# The Detection of Aquatic Species using Environmental DNA – a review of eDNA as a survey tool in Ecology

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**Summary**

1. Knowledge of species distribution is critical to ecological management and conservation biology. Effective management requires the detection of populations which can sometimes be at low densities and is conventionally based on visual detection and counting.
2. Recently there has been considerable interest in the detection of short species-specific environmental DNA (eDNA) fragments to allow aquatic species monitoring within different environments. eDNA can be used to illustrate species distribution by way of presence/absence data and can allow the detection of rare or invasive species due to its greater sensitivity than traditional survey methods which can be time-consuming and costly.
3. eDNA analysis has been used to successfully detect specific rare and invasive species and when combined with next generation sequencing has demonstrated that entire faunas can be identified.
4. eDNA detection has also been applied to persistence studies and to estimations of species biomass and distribution. However, there remain areas where methodologies could be standardised to allow results to be compared across studies.
5. *Synthesis and applications.* eDNA analysis is increasingly being used in species-specific detection. We review recently published studies that use eDNA to monitor aquatic populations and discuss the methodologies used along with the application of eDNA analysis as a survey tool in ecology.

**Keywords**

DNA barcoding, ecosystem management, invasive species, mtDNA, rare or threatened species, species-detection, species-specific, water bodies, water sampling.

**Introduction**

Environmental DNA (eDNA) is the DNA that can be extracted from a particular environmental sample (soil, water or air), and in this review is used in the context of monitoring of aquatic populations by analysis of DNA in water bodies e.g. a pond, stream or lake. The total eDNA present will have originated from animals occupying that water body via their faeces, saliva, urine, skin cells, etc. and similarly also from animals that visit the environment such as birds and mammals which, for example may use the water for drinking. The analysis of water for eDNA specific to different animal species is a new and emerging technique that will have application to aquatic organism surveys and conservation projects.

The rapid spread or diffusion of the DNA from its source means that in theory the presence of a specific animal can be detected anywhere within the water body and not just at its point of origin. DNA released into the environment is likely to be broken down and eventually lost by the action of UV light and microbial activity over a period of around two to four weeks (Dejean *et al.* 2011; Thomsen *et al.* 2012a; Thomsen *et al.* 2012b). The presence of the eDNA from a target species of interest allows us to detect the presence or very recent presence of that species without the need for direct observation or trapping, thus, this is an applicable tool for the detection of aquatic species. Furthermore, this is particularly useful for those species that are difficult to detect using conventional methods e.g. those that need trapping or require special licences, as in the case of some endangered or under-threat species.

The monitoring and conservation of aquatic populations using eDNA analysis arose from the use of the method to assess the diversity of macro-organisms in ancient sediments (Willerslev *et al.* 2003). Several different ancient and modern environments have been subject to this approach, with the first study on freshwater samples based on the persistence of DNA in the environment from the invasive American bullfrog (Ficetola *et al.* 2008). These studies are based on the detection of a short fragment of mitochondrial DNA (mtDNA) for rapid and accurate species identification and as such can be grouped with other methods used for 'DNA barcoding' or taxon identification using a standardized DNA region (Hebert & Gregory 2005). For animals, the proposed standardised

'barcode' is a 658bp region of the mitochondrial cytochrome c oxidase 1 (COI) (Hebert, Ratnasingham & deWaard 2003) and there is also an effort to develop short sequence (~100bp) DNA barcodes for species identification in degraded samples (Hajibabaei *et al.* 2006) which could have application in environmental monitoring. A recent study has designed 36 primer pairs within the barcoding segment of the COI gene for four endangered freshwater species and 10 invasive aquatic species, 26 of these primer pairs have been tested for cross species amplification and 18 were shown to be species-specific (Bronnenhuber & Wilson 2013). mtDNA markers are targeted due to the substantially greater number of copies of mtDNA per cell than nuclear DNA and in environments where DNA is present at low concentrations and/or is degraded this becomes especially important for its detection. The studies reviewed here have focused on the detection of various regions of mitochondrial DNA, with cytochrome b being used by the majority (Ficetola *et al.* 2008; Goldberg *et al.* 2011; Dejean *et al.* 2012; Minamoto *et al.* 2012; Olson, Briggler & Williams 2012; Takahara *et al.* 2012; Takahara, Minamoto & Doi 2013; Wilcox *et al.* 2013). The fragment to be amplified ranges in size from 62bp for detection of the harbour porpoise (Foote *et al.* 2012) to 312bp for the detection of the bighead carp (Jerde *et al.* 2011), this is, however, unusually long for this type of study. Most studies use fragments sizes falling within the range of 90 to 120bp which is recommended by a USGS Factsheet created to help in the understanding of the methods involved in eDNA analysis (Darling & Mahon 2011). Due to the rapid degradation of eDNA within water it is important to use a small fragment size as larger fragments will be less likely to persist long enough to allow species detection.

