors that influence detection. Some participants raised concerns over potentially false positive results from eDNA: this hinged on the observation that the results must be false if follow-up surveys using traditional methods yield negative results. However, traditional methods also suffer from imperfect detection, and it should not be assumed that negative traditional surveys following a positive eDNA result indicate a false positive eDNA sample. Nevertheless, there is potential for contamination both in the field and in the lab which may lead to positive results when the species is not present. Protocols to reduce contamination risk therefore need to be emphasised, and work is ongoing to assess the scale of both false positive and false negative eDNA results.

The timing of eDNA surveys and the lag between sample collection and the return of results was frequently raised as an issue, particularly when follow-up surveys were required. Cost and delay implications when waiting for the next season to carry out follow-up surveys was viewed as unacceptable. Many practitioners appear to be either (1) running eDNA in parallel with traditional surveys; or (2) undertaking an early eDNA survey to attempt to fit traditional surveys into the same survey window. The first option runs the risk of contamination from equipment, while the second option

risks non-detection of eDNA, due to low concentrations in the early part of the season (Buxton et al., 2017).

Inconclusive results can arise from both inhibited and degraded samples. Discussions suggested that inconclusive results were sometimes erroneously being interpreted as negative. There also appeared to be inconsistencies between the labs in the reporting of inhibited and degraded samples, which can lead to disparities in conclusions. Guidance is required for the labs on minimum reporting of inhibition and degradation within a sample, and guidance on the causes (such as sediment in the sample) and interpretation of inconclusive results need to be issued to practitioners.

The number of positive qPCR replicates was found to be interpreted in different ways by different individuals, and there was a suggestion that the number of positive qPCR replicates was being used as a proxy for population size class assessment. Although weak relationships between counts of newts and the number of positive qPCR replicates in eDNA samples have been identified (Biggs et al., 2014), the number of replicates to amplify is related to the amount of DNA within the water sample, rather than the number of individual newts. The amount of DNA within the water varies seasonally and is influenced by a number of factors including number of newts present, but can also change dramatically within a season without a change in newt numbers (Buxton et al., 2017). It is therefore unwise to interpret the number of qPCR replicates amplifying as an index of population size.

There were also concerns over the understanding of limitations of the method particularly by developers and local authorities. There was a view that there was overreliance and/or misinterpretation of the results by stakeholders. The new approach eDNA takes to species distribution assessment may make it more difficult for ecologists lacking detailed knowledge of the method to communicate effectively, both with ecologist colleagues and with other stakeholders. An inconsistent approach to interpretation by local authorities and a perception that the developers see the

method as a ‘silver bullet’ solution has been leading to tensions between practitioners and their clients. We recommend that documentation aimed at both ecologists and non-specialists are produced that outline the eDNA process, its limitations, how to interpret results and the consequences of different results. This will allow better communication between ecologists, developers and local planning authorities.

It is recognised that updating the current eDNA protocol will be required to incorporate new technological developments that may improve reliability. The majority of participants were receptive to additional time being required at the pond in order to achieve more reliable detections. Concentrating eDNA using manual filtration can require physical exertion due to the tendency of filters to become rapidly blocked. The increased physical effort required using filters may deter volunteers, but could be acceptable for commercial ecologists. Increasing the number of samples may increase the accuracy of detection, but comes at an additional economic cost. Opinion was evenly split as to whether the benefit of additional samples in increasing the chance of detection would outweigh any financial implications. Research is needed to demonstrate the levels of gains in detection by altering the methodologies, and justify this financially.

Recommendations

- Lab performance/quality assurance exercise completed
- eDNA interpretation manual for ecologists – including guidance on inhibition and degradation
- eDNA interpretation manual for developers and planning authorities
- Refinement of values for detection probabilities using eDNA and the factors that influence it
- Identification of limit of detection, in terms of eDNA concentration in water

- Evaluation of emerging eDNA survey and analysis methods, with robust assessment of costs and benefits, financially and practically as well as on detectability
- Improved sampling kit – e.g. transparent dippers; wider more stable Whirl-Pak® bags; more pairs of gloves
- Improved advice on standardised protocols for sample storage and transport

References

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R., Foster, J., Wilkinson, J., Arnett, A., Williams, P. & F, D. (2014). Analytical and methodological development for improved surveillance of the great crested newt. Defra Project WC1067. Oxford.

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

Natural England (2017). Wildlife licensing newsletter March 2017. Peterborough, UK.

Rees, H. (2017). eDNA proficiency testing. [Online]. 2017. ADAS. Available from: http://www.adas.uk/News/edna-proficiency-testing. [Accessed: 21 September 2017].

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been possible. We emphasize that this report describes the output from the workshop rather than the views of the authors, organisers or other organisations linked to the workshop.

Appendix III – A Bayesian model for assessing factors influencing the detection of environmental DNA

This appendix is In Prep and is a joint project between the School of Anthropology and Conservation and the School of Mathematics, Statistics and Actuarial Science. A.S. Buxton is the second author, who was involved with the concept, ecological interpretation of the results and produced the ecological perspective of the manuscript, however the modelling was developed and conducted in the School of Mathematics, Statistics and Actuarial Science.

Running Head: Bayesian - influences on eDNA detection

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Keywords: Bayesian inference; Detectability; Environmental DNA; False Positive; False Negative; Occupancy.

Summary

1. Environmental DNA (eDNA) is a survey tool with rapidly expanding applications for assessing occupancy of species. It is known that eDNA methods produce both false negative and false positive errors. Methods for estimating eDNA presence that account for error rates have been developed and applied to eDNA data. However, environmental characteristics that influence eDNA presence - as well as false presences and false absences - have not been identified.

2. We develop a novel Bayesian approach for estimating the probability of eDNA presence, as well as the probabilities of false positive and false negative errors, whilst accounting for covariates which may affect these three probabilities. Our model formulation enables us to perform model selection efficiently, without the need to employ trans-dimensional algorithms, while at the same time overcoming identifiability issues. We apply our approach to a real commercially-collected great crested newt (*Triturus cristatus*) eDNA data set.

3. We identify detection probabilities and false positive rates comparable with estimates from other eDNA studies. Waterbody characteristics were of only limited importance to eDNA presence rates. However, the probability of a false negative error (8-19%) was strongly influenced by water depth and to a lesser extent by pond density and the surrounding habitat, whereas the probability of a false positive error (4-9%) was considerably reduced by the presence of fish in the pond.

4. This approach has wide-ranging applications when using eDNA to assess species presence, but is also applicable to other survey methods. It allows practitioners to estimate error rates and identify the factors that influence them in order to design more robust sampling strategies. The approach used here can be applied retrospectively and requires no calibration data.

Introduction

Since the initial proof of concept by Ficetola et al. (2008), the use of environmental DNA (eDNA) for the assessment of aquatic biodiversity has been rapidly expanding. In essence, the eDNA survey method isolates DNA that has become separated from an organism and suspended within the water column, to identify the recent presence of that species within a waterbody (Jane et al., 2015). Surveyors opt for the new technique over traditional survey methods for two reasons. First, eDNA offers a rapid assessment tool with potential cost (Rees et al., 2014b) and logistical savings, allowing large-scale monitoring programs to be implemented, that would be too onerous using traditional methods such as trapping or electrofishing (Jerde et al., 2011; Biggs et al., 2015). Second, some studies have indicated a decrease in the probability of a false negative error over traditional methods, increasing the accuracy of the results (Jerde et al., 2011; Biggs et al., 2015), particularly for rare and cryptic species that are difficult to detect (Sigsgaard et al., 2015).

The rapid adoption of eDNA has left several questions unaddressed. Considering that eDNA analyses are not error-free (e.g. for great crested newts (*Triturus cristatus*) naïve estimates of a false negative error range from 1% to 40% (Thomsen et al., 2012; Rees et al., 2014a; Biggs et al., 2014, 2015)), one of the most glaring gaps is the estimation of error rates as functions of covariates. Errors from eDNA samples can be either false positive results (perceived detection when the target species is not present), or false negative results (failure to detect the species when it is present; Roussel et al., 2015). False positive results may originate from non-specificity of the laboratory test, natural water movement between ponds or contamination during sample collection or analysis (Biggs et al., 2014).

Potential origins of false negative results are more diverse but largely involve the failure to collect and extract sufficient DNA from the target species above a minimum limit of detection (Biggs et al., 2014; Tréguier et al., 2014). The ability to identify the

degree of error from eDNA surveys and to link that to environmental covariates would be hugely valuable in demonstrating the accuracy of the technique and assigning confidence in individual samples (Barnes et al., 2014; Barnes and Turner, 2016; Willoughby et al., 2016). Most studies assume constant probabilities of false positive and negative errors, while it is well-known that these parameters may be influenced by environmental and waterbody characteristics (Ficetola et al., 2015). For example, dense mats of vegetation or wide shallow drawdown zones around ponds may both prevent the thorough mixing of eDNA into the water column, potentially resulting in a failure to collect target DNA (Biggs et al., 2014). Similarly, water flows between ponds may allow for the transport of eDNA from one pond to another leading to a false positive result, or the removal of eDNA from a survey area leading to false negative results (Biggs et al., 2014).

Estimating error rates and identifying influences on detection will improve the reliability of surveys. This is particularly true as eDNA surveys are now being enshrined within policy and commercial practice. Commercial and political decision making has started to rely solely on results from eDNA surveys, whether this be in management decisions around the introduction of invasive species of Asian carp in the USA (Jerde et al., 2011) or development mitigation decisions surrounding protected species such as the great crested newt in the UK (Natural England, 2017). However, no formal analyses of error rates as functions of covariates have been undertaken and, as a result, decisions with prominent commercial and political consequences are being made with unknown levels of confidence in the results.

