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Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*)

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ABSTRACT

The use of environmental DNA (eDNA) is rapidly emerging as a potentially valuable survey technique for rare or hard to survey freshwater organisms. For the great crested newt (*Triturus cristatus*) in the UK, the substantial cost and manpower requirements of traditional survey methods have hampered attempts to assess the status of the species. We tested whether eDNA could provide the basis for a national citizen science-based monitoring programme for great crested newts by (i) comparing the effectiveness of eDNA monitoring with torch counts, bottle trapping and egg searches and (ii) assessing the ability of volunteers to collect eDNA samples throughout the newt's UK range. In 35 ponds visited four times through the breeding season, eDNA detected newts on 139 out of 140 visits, a 99.3% detection rate. Bottle traps, torch counts and egg searches were significantly less effective, detecting newts 76%, 75% and 44% of the time. eDNA was less successful at predicting newt abundance being positively, but weakly, correlated with counts of the number of newts. Volunteers successfully collected eDNA samples across the UK with 219 of 239 sites (91.3%) correctly identified as supporting newts. 8.7% of sites generated false negatives, either because of very small newt populations or practical difficulties in sample collection. There were no false positives. Overall, we conclude that eDNA is a highly effective survey method and could be used as the basis for a national great crested newt monitoring programme.

1. Introduction

The use of environmental DNA (eDNA), nuclear or mitochondrial DNA that is released from an organism into the environment, is rapidly emerging as a potentially valuable survey technique for detecting cryptic or difficult to survey freshwater organisms (Lodge et al., 2012; Sutherland et al., 2013). Proof of concept studies have shown that eDNA can be used to detect the presence of amphibians, fish, invertebrates (including dragonflies and crustaceans), mammals and water birds (Ficetola et al., 2008; Goldberg et al., 2011; Olson et al., 2012; Thomsen et al., 2012), and to assess fish and amphibian abundance (Takahara et al., 2012; Pilliod et al., 2013a). Depending on the taxonomic group and habitat, results so far indicate that eDNA may be more, equally or slightly less effective at detecting species than traditional methods (respectively, Dejean et al., 2012 for American bullfrog (*Lithobates catesbeianus*); Pilliod et al., 2013a for two riverine amphibians; Thomsen et al., 2012 for large white-faced darter dragonfly (*Leucorrhinia pectoralis*)). Thus although eDNA may have a future use as an important survey and monitoring method for freshwater organisms, it is essential to test its effectiveness compared to existing survey methods (Thomsen et al., 2012) to establish the taxa to which it can be validly applied.

A particularly important attribute of eDNA methods is that the water samples needed for analysis are usually quicker and more technically straightforward to collect than are biotic data (Ficetola et al., 2008. Thomsen et al., 2012). This opens a new opportunity for credible monitoring surveys to be undertaken by volunteer surveyors in citizen science programmes as volunteer surveyors often have less time, or more limited taxonomic skills, than professional ecologists. The use of volunteers for water and biodiversity monitoring is growing worldwide (Silvertown, 2009; Schmeller et al., 2009; Roy et al., 2012) reflecting both a need for

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data which far outstrips the resources available for professional monitoring programmes and a recognition of the benefits of engaging 'ordinary citizens' with science and nature. In practice, however, the validity and effectiveness of a volunteer-based eDNA survey programme has yet to be tested.

In this study we compared the use of eDNA with traditional methods for surveying the great crested newt (*Triturus cristatus*), a protected species listed in Annexes II and IV of the EC Habitats Directive (European Commission, 1992). At present, substantial survey effort is required to assess whether great crested newts are absent from a site, with typically 4-6 annual visits needed, using at least three survey methods on each occasion (Sewell et al., 2010). The survey effort required to collect adequate data has so prevented the establishment of a robust national monitoring programme for the species in the United Kingdom either by professional surveyors, where the cost would be considerable, or by volunteers working on the UK National Amphibian and Reptile Recording Scheme (Wilkinson and Arnell, 2013), for whom the substantial time commitment, combined with lack of funds for professional support to arrange site access permission, has prevented the establishment of a survey programme.

Our study firstly assessed the effectiveness of eDNA methods for the detection of the great crested newt compared to the standard survey techniques used for this species: torch counting, bottle trapping and egg searching, and secondly, investigated the ability of volunteers to collect eDNA samples from the known range of the great crested newt in the United Kingdom, to inform the development of a national surveillance scheme for the species involving the use of volunteer collected data.

2. Materials and methods

2.1. Field survey methods

2.1.1. Traditional great crested newt survey methods

We compared the eDNA method with the three main methods recommended in the United Kingdom for detecting presence and/or relative abundance of great crested newts (English Nature, 2001; Sewell et al., 2013): night-time torch counting and overnight bottle trapping of adults and visual egg searches. We did not use netting as a technique because it may damage vegetation which is used by great crested newts, is considerably less effective at detecting adults than the other methods (Griffiths et al., 1996) and because many experienced surveyors now consider this technique is likely to lead to unacceptable levels of injury to the larval stages of great crested newts.

We selected 35 ponds for the comparison of eDNA with traditional methods, 20 in south Hampshire in southern England and 15 in north-east Wales near the town of Buckley in Flintshire. The two regions were selected to represent contrasting parts of the range of the great crested newt: in south Hampshire, ponds are at relatively low density (2–4 ponds per km^2) with scattered great crested newt populations, whereas in north-east Wales ponds density can exceed 20 per km^2, with a relatively high density of great crested newt populations. All selected ponds were known in advance to support great crested newts, so that all surveys were a test of the extent to which the survey methods generated false negative results. In south Hampshire most survey work was undertaken by a small team of professional surveyors; in north-east Wales surveys were undertaken by a team of 50 volunteers co-ordinated by professional staff.

