Use of Environmental DNA Analysis to Detect the Presence of Water Vole

Keywords: conservation, molecular ecology, real-time PCR, species-detection, survey

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The UK water vole population has fallen dramatically in recent years. Accurate and reliable methods of detecting the presence or absence of water vole at specific locations are critical to conservation efforts. Traditional survey methods can, in some cases, be invasive, inaccurate or difficult to carry out. This study aimed to develop a novel method based on identification of environmental DNA (eDNA) to detect the presence of water vole via analysis of water samples. The results demonstrate that the technique offers an accurate method of detection. However, this study was based on a relatively small sample and certain limitations of the technique have been identified, which will be explored with further research. Nevertheless, used and interpreted correctly, the technique can provide reliable evidence of presence or absence.

Water vole *Arvicola amphibius*. Photo credit Peter Trimming.

Introduction

Environmental DNA

Wildlife conservation has entered a new era. The development of molecular genetic tools is providing novel methods to study species, leading to insights and information that would have been unobtainable just a few years ago.

The development of these techniques has been driven by the need for improved biological records and a demand for more effective methods to monitor species' populations. Often, traditional methods of determining presence/absence, based on identification of the physical signs of a species' presence, are expensive, inaccurate or harmful. The analysis of environmental DNA (eDNA) is now well established as an alternative technique that allows researchers to detect the presence of rare, secretive or invasive species, rapidly, non-destructively and accurately.

eDNA refers to the genetic material isolated from environmental samples, such as water and soil. Aquatic species release DNA into the environment that they inhabit in various ways including excretions and by shedding skin cells. This DNA disperses within the body of water as suspended particles, within cells and mitochondria or as free DNA (Turner *et al*. 2014). eDNA analysis involves the collection of an environmental sample (e.g. water, soil) from which the DNA is extracted and analysed to identify the presence of one or more target species (Rees *et al*. 2014).