Occupancy models use repeat observations to estimate the probability that a site is occupied by the target species, (occupancy probability), accounting for imperfect detection (detection probability; MacKenzie et al., 2002, 2003; Tyre et al., 2003). Classic occupancy models assume that the probability of a false positive result is equal to zero. Nonetheless, even low false positive rates can bias occupancy and

detection probability estimates (Lahoz-Monfort et al., 2015). Royle and Link (2006) presented the first occupancy model accounting for both false positive and false negative probabilities. However, due to a symmetry in the likelihood, the model is unidentifiable and Royle and Link (2006) suggested constraining the probability of a true positive to be greater than the probability of a false positive in order to obtain a unique solution. Alternative models have been considered for eDNA data, incorporating both false positive and negative probabilities. Specifically, Ficetola et al. (2015) assumed that a species was absent if its presence was uncertain. Clearly, this approach artificially reduces detection rates as some uncertain results will in fact not be false positives. Lahoz-Monfort et al. (2015) address the identifiability issue in two alternative ways, firstly through calibration with survey methods that are not susceptible to false positive errors, as presented by Miller et al. (2011), and secondly through a Bayesian framework with prior distributions for detection probabilities reflecting the assumption that false detections are relatively rare compared to true detections. A two stage occupancy detection model has also been developed to account for false positive and false negative errors when replication has been undertaken at both the field and laboratory stages (Guillera-Arroita et al., 2017). However none of the models take into account site characteristics as covariates and therefore do not attempt to identify the origins of error.

Model selection for occupancy models to identify covariates that are linked to changes in occupancy or detection probabilities has primarily been performed in a classical framework, using for example information criteria (IC), such as the Akaike IC (Akaike, 1976; AIC), which is part of the output in MARK (White and Burnham, 1999), PRESENCE (https://www.mbr-pwrc.usgs.gov/software/presence.html) and unmarked (Fiske et al., 2011). AIC is known to select more complicated models than necessary and in order to select the model with the smallest AIC value out of the list of possible models, one has to fit a potentially very large number of models. Recently,

Broms et al. (2016) demonstrated the use of cross-validation in a Bayesian inference framework to perform model selection in multi-species occupancy models and mentioned reversible jump (RJ) MCMC (Green, 1995) as an alternative model-selection method. Their results suggested that within-sample criteria, such as the Watanabe-Akaike IC (Watanabe, 2010) lead to overfitting. However, cross-validation is computationally intensive and practically non-feasible when there is a large number of models to be considered. RJMCMC has been used extensively in the statistical ecology literature (King and Brooks, 2003; King et al., 2006, 2008; Matechou et al., 2015, 2016) but is again computationally intensive, requires derivation of complicated acceptance probabilities and tuning. Finally, Taylor-Rodríguez et al. (2016) presented objective Bayesian priors and an automated algorithm for model fitting for classic occupancy models that overcomes some of the aforementioned problems. However, to the best of our knowledge, the only model-selection tool employed for extended occupancy models has been AIC.

We develop a novel Bayesian modelling approach for occupancy data that estimates site-specific occupancy probabilities accounting for both false negative and false positive errors when all model parameters are potentially functions of covariates. We propose a set of prior distributions that overcomes the identifiability issue of the model, introduced by the likelihood function, and we demonstrate how this novel model formulation allows us to elegantly perform Bayesian model selection, even when the number of possible models to be considered is large, avoiding RJMCMC. Our approach does not require additional data, such as certain presences, nor does it rely on arbitrarily classifying observed positives as certain or uncertain.

We apply our proposed methods to a commercially collected and analysed eDNA data set, commissioned by Natural England. We identify pond-related covariates that impact the probability of presence of great crested newt eDNA, while accounting for pond-specific false positive and false negative error rates (Ficetola et al., 2015; Rees

et al., 2014b; Roussel et al., 2015), with effects of covariates in all cases assessed using Bayesian model selection. We provide valid estimates of eDNA presence rates, while assessing the reliability of the currently accepted commercial eDNA collection and analysis methods used within the UK.

Materials and methods

eDNA sample collection and analysis

eDNA has been extensively used with great crested newts in research (Thomsen et al., 2012; Rees et al., 2014a; Biggs et al., 2014, 2015; Buxton et al., 2017; Rees et al., 2017) and commercial surveys, with a vast number of samples collected making them an ideal target species for our analysis. eDNA samples were collected from 195 ponds from the Ashford area of central Kent, UK, a known stronghold for the target species, between the 30 of April 2014 and the 26 May 2014. Samples were collected as part of a national distribution modelling assessment for great crested newts, commissioned by Natural England (Bormpoudakis et al., 2016). Sample collection and analysis followed a precipitation in ethanol protocol, exactly following those outlined in Biggs et al. (2014, 2015). Twelve quantitative real-time PCR (qPCR) replicates were performed per sample following the assay outlined in Biggs et al. (2014, 2015) using primers TCCBL and TCCBR, with hydrolysis probe TCCB developed by Thomsen et al. (2012). Appropriate positive and negative control samples were included. An amplification replicate was considered to be positive if an exponential phase was observed during qPCR.

Habitat Suitability Index

A habitat suitability index (HSI) was developed for great crested newts by Oldham et al. (2000) as a measure of habitat quality for the species. The standard great crested newt HSI combines ten factors which are scored in the field, with the geometric mean of the factors taken as the index of habitat suitability. The standard HSI requires

information to be collected on geographic area, pond area, pond performance (pond drying), water quality (assessed through invertebrate diversity), pond shading, waterfowl presence, fish presence, pond count (the number of ponds within 1km), quality of terrestrial habitat and macrophyte cover. Although HSI scores were initially developed to assess the habitat quality for great crested newts, these pond characteristics may also influence eDNA detection.

Along with the collection of an eDNA sample the commercial surveyors were asked to undertake the standard great crested newt HSI assessment (Oldham et al., 2000). A qualitative assessment based on expert judgement of the 10 HSI categories was undertaken. In addition, surveyors were asked to collect additional variables i.e. areas of surrounding woodland, rough grassland, scrub and hedge, and ruderal habitat types in the vicinity of the ponds to give a more detailed assessment of terrestrial habitat. Any visible pollution in the pond was noted, as this may not be incorporated in the water quality assessment within the standard HSI and may have an influence on both eDNA detection and great crested newt presence. Pond dimensions, max depth, max width and max length, the presence of an inflow or outflow to or from the pond were also noted, as the shape of the pond may influence the ability to collect eDNA and inflow and outflow may dilute eDNA or transport eDNA to or from the pond. Additionally the extent of overhanging vegetation was recorded as this indicates potential organic input to the pond. Nineteen of these pond characteristic variables were taken forward into our analysis and can be found along with assessment categories in Table 1. It is acknowledged that the selection of criteria to include in HSI assessment is more aimed at the criteria influencing great crested newt utilization of a pond rather than factors that would influence eDNA error rates; this has resulted from the original purpose of the data set.

Bayesian model

Occupancy models have been used extensively to estimate the probability that a site is occupied, denoted by ψ, by the target species. The classic occupancy model by MacKenzie et al. (2002) and the related models, as for example in MacKenzie et al. (2003), allow for imperfect detection by defining the probability p that a species is detected at an occupied site, but assume that the probability of falsely detecting a species at an unoccupied site equals zero.

Extended occupancy models accounting for both false negative and false positive probabilities were developed by Royle and Link (2006), who, in addition to parameters ψ and p, introduced parameter q to denote the probability of a false detection at an unoccupied site.

We assume that data are collected on K visits, or in this case, eDNA qPCR replicates, from S sites and that all parameters can be site-specific, enoted by ψ_i, p_i and q_i, $i = 1, \ldots, S$. If we denote the number of positives obtained at site i out of the K samples by y_i, for $i = 1, \ldots, S$, assume independence between samples and between sites, we obtain the following expression for the likelihood function (Royle and Link, 2006)

$$L(p, q, \psi|y) \propto \prod_{i=1}^{S} \left[\left\{ p_i^{y_i} (1 - p_i)^{K - y_i} \right\} \psi_i + \left\{ q_i^{y_i} (1 - q_i)^{K - y_i} \right\} (1 - \psi_i) \right]. \qquad (1)$$

However, the model suffers from a likelihood symmetry, since it can be seen that $L(p, q, \psi) = L(q, p, 1 - \psi)$ and Royle and Link (2006) suggested setting a constraint such that $p > q$ to choose one of these two equally supported solutions.

In a Bayesian framework, the posterior distribution will suffer from the same problem of symmetry if p_i and q_i are given the same prior distribution. To address this problem, Lahoz-Monfort et al. (2015) considered a prior distribution that ensures $p > q$, which

is straightforward in the absence of covariates. However, specifying a prior which imposes a $p > q$ constraint is more difficult when p and q are functions of covariates (for example linear functions of the covariates on the logit scale). The challenge becomes even greater when model selection needs to be performed, as the required constraint may be satisfied for one set of covariates, but not for another. To solve this problem, we consider prior distributions for which the prior probability that $p < q$ can be chosen to be arbitrarily small (rather than zero as in Lahoz-Monfort et al., 2015).