The ponds were visited on four occasions during the great crested newt breeding season from late April to late June with visits at 2–3 week intervals, giving a total of 140 visits to the 35 ponds. Full details of the locations of the survey sites and visit dates are given in Table S1 of the Supplementary Content. On arrival at sites, surveyors first undertook torch counts of newts, then collected an eDNA water sample (for methods see 2.1.2 below) and finally set bottle traps around the ponds. Torch counts were undertaken by walking the perimeter of the pond after dark, and used Clulight CB2 1,000,000 candle power torches. Torch counts were undertaken in south Hampshire mainly between 21:30 and 23:30 h and took on average 24 min. On average 84% of the ponds' shorelines were accessed. In north-east Wales two-thirds of torch counts were carried out between 21.50 and 22.50 h and most of the remaining third between 22.50 and 23.50 h. On average counts also took 24 min and 97% of the ponds' shorelines were accessed. Bottle traps followed the design of Griffiths (1985) and were set at 2 m intervals around the pond perimeter, the standard recommended for newt surveys in the UK by English Nature (2001), with between 17 and 62 traps per pond, reflecting the variation in pond sizes. Traps were left overnight and emptied the following morning, with newts counted, aged and sexed. Once bottle traps were emptied a visual egg search was undertaken if eggs had not already been noted during the course of other survey work. The survey order: torch count, eDNA sample, bottle trapping and egg searching was designed to ensure that, as far as practically possible, the methods used did not interfere with each other. We assessed the size of newt counts in terms of the standard classification adopted by UK statutory bodies, 'small' populations being those with up to 10 animals counted, 'medium' populations being those with 11–100 animals counted and 'high' populations being counts exceeding 100 animals (English Nature, 2001).

2.1.2. eDNA water sample collection

A single water sample was collected for eDNA analysis at each pond at the same time as the traditional survey data were collected. The field survey method was modified from that developed by Ficetola et al. (2008) and Thomsen et al. (2012) using a simple sampling kit comprising a sterile water sampling ladle, a self-supporting sterile Whirl-Pak® bag, a sterile pipette, plastic gloves to minimise contamination and six 50 ml sample tubes containing the DNA preservative. To preserve DNA, each sample tube contained 33 mL of absolute ethanol and 1.5 ml of sodium acetate 3 M. Using the ladle, a 30 mL water sample was collected at each of 20 locations around the pond margin to create a pooled water sample of approximately 600 mL in the sterile self-supporting plastic bag. Samples were collected whilst the surveyor stood only on the pond bank or on muddy pond edges without entering the water to avoid possible contamination from the surveyors boots, or by stirring up sediment. Sampling locations were spread evenly around the pond including, as far as possible, locations where great crested newts were expected to occur in the ponds: for example, near to vegetation used for egg laying or in open areas suitable for displaying. However, it should be noted that at present we have very little detailed understanding of the distribution of newt DNA in ponds. This water sample was homogenised by gently shaking the bag to ensure that eDNA was evenly mixed through the sample and then 90 ml of pond water taken, in six subsamples of 15 mL each, using the sterile pipette, and added to each of the sample tubes. It was necessary to collect water samples in six separate 50 mL tubes because only centrifuge rotors for 50 mL tubes can reach the required speed of 15,000 x g, and only 50 mL tubes can withstand this force. Samples were stored in a cold room, but not frozen, before shipping at ambient temperature to the analysis laboratory where samples were stored at −20 °C prior to DNA extraction. Analyses were undertaken within 1 month of sample collection.

We did not collect distilled water negative controls (*sensu* Pilliod et al., 2013b) at the main professional or volunteer survey sites because at all sites we had good evidence in advance of the survey that great crested newts were either present in the current

field season, or had not been recorded (see 2.1.4 below). Distilled water blanks would not therefore provide evidence of cross-contamination in the field samples. Additionally, in the volunteer survey cross contamination was unlikely because most volunteers were working separately from each other and visited single sites.

2.1.3. eDNA laboratory methods

2.1.3.1. Primer validation. eDNA samples were analysed using primers and probes designed by Thomsen et al. (2012). Before analysis these markers were first tested *in silico, in vitro* and *in situ.*

The *in silico* analysis was performed with an electronic PCR using ecoPCR software, allowing three mismatches between each primer and the template (Taberlet et al., 2007, available at http://www.grenoble.prabi.fr/trac/ecoPCR) on the EMBL-Bank release 114 (released in December 2012) and the SPYGEN reference database which includes 56 European amphibian species.

Primers and probes were tested *in vitro* against tissue samples collected from 16 individual great crested newts by external swabbing from three different populations the south (Hampshire), north-west (Lancashire) and north-east (Yorkshire) of England. Swabbing, which was non-invasive, was undertaken by herpetologists licensed by Natural England (the statutory authority in England) to undertake this procedure. DNA was extracted using the DNA Blood and Tissue kit (Qiagen®) following the manufacturer's instructions. The DNA extracted was quantified using a Qubit (Life Technologies ®). Additionally, primers and probes were also tested on tissue samples of the marbled newt (*Triturus marmoratus*) and the Italian crested newt (*Triturus carnifex*).

The limit of detection (LOD, i.e. the minimum amount of target DNA sequence that can be detected in the sample) and the limit of quantification (LOQ, i.e. the lowest amount of target DNA that yields an acceptable level of precision and accuracy) were calculated by running a dilution series of a known amount of great crested newt DNA, ranging from 10^{-1} ng μL^{-1} to 10^{-10} ng μL^{-1} with 12 replicates per concentration.

Following *in vitro* testing, primers and probe were tested *in situ.* Nine samples were collected between April and May in ponds where great crested newt densities were known. Three samples were collected from ponds with low great crested newt counts (*sensu* English Nature, 2001), three with medium counts and three from ponds where the species was absent. The low and medium count populations were in south Hampshire and the zero count ponds were outside the species UK range in the Shetland Isles in the north of Scotland.

2.1.3.2. eDNA sample analysis. When returned to the laboratory for DNA analysis, all samples were identified only by a unique numerical code which contained no information about the sampler, the site or whether the site supported great crested newts, ensuring that DNA analyses were undertaken 'blind'. This approach was applied both to samples collected in the methodological study and the volunteer survey programme (see Section 2.1.5).

The DNA extraction was performed following the method describe in Treguier et al. (2014). DNA extraction was performed in a dedicated room for water DNA sample extraction, the six subsamples being pooled in the extraction room. This room was equipped with positive air pressure, UV treatment and had frequent air renewal. Before entering the extraction room laboratory personnel changed into full protective clothing comprising disposable body suit with hood, mask, laboratory shoes, overshoes and gloves. Extraction of negative controls was systematically performed to monitor possible contamination.