**Detection of Aquatic Species**

Analysis of eDNA involves: the collection of a sample of water, which does not need to be under license; extraction of the eDNA; and standard or real-time PCR to amplify the DNA of the species of interest. DNA extraction and real-time PCR can be carried out within a few hours, making this technique a rapid method for detecting the presence of a target species. An increasing number of protocols for these different steps are being described for the analysis and are outlined as follows.

**Sampling**

Water samples have been collected from a variety of water bodies including: laboratory tanks (Ficetola *et al.* 2008; Dejean *et al.* 2011; Minamoto *et al.* 2012; Olson, Briggler & Williams 2012; Takahara *et al.* 2012; Collins *et al.* 2013); experimental/artificial ponds (Dejean *et al.* 2011; Takahara *et al.* 2012; Thomsen *et al.* 2012b); natural ponds/lakes (Ficetola *et al.* 2008; Dejean *et al.* 2012; Thomsen *et al.* 2012b; Takahara, Minamoto & Doi 2013); lagoons (Takahara *et al.* 2012); streams/rivers (Goldberg *et al.* 2011; Jerde *et al.* 2011; Minamoto *et al.* 2012; Olson, Briggler & Williams 2012; Thomsen *et al.* 2012b; Jerde *et al.* 2013; Mahon *et al.* 2013; Wilcox *et al.* 2013); and seawater (Foote *et al.* 2012; Thomsen *et al.* 2012a). The number of samples taken per water body varies, in some cases only one water sample has been taken e.g. for DNA persistence studies where multiple samples are not required due to the experimental setup (e.g. small water volumes used in beakers and tanks). To improve the coverage of the water system and the chances of species detection several studies have taken three samples from different sites in the water body (Ficetola *et al.* 2008; Dejean *et al.* 2012; Takahara *et al.* 2012; Thomsen *et al.* 2012b). The Thomsen group looked at the probabilities of detecting the target species with increasing number of samples taken, when they set the probability of detection upon considering three samples to 100% they showed significant reductions in rates of detection upon looking at only one or two samples e.g. for great crested newts around 70% and around 95% respectively. It would appear that taking three samples per water body is a sensible sampling regime that could be standardised for certain environments such as ponds (experimental/artificial and natural) and lakes. The numbers of samples required for rivers/streams, lagoons, and seawater will be dependent on the size of the environment under study and is difficult to specify but examples include: collecting approximately 1000 2L samples from a large canal and waterways system (Jerde *et al.* 2011); collecting 2L samples from three sites along the Yura river (Minamoto *et al.* 2012); and collecting 2L samples from 21 sites within a lagoon (Takahara *et al.* 2012). Sample sizes varied from 15ml, taken from laboratory tanks, natural ponds/lakes, streams, and seawater (Ficetola *et al.* 2008; Dejean *et al.* 2011; Dejean *et al.* 2012;

Foote *et al.* 2012; Thomsen *et al.* 2012a; Thomsen *et al.* 2012b; Collins *et al.* 2013), to 10L taken from a stream (Goldberg *et al.* 2011), with a more standard sample size being 1 - 2L from streams/rivers, lagoons, and seawater (Jerde *et al.* 2011; Minamoto *et al.* 2012; Olson, Briggler & Williams 2012; Takahara *et al.* 2012; Thomsen *et al.* 2012a; Jerde *et al.* 2013; Mahon *et al.* 2013; Takahara, Minamoto & Doi 2013) (see summary in Table 1).

Due to the diversity of water bodies and different levels of eDNA, dictated by species abundance, methods for sample collection will vary. Water samples can be collected by pumping a volume of water through a filter with a peristaltic or in-line pump using either cellulose nitrate filters (Goldberg *et al.* 2011), glass fibre filters (Jerde *et al.* 2011; Jerde *et al.* 2013; Mahon *et al.* 2013; Wilcox *et al.* 2013), carbonate filters (Takahara *et al.* 2012), or nylon filters (Thomsen *et al.* 2012a) (Table 1). A further method is the collection of surface water in a bottle by partially submersion followed by filtration (Minamoto *et al.* 2012; Olson, Briggler & Williams 2012; Takahara, Minamoto & Doi 2013). When taking samples from boats, it is usually favoured to collect water from rivers/streams and lagoons towards upstream sites to prevent the mixing of water by movement of the boat. Samples of seawater have been collected by taking 50ml sub-samples from the water at a depth of 1.5 to 6m along a 145m transect prior to pooling samples (Thomsen *et al.* 2012a); or by sampling at a depth of 50cm in the direction of the ebbing tide (Foote *et al.* 2012).