In the absence of covariates, $p_i = p$ and $q_i = q$ and we propose the following prior distributions

$$\text{logit}(q) \sim \text{N}\left(\text{logit}(a), \frac{(\text{logit}(b) - \text{logit}(a))^2}{2\delta^2}\right) \tag{2}$$

and

$$\text{logit}(p) \sim \text{N}\left(\text{logit}(b), \frac{(\text{logit}(b) - \text{logit}(a))^2}{2\delta^2}\right) \tag{3}$$

where $a < b$ and $\delta > 0$ are hyperparameters that need to be pre-specified. The prior probability that $p < q$ is $\Phi(-\delta)$ (see details in the appendix) and, after choosing values for a and b (the prior medians of q and p respectively), a sufficiency large value of δ can be chosen to make the probability that $p < q$ as small as required. Figure A3.1 shows examples of different possible prior distributions for p and q using different hyperparameter values, for which the probability that $p < q$ is small. For fixed a and b, as δ increases the overlap between the prior densities of p and q, and hence the prior probability that $p < q$, decreases. For fixed δ and a, as b increases, the median of the prior distribution for q shifts to the right, while for fixed δ and b, as a increases, the median of the prior distribution for p shifts to the left.

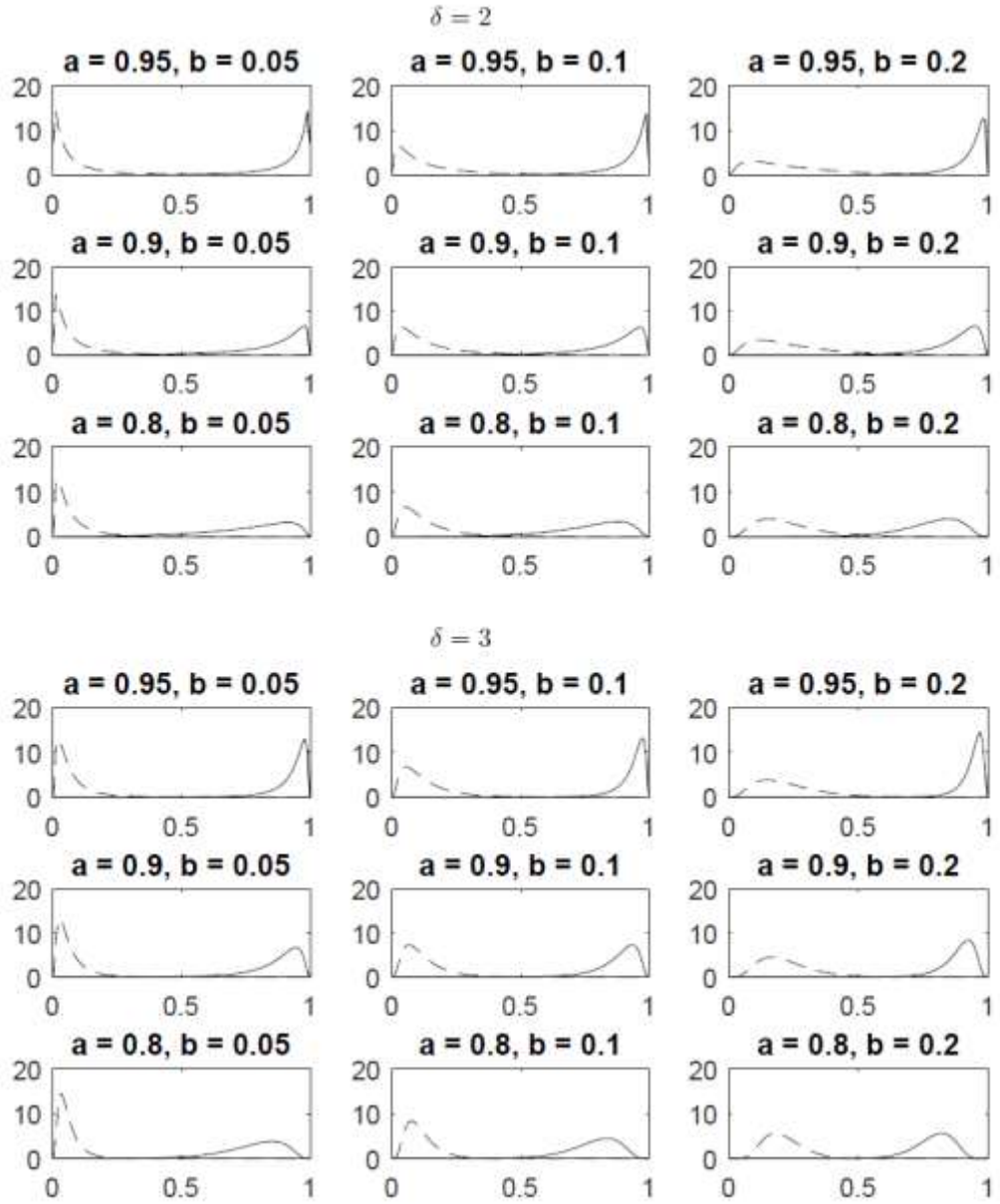

Figure A3.1: The prior density for *p* (solid line) and *q* (dashed line) for different choices of *a*, *b*, and *δ*.

We assume that potentially different sets of covariates are available for *p, q* and *ψ* and that these can be either continuous or categorical. There are a_p continuous covariates for p with values at site *i* $X^p_{i,1}, \ldots, X^p_{i,a_p}$ and b_p categorical covariates with values $Z^p_{i,1}, \ldots, Z^p_{i,b_p}$, giving a total of $d_p = a_p + b_p$ covariates. The categorical covariates are included using dummy variables relative to a baseline class. For the *j*-

th categorical covariate with L_j^p levels we define $X^p_{i,a_{p+j}}$ to be a L_j^p−1-dimensional vector containing the values of the dummy variables for the *i*-th observation. We assume a logistic regression model for p_i,

$$\text{logit}(p_i) = \eta_i^p = \mu^p + \sum_{j=1}^{a_p} X_{i,j}^p \beta_j^p + \sum_{j=a_p+1}^{d_p} X_{i,j}^p \beta_j^p$$

where μ is an intercept parameter, β_j^p are scalars (for $j = 1, \ldots, a_p$) and $\beta^p_{a_{p+j}}$ is a L_j^p–dimensional vector (for $j = 1, \ldots, b_p$). We write X_p as the matrix whose *i*-th row is formed by concatenating $X_{i,j}^p$

Similarly, we assume logistic regression models for q_i and ψ_i,

$$\text{logit}(q_i) = \eta_i^q = \mu^q + \sum_{j=1}^{a_q} X_{i,j}^q \beta_j^q + \sum_{j=a_q+1}^{d_q} X_{i,j}^q \beta_j^q$$

and

$$\text{logit}(\psi_i) = \eta_i^\psi = \mu^\psi + \sum_{j=1}^{a_\psi} X_{i,j}^\psi \beta_j^\psi + \sum_{j=a_\psi+1}^{d_\psi} X_{i,j}^\psi \beta_{i,j}^\psi$$

where X^q and X^ψ are formed using the covariate values associated with q and ψ respectively. We also assume that all continuous covariates have zero mean and are measured on the same scale (for example, by standardizing the covariates to have mean 0 and variance 1, which is standard practice).

We extend the prior distributions in (2) and (3) to allow for covariates by assuming that μp, $\beta_1^p, \ldots, \beta_{dp}^p$ are independent and that

$$\mu_p \sim \mathrm{N}\left(\mathrm{logit}(b), \frac{\alpha_0(\mathrm{logit}(b)-\mathrm{logit}(a))^2}{2\delta^2(\alpha_0+d_p)}\right),$$

$$\beta_i^p \overset{i.i.d.}{\sim} \mathrm{N}\left(0, \frac{(\mathrm{logit}(b)-\mathrm{logit}(a))^2}{2\delta^2(\alpha_0+d_p)}\right), \quad i=1,\ldots,a_p$$

and

$$\beta_{i+a_p}^p \overset{ind.}{\sim} \mathrm{N}\left(0_{L_i^p-1}, \frac{(\mathrm{logit}(b)-\mathrm{logit}(a))^2}{4\delta^2(\alpha_0+d_p)}\left(J_{L_i^p-1}+I_{L_i^p-1}\right)\right), \quad i=1,\ldots,b_p$$

where 0_m represents an (m × 1)-dimensional vector of 0's, Jm represents an (m × m)-dimensional vector of 1's and Im represents the (m × m)-dimensional identity matrix. This choice of prior covariance matrix for the regression coefficients associated with categorical covariates makes the prior invariant to the choice of the baseline class (Fearn et al., 1999). The hyperparameter α_0 controls the variance of the prior distribution on the intercept relative to the variance of the prior distribution on the regression coefficients. Then

$$\mathrm{E}[\eta_i^p] = \mathrm{logit}(b) \text{ and } \mathrm{V}[\eta_i^p] = \frac{(\mathrm{logit}(b)-\mathrm{logit}(a))^2}{2\delta^2}.$$

as the covariates have been standardized and so the prior distribution in the presence of d_p covariates replicates the properties of the prior distribution in the no covariate case. Similarly, we define

$$\mu^q \sim \mathrm{N}\left(\mathrm{logit}(a), \frac{w_0(\mathrm{logit}(b)-\mathrm{logit}(b))^2}{2\delta^2(w_0+d_q)}\right),$$

$$\beta_i^q \sim \mathrm{N}\left(0, \frac{(\mathrm{logit}(b)-\mathrm{logit}(b))^2}{2\delta^2(w_0+d_q)}\right), \qquad i=1,\ldots,a_q$$

and

$$\beta^q_{i+a_q} \overset{ind.}{\sim} \mathrm{N}\left(0_{L^q_i-1}, \frac{(\mathrm{logit}(b)-\mathrm{logit}(a))^2}{4\delta^2(\alpha_0+d_q)}\left(J_{L^q_i-1}+I_{L^q_i-1}\right)\right), \quad i=1,\ldots,b_q.$$