After the DNA extraction the samples were tested for inhibition by qPCR. The quantitative PCR was performed in a final volume of 25 μL, using 3 μL of template DNA, 3 μL of 10–3 ng/μL of DNA of a synthetic gene, 12.5 μL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®), 3.5 μL of ddH2O, 1 μL of each specific primer for the synthetic gene (10 μM) and 1 μL of probe (2.5 μM) under thermal cycling 50 °C for 5 min and 95 °C for 10 min, followed by 55 cycles of 95 °C for 30 s and 52 °C for 1 min. All the samples were analysed in duplicate. If at least one of the replicates showed a different Ct than expected (at least 2 Ct), the sample was considered inhibited and diluted 2-fold before the amplification with great crested newt primer and probes. Of the total 439 eDNA samples extracted, 52 (c.a. 11% of the total) were found to be inhibited. After dilution all the samples were found to be not inhibited, demonstrating that sample dilution effectively reduced qPCR inhibition, an observation also made by McKee et al. (in press).

Samples were amplified using primers and probes designed by Thomsen et al. (2012) and validated in this study. The qPCR was performed in a final volume of 25 μL, which included 3 μL of template DNA, 12.5 μL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®), 6.5 μL of ddH2O, 1 μL of each primer (TCCBL: CGT AAA CTA CGG CTG ACT AGT ACG AA, TCCBR: CCG ATG TGT ATG TAG ATG CAA ACA, 10 μM each) and 1 μL of probe (TCCB_Probe: FAM-CAT CCA CGC TAA CGG AGC CTC GC-BHQ1 2.5 μM) all under thermal cycling at 50 °C for 5 min and 95 °C for 10 min, followed by 55 cycles of 95 °C for 30 s and 56.3 °C for 1 min. Samples were run on a BIO-RAD® CFX96 Touch real time PCR detection system. Each sample was run in 12 replicates. A dilution series of great crested newt DNA, ranging from 10^{-1} ng μL^{-1} to 10^{-4} ng μL^{-1}, was used as a qPCR standard. The qPCR standards were added to the qPCR plate in a separate room from the eDNA extraction room. The tubes containing the eDNA samples were sealed and then the qPCR standards were added to the qPCR plate in a separate room from the eDNA extraction room. Four negative (UHQ water) controls were added, at the same location in the plate, during the qPCR step. qPCR analysis was performed in a third room, dedicated to amplified DNA analysis with negative air pressure and physically separated from the eDNA extraction room. In all cases, the estimated concentrations of DNA were below the limit of quantification (LOQ, i.e. less than 10^{-3} ng/l^{-1}), meaning that eDNA quantification was not possible. For this reason we reported the number of replicates amplified in a sample run, from 0 out of 12 to 12 out of 12, as the 'eDNA score' rather than reporting the amount of eDNA detected quantitatively. Because we could not accurately quantify the amount of DNA below the Limit of Quantification we cannot be certain that the number of positive qPCR replicates reflects the amount of DNA in the sample. However, our working assumption is that the two are positively related. There was no evidence that the limit of quantification was related to sample inhibition.

2.1.4. Tests for eDNA false positives

To assess whether our eDNA method generated false positives we collected water samples at two subsets of 30 sites where we had good reason to believe that newts were absent. The first subset of sites was located just beyond the edge of the known range of the great crested newt in south-west England (Cornwall). Sites were selected after compiling all available records in the UK for the great crested newt which we used to develop a modelled national distribution map for the species. The second subset of thirty sites were within the known range of the great crested newt and were locations where we had good reason from local knowledge to expect newts to be absent. Sites included garden ponds belonging to members of the project team where great crested newts had never been seen, ponds at education centres where regular pond surveys had never encountered great crested newts and ponds with substantial fish populations which were unsuitable for newts and had been surveyed in previous years and shown to lack newts. Thus although we did not specifically survey sites we were confident that newts would be absent from the sites.

2.1.5. Use of the eDNA method by volunteers

To evaluate the suitability of the eDNA method for use by volunteers we set up a national feasibility study to test the ability of volunteers to collect eDNA samples from ponds known to support great crested newts throughout the range of the species in England, Wales and Scotland. A total of 86 volunteers, who comprised a mixture of professional and amateur workers involved in voluntary wildlife recording programmes, particularly the UK National Amphibian and Reptile Recording Scheme and the PondNet programme (Wilkinson and Arnell, 2013; Williams et al., 2012) were recruited and collected eDNA samples from 239 ponds. Samples were collected as close as possible to the peak of the great crested newt breeding season (mid May) with most samples collected (86%) between 15 May and 17 June 2013 (Fig. 1). Sites were distributed throughout the national range of the species (Fig. 2), and were typical of the altitudinal range and size of ponds used by great crested newts and the type of landscapes in which great crested newt ponds occur in Britain (Supplementary Content Fig. 1a–c). A full list of the sites surveyed is available in the Table S2 of the Supplementary Content. Volunteer surveyors were given simple written instructions on the use of the survey method (Biggs et al., 2014) but were not otherwise trained or supervised in the field. At each site volunteers collected a single 600 mL pooled eDNA water sample (30 mL from 20 locations), from which six subsamples of 15 mL were taken and preserved, using the standard kit and sampling procedure described in Section 2.1.2. We resurveyed 26 (11%) of the volunteer sites to quality assure the volunteer surveyors' eDNA sample collection technique.

There is relatively little information available to date on the influence of environmental factors on the breakdown and persistence of eDNA in freshwaters. Dejean et al. (2011) noted that endogenous nucleases, water, UV radiation and the action of bacteria and fungi in the environment contribute to DNA decay and Pilliod et al. (2014) showed experimentally that both light and temperature influenced eDNA persistence. Thomsen et al. (2012) showed experimentally that great crested newt DNA persisted in mesocosms for 1–2 weeks. We investigated whether broad relationships could be detected between environmental factors likely to affect eDNA persistence or breakdown, such as shade (a surrogate of light intensity and tannins derived from leaf fall), altitude (a surrogate of water temperature and the pH gradient, with high altitude sites in the UK generally being located on more acid rocks) and landuse (a surrogate of the occurrence of agricultural and other types of water pollution). To broadly characterise pond environments volunteers recorded ten metrics of pond quality used to calculate the great crested newt Habitat Suitability Index (Oldham et al., 2000): pond area, amount of shading, vegetation density, a subjective assessment of water quality, waterbody permanence, surrounding terrestrial habitat quality, number of adjacent ponds and the impact of fish and waterfowl. We derived waterbody altitude from national mapping.