Several methods have now been described for the extraction of DNA from water samples. Four different commercial DNA extraction kits have been described with application to eDNA: the DNeasy Tissue and Blood DNA extraction kit (Qiagen, Inc.); the QIAamp Blood and Tissue extraction kit (Qiagen, Inc.); the MoBio Power Water DNA extraction kit (MoBio Laboratories, Inc.); and the Quick-gDNA spin-column kit (Zymo Research Corporation). A fifth kit, the UltraClean®Soil DNA isolation kit (MoBio Laboratories, Inc.) has been found unsuitable on the grounds that DNA was not successfully extracted from the filter samples in one study (Goldberg *et al.* 2011). Despite only three commonly used methods being employed for DNA extraction, there is considerable variation as to how water samples are processed prior to the extraction step. Methods for sample preservation, filtration, and

DNA extraction vary depending on the sample type and are summarised in Table 1. To date, five methods have been used for sampling: (1) the collection of 15ml of water and preservation of the sample with sodium acetate and 100% ethanol prior to DNA extraction (Ficetola *et al.* 2008; Dejean *et al.* 2011; Dejean *et al.* 2012; Foote *et al.* 2012; Minamoto *et al.* 2012; Collins *et al.* 2013); (2) filtering of the water sample through a 0.45µm cellulose nitrate filter which is then preserved in 95% ethanol prior to DNA extraction (Goldberg *et al.* 2011); (3) filtering of the water sample through a 1.5µm glass fibre filter which is preserved by storage on ice or at -20°C prior to DNA extraction (Jerde *et al.* 2011; Olson, Briggler & Williams 2012; Jerde *et al.* 2013; Mahon *et al.* 2013; Wilcox *et al.* 2013); (4) filtering of the water sample through a 3µm or a 12µm plus an 0.8µm polycarbonate filter followed by either immediate DNA extraction or a second filtration with an Amicon Ultra 15 centrifugal filter unit and storage of the filtrate at -25°C prior to DNA extraction (Minamoto *et al.* 2012; Takahara *et al.* 2012; Takahara, Minamoto & Doi 2013); and (5) filtering of the water sample through a 0.45µm nylon filter followed by bead beating of the filter and DNA extraction (Thomsen *et al.* 2012a). The methods described have all been shown to be successful; however, a direct comparison of these methods to monitor the effects of different processing methods on eDNA extraction and detection would be useful.

**eDNA Persistence**

The predominant sources of eDNA are thought to be faeces, urine, and epidermal cells, and eDNA can survive from hours in freshwater to thousands of years in fossil ice and permafrost (Willerslev, Hansen & Poinar 2004). Experiments have shown the rapid degradation of DNA in freshwater e.g. plasmid DNA degraded in a few hours in non-sterile water (Alvarez *et al.* 1996; Kim, Kwak & Lee 1996; Matsui, Honjo & Kawabata 2001) plasmid and plant DNA was degraded within 96 hours (Zhu 2006); and baculoviral DNA degraded in around 24 hours (England *et al.* 2005); however, species specific DNA persistence has only recently been investigated. Experiments investigating the persistence of DNA have been in freshwater for bullfrog tadpoles and sturgeon (Dejean *et al.* 2011), or newt larvae and spadefoot toad tadpoles (Thomsen *et al.* 2012b); and also on seawater for the

three-spined stickleback and European flounder (Thomsen *et al.* 2012a). The freshwater studies were carried out by housing/rearing animals in beakers or experimental ponds/mesocosms at different densities. Following this the animals were removed and water samples taken at this time and at defined times for up to 48 days. Both experiments agreed with the previous studies and showed that DNA detectability decreased with time after the removal of the species of interest i.e. DNA was negatively correlated with time. DNA was also shown to become undetectable between two weeks or one month of the removal of the animals (Thomsen, Dejean respectively). Experiments on seawater mirrored the results found in freshwater experiments. The short persistence time of DNA in water bodies illustrates the potential for conservation biology, as DNA traces are near contemporary with the presence of a species which could be rare, secretive, potentially invasive, or at low density.