In the absence of any prior information on ψ, the prior distributions for μ^ψ and β^ψ are chosen to be

$$\mu^\psi \sim \mathrm{N}(0,\phi_\mu), \qquad \beta^\psi_i \overset{i.i.d.}{\sim} \mathrm{N}(0,\phi_\beta), \qquad i=1,\ldots,a_\psi.$$

and

$$\beta^\psi_{i+a_\psi} \overset{ind.}{\sim} \mathrm{N}\left(0_{L^\psi_i-1}, \frac{\phi_\beta}{2}\left(J_{L^\psi_i-1}+I_{L^\psi_i-1}\right)\right), \quad i=1,\ldots,b_\psi.$$

Inference in the model in (1) can be made by employing Markov chain Monte Carlo methods using the hierarchical representation

$$\begin{aligned} z_i &\sim \mathrm{Bernoulli}(\psi_i) \\ y_i|z_i=1 &\sim \mathrm{Binomial}(K,p_i) \\ y_i|z_i=0 &\sim \mathrm{Binomial}(K,q_i) \end{aligned} \tag{4}$$

and treating $z = (z1, \ldots, zs)$ as latent variables, where $z_i = 1$ indicates presence and $z_i = 0$ absence of eDNA from site i. This leads to the following complete-data likelihood function

$$L(p,q,\psi,z|y) \propto \prod_{i=1}^{S}\left[p_i^{z_i y_i}(1-p_i)^{(K-y_i)z_i}q_i^{(1-z_i)y_i}(1-q_i)^{(K-y_i)(1-z_i)}\psi_i^{z_i}(1-\psi_i)^{1-z_i}\right]. \tag{5}$$

This complete-data likelihood is the product of three logistic regression likelihood terms (for p, q and ψ respectively). This representation combined with the Pólya-Gamma sampling method for logistic models (Polson et al., 2013) allows a simple

Markov chain Monte Carlo scheme to be defined that enables Bayesian inference (further details are provided in the supplementary information).

The previous description assumes that the covariates in x^p, x^q and x^ψ are pre-specified. We wish to consider the slightly different problem where x^p, x^q and x^ψ contain potentially different covariates chosen from a fixed set of D possible covariates leading to three covariate or model selection problems. We will assume that all levels of categorical covariates are either all included or excluded in the model selection and introduce variables γ^p, γ^q and γ^ψ, D_p, D_q and D_ψ–dimensional vectors, respectively, for which $\gamma_k^q = 1$ if the k-th covariate is included in the linear predictor for p and 0 otherwise, $\gamma_k^q = 1$ if the k-th covariate is included in the linear predictor for q and 0 otherwise, and $\gamma_k^\psi = 1$ if the k-th covariate is included in the linear predictor for ψ and 0 otherwise. The prior on the included covariates follows the suggestion of Ley and Steel (2009),

$$\gamma_k^p \overset{\text{i.i.d.}}{\sim} \text{Bernoulli}(\pi_p) \text{ and } \pi_p \sim \text{Be}\left(1, \frac{D_p - \bar{d}_p}{\bar{d}_p}\right)$$

$$\gamma_k^q \overset{\text{i.i.d.}}{\sim} \text{Bernoulli}(\pi_q) \text{ and } \pi_q \sim \text{Be}\left(1, \frac{D_q - \bar{d}_q}{\bar{d}_q}\right)$$

$$\gamma_k^\psi \overset{\text{i.i.d.}}{\sim} \text{Bernoulli}(\pi_\psi) \text{ and } \pi_\psi \sim \text{Be}\left(1, \frac{D_\psi - \bar{d}_\psi}{\bar{d}_\psi}\right).$$

This choice of prior implies that the prior mean of the number of included covariates for p, dp, is $\bar{d}_p$ and leads to a heavy tailed prior on dp. The value of $\bar{d}_p$ is set to a prior guess for the number of covariates included in the logistic regression model for p. The parameters of the priors for q and ψ can be chosen in the same way. Posterior inclusion probabilities are often used to summarize the inference about the importance of the covariates. The posterior inclusion probability (PIP) for the j-th variable to predict p is $p(\gamma_i^p = 1|y)$ and this can be easily estimated from MCMC output by the proportion of iterations for which the j-th variable is included in the model for p.

Clearly, the PIP's for the difference for the prediction of ψ and q can be similarly defined.

We again use the Pólya-Gamma sampling method for logistic models (Polson et al., 2013) to define an efficient MCMC scheme for Bayesian inference in this model selection problem without using RJMCMC. Details are provided in the supplementary information.

Results

We analysed the data using our Bayesian method with the following choices of hyperparameters. The posterior median probability of a true positive, a, was set to 0.9, the posterior median of the probability of a false positive, b, was set to 0.1 and we choose $\delta = 3$. This corresponds to the prior density shown in the centre of the middle row of graphs for $\delta = 3$ in Figure A3.1. The parameter α_0 was set equal to 1 which reflects a prior belief that half of the variation in the response can be explained by the regressors. In the logistic regression for ψ, the prior variance of the intercept, φ_μ, was set to 4 and the prior variance of the regression coefficient, φ_β was set 0.25. The prior distribution of the intercept reflects a belief that the probability of detection is roughly uniformly distributed and the prior on the regression coefficient represents a belief that the regression effects will be in (−1, 1) with high probability. The prior expected numbers of included covariates $\bar{p}$, $\bar{q}$ and $\bar{\psi}$, were set to 4.

Since the covariates are centred, the estimated intercepts can be interpreted as probabilities for an observation at the baseline value of each of the categorical covariates and the average level of each of the continuous covariates. Therefore, at these covariate values, the posterior median level of the probability of occupancy, ψ, is 0.21 (with a 95% highest probability density region of (0.11, 0.36)), of the true positive probability (detection probability, p) is 0.88 (with a 95% highest probability density region of (0.81, 0.92)) and of the false positive probability, q is 0.06 (with a

95% highest probability density region of (0.04, 0.09)). As expected, both the probabilities of a false negative and a false positive error are estimated close to 0. However, they are not exactly equal to zero, a result which highlights that even though eDNA methods may lead to lower false negative errors compared to standard sampling methods, they are still not error-free.

The inference about covariate selection is shown in Figures A3.2 for ψ, A3.3 for p, and A3.4 for q.

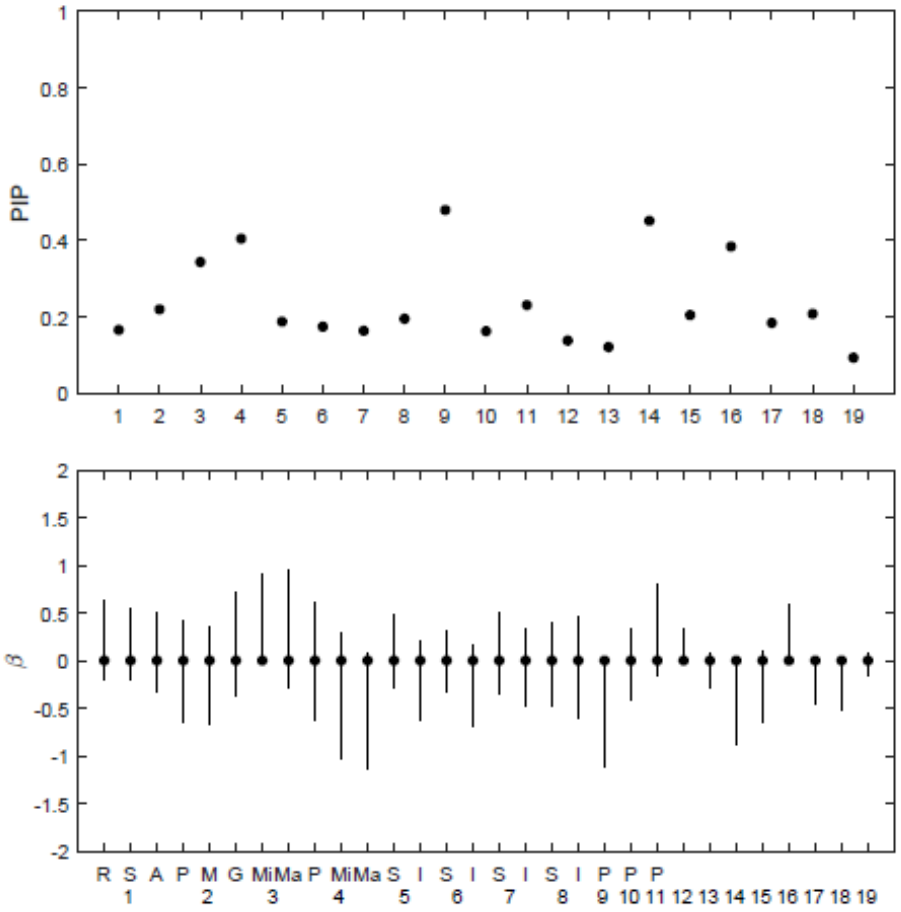

Figure A3.2: Prediction of ψ: posterior inclusion probabilities of each variable (top row) and inference about the regression coefficients (shown as posterior median and 95% highest probability density region; bottom row) with the label of the *x*-axis showing the variable numbers underneath the levels of each variable as indicated in Table A3.1.