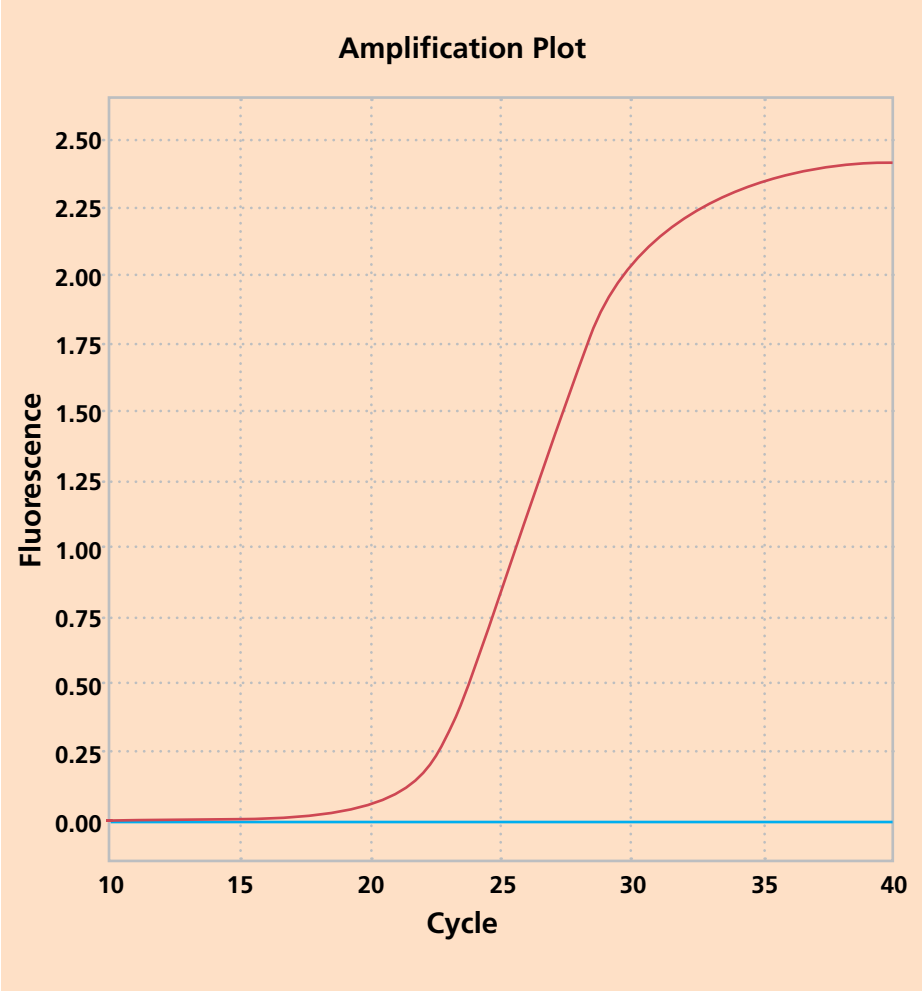

Figure 1. Graphical representation of real-time PCR data. The graph plots the magnitude of fluorescence from the qPCR, measured in relative fluorescence units (ΔR) against the number of qPCR cycles. In samples containing the target DNA sequence, the fluorescence intensity increases due to the release of a fluorescent molecule as the DNA is copied, resulting in an amplification curve (red curve). In negative samples, the DNA target is not present so that there is negligible increase in fluorescence and an amplification curve is not produced (blue line).

The technique relies on targeting the variation in DNA sequences between different species. A sensitive method for the detection and quantification of nucleic acids, known as quantitative real-time Polymerase Chain Reaction (qPCR) is used; qPCR uses a mixture of short DNA molecules and an enzyme to copy specific sections of DNA through the cyclical heating and cooling of the reaction mixture (Heid *et al*. 1996). As the DNA is copied, a fluorescent molecule is released and detected (see Figure 1).

In 2008, eDNA analysis was used to detect a freshwater vertebrate species for the first time when DNA was extracted from water samples and analysed to detect the American bullfrog *Lithobates catesbeianus* (Ficetola *et al*. 2008). The study showed that the detection of eDNA could accurately determine the presence or likely absence of the species. Variations of the technique have since been applied to a range of habitats and species, including amphibian (Thomsen *et al*. 2012) and fish species (Sigsgaard *et al*. 2015), amongst others.

Applying eDNA detection to the water vole

These advances in molecular detection using eDNA have come at an opportune time. Factors including climate change, habitat loss and the spread of invasive species are negatively impacting many species and ecosystems.

The water vole *Arvicola amphibius* (Figure 2) exemplifies this global issue. The population has suffered severe declines over the last century (Jefferies *et al*. 1989) with estimates of a 90% reduction in the UK, attributed to the introduction of the invasive American mink *Neovison vison* and the loss of suitable habitats (Barreto *et al*. 1998). Traditional survey methods rely upon the recording of field signs indicative of the presence of water voles, as described in the *Water Vole Conservation Handbook* (Strachan *et al*. 2011). However, in certain situations, these methods can be inaccurate, labour intensive and expensive to undertake. Consequently, there is a need for additional survey methods. Despite the demonstrated successes of eDNA analysis for the detection of a wide range of species, the technique has, up to now, not been applied to detect the water vole. The way in which their varying habitats, environmental conditions and behaviours would affect the ability to detect the species using this method was largely unknown. In addition, due to several known limitations of the technique, such as the technique not accurately measuring the population size of a target species at a location and the transportation of eDNA in flowing water, it was not known how useful the results would be.

Aim of the study

This study aimed to develop a species-specific qPCR assay for the detection of water vole and to field-test this method, alongside traditional sampling methods, to assess its suitability as a presence-absence survey technique that can ultimately be used to better inform the conservation efforts to preserve the species.

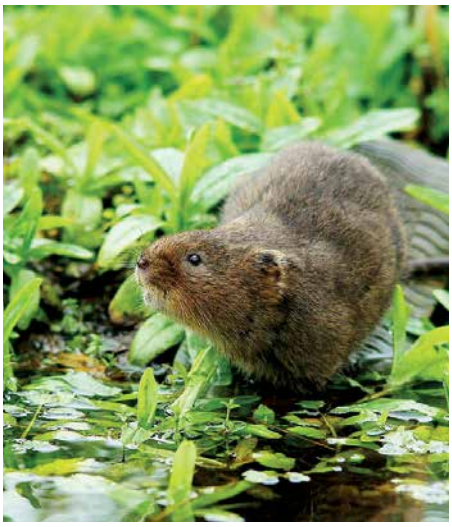

Figure 2. Water vole *Arvicola amphibius*. Photo credit Peter Trimming.

Table 1. List of non-target sympatric species tested during qPCR assay validation.