**Validation and Quality Control**

eDNA analysis has been performed by both standard PCR and quantitative PCR. In terms of primer design TaqMan™ assays with minor groove binding probes have shown that the number and placement of base pair mismatches between the target and non-target templates is important (Wilcox et al. 2013). Non-target competition has been found to affect Ct values, which in turn produce inaccurate estimates of DNA quality and thus species abundance. The study found that this non-target competition becomes weaker as the number of primer base pair mismatches increases i.e. as the primers became more specific for the target eDNA. Target specificity was most influenced by base pair mismatches in the primers, rather than in the probe. These findings underscore the importance of careful primer design and it has been recommended that primers should incorporate as many differences as possible between the primer/probe design for the target species and any other species where the analogous sequence is known (Darling & Mahon 2011).

Primers (and associated probes if required for qPCR) have been validated in all studies by careful design e.g. using Primer3 software (Rozen & Skaletsky 2000) and alignment searches against other sequences stored in GenBank such that they test negative when/if tested on other related or

206 common species that might occur in that area of sampling. To reduce the likelihood of occurrence of
207 false positives; primers have also been tested *in silico* using the ecoPCR software (Dejean *et al.*
208 2011).
209 In general, validation of the eDNA methods has involved sequencing of a selection of positive PCR
210 products to demonstrate primer specificity to the species of interest. The first study of this type
211 found that almost all PCR products sequenced corresponded perfectly to the published sequence for
212 bullfrog *cyt-b* (Ficetola *et al.* 2008). This study also used 454 deep sequencing of 673 fragments
213 derived from the PCR product of one sampled pond and found that apart from clearly recognizable
214 sequencing and PCR errors, all the resulting sequences were for the species of interest and these did
215 not contain mixed sequences indicating specificity for a single species.
216 PCR reaction volumes vary from 10 to 40µl, containing 1 to 10µl of DNA template. Only one study
217 has tried to quantify the DNA prior to the PCR step, the quality and quantity of DNA was determined
218 by agarose gel electrophoresis (Wilcox *et al.* 2013). Quantification of DNA or at least
219 presence/absence of DNA in a sample measured prior to PCR amplification would help to inform on
220 the reasons for PCR failures i.e. failure due to absence of DNA rather than matrix effects or inhibition
221 for example. Measurement may not be possible, however, due to the small quantities of DNA being
222 extracted from water samples and as such spiked positive controls should be used to illustrate the
223 potential of the water sample to amplify under the conditions being used.
224 Although some studies have only performed a single PCR reaction per sample e.g. samples taken
225 from aquaria/beakers (Dejean *et al.* 2011; Minamoto *et al.* 2012), most perform three, eight, or 10
226 PCR replicates per water sample. The results of such approaches then need to be scored as positive
227 or negative, although the criteria used for this is not always stated. In terms of real time PCR a
228 sample will only be scored as positive if amplification goes above the fluorescence threshold (Ct)
229 (Foote *et al.* 2012; Takahara *et al.* 2012; Thomsen *et al.* 2012a; Thomsen *et al.* 2012b; Takahara,
230 Minamoto & Doi 2013; Wilcox *et al.* 2013), and for standard PCR one criterion was that a sample was
231 found to be positive if any one of eight PCR replicates was positive (Jerde *et al.* 2011; Jerde *et al.*

2013; Mahon *et al.* 2013). The studies went on to state that when a sample was found to be positive it had to be screened twice more, i.e. duplicate sets of eight PCR reactions, and must screen positive a second time from these before the sample could be confirmed as positive. Most studies merely state that samples were found to be positive for the eDNA of interest and as such do not have this high level of stringency. A standard PCR methodology based on a repeat analysis approach (Taberlet *et al.* 1996) could be achieved across all studies to allow for better comparisons of data and we propose should include at least three PCR replicates, preferably eight per sample, which can be scored as positive if any one of the replicates is positive/above the fluorescence threshold.

False positives and false negatives are especially important in surveys for rare, low density, or possibly extinct animals and steps should be taken to minimise the risk of these occurring. For example samples should be collected and transported with care so as not to contaminate them, and all eDNA extractions and PCR setups should be conducted in a laboratory where PCR products have not been handled, and PCR machines should be located outside of this space.