Table A3.1: List and description of pond-specific covariates.

No.	Covariate	Type
1	Permanence	Discrete (Never Dries, (R)arely Dries, (S)ometimes Dries, Dries (A)nnually)
2	Water Quality	Discrete (Bad, (P)oor, (M)oderate, (G)ood)
3	Water Fowl	Discrete (Absent, (Mi)nor, (Ma)jor)
4	Fish	Discrete (Absent, (P)ossible, (Mi)nor, (Ma)jor)
5	Woodland	Discrete (None, (S)ome, (I)mportant)
6	Rough Grass	Discrete (None, (S)ome, (I)mportant)
7	Scrub Hedge	Discrete (None, (S)ome, (I)mportant)
8	Ruderals	Discrete (None, (S)ome, (I)mportant)
9	Inflow	Discrete (Absent, (P)resent)
10	Outflow	Discrete (Absent, (P)resent)
11	Pollution	Discrete (Absent, (P)resent)
12	Max Depth	Continuous
13	Width	Continuous
14	Length	Continuous
15	Area	Continuous
16	Macrophytes	Continuous
17	Overhang	Continuous
18	Shade	Continuous
19	Pond Density	Continuous

We have not identified any covariates that are linked to the probability of eDNA presence, as they all have PIP below 50%. Note that presence of waterfowl, fish or inflow, the length of the pond and macrophyte cover were each present in between 30% and 50% of iterations of the algorithm, so they are potentially useful predictors for great crested newt eDNA presence in a pond.

Given eDNA presence, Figure A3.3 shows very strong evidence that the depth of the pond (PIP: 0.98) has a positive effect on detection probability. On the other hand,

pond density (PIP: 0.65) decreases the probability of detection, as does, potentially, the presence of rough grass (PIP: 0.50).

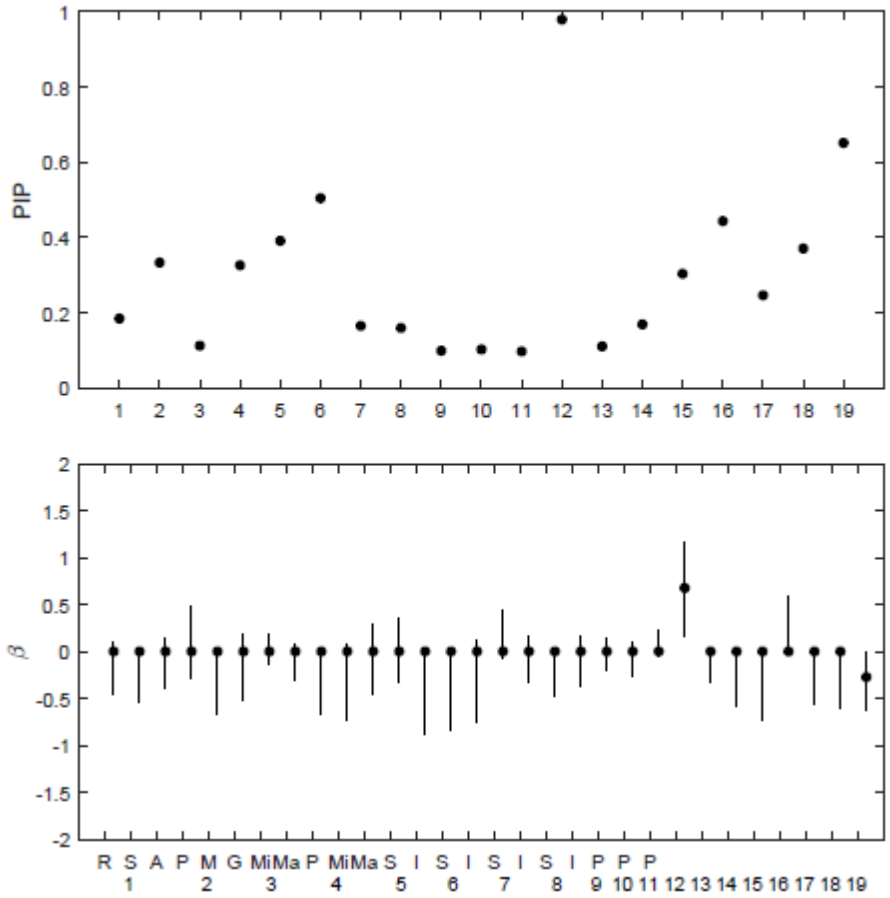

Figure A3.3: Prediction of *p*: posterior inclusion probabilities of each variable (top row) and inference about the regression coefficients (shown as posterior median and 95% highest probability density region; bottom row) with the label of the *x*-axis showing the variable number underneath the levels of each variable as indicated in Table A3.1.

Lastly, Figure A3.4 shows that, given non-presence of eDNA, there is very strong evidence (PIP: 0.97) that the presence of fish decreases the probability of a false positive result.

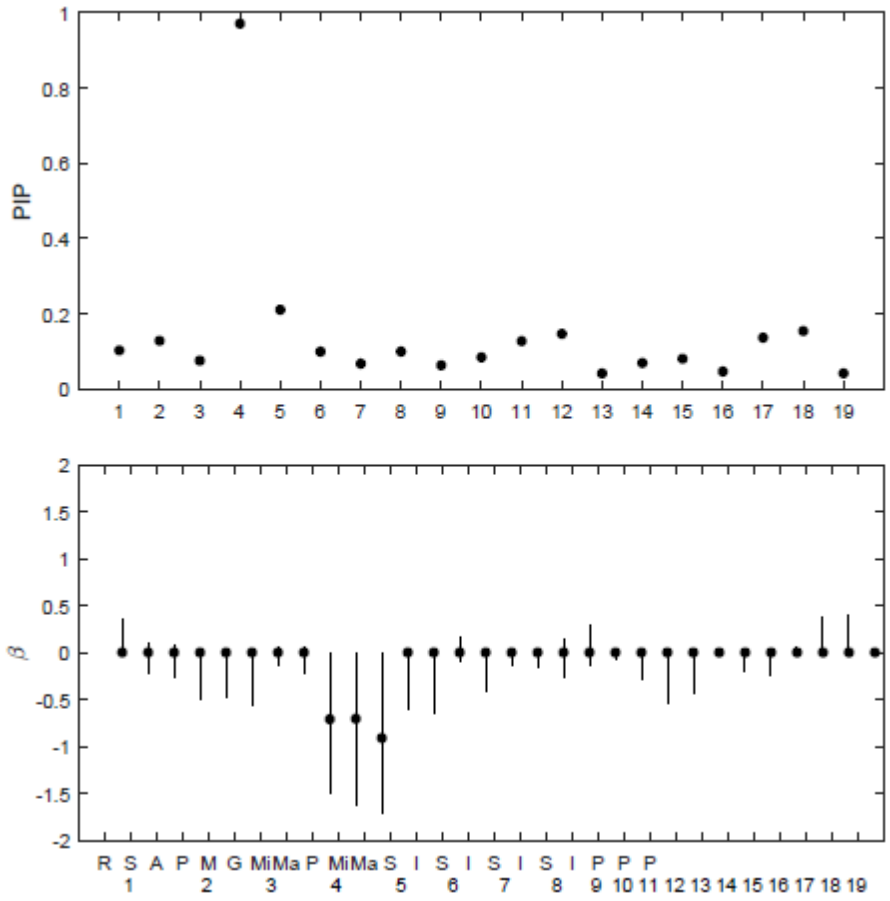

Figure A3.4: Prediction of *q*: posterior inclusion probabilities of each variable (top row) and inference about the regression coefficients (shown as posterior median and 95% highest probability density region; bottom row) with the label of the *x*-axis showing the variable numbers underneath the levels of each variable as indicated in Table A3.1.

Discussion

Our Bayesian approach provides estimates of both true and false positives in eDNA surveys, while exploring potential influences on occupancy and error probabilities. A novel prior distribution for error probabilities with covariates is introduced which assumes that the prior probability of a true positive is greater than the probability of a false positive. The use of the Pólya-Gamma sampler allows us to define an efficient

MCMC algorithm for posterior inference where the models for *p, q* and *ψ* can be updated using a Metropolis-Hastings step.

Incorporating both false positive and false negative estimates in previous models has proved problematical. Our novel approach allows: 1) true and false positive presence occupancy models that account for the influence of covariates on error rates; 2) improved survey designs utilising true and false positive models; 3) exploration of the occurrence of eDNA in relation to site characteristics. Finally, 4) considering that false positives are common in other settings too (e.g. bird or frog aural surveys; Guillera-Arroita et al., 2017) our method can explore drivers of imperfect detection and is applicable to other survey methods. Occupancy of a pond by great crested newts and presence of eDNA within a sample from that pond are intrinsically linked but distinct from one another. Within our analysis we only observe presence of eDNA; however its presence is reliant on the presence of great crested newts, therefore a single covariate may influence occupancy either by influencing the presence of the species or by influencing the presence of eDNA of that species, or both. As mentioned above eDNA presence was found to be 0.21 (0.11-0.36) slightly lower than previous occupancy estimates for great crested newts in the Kent area of between 0.31 and 0.35 (Sewell et al., 2010).