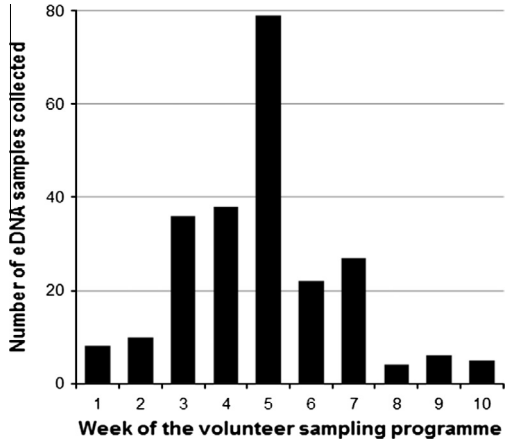

Fig. 1. Week during the great crested newt breeding season when volunteer eDNA samples were collected. Week 1 = 1–7 May 2013; Week 10 = 3–9 July 2013.

2.2. Data analysis

In the detailed comparison of eDNA and traditional survey methods we compared the effectiveness of eDNA and traditional methods using McNemar's test which evaluates whether differences in discordant pairs is greater than would be expected by chance alone (McNemar, 1947). In this context, a discordant pair occurs when eDNA detected newts but other methods did not, or vice versa. We did not apply occupancy modelling approaches in this work (c.f. Sewell et al., 2010) because we knew in advance that all sites supported newts so that all methods could potentially generate 100% positive records.

We assessed whether eDNA score was related to the abundance of newts, as described by the torch counting or bottle trap counts, using the Cochran Armitage Trend test (Cochran, 1954; Armitage, 1955) for trends in binomial proportions across the levels of a single ordinal variable. The two-level variable represents the response, eDNA score out of 12, and the other represents the explanatory variable, newt abundance, as ordinal data. The null hypothesis is the hypothesis of no trend, which means that the binomial proportion is the same for all levels of the explanatory variable.

Volunteer survey effectiveness was evaluated in terms of the number of false negative records generated. Data from the volunteer survey were highly non-normal and could not be corrected by transformation (Anderson–Darling normality test). To analyse relationships between eDNA score and environmental factors we ran a series of Spearman rank order correlations with a Bonferroni correction to counteract the problem of multiple comparisons such that the minimum significance level for tests was $p < 0.004$. We tested whether location within the range of the great crested newt (core or marginal areas of the range) or date of sampling affected eDNA scores in volunteer samples using a Kruskall–Wallis H test.

All statistical analyses were performed using XLSTAT Version 2014.1.8 (Addinsoft 1995–2014).

3. Results

3.1. Primer validation

When analysing only the primer pairs, without taking into account the probe, the primers amplified 63 species present in GenBank. When the bioinformatic analysis was performed using the combination of primers and probe they were found to bind perfectly with great crested newt DNA, but also, with some mismatches, to the eastern rainbowfish (*Melanotaenia splendida*), a warmwater species native to Australia, the California newt (*Taricha torosa*), the Italian crested newt and the southern crested newt (*Triturus karelinii*). Because of the number of mismatches and their position on the primer binding sites, the chance of amplifying these species is very low, but we cannot exclude their amplification. All of these species are absent from the UK, except for the Italian crested newt. None of the sites at which eDNA samples were collected were suspected to be supporting this species, which has been introduced to a small number of locations in the UK (Jehle et al., 2011), and the *in vitro* test demonstrated that none of the Italian crested newt or marbled newt samples were amplified, showing the suitability of the primer pair and probe. In the *in situ* tests, great crested newts were detected at all sites where they

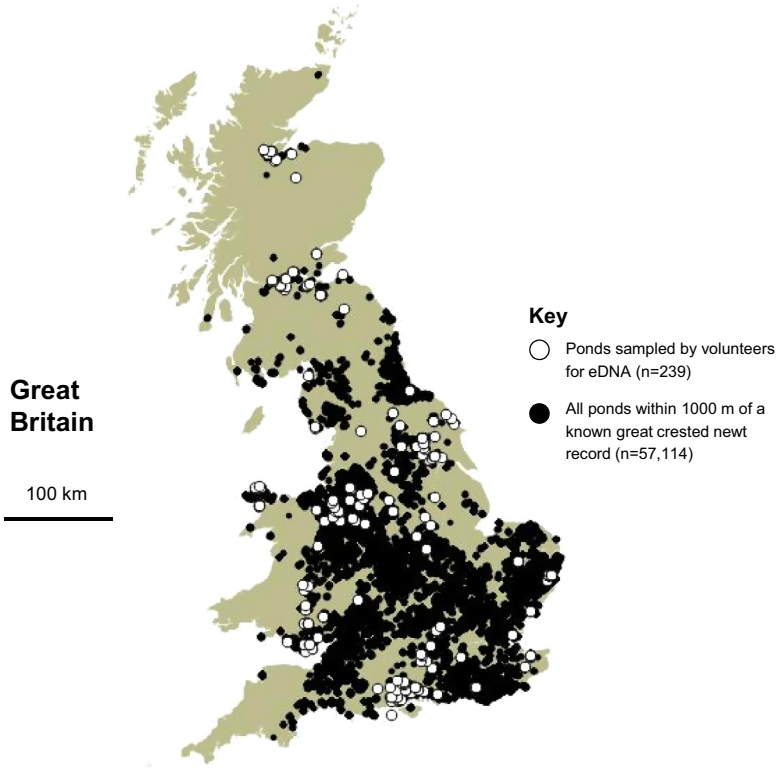

Fig. 2. Location of ponds sampled by volunteers for eDNA compared to distribution of all ponds within 1000 m of a known great crested newt record. Open circles indicate ponds sampled by volunteers for eDNA. Filled circles indicate all ponds within 1000 m of a known great crested newt record.

were present and in none of the sites where they were absent, completing validation for the primer and probes for this study.