False positives tend to result from low specificity of the primers and probes and non-target template competition as discussed previously. Next generation sequencing of positive PCR reactions can be carried out to confirm sequence identity (Ficetola *et al.* 2008) and a further suggestion to counter false positives is to place a higher threshold on the copy number representing a positive detection (Darling & Mahon 2011). It is also possible that target species eDNA could enter the environment by sources other than living animals e.g. sewage and wastewater, or faeces from other predatory animals leading to false positives. There also remains the issue of whether eDNA from dead animals can interfere with detection, this is something which has not been fully discussed in the papers reviewed here so the impact of this is not yet known, although it has been suggested to be a possible but unlikely source of eDNA in Asian Carp studies in Chicago, Illinois waterways and canals (Jerde *et al.* 2011; Jerde *et al.* 2013; Mahon *et al.* 2013). Interference from dead animals could occur if estimates of biomass are to be made. eDNA from dead animals becomes less of an issue when looking at spatial patterns in a large water system or where repeated detections are made over

multiple time points. In the case of rare/elusive or threatened species and alien invasive species (AIS) it is likely that basic species presence and distribution data would be used to target specific water bodies for full ecological surveys thus reducing any impact from this type of false positive. False negatives can occur when the quantity of target eDNA falls below a detection threshold, because non-target eDNA species interfere with the reaction, or due to sample matrix effects; thus mitigating against these factors is important. Extraction of DNA from large volumes of water samples and capitalising on the extreme sensitivity of PCR along with careful primer design to ensure specificity to the target eDNA will minimise this risk. The effect of the sample matrix on both DNA extraction and PCR amplification e.g. PCR inhibition due to salinity in seawater samples (Foote *et al.* 2012) is an area which requires further investigation. Matrix effects can be monitored by use of appropriate controls e.g. spiked distilled water as a PCR positive control and the inclusion of spiked water samples for DNA extraction and amplification to ensure that PCR is possible in the matrix. However, a study of different water types e.g. chemistry, pH, sediment content etc. and their applicability to eDNA analysis could help to inform on sample suitability/selection and methodological adaptations.

An area of considerable variation in the published studies is the number and variety of negative control samples being utilised. Some studies make no mention of negative controls (Dejean *et al.* 2011; Mahon *et al.* 2013) or have included negative controls (no template controls) only in the PCR stage of the study (Wilcox *et al.* 2013); whereas others have included both PCR and DNA extraction controls on water samples taken from areas/aquaria where the species of interest is not known to be present (Ficetola *et al.* 2008; Goldberg *et al.* 2011; Jerde *et al.* 2011; Dejean *et al.* 2012; Minamoto *et al.* 2012; Olson, Briggler & Williams 2012; Takahara *et al.* 2012; Thomsen *et al.* 2012a; Thomsen *et al.* 2012b; Collins *et al.* 2013; Takahara, Minamoto & Doi 2013). 'Equipment blank' bottles containing ultra-pure water from the laboratory which is filtered using the filtration equipment prior to filtering any sampled water have also been used as negative/extraction controls in conjunction with some or all of the controls described above (Jerde *et al.* 2011; Foote *et al.* 2012;

Olson, Briggler & Williams 2012; Jerde *et al.* 2013). Finally, so called 'cooler blanks' bottles, these being bottles of deionised or ultra-pure water filled and sealed in the laboratory and taken out into the field but not opened have also been used to test for contamination (Jerde *et al.* 2011; Olson, Briggler & Williams 2012; Jerde *et al.* 2013). The use of a standard set of controls could easily be achieved across all studies to allow for better comparisons of data and we propose should include DNA extraction and PCR negative and positive controls (which in turn will inform on sample matrix effects), cooler blanks, and where additional equipment is used in sample collection, equipment blanks.

**eDNA as a survey tool in ecology**

**Biomass Estimation**

To date, two studies using the eDNA technique as a tool to estimate species biomass in water bodies have been published. Thomsen et al (2012a) were able to correlate DNA concentration and estimated population density based on traditional counts for amphibians in their study. Using freshwater mesocosms, they showed that there is a highly significant relationship between animal density, time (after the introduction of the animals) and the resulting DNA concentrations. The group speculated that there is a simple relationship between DNA excretion (which is dependent on animal density and size) and DNA degradation (a constant rate) such that there is a consistent quantitative relationship between the density of animals and DNA molecules.

Takahara *et al* (2012), worked on the hypothesis that the rate at which fish release DNA into the water is commensurate with their biomass. Using the common carp as a model organism, laboratory experiments were conducted to monitor eDNA persistence and concentrations to allow the development of a model to estimate the carp biomass based on the number of eDNA copies. Additionally, methods to evaluate eDNA concentrations in a large body of water were developed using outdoor artificial ponds and both experiments showed that the concentration of eDNA was positively correlated with carp biomass such that eDNA concentrations were suggested to reflect the