No waterbody characteristics were found to affect eDNA presence, which is surprising given the reliance on HSI-related covariates in the analysis. We could argue that given small geographic area the sample was collected from, perhaps the variability of pond characteristics is low in comparison to the range of the species (i.e. the occupancy-related abiotic environment is relatively homogeneous). Secondly, newts are perhaps being displaced into less optimal habitat due to high population densities, thereby masking the influence of pond characteristics on occupancy. Thirdly, the assessment of HSI variables in the field, is imprecise and subject to a level of surveyor subjectivity. Finally, waterbody characteristics may have been recorded at too broad

a scale, e.g. had water quality been broken down into the composition of individual components it may have been important (Gustafson et al., 2009).

Overall false negative rates (i.e. 1-*p*) were found to be between 8% and 19%, much lower than that for a combination of four traditional survey methods and a single visit (p=0.68, SE=0.081 in one year and p=0.56, SE=0.082 in another; Sewell et al., 2010). However, false negative rates were potentially higher for eDNA than when 4 to 6 visits are made using the traditional methods, as is required for a commercial survey, where 95% confidence in a negative is expected (English Nature, 2001; Sewell et al., 2010). Using experienced surveyors the probability of false positives (i.e. *q*) should be 0 with traditional methods, but we identify a false positive rate of between 4% and 9% using eDNA within this study. When classic occupancy models have been applied to other eDNA datasets, detection probabilities of between 0.74 and 0.96 have been found, equating to a false negative rate of between 4% and 26% (Schmidt et al., 2013; Hunter et al., 2015; Schmelzle and Kinziger, 2016; Guillera-Arroita et al., 2017; Vörös et al., 2017). To our knowledge Guillera-Arroita et al. (2017) is the only study to have identified false positive rates using real eDNA data, with false positive rates of between 0.03 and 0.05 (or between 3% and 5%). The false negative error rate we identify of between 8% and 19% with the false positive error rate of between 5% and 9% are not too dissimilar from the previously published results, and will be expected to vary depending on species and eDNA methodology used. The high detection probabilities, of between 81% and 92%, within our study may result from the sampling area constituting the core range of the species where population densities are high. Although not the only factor influencing the concentration of eDNA within a pond (Buxton et al., 2017), greater densities of individuals can lead to increases in eDNA concentration (Thomsen et al., 2012), with the more target DNA present the greater the chance of detecting it. Consequently, detection probability may vary with population density across the species range. We speculate that error rates such as

these will be less of an issue when eDNA is incorporated into distribution models, but may be more of an issue when individual site accuracy is required, such as the identification of single sites where a protected species may be present (e.g. for planning or conservation decision making).

Pond characteristics influence the detection of newt eDNA. The maximum depth of the pond has a positive influence on detection probability, whereas pond density and to a lesser extent the vicinity of ruderal grassland reduce the probability of detection. Increased depth was found to have a very strong influence on the increase in detection; increased depth may suggest a pond at an earlier stage in succession with abundant open water allowing for homogenisation of eDNA within the water column, making target DNA more uniformly available for survey. The limited distribution of the sample collection may have resulted in samples not being truly independent from one another.

Fish presence was linked to a decrease in the probability of a false positive result if sites are not occupied, with major fish presence reducing false positive results to a greater extent than possible or minor fish presence categories. Presence of fish at very high densities may also collectively produce quantities of target DNA so great that they inhibit the amplification of DNA at lower concentrations, such as that from contaminants. This may however be an artefact of few samples containing both fish and target eDNA as we find very low target eDNA presence (5/36 samples) when fish were classed as either a minor or major impact on a pond.

The amount of eDNA within a sample has a strong influence on detection (Furlan et al., 2015); it has been shown that this is influenced by both the breeding status of the pond as well as to a lesser extent the abundance or density of individuals (Buxton et al., 2017). Within this study no attempt was made to collect data on the target species density or breeding status, which may have an overreaching influence on the amount of DNA and therefore the probability of eDNA detection. Future studies should clarify

this by collecting and using estimates of abundance or density as additional covariates in models, given that it is quite possible the effect of the covariates identified as having an influence on detection would be minimised if individual density estimates were included.

All survey methods have biases and uncertainties when applied in the field, and it is imperative to try to reduce them. Care should be taken to evaluate eDNA surveys, before they are over-relied upon for critical species conservation decision-making. It is evident that to reduce false negative results one needs to increase the amount and quality of the target DNA within an eDNA sample and improve the efficiency of extraction methods (Hinlo et al., 2017). eDNA methodological advances in both field and laboratory are ongoing and these are likely to both reduce and identify sample contamination. This will reduce false positive results and allow larger samples of eDNA that will reduce false negatives (Spens et al., 2016; Wilson et al., 2016). Further refinement of field, laboratory and statistical protocols will also lead to improved *p* and *q* values and identify environmental characteristics which may influence these methods.

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References

Akaike, H. 1976. Canonical correlation analysis of time series and the use of an information criterion. *Mathematics in Science and Engineering*, 126:27–96.

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

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

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

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R., Foster, J., Wilkinson, J., Arnett, A., Williams, P., and F, D. 2014. *Analytical and methodological development for improved surveillance of the great crested newt. Defra Project WC1067*. Technical report, Freshwater Habitats Trust, Oxford, UK.

Bormpoudakis, D., Foster, J., Gent, T., Griffiths, R. A., Russell, L., Starnes, T., Tzanopoulos, J., and Wilkinson, J. 2016. *Developing models to estimate the occurrence in the English countryside of great crested newts, a protected species under the Habitats Directive. Defra Project WC1108*. Technical report, DICE, University of Kent, Canterbury, UK.

Broms, K. M., Hooten, M. B., and Fitzpatrick, R. M. 2016. Model selection and assessment for multi-species occupancy models. *Ecology*, 97:1759–1770.

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

English Nature 2001. *Great crested newt mitigation guidelines*. English Nature, Peterborough.

Fearn, T., Brown, P. J., and Haque, M. S. 1999. Logistic discrimination with many variables. *Revistade la Real Academia de Ciencias, Exactas, Fiscias y Naturales*, 93:372–342.

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

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly L., Lopes, C. M., Boyer, F., Pompanon, F., et al. 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, 15:543–556.

Fiske, I., Chandler, R., et al. 2011. unmarked: An R package for fitting hierarchical models of wildlife occurrence and abundance. *Journal of Statistical Software*, 43:1–23.

Furlan, E. M., Gleeson, D., Hardy, C. M., and Duncan, R. P. 2015. A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*, 16:641–654.

Green, P. 1995. Reversible jump MCMC computation and Bayesian model determination. *Biometrika*, 82:711.

Guillera-Arroita, G., Lahoz-Monfort, J. J., Rooyen, A. R., Weeks, A. R., and Tingley, R. 2017. Dealing with false-positive and false-negative errors about species occurrence at multiple levels. *Methods in Ecology and Evolution*,doi:10.1111/2041–210X.12743.

Gustafson, D. H., Andersen, A. S., Mikusiński, G., and Malmgren, J. C. 2009. Pond quality determinants of occurrence patterns of great crested newts (*Triturus cristatus*). *Journal of Herpetology*, 43:300–310.

Hinlo, R., Gleeson, D., Lintermans, M., and Furlan, E. 2017. Methods to maximise recovery of environmental DNA from water samples. *PLoS ONE*, 12:e0179251.

Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., Reed, R. N., and Hart, K. M. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PloS ONE*, 10:e0121655.

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

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

King, R. and Brooks, S. P. 2003. Survival and spatial fidelity of mouflons: The effect of location, age, and sex. Journal of Agricultural, *Biological, and Environmental Statistics*, 8:486–513.

King, R., Brooks, S. P., Mazzetta, C., Freeman, S. N., and Morgan, B. J. 2008. Identifying and diagnosing population declines: a Bayesian assessment of lapwings in the UK. *Journal of the Royal Statistical Society: Series C (Applied Statistics)*, 57:609–632.

King, R., Brooks, S. P., Morgan, B. J., and Coulson, T. 2006. Factors influencing Soay sheep survival: a Bayesian analysis. *Biometrics*, 62:211–220.

Lahoz-Monfort, J. J., Guillera-Arroita, G., and Tingley, R. 2015. Statistical approaches to account for false-positive errors in environmental DNA samples. *Molecular Ecology Resources*, 16:604–607.

Ley, E. and Steel, M. F. J. 2009. On the effect of prior assumptions in Bayesian model averaging with applications to growth regression. *Journal of Applied Econometrics*, 24:651–674.

MacKenzie, D. I., Nichols, J. D., Hines, J. E., Knutson, M. G., and Franklin, A. B. 2003. Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly. *Ecology*, 84:2200–2207.

MacKenzie, D. I., Nichols, J. D., Lachman, G. B., Droege, S., Andrew Royle, J., and Langtimm, C. A. 2002. Estimating site occupancy rates when detection probabilities are less than one. *Ecology*, 83:2248–2255.

Matechou, E., McCrea, R. S., Morgan, B. J., Nash, D. J., Griffiths, R. A., et al. 2016. Open models for removal data. *The Annals of Applied Statistics*, 10:1572–1589.

Matechou, E., Nicholls, G., Morgan, B. J., Collazo, J. A., and Lyons, J. E. 2015. Bayesian analysis of Jolly-Seber type models; incorporating heterogeneity in arrival and departure. *arXiv preprintarXiv*:1512.05170.

Miller, D. A., Nichols, J. D., McClintock, B. T., Grant, E. H. C., Bailey, L. L., and Weir, L. A. 2011. Improving occupancy estimation when two types of observational error occur: non-detection and species misidentification. *Ecology*, 92:1422–1428.

Natural England 2017. *Wildlife licensing newsletter March 2017*. Technical report, Natural England, Peterborough, UK.