The LOQ in this study was $3 * 10^{-3}$ ng, with great crested newt DNA still detected at a concentration of $3 * 10^{-9}$ ng, with at least one qPCR replicate in twelve showing a positive result. This concentration was set as the LOD.

3.2. Comparison of eDNA with traditional survey methods

In the 35 south Hampshire and north-east Wales ponds, eDNA methods detected great crested newts on 139 out of 140 survey visits, a detection rate of 99.3% (Fig. 3a). eDNA was more effective than bottle trapping and torch counts which detected newts on 76% and 75% of the survey visits, respectively. Egg searches detected animals on 44% of survey visits. Differences between eDNA and the other methods were highly significant ($p < 0.0005$, McNemar's test).

Amphibian surveyors typically combine survey methods during work to assess newt presence or absence (English Nature, 2001; Sewell et al., 2010). When we combined torch counting and bottle trapping, the two methods were able to detect newts on 95% of survey visits (Fig. 3b) close to, but still significantly different from, the eDNA method (McNemar's test, $p < 0.05$).

Counts of animals using traditional methods (torch counting and bottle trapping) varied from 1 to 45 individuals on each survey visit, except for two visits when no animals were recorded using traditional methods. 75% of counts were in the 'small' population category of English Nature (2001) and 25% in the 'medium' category. eDNA score was broadly correlated with newt counts (Fig. 4; Cochran-Armitage test: eDNA score vs torch counts $p < 0.001$, eDNA score vs bottle trapping $p < 0.0001$). However, although low eDNA scores were always associated with 'low' counts of newts, a high eDNA score was not always associated with higher newt populations. eDNA did not, therefore, prove a consistent predictor of great crested newt abundance.

3.3. Volunteer eDNA survey

Volunteers eDNA surveys were positive at 218 of the 239 (91.3%) of the survey sites, all of which were sites known to support great crested newts. 8.7% of sites generated false negative records. There was no evidence that the date of sample collection affected the ability to detect eDNA, with median eDNA score showing no significant differences over the 11 weekly sampling periods of the study (Fig. 5; Kruskall–Wallis H Test $(10,221) = 6.33$, $p = 0.787$). There was also little evidence to suggest that eDNA scores were influenced by variation in the pond environmental factors (Table 1). There were no significant correlations between eDNA score and pond shade, pond area, pond permanence, abundance of aquatic vegetation, water quality, presence of waterfowl, surrounding terrestrial habitat quality, number of adjacent ponds or pond altitude. The only significant correlation between eDNA score and environmental factors was for the total HSI score, suggesting that the main factor affecting eDNA detection was the overall suitability of the site for great crested newts.

Tests for inhibition of eDNA samples indicated that there was little overall difference in inhibition rates in the detailed methodological study and the volunteer survey with, respectively, 10% and 11% of samples showing evidence of inhibition. Of the false negative sites in the volunteer survey just over a quarter (28%) showed evidence of inhibition. There was no significant difference in eDNA scores at sites with and without inhibition (Mann–Whitney U Test, $Z = -0.03$, $p = 0.97$).

The 21 false negative ponds comprised 4 sites where we believe methodological errors were probably made in the collection of

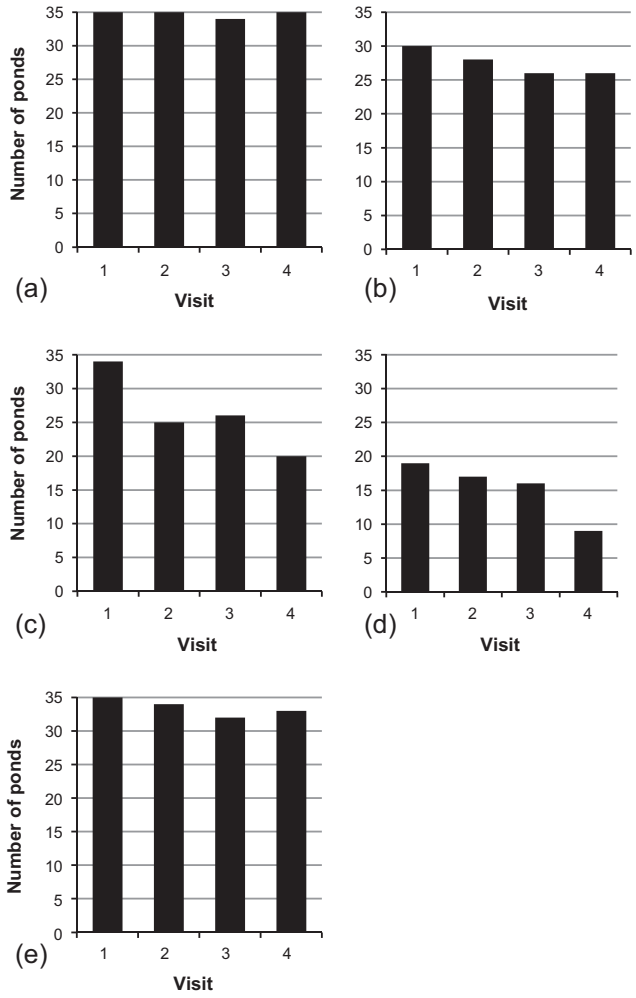

Fig. 3. Number of ponds (total: $n = 35$) in which great crested newts detected by eDNA and traditional survey techniques, surveyed on four occasions during the breeding season, from late April to late June 2013. Detection methods: (a) eDNA, (b) bottle trapping, (c) torch counting, (d) visual egg searches, (e) bottle trapping and torch counting combined.

water samples, with surveyors only collecting water from part of the pond perimeter or from areas which were inaccessible to newts, such as very shallow water or dense marginal vegetation. 6 sites were locations where newts were present in very small numbers. At the remaining 11 false negative sites there was no obvious explanation for the result.

3.4. Generation of false positives using eDNA and quality assurance of volunteer eDNA sampling

No false positive records were generated using the eDNA method at either the out of range or within range sites. We were confident that there were no within range false positives because (i) because all positives came sites where field survey data or local expert knowledge from the 2013 field season indicated that great crested newts were present and (ii) at the 30 sites selected within range where newts were known, or very likely to be absent, there were no DNA positives.