biomass of the target species. When applied to a lagoon system, it was shown that eDNA concentrations were highly variable across the sites sampled (different areas of the lagoon) and that this had a significant correlation to water temperature, with warmer areas having more eDNA. During laboratory experiments it was found that water temperature had no effect on the number of eDNA copies. These findings led the group to speculate that the eDNA concentration at each site could represent the temperature dependent preferences of the carp and thus also reflected the potential distribution of common carp in the natural environment. The group concluded that using this method, the species biomass in natural environments could be estimated more easily and rapidly than using traditional methods, and may be used to monitor seasonal changes in eDNA concentrations. However, they also noted that to improve accuracy, further experiments should focus on collecting more field data and comparing this method with other estimation methods. These data provide evidence that it may be possible to estimate population densities so long as you know the volume of water being monitored and have a standard curve of animal density versus eDNA copies. There are several methods for determining the volume of water in a lake or reservoir, (Taube 2000; Herschy 2012) e.g. by determination of the average depth of the lake and multiplying this by the area of the lake, thus this would not be a limiting factor for biomass estimations. Both studies represent an important development in the monitoring of animal populations as information on biomass is often difficult to accurately estimate and it plays a significant role in the conservation of rare and endangered species and population management.

**Invasive Species**

Detecting alien invasive species (AIS) at the early stages of introduction or when they are at low density is key to control and eradication strategies (Hulme 2006). In these cases detection rates using traditional census techniques can be of low-quality; require a huge sampling effort; or be impossible until the density reaches a certain threshold, therefore, the use of the eDNA technique could be of enormous importance given its ability to detect specific target species even at low densities. eDNA studies on AIS have focused on the American bullfrog, considered to be one of the

world's most harmful invasive species (Ficetola *et al.* 2008; Dejean *et al.* 2011; Dejean *et al.* 2012); the Bluegill sunfish, one of the most widely distributed fish in Japan (Takahara, Minamoto & Doi 2013); and Asian carps, which are invasive in many North American rivers (Jerde *et al.* 2011; Jerde *et al.* 2013; Mahon *et al.* 2013). The technique has been used to: reliably detect AIS eDNA at low densities (Ficetola *et al.* 2008); detect AIS at sites where traditional sampling did not (Dejean *et al.* 2012); determine invasion fronts (Jerde *et al.* 2011; Jerde *et al.* 2013; Mahon *et al.* 2013); estimate AIS distribution (Takahara, Minamoto & Doi 2013); and to further develop the eDNA method as a biosecurity tool for ornamental fishes as the international trade in ornamental aquatic organisms represents an important vector in the spread of invasive species worldwide (Collins *et al.* 2013). The eDNA technique could also be included in eradication strategies to monitor invasive species before and after removal and thus could assist in monitoring the efficacy of such strategies.

**Rare or Threatened Species**

In cases of elusive, secretive and rare species, a non-invasive genetic sampling technique allows data to be collected that would otherwise be very difficult to obtain due to constraints in being able to physically locate these species. This is of great use in the conservation and management of these species providing presence data and can be used to estimate population sizes. Worldwide over 5900 fresh-water and marine animal species are listed as endangered (IUCNredlist 2012) therefore, the development and validation of techniques such as eDNA analysis are of great interest for these vulnerable species.

The technique has been used to detect populations of the eastern hellbender *Cryptobranchus a. alleganiensis*, an amphibian of conservation concern (Olson, Briggler & Williams 2012); and monitor freshwater biodiversity of locally rare and low abundance species that require strict protection in their natural habitats and substantial monitoring efforts in the EU e.g. the great crested newt (Thomsen *et al.* 2012b). eDNA methods can be seen as a cost effective means by which to obtain basic distribution and abundance data and will enable limited conservation resources and taxonomic

361 expertise to be efficiently deployed to maximise returns. As such it can be considered a valuable tool
362 for detecting many species that are difficult to study by traditional methods.

363 **Species composition**

364 The use of eDNA in combination with next generation sequencing to detect multiple species
365 simultaneously is a powerful tool for monitoring species diversity in water bodies and will allow
366 more accurate estimates of species diversity rather than targeted surveillance of one, or a handful of
367 species. To date, only one group has used this combination of techniques: to demonstrate that
368 entire faunas of amphibians and fish can be detected from pond water (Thomsen *et al.* 2012b); and
369 to identify fish species from seawater samples (Thomsen *et al.* 2012a). The group performed 454
370 pyrosequencing on pooled PCR products; those from ponds were amplified with a range of different
371 primers for fish and amphibian communities and those from seawater were amplified with generic
372 primer sets for fish. The resulting DNA sequences were then analysed using custom scripts and
373 compared to the BLAST database. For the pond water study, this procedure allowed the recovery of
374 species-specific DNA fragments with 100% sequence match for all species of fish/amphibians
375 previously recorded by conventional pond surveys as well as DNA sequences from species living in
376 close proximity to the sampling sites. For the seawater study, this procedure allowed 15 species-
377 specific fish eDNA sequences to be recovered which was the highest number of different fish
378 recorded in that area when compared to the results of 9 conventional survey methods. The DNA
379 analysis offered a coverage that was comparatively better or at least as good as any single
380 conventional method used. Due to studies such as these, next generation sequencing is becoming an
381 area of growing interest to regulatory bodies and ecologists (personal communications) and as costs
382 continue to decrease this high-throughput method of species monitoring will become a very
383 attractive proposition.