Species	Latin name	qPCR test result
Bank vole	*Myodes glareolus*	Negative
Field vole	*Microtus agrestis*	Negative
House mouse	*Mus musculus*	Negative
Water shrew	*Neomys fodiens*	Negative
Brown rat	*Rattus norvegicus*	Negative
Wood mouse	*Apodemus sylvaticus*	Negative
European otter	*Lutra lutra*	Negative
Eurasian beaver	*Castor fiber*	Negative
American mink	*Neovison vison*	Negative
Human	*Homo sapiens*	Negative
Domestic dog	*Canis lupus familiaris*	Negative
Domestic cat	*Felis catus*	Negative
Domestic pig	*Sus scrofa domesticus*	Negative
European badger	*Meles meles*	Negative
Pine marten	*Martes martes*	Negative

Methods

qPCR test development

The qPCR test was designed to detect a region of the water vole cytochrome b *(cyt b)* gene and was verified using the PrimerBlast online tool (Ye *et al*. 2012) to confirm that water vole DNA would be specifically targeted. Hair samples were collected by trained staff from eight adult water voles in captive populations at the Derek Gow Consultancy and Wildwood Trust. Hair samples were also collected from a range of non-target species either related to the water vole or likely to be present in the same habitats. DNA was extracted from the hair samples at Crestwood Environmental's eDNA analysis laboratory. These DNA samples were tested by qPCR to ensure that only water vole DNA produced a positive result and that the non-target species' DNA produced a negative result. Table 1 lists the tested non-target species. The assay was then optimised, including altering the reaction mixture and qPCR thermal cycling temperatures to increase the sensitivity of water vole DNA detection whilst maintaining species-specificity by preventing non-target DNA detection.

Field testing

Traditional surveys: Ten sites, situated within the West Midlands, England, were selected for field-testing. Each site was first surveyed for water vole field signs to determine presence or absence. A transect was walked along the survey area to identify and record water vole field signs including droppings, feeding signs such as distinctive vegetation cuttings, burrows and associated vegetation lawns and nests. The field sign search results were withheld from laboratory staff until all laboratory analyses had been completed.

eDNA water sampling: Immediately following completion of the field sign search, the surveyor selected a suitable site for water sample collection, targeting water vole habitat. The surveyor then walked 100 m downstream and using a sterile 30 ml plastic ladle, collected 100 ml of water into a 1 litre plastic laboratory bottle. Nine further 100 ml water samples were collected at 10 m intervals, moving upstream towards the original start point. These were added to the bottle until 1 litre of water had been collected in total. The 1 litre water sample was stored in a Coolbox and transported back to Crestwood Environmental's eDNA analysis laboratory and stored at 4°C for a maximum of 24 hours prior to analysis. All analyses were undertaken by trained and experienced molecular biologists. The surveys and collection of water samples were carried out during August 2017.

Laboratory analysis

Water samples were filtered through a 47 mm, 0.7 µM-pore, glass fibre filter paper to capture the DNA. The DNA was extracted from the filter paper using a modified version of the Qiagen DNeasy Blood and Tissue extraction protocol, as described in Goldberg *et al*. (2011). The DNA samples were then tested for the presence of water vole DNA by qPCR (as described above) using an Aria Mx qPCR system (Agilent Technologies, Santa Clara, CA, USA). Six replicates were run for each DNA sample from each site, with detection of water vole DNA in any of the replicates, shown by the release of a fluorescent molecule (see above), indicating a positive result for that site.

Results

qPCR test development

The qPCR test developed in this study successfully detected water vole DNA from all the DNA samples extracted from known water vole hair samples (n=8). In addition, following optimisation of the assay, there was no detection of DNA from any tested non-target species' DNA samples, extracted from hair samples (Table 1).

Field testing

Ten field sites were assessed (Table 2) and water vole field signs were identified at six of these sites. Water vole eDNA was positively detected at these same six sites. Field signs were not observed at the remaining four sites, indicating that water voles were not present or their field signs were not detected. At three of these four sites, water vole eDNA was not detected, matching the field survey results at those locations. At one site, Newport Brook, field signs were not observed but a positive result was returned for the presence of water vole eDNA. Overall, 90% of eDNA results matched the results from the original field survey with no negative eDNA result from any site with water vole field signs.