Quality assurance of volunteer surveyors found that the same eDNA result was obtained at 25 of the 26 (96%) volunteer survey sites which were resurveyed by a professional team member.

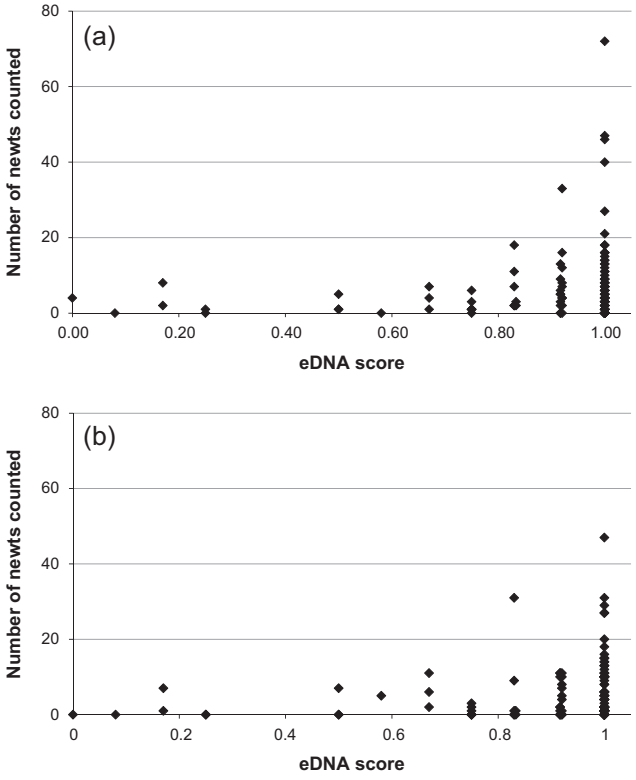

Fig. 4. Relationship between eDNA score and great crested newt abundance. (a) eDNA vs torch counts; (b) eDNA vs bottle trapping. eDNA scores range from 0 = 0/12 positive qPCR replicates to 1 = 12/12 positive qPCR replicates.

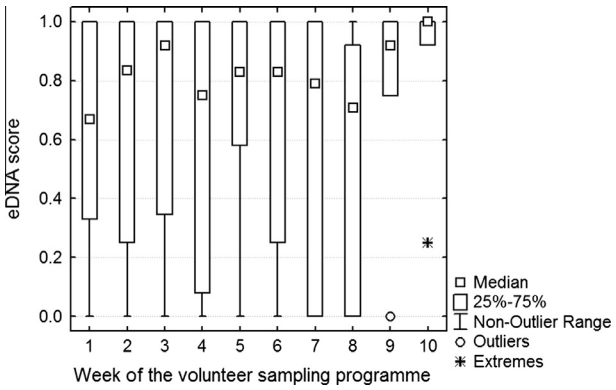

Fig. 5. Median eDNA scores of volunteer water samples collected during the great crested newt breeding season. Week 1 = 1–7 May 2013; Week 10 = 3–9 July 2013.

4. Discussion

4.1. Effectiveness of the eDNA method

eDNA was highly effective at detecting the presence of great crested newts, with near perfect detection of the species in the intensive study of 35 ponds. eDNA was also more effective than traditional sampling methods. Our observations confirm and extend the results of Thomsen et al. (2012) who detected great crested newts in 10 out of 11 ponds examined, a 91% detection rate and of Rees et al., 2014 who tested 38 ponds (19 with great crested newts, and 19 without) obtaining 84% detection at positive sites. Our results are similar to those of Pilliod et al. (2013a) who achieved detection rates of 83–100% in studies of two

Table 1
Correlations between eDNA score and environmental factors potentially able to influence the detection of eDNA.

Environmental factors	Spearman's rank correlation	*P* (significant values in bold)	*n*
Overall HSI score	0.221	**0.001**	231
Shade	0.106	0.109	230
Fish (absence)	0.146	0.027	230
Terrestrial habitat quality	0.071	0.282	230
Presence of waterfowl	0.018	0.781	230
Number of adjacent ponds	0.049	0.463	230
Water quality	0.095	0.150	230
Pond dries	0.015	0.817	230
Pond area	0.023	0.727	230
Abundance of aquatic vegetation	0.006	0.930	230
Altitude	0.026	0.688	237
	Kruskal–Wallis H	*p*	*n*
Range areas of great crested newt: A, B and C Oldham et al. (2000)	4.6 (df = 2)	>0.1	230

stream-dwelling amphibians at 13 sites. Overall the results of our intensive study support the suggestion by Pilliod et al. (2013a) that, because eDNA detection rates are so high, naive estimates of occupancy derived simply from eDNA results may be acceptable for monitoring surveys, eliminating the need for multiple visits to sites to confirm the absence of the species. Thus a single eDNA sample collected during the breeding season appears to be equivalent to the 5 survey visits, using three survey methods, recommended by Sewell et al. (2010) to achieve 95% confidence that great crested newts are absent from a site.

The results of the volunteer feasibility study indicate that the method is suitable for application by large teams of surveyors with limited training and experience. There was a higher rate of false negatives than in the intensive study, although the rate of 91% positive records remained similar to the levels achieved in the studies of Thomsen et al. (2012), Rees et al. (2014) and Pilliod et al. (2013a), suggesting that the method is remarkably robust, even when applied by relatively inexperienced surveyors. A 91% rate of detection would not be good enough to allow a survey based on a single site visit. However, given that volunteer surveyors received no specific training in the sampling technique, working only from a written instruction sheet, we expect that it will be possible for volunteers, with simple training, to achieve detection rates closer to those of the methodological comparison study where professional biologists either supervised or undertook the sample collection. Detailed inspection of the volunteer site data suggests that, with methodological errors corrected, around 95% of sites would have been correctly identified as supporting newts, close to the level of detection proposed by Sewell et al. (2010) for volunteer surveys. There were too few false negatives for statistical analysis of environmental factors that might influence the occurrence of false negatives. However, discussion with individual volunteers implicated three potential causal factors: (i) ponds where numbers of newts were very small and the water sample failed to collect any eDNA; (ii) ponds where surveyors could only access a small proportion of the pond perimeter and (iii) sites with very broad shallow drawdown zones where surveyors (who were instructed not to enter the water) could not get close to the deeper water areas often favoured by newts. We also suspect that false negatives were more likely where these factors operated together.