384 **Potential Limitations of the Methodology**

385 The use of eDNA as a survey tool does have some issue to overcome, one of which is the transient
386 nature of some animal species within the water system e.g. the Great Crested Newt (GCN). The GCN

387 is both elusive and threatened and the adults and juveniles normally live on land, hibernating
388 between October and March, but have a breeding season, peaking in March to May, where they
389 breed in ponds and pools. This results in a survey 'window' within which the detection of the GCN is
390 possible via both conventional survey methods (trapping, torch-light surveys, and egg counts) and
391 eDNA analysis (Thomsen *et al.* 2012b)(Rees et al, unpublished data). With animals such as these one
392 of the hopes of eDNA analysis is that this survey window can be extended to include the time that
393 larvae/juveniles inhabit the ponds, and therefore that eDNA analysis can be a suitable addition to
394 current survey methods.
395 Another area of difficulty with eDNA analysis is in the nature of the water body to be sampled. Some
396 environments such as ponds and lakes lend themselves more easily to these techniques. eDNA
397 detection in running water where the flow may move shed cells away from their source at a rate
398 prohibitive to eDNA collection is one such environment. Several studies have looked at the potential
399 of eDNA analysis in the survey of stream species and have successfully monitored Rocky Mountain
400 Tailed Frogs and Idaho Giant Salamanders (Goldberg *et al.* 2011), various fish species (Jerde *et al.*
401 2011; Minamoto *et al.* 2012; Jerde *et al.* 2013; Mahon *et al.* 2013; Wilcox *et al.* 2013), and the
402 European weather loach and Eurasian otter (Thomsen *et al.* 2012b). All of these studies used large
403 volumes of water (2-10L) when sampling from the streams and rivers to compensate for the removal
404 of eDNA by water flow and this points to the importance of different methodologies for different
405 environments rather than a 'one size fits all' approach.
406 **eDNA versus traditional survey methods**
407 Non-invasive survey methods such as eDNA analysis could have considerable advantages over
408 traditional survey methods where the species of interest has to be disturbed or even caught to get a
409 positive identification, thus encroaching on animal welfare. eDNA analysis has been shown by
410 various laboratories to be a reliable detection method and appears to correlate with conventional
411 survey results, in some cases being a more sensitive method of detection, and could extend the
412 traditional survey windows for transient species.

In terms of sampling effort eDNA analysis can have considerable time and therefore cost benefits over traditional survey methods, especially when looking at the distribution of rare or threatened species where conventional methods of survey require a huge sampling effort. In a study of invasive Asian carp in USA canals and waterways (Chicago, Illinois) eDNA analysis always had a higher 'catch per unit effort' than the traditional electrofishing where in one example it took 93 days of person effort to detect one silver carp by electrofishing at a site where eDNA analysis had found Asian carp eDNA to be present, with the authors calculating the person effort per eDNA sample as 0.174 days (Jerde *et al.* 2011). This is obviously an extreme example when looking for small numbers of a species, but could be comparable to the sampling effort required for the monitoring of rare species. Although this technique is unlikely to replace current survey methods, it could have future applications for reduced field survey effort e.g. current best practice in the UK for great crested newt surveys suggests six to eight visits per pond for population counts using conventional methods. The survey methods that are currently stipulated by Natural England, consist of aquatic funnel traps (including bottle traps), netting, torch-light surveys and egg counts. Sampling for eDNA first could be used as a relatively quick, inexpensive tool for collecting species presence and distribution data. This is especially pertinent if the sampling area is very large and data could then be used to target specific water bodies for full ecological surveys.