Oldham, R., Keeble, J., Swan, M., and Jeffcote, M. 2000. Evaluating the suitability of habitat for the great crested newt (*Triturus cristatus*). *Herpetological Journal*, 10:143–156.

Polson, N. G., Scott, J. G., and Windle, J. 2013. Bayesian inference for logistic models using Pólya-Gamma latent variables. *Journal of the American Statistical Association*, 108:1339–1349.

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

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

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

Roussel, J.-M., Paillisson, J.-M., Treguier, A., and Petit, E. 2015. The downside of eDNA as a survey tool in water bodies. *Journal of Applied Ecology*, 52:823–826.

Royle, J. A. and Link, W. A. 2006. Generalized site occupancy models allowing for false positive and false negative errors. Ecology, 87:835–841.

Schmelzle, M. C. and Kinziger, A. P. 2016. Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. *Molecular Ecology Resources*, 16:895–908.

Schmidt, B. R., Kery, M., Ursenbacher, S., Hyman, O. J., and Collins, J. P. 2013. Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, 4:646–653.

Sewell, D., Beebee, T. J., and Griffiths, R. A. 2010. Optimising biodiversity assessments by volunteers: the application of occupancy modelling to large-scale amphibian surveys. *Biological Conservation*, 143:2102–2110.

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

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S., Sigsgaard, E. E., and Hellström, M. 2016. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*, 8:635–645.

Taylor-Rodríguez, D., Womack, A. J., Fuentes, C., Bliznyuk, N., et al. 2016. Intrinsic Bayesian analysis for occupancy models. *Bayesian Analysis*, 12:855–877.

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

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., and Roussel, J.-M. 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, 51:871–879.

Tyre, A. J., Tenhumberg, B., Field, S. A., Niejalke, D., Parris, K., and Possingham, H. P. 2003. Improving precision and reducing bias in biological surveys: estimating false-negative error rates. *Ecological Applications*, 13:1790–1801.

Vörös, J., Márton, O., Schmidt, B. R., Gál, J. T., and Jelić, D. 2017. Surveying Europe's only cavedwelling chordate species (*Proteus anguinus*) using environmental DNA. *PloS ONE*, 12:e0170945.

Watanabe, S. 2010. Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. *Journal of Machine Learning Research*, 11:3571–3594.

White, G. C. and Burnham, K. P. 1999. Program MARK: survival estimation from populations of marked animals. *Bird Study*, 46:S120–S139.

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

Wilson, C. C., Wozney, K. M., and Smith, C. M. 2016. Recognizing false positives: synthetic oligonucleotide controls for environmental DNA surveillance. *Methods in Ecology and Evolution*, 7:23–29.

Appendix III – Supplementary Information

1 Calculating the prior probability that $p < q$

$$p(p < q] = p(\text{logit}(p) < \text{logit}(q)) = p(\text{logit}(p) - \text{logit}(q) < 0)$$

Clearly,

$$\text{logit}(p) - \text{logit}(q) \sim \text{N}\left(\text{logit}(b) - \text{logit}(a), \frac{(\text{logit}(b) - \text{logit}(a))^2}{\delta^2}\right)$$

and so

$$p(p < q] = p(\text{logit}(p) - \text{logit}(q) < 0) = \Phi(-\delta).$$

2 Markov chain Monte Carlo algorithm

The complete-data likelihood defined in (5) is combined with the logistic regression models for p, q and ψ and the variable selection priors to define the posterior density

$$p(\theta^p, \theta^q, \theta^\psi, \gamma^p, \gamma^q, \gamma^\psi, z|y) \propto \prod_{i=1}^{S} \left[\frac{\exp\{\eta_i^p\}^{z_i y_i}}{(1+\exp\{\eta_i^p\})^{z_i K}} \frac{\exp\{\eta_i^q\}^{(1-z_i) y_i}}{(1+\exp\{\eta_i^q\})^{(1-z_i) K}} \frac{\exp\{\eta_i^\psi\}^{z_i}}{1+\exp\{\eta_i^\psi\}} \right]$$
$$\times p(\theta^p|\gamma^p) p(\theta^q|\gamma^q) p(\theta^\psi|\gamma^\psi) p(\gamma^p) p(\gamma^q) p(\gamma^\psi),$$

where $\theta^p = (\mu^p, \beta^p)$, $\theta^q = (\mu^q, \beta^q)$ and $\theta^\psi = (\mu^\psi, \beta^\psi)$. The idea of Pólya-Gamma sampling for logistic regression models (Polson et al., 2013) can be used to define an efficient MCMC scheme to sample from this posterior distribution.

The Pólya-Gamma sampling method uses the identity

$$\frac{(\exp\{\xi\})^a}{(1+\exp\{\xi\})^b} = 2^{-b} \int_0^\infty \exp\{-\omega(\xi^2 - 2\kappa\xi)/2\} p(\omega)\, d\omega \tag{6}$$

where $\kappa = a - b/2$, $\omega \sim$ PG(1, 0) and PG(b, 0) represents the Pólya-gamma distribution. This distribution is defined as an infinite sum, so that if $X \sim$ PG(b, c) then

$$X = \frac{1}{2\pi^2} \sum_{k=1}^{\infty} \frac{g_k}{(k-\frac{1}{2})^2 + \frac{c^2}{4\pi^2}}$$

where $g_k \overset{i.i.d}{\sim} Ga(b, 1)$. Although the infinite sum makes the density hard to work with directly, Polson et al. (2013) describe efficient methods for simulating draws from this distribution.

The identity in (6) allows us to write each element of (5) in terms of an integral. For example,

$$\prod_{\{i|z_i=1\}} \frac{\exp\{\eta_i^p\}^{z_i y_i}}{(1+\exp\{\eta_i^p\})^{z_i K}} = \prod_{\{i|z_i=1\}} \left[2^{-K z_i} \int_0^\infty \exp\left\{ -\omega_i^p \left({\eta_i^p}^2 - 2 z_i (y_i - K/2) \eta_i^p \right) /2 \right\} p(\omega_i)\, d\omega_i \right] \tag{7}$$

where $\omega_i \sim$ PG(K, 0). Similar expressions can be derived for the contribution of the q and ψ terms to the complete-data likelihood. This allows us to define the extended posterior density

$$
\begin{aligned}
&p(\theta^p, \theta^q, \theta^\psi, \gamma^p, \gamma^q, \gamma^\psi, z, \omega^p, \omega^q, \omega^\psi | y) \\
&\propto \prod_{i=1}^{S} \left[\left(\exp\left\{ -\omega_i \left(\eta_i^{p2} - 2(y_i - K/2)\eta_i^p \right) /2 \right\} \right)^{z_i} \left(\exp\left\{ -\omega_i \left(\eta_i^{q2} - 2(y_i - K/2)\eta_i^q \right) /2 \right\} \right)^{1-z_i} \right. \\
&\left. 2^{-z_i} \exp\left\{ -\omega_i^\psi \left(\eta_i^{\psi 2} - 2(z_i 1 - 1/2)\eta_i^\psi \right) /2 \right\} p(\omega_i) p(\omega_i^\psi) \right] p(\theta^p | \gamma^p) p(\theta^q | \gamma^q) p(\theta^\psi | \gamma^\psi) \\
&p(\gamma^p) p(\gamma^q) p(\gamma^\psi)
\end{aligned}
$$

where $\omega_j \sim \text{PG}(K, 0)$ and $\omega_j^\psi \sim \text{PG}(1, 1)$. The identity in (6) implies that integrating ω and ω^ψ from this posterior leads to the posterior density in (7). The linear predictors now enter this posterior distribution in a form which implies that the full conditionals of the regression parameters will be normal and which allows us to integrate the regression coefficients to perform variable selection.

Recall that we define X^p, X^q and X^ψ to be the design matrix associated with covariates included in the regression models for p, q and ψ respectively (including a first column of ones for the intercept). We define $\tilde{X}^p$ to be the submatrix of X^p only including the rows for which $z_i = 1$ and, similarly, $\tilde{X}^q$ to be the submatrix of X^q only including the rows for which $z_i = 0$. We also define Y^p to be the response for which $z_i = 1$ and Y^q to be the response for which $z_i = 0$. The included covariates can be updated using a Metropolis-Hastings step where covariates are either added to the model, deleted from the model or a variable currently included in the model is replaced by a variable currently not included in the model. The steps of the Gibbs sampler are given below.