Of the three factors thought likely to have caused false negatives, there is potential to address two with modest additional training, advice or equipment. For example, areas of deeper water further from the pond shoreline can be sampled using extension poles to reach beyond vegetation mats or into more areas of the pond where some part of the perimeter are difficult to access. We also believe that further training of volunteers would help to reduce false negatives: in collecting water samples we recommended that surveyors sampled areas of the ponds used by newts. Many of the volunteers in the project had some previous experience of amphibian or pond surveys, and would be able to recognise the preferred habitats of great crested newts, but those with less experience would find it more difficult to interpret this instruction correctly. The third factor, ponds with very small newt populations where too little DNA is produced for the current method to detect, will require further detailed field and laboratory investigation to better understand the limits of the method (see Section 4.3).

Although our study did not use field negatives because they were not required in our design (Pilliod et al., 2013b), they may be valuable in the implementation of large volunteer programmes where relatively inexperienced surveyors are collecting eDNA samples. Although it is probably not justified in cost terms to collect field negatives at every site, they should probably be part of standard quality assurance work on a proportion of sites.

Given that there was only one false negative site in the methodological study (0.7%) compared to 21 (9%) in the volunteer survey, this suggests that inhibition was not a cause of false negatives. Added to this eDNA scores did not differ significantly in samples that were inhibited compared to those that were not. However, given the known role of inhibitors in eDNA detection further work on inhibitors in great created newt ponds would be valuable, especially in ponds with small newt populations.

4.2. Assessing great crested newt abundance with eDNA

Although eDNA is very effective for detecting great crested newt presence, our results indicate that the method is currently less valuable for assessing abundance. Although there was a broad and statistically significant relationship between eDNA score and newt counts, the relationship was strongest where eDNA scores were low, these sites always having low newt counts. In contrast high eDNA scores were generated at sites with both low and high newt counts. Assessing the abundance of great crested newts using conventional methods is generally difficult so it is not surprising that there is a rather noisy relationship between eDNA scores and counts based on torching and bottle trapping. Accurate estimates of newt population sizes requires either drift fencing with pitfall trapping or mark / recapture studies, and where counts are used they may considerably over or under-estimate the true population size (Sewell et al., 2013). In addition, we were not able to relate newt counts to quantified amounts of DNA because all successful amplifications occurred at DNA concentrations below the limit of quantification, the level at which we were able to estimate DNA abundance with an acceptable level of precision and accuracy. It is possible that with an eDNA assay that had greater precision and accuracy (i.e. able to quantify eDNA amounts at lower concentrations than the present study) we would see a clearer relationship between eDNA and newt abundance. However, better estimates of eDNA quantities are not the only factor affecting assessment of newt abundance: environmental factors influencing eDNA degradation, distribution of newts in the pond and water sampling methods probably all play a role in accurately relating eDNA amounts to newt abundance. Elsewhere in this special issue, Strickler et al. (2014) report on the effects of temperature, UV-B radiation and pH on the degradation of American bullfrog tadpole eDNA in the laboratory. Their results suggest that aquatic habitats that are colder, more protected from solar radiation (i.e. shaded), and more alkaline are likely to hold detectable amounts of bullfrog eDNA for longer than those that are warmer, sunnier, and neutral or acidic. Given that great crested newts occur

in ponds which span a considerable spectrum of shade, pH and temperature conditions, it will be important to translate these laboratory observations to field conditions as soon as practically possible.

Our results contrast with those of Thomsen et al. (2012) who found a positive correlation between DNA concentration and estimated population density based on conventional monitoring. Similarly Pilliod et al. (2013a) also reported that eDNA amounts could be related to density and biomass of tailed frog tadpoles and, to a lesser extent, giant salamander larvae and paedomorphic adults.

4.3. Methodological refinements of the eDNA method

The current study shows the value of eDNA for detecting presence/absence of great crested newts in ponds. However, as noted by Lodge et al. (2012) there remain 'striking gaps' in our understanding of the way in which field and laboratory protocols influence the detection of great crested newt eDNA, and how different environmental conditions affect the production, degradation and detection of the species' eDNA. Filling these gaps will make the use of the method, and its interpretation, more robust. Practically, important questions are 'What is the smallest number of newts that can be detected by the method?', 'What is the role of inhibitors in detecting eDNA?' and 'How likely is the method to detect transient visits to ponds when newts move between waterbodies in spring and summer for foraging?'. Methodologically, field techniques for pond sampling are still something of a black box. We have little understanding of the optimal locations for sampling eDNA in ponds, how eDNA is produced by different life stages of the great crested newt, how concentrations vary during the year (i.e. can newts be surveyed outside the breeding season) and whether it can be retrieved from shallow pond sediments (potentially an important source of both contamination and historical information on distributions). Pilliod et al. (2014) have advanced understanding of the factors that affect persistence of DNA in the water, but more information on persistence and distribution of within ponds is still needed. Recent observations on the distribution of carp eDNA in sediments, reported in this special issue by Turner et al. (2014), indicate that sediments are likely to be an important compartment for eDNA preservation. These authors found that carp DNA was 8 to 1800 times more concentrated in sediments than water and persisted for up to 152 days in sediments, about 5 times longer than any previously reported water column microbial eDNA persistence. If applicable to great crested newt eDNA, these observation have important implications in (a) confirming the need to avoid sediment contamination of water samples where the short-lived nature of eDNA is an advantage and (b) offering an alternative sampling compartment at sites where water column eDNA may be very sparse (e.g. sites anticipated to have small great crested newt populations). Empirical observations in the present project indicated that DNA is not evenly distributed throughout ponds. For example at one site where we suspected vegetation density could be a problem, we collected a water sample from very dense marginal floating mats of vegetation in which great crested newt DNA was not detected (a false negative), whereas water collected, using a pole, from the open centre of the pond did contain great crested newt DNA.