**Conclusions and Future Perspectives**

There is growing interest in the use of eDNA where DNA-based single-species identification is of importance in ecosystem management. The eDNA technique is a rapid method for the detection of target species which could lend itself to use within a mobile sampling unit to allow for quick on site determination of presence of a target species so long as all necessary steps could be taken to prevent the occurrence of false negatives and false positives. Due to the wide range of methodologies used there does not appear to be a single study which could be used as a template for the standardisation of this technique. The wide range of protocols used in all aspects of this

technique has not yet been compared to identify how different processing methods can affect the detection of eDNA. In terms of sample size, water collection will be dependent on the water body being sampled, with far larger samples needing to be taken from running water than from their standing water counterparts, and three samples being taken per water body being a sensible strategy in many cases. There is also a large disparity in the way samples are scored as positive, by adherence to a standardised PCR/qPCR method based on a repeat analysis approach (Taberlet *et al.* 1996) and confirmation of species identity by sequencing of a number of positive reactions, a universal scoring system could be achieved. The technique has been used as an accurate indicator of the presence of species in a range of aquatic environments, and shows great potential in measuring population abundance which is especially important for monitoring invasion by harmful species, and rare or threatened species. Where it is appropriate to do so, the coupling of eDNA to next generation sequencing technology opens up new avenues of ecosystem monitoring by allowing the species richness of aquatic environments to be quantified. Thus, eDNA analysis is fast becoming an important tool in the study of aquatic species and could be used in cost-efficient multi-species inventory and monitoring programmes.

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551 Table 1. Summary of sample collection and preparation methods used to detect eDNA in water

552 bodies

| **Reference** | **Environment** | **Volume of Samples** | **Filter Type** | **Preservation Method** | **DNA extraction method** |
|---|---|---|---|---|---|
| (Collins *et al.* 2013) | Containers | 15ml | N/A | Addition of 3M sodium acetate and 100% ethanol | Quick-gDNA spin-column kit |
| (Dejean *et al.* 2011) | Beaker, artificial/experimental pond | 15ml | N/A | Addition of 3M sodium acetate and 100% ethanol | DNA precipitation followed by QIAamp kit |
| (Dejean *et al.* 2012) | Natural pond | 15ml | N/A | Addition of 3M sodium acetate and 100% ethanol | DNA precipitation followed by QIAamp kit |
| (Ficetola *et al.* 2008) | Aquaria, natural pond | 15ml | N/A | Addition of 3M sodium acetate and 100% ethanol | DNA precipitation followed by QIAamp kit |
| (Foote *et al.* 2012) | Seawater | 15ml | N/A | Addition of 3M sodium acetate and 100% ethanol | DNA precipitation followed by DNeasy kit |
| (Goldberg *et al.* 2011) | Stream | 5L or 10L | 0.45µM cellulose nitrate filter | Filter stored in 95% ethanol | Filter air dried then DNeasy kit |
| (Jerde *et al.* 2011) | River | 2L | 1.5µM glass fibre filter | Filter stored at -20°C | MoBio kit |
| (Mahon *et al.* | River | 2L | 1.5µM glass fibre | Filter stored | MoBio kit |

| | | | | | |
|---|---|---|---|---|---|
| 2013) | | | filter | at -20°C | |
| (Minamoto *et al.* 2012) | River | 2L | 3µM isopore polycarbonate filter | Sample immediately transferred to laboratory for filtration and DNA extraction | DNeasy kit |
| | Tank | 120ml | N/A | Addition of 3M sodium acetate and 100% ethanol | DNeasy kit |
| (Olson, Briggler & Williams 2012) | Tank | 2, 4, 8L | 1.5µM glass fibre filter | Filter stored at -20°C | MoBio kit |
| | River | 2L | | | |
| (Takahara *et al.* 2012) | Tank | 20ml | Amicon Ultra 15 centrifugal filter unit | Filtrate stored at -25°C until DNA extraction performed | DNeasy kit |
| | Pond | 2L | 3µl polycarbonate filter OR 12µM polycarbonate prefilter + 0.8µM polycarbonate filter followed by Amicon Ultra 15 centrifugal filer unit | | |
| | Lagoon | 2L | 3µl polycarbonate filter followed by Amicon Ultra 15 | | |

| | | | | | |
|---|---|---|---|---|---|
| | | | centrifugal filter unit | | |
| (Takahara, Minamoto & Doi 2013) | Pond | 1L | 3µl polycarbonate filter followed by Amicon Ultra 15 centrifugal filter unit | Filtrate stored at -25°C until DNA extraction performed | DNeasy kit |
| (Thomsen *et al.* 2012a) | Seawater | 1.5L (pool of 30 x 50ml subsamples) | 0.45µM nylon filter | Samples stored at -20°C until filtration performed | Bead beating of filter followed by DNeasy kit |
| (Thomsen *et al.* 2012b) | Pond/Lake, stream, mesocosm | 15ml | N/A | Samples stored at -20°C followed by addition of 3M sodium acetate and 100% ethanol | DNA precipitation followed by DNeasy kit |
| (Wilcox *et al.* 2013) | Stream | 6L | 1.5µM glass fibre filter | Filters stored on ice and transferred to laboratory for DNA extraction | MoBio |

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