Updating γ^p and θ^p

The parameter γ^p is updated integrating over θ^p using a standard Add-Delete-Swap Metropolis-Hastings sampler. In this sampler, a proposed value c^p is sampled by either: an *Add* move, where *j* such that $\gamma_j^p = 0$ is chosen at random and $c_j^p = 1$ and $c_k^p = \gamma_k^p$ for $k \neq j$, a *Delete* move, where *j* such that $\gamma_j^p = 1$ is chosen at random and $c_j^p =$

0 and $c_k^p = \gamma_k^p$ for $k \neq j$, or a *Swap* move, where *j* such that $\gamma_j^p = 0$ is chosen at random and *m* such that $\gamma_m^p = 1$ is chosen at random then $c_j^p = 1$, $c_m^p = 0$ and $c_k^p = \gamma_k^p$ for $k \neq j$, *m*. The proposed value is accepted with the following probabilities

$$\begin{cases} \min\left\{1, \frac{L_p(c_p)\pi^{d'_p}(1-\pi)^{d-d'_p}(d-d_p)}{L_p(\gamma_p)\pi^{d_p}(1-\pi)^{d-d_p}(d_p+1)}\right\} & \text{Add} \\ \min\left\{1, \frac{L_p(c_p)\pi^{d'_p}(1-\pi)^{d-d'_p}d)p}{L_p(\gamma_p)\pi^{d_p}(1-\pi)^{d-d_p}(d-d_p+1)}\right\} & \text{Delete} \\ \min\left\{1, \frac{L_p(c_p)\pi^{d'_p}(1-\pi)^{d-d'_p}}{L_p(\gamma_p)\pi^{d_p}(1-\pi)^{d-d_p}}\right\} & \text{Swap} \end{cases}$$

where

$$L_p(\gamma_p) = \frac{|B_p|^{-1/2}}{|\tilde{X}_p^T \Omega_p \tilde{X}_p + B_p^{-1}|^{1/2}} \exp\left\{-\frac{1}{2}\left[b_p^T B_p^{-1} b_p - \left(\tilde{X}_p^T \kappa_p + B_p^{-1} b_p\right)^T (\tilde{X}_p^T \Omega \tilde{X}_p + B_p^{-1})^{-1}\left(\tilde{X}_p^T \kappa_p + B_p^{-1} b_p\right)\right]\right\},$$

Ω is a diagonal matrix containing the $\omega = \text{diag}(\{\omega_i | z_i = 1\})$, $B_p = \text{diag}(\alpha_0 \sigma_p^2, \{\sigma_p^2 / 2(J_{L_j} - 1 + I_{L_j} - 1) | \gamma_j^p = 1\})$ (where $\sigma_p^2 = \frac{(logit(b) - logit(a))^2}{2\delta^2(\alpha 0 + dp)}$), $\kappa = \{y_i - K/2 | z_i = 1\}$ and $b_p = (b, 0, \ldots, 0)$.

The parameters θ^p are sampled from their conditional distribution

$$\theta^p \sim \mathrm{N}\left(\left(\tilde{X}_p^T \Omega \tilde{X}_p + B_p^{-1}\right)^{-1}\left(\tilde{X}_p^T \kappa_p + B_p^{-1} b_p\right), \left(\tilde{X}_p^T \Omega\ \tilde{X}_p + B_p^{-1}\right)^{-1}\right).$$

Updating γ^q and θ^q

The parameter γ^q is updated integrating over θ^q using a standard Add-Delete-Swap Metropolis-Hastings sampler. In this sampler, a proposed value c^q is sampled by either: an *Add* move, where *j* such that $\gamma_j^q = 0$ is chosen at random and $c_j^q = 1$ and $c_k^q = \gamma_k^q$ for $k \neq j$, a *Delete* move where j such that $\gamma_j^q = 1$ is chosen at random and $c_j^q = 0$ and $c_k^q = \gamma_k^q$ for $k \neq j$, or a *Swap* move where *j* such that $\gamma_j^q = 0$ is chosen at random

and m such that $\gamma_m^q = 1$ is chosen at random then $c_j^q = 1$, $c_m^q = 0$ and $c_k^q = \gamma_k^q$ for $k \neq j$, m. The proposed value is accepted with the following probabilities

$$\begin{cases} \min\left\{1, \frac{L_q(c_q)\pi^{d'_q}(1-\pi)^{d-d'_q}(d-d_q)}{L(\gamma_q)\pi^{d_q}(1-\pi)^{d-d_p}(d_q+1)}\right\} & \text{Add} \\ \min\left\{1, \frac{L_q(c_q)\pi^{d'_q}(1-\pi)^{d-d'_q}d)p}{L(\gamma_q)\pi^{d_q}(1-\pi)^{d-d_p}(d-d_q+1)}\right\} & \text{Delete} \\ \min\left\{1, \frac{L_q(c_q)\pi^{d'_q}(1-\pi)^{d-d'_q}}{L(\gamma_q)\pi^{d_q}(1-\pi)^{d-d_q}}\right\} & \text{Swap} \end{cases}$$

where

$$L(\gamma_q) = \frac{|B_q|^{-1/2}}{|\tilde{X}_q^T\Omega\tilde{X}_q + B_q^{-1}|^{1/2}} \exp\left\{-\frac{1}{2}\left[b_q^T B_q^{-1} b_q - \left(\tilde{X}_q^T\kappa_q + B_q^{-1}b_q\right)^T (\tilde{X}_q^T\Omega\tilde{X}_q + B_q^{-1})^{-1}\left(\tilde{X}_q^T\kappa_q + B_q^{-1}b_q\right)\right]\right\},$$

Ω is a diagonal matrix containing the $\omega = \text{diag}(\{\omega_i | z_0 = 1\})$, $B_p = \text{diag}(\alpha_0\sigma_q^2, \{\sigma_q^2/2(J_{L_j} - 1 + I_{L_j} - 1)|\gamma_j^q = 1\})$ (where $\sigma_q^2 = \frac{(logit(b)-logit(a))^2}{2\delta^2(\alpha 0 + dq)}$), $\kappa = \{y_i - K/2 | z_i = 0\}$ and $b_q = (a, 0, \ldots, 0)$.

The parameters θ^q are sampled from their conditional distribution

$$\theta^p \sim \mathrm{N}\left(\left(\tilde{X}_q^T\Omega\tilde{X}_q + B_q^{-1}\right)^{-1}\left(\tilde{X}_q^T\kappa_p + B_q^{-1}b_q\right), \left(\tilde{X}_q^T\Omega\tilde{X}_q + B_q^{-1}\right)^{-1}\right).$$

Updating γ^ψ and θ^ψ

The parameter γ^ψ is updated integrating over θ^ψ using a standard Add-Delete-Swap Metropolis-Hastings sampler. In this sampler, a proposed value c^p is sampled by either: an *Add* move, where j such that $\gamma_j^\psi = 0$ is chosen at random and $c_j^\psi = 1$ and $c_k^\psi = \gamma_k^\psi$ for $k \neq j$, a *Delete* move, where j such that $\gamma_j^\psi = 1$ is chosen at random and $c_j^\psi = 0$ and $c_k^\psi = \gamma_k^\psi$ for $k \neq j$, or a *Swap* move, where j such that $\gamma_j^\psi = 0$ is chosen at

random and m such that γ_m^{ψ} = 1 is chosen at random then c_j^{ψ} = 1, c_m^{ψ} = 0 and $c_k^{\psi} = \gamma_k^{\psi}$ for $k \neq$ j, m. The proposed value is accepted with the following probabilities

$$\begin{cases} \min\left\{1, \frac{L_\psi(c_\psi)\pi^{d'_\psi}(1-\pi)^{d-d'_\psi}(d-d_\psi)}{L_\psi(\gamma_\psi)\pi^{d_\psi}(1-\pi)^{d-d_\psi}(d_\psi+1)}\right\} & \text{Add} \\ \min\left\{1, \frac{L_\psi(c_\psi)\pi^{d'_\psi}(1-\pi)^{d-d'_\psi}d_\psi}{L_\psi(\gamma_\psi)\pi^{d_\psi}(1-\pi)^{d-d_\psi}(d-d_\psi+1)}\right\} & \text{Delete} \\ \min\left\{1, \frac{L_\psi(c_\psi)\pi^{d'_\psi}(1-\pi)^{d-d'_\psi}}{L_\psi(\gamma_\psi)\pi^{d_\psi}(1-\pi)^{d-d_\psi}}\right\} & \text{Swap} \end{cases}$$

Where

$$L_\psi(\gamma_\psi) = \frac{|B_\psi|^{-1/2}}{|\tilde{X}_\psi^T\Omega_\psi X_\psi + B_\psi^{-1}|^{1/2}} \exp\left\{-\frac{1}{2}\left[b_\psi^T B_\psi^{-1} b_\psi - \left(\tilde{X}_\psi^T\kappa_\psi + B_\psi^{-1}b_\psi\right)^T(\tilde{X}_\psi^T\Omega_\psi\tilde{X}_\psi + B_\psi^{-1})^{-1}\left(\tilde{X}_\psi^T\kappa_\psi + B_\psi^{-1}b_\psi\right)\right]\right\}$$

Ω_ψ is a diagonal matrix containing the ω^ψ = diag(ω_1^{ψ}, . . . , ω_S^{ψ}), B_ψ = diag(φ_μ, {φ_β/2(J_{L_j} −1 + I_{L_j} −1)|γ_j^{ψ} = 1}), κ = {z_i − 1/2}.

The parameters θ^ψ are sampled from their conditional distribution

$$\theta^\psi \sim \mathrm{N}\left(\left(X_\psi^T\Omega X_\psi + B_\psi^{-1}\right)^{-1}X_\psi^T\kappa_p, \left(X_\psi^T\Omega X_\psi + B_\psi^{-1}\right)^{-1}\right).$$

Updating ω and ω^ψ

The full conditional distributions are ω_i ∼ PG(K, |μ^p + $X_i^p\beta^p$|) if z_i = 1, ω_i ∼ PG(K, |μ^q+$X_i^q\beta^q$| if z_i = 0 and ω^ψ ∼ PG(1, |μ^ψ + $X_i^\psi\beta^\psi$|). Efficient algorithms for simulating Pólya-Gamma random variables are provided in (Polson et al., 2013).

Updating z

The full conditional distribution of z_i is

$$p(z_i = 1) = \frac{\psi_i p_i^{y_i} (1 - p_i)^{K - y_i}}{\psi_i p_i^{y_i} (1 - p_i)^{K - y_i} + (1\psi_i) q_i^{y_i} (1 - q_i)^{K - y_i}}.$$