Table 2. Presence of water voles at ten sites established through i) traditional field survey and ii) eDNA analysis of water samples according to the protocol described in the text. All samples were taken from flowing (i.e. lotic) water. eDNA results are expressed as the proportion of replicates analysed that gave a positive result, e.g. 4/6 means 4 replicates confirmed presence of water voles out of 6 replicates from each sample; one sample was taken from each site.

Site	Grid reference	Date of survey	Field signs observed	eDNA result	Positive eDNA replicates
Cecilly Brook	SE 525911 015849	03/08/17	Droppings Latrines Vegetation cuttings	Positive	6/6
River Tame	SP 03519 92579	05/08/17	Vegetation cuttings	Positive	6/6
Battlefield Brook	SO 94928 70603	13/08/17	Vegetation cuttings Burrows	Positive	4/6
Newport Brook	SJ 75772 18666	12/08/17	None	Positive	2/6
Staffs and Worcs Canal	SJ 90233 01056	15/08/17	None	Negative	0/6
Spadesbourne Brook	SO 96495 71248	24/08/17	Vegetation cuttings Droppings	Positive	2/6
New Hall Country Park Brook	SP 12957 94922	28/08/17	Droppings Vegetation cuttings Burrows	Positive	1/6
Smestow Brook	SO 89173 99882	29/08/17	None	Negative	0/6
Greenfield Local Nature Reserve	SJ 52942 41645	31/08/17	Vegetation cuttings Droppings	Positive	6/6
Forge Mill Lake	SP 03493 92567	16/08/17	None	Negative	0/6

Discussion

The successful development of an eDNA detection assay for water vole demonstrates the potential of the technique as a reliable survey method for determining the presence or likely absence of water voles at aquatic sites.

The results from the traditional surveys and eDNA analysis were in agreement at nine of the ten sites surveyed by both methods. The exception was Newport Brook where no field signs were found yet a positive eDNA result was obtained. It is possible that the eDNA technique detected the presence of water vole where the traditional methods did not. However, it is also plausible that the water vole eDNA had been transported from a different location by flowing water, resulting in a false positive result. Due to the limitations of this study, it is not possible to differentiate between these causes. Further work is required to confirm the origin of the water vole eDNA detected at this site.

Following these encouraging results, the next step is to identify how to apply the technique to improve the conservation of the water vole. We envisage that sampling for eDNA will be utilised as an additional survey technique for water vole to complement the traditional methods, or as a stand-alone survey method when appropriate. In addition, the technique will be useful when a large geographical area or many separate sites need to be surveyed within a limited time (e.g. along a watercourse). Key advantages include the relative cost, speed and labour efficiency required to collect eDNA water samples when compared to traditional survey techniques.

While the results of this study are very encouraging, several limitations should be noted. For example, the water samples were collected during August, considered to be the time of year when the water vole population is at its peak and is most active (Wildlife Trusts 2017), resulting in higher concentrations of eDNA. Additional field trials are needed to confirm the level of effectiveness and reliability of the technique at different times of the year and to refine the application of the technique.

Water voles are known to inhabit a range of aquatic habitats, including flowing and stationary water systems. It is not yet known how different habitats will affect the transportation and degradation of water vole eDNA and the subsequent effect on detection rates. Therefore, water sampling methods will need to be adapted to different types of habitat, taking into account factors such as the flow-rate and inlet location to ensure that the optimum method is used. Further testing of the technique in a wide variety of aquatic habitats is planned to optimise its use in different situations.

In addition, eDNA analysis cannot give an accurate measure of the water vole population size. Therefore, while it can determine whether water voles are present or most likely to be absent from a location, it cannot provide information on the health of the population. This limits its potential for conservation.

In conclusion, the results presented in this study form part of a growing body of evidence demonstrating that the analysis of eDNA is a very useful tool in

species conservation, although certain limitations of the technique must be taken into consideration. Specifically, we have demonstrated that the detection of water vole eDNA in water samples is an accurate indicator of presence. As the UK water vole population continues to decline, this technique has the potential to contribute important data to conservation strategies aimed at promoting the species' recovery.

Acknowledgements

The authors thank Matthew Bissett for the collection of water samples; the UK Water Vole Steering Group for expertise and advice; and the Wildwood Trust, Ms. Emily Elwell, Aim Environmental and the Derek Gow Consultancy for providing hair samples.

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