For volunteer surveys specifically an important methodological issue is the technique used for eDNA preservation. In the present project pure ethanol was the main preservative but is classified as a highly flammable substance by the Dangerous Substances Directive (67/548/EEC) and it is subject to a strict regulation for handling and shipping. Although suitable for professionally organised surveys safety and licensing regulations make the use of ethanol ultimately more problematic in volunteer-based surveys. The alternatives to ethanol – in which samples are filtered rather than preserved - may require pumps in the field (e.g. Goldberg et al., 2011) which can substantially increase the cost of sampling. Alternatively, if filtration is performed in the laboratory (Jerde et al., 2011; Takahara et al., 2012), samples must be delivered to the genetics facility within a few hours after sampling to avoid DNA degradation, which would pose significant practical constraints in a volunteer programme. Further development of the sampling methods will probably be required to avoid the use of chemicals, such as ethanol, which require special measures for shipping, storage and handling.

Given the likely increase in the use of eDNA methods in biodiversity monitoring we echo the comment of Lodge et al. (2012) that there is a need to systematically compare field and laboratory eDNA protocols. This includes the need to further evaluate the efficiency of different eDNA capture and extraction methods, and of water sample processing techniques. Thus Deiner et al. (2014) in this special issue have shown that there are significant differences in the amounts of eDNA detected by different eDNA analysis protocols. Also in this special issue, Takahara et al. (2014) found that less eDNA was extracted from water samples that were frozen, compared to unfrozen samples. Of particular importance is the need to ensure comparability of methods, both in field sampling techniques and in laboratory procedures, if consistently low false negative rates and low (or zero) false positive rates are to be maintained. In the field, cross-contamination seems the most likely route by which false positives may be generated, either by operator errors or 'natural' cross contamination (for example, by biological vectors such as water birds or inflows from other waterbodies). In the laboratory, high operating standards are needed to ensure low false negative rates (see for example the difference in positive detections between Rees et al., 2014 and the present work, dealing with the same species, in the same country and using modifications of the same method) and to maintain zero false positive rates, as the throughput of samples increases, particularly by ensuring that laboratory design, operation and protocols are similar to those originally developed for ancient DNA studies (Cooper and Poinar, 2000; Knapp et al., 2012). The maintenance of high standards is particularly important in environmental impact assessment where costly decisions about human infrastructure projects may be influenced by the recording (or not) of the species concerned.

4.4. Practical implications for the conservation of great crested newts and other aquatic species

The present study is first to demonstrate that eDNA methods can provide the basis for a practical large-scale monitoring program operating across the full national range of an endangered species, with the study sites spanning about 75% of the great crested newt's 217,000 km^2 range in Great Britain (Biggs et al., 2014), in an area with substantial geological, land-use and habitat diversity (c.f. Jerde et al. 2011 who studied an area of about 4000 km^2 in north-east Illinois in the United States and Takahara et al., 2012 working in an area of around 3500 km^2 on the south coast of Honshu island, Japan). Our results indicate that a single eDNA sample is as effective in determining whether newts are absent from a site as an intensive 4 visit/3 method (torching, bottle trapping, egg search) survey. However, eDNA surveys are much quicker in the field and have modest laboratory costs, already similar to those of conventional chemical water quality analysis. We estimate that it takes about 0.5 h (excluding time to get to the site) to collect an eDNA sample, compared to a total of about 40 h to undertake a conventional 4 visit survey. The traditional survey takes substantially longer in part because of safety legislation which requires that surveyors entering the water or working at night-time must operate in pairs (i.e. the survey requires 2 people

working 20 h each). At standard UK field staff rates this represents about €20/site for eDNA sampling compared to €1450 for a traditional field survey. In the present study set-up costs for target species tissue collection and primer design were equivalent to €10/site and commercial charges for sample transport, analysis (extraction and qPCR) were about €120/sample, including the costs of laboratory establishment and running costs. In total, eDNA field and laboratory costs were about €140/site compared to €1450/site for traditional field sampling. The comparison of eDNA and traditional survey costs in the review by Herder et al., 2014 provides further evidence that eDNA surveys are often (although not always) more cost effective for aquatic vertebrates than traditional surveys.

We have shown elsewhere that, to detect a 30% change in pond occupancy by great crested newts with 80% power would require data on newt presence from 1100 ponds in England, 600 ponds in Wales and 380 ponds in Scotland on each occasion a survey was undertaken (Biggs et al., 2014). It is very unlikely that such a survey could be organised, either by professional biologists or volunteers, if it was necessary to undertake four visits using 3 survey methods to be certain that newts were absent from sites (*sensu* Sewell et al., 2010). This practical difficulty reflects the limitations both of funding for a professional survey and the number of volunteer surveyors with sufficient time and skills to undertake the work voluntarily. Volunteer surveys on this scale have also been hampered by a lack of funds for professional organisers to arrange permissions to visit sites, which otherwise present a very substantial barrier to volunteers undertaking surveys (Williams et al., 2013). As a result it has proved difficult for existing volunteer survey programmes in the UK to create large enough datasets for great crested newt monitoring, even though these programmes do have enough sites and surveyors (and therefore statistical power) for assessing change in more widespread species such as the Common Frog (*Rana temporaria*) or Smooth Newt (*Lissotriton vulgaris*) (Wilkinson and Arnell, 2013).

The results of the present survey suggest that it may be practical to develop large scale eDNA monitoring programmes for other groups of aquatic organisms (e.g. micro and macroinvertebrates, fish) as the effectiveness of the method for different species and environments is established (e.g. Goldberg et al., 2013; Deiner and Altermatt, 2014). The technique may be particularly important for small waterbodies (ponds, small lakes, ditch networks, headwaters and small streams) which are enormously abundant but currently little monitored (Downing, 2010; Meyer et al., 2003), even though they support a large proportion of freshwater species and make up a large part of the water environment (Williams et al., 2004; Verdonschot et al., 2011). There is an urgent need to follow on from the initial studies such as those of Ficetola et al. (2008), Goldberg et al. (2011), Thomsen et al. (2012) and Pilliod et al. (2013a) who provided initial proofs of the concept.

4.5. Practical implications of eDNA for volunteer surveys programmes

Volunteer survey programmes are growing in popularity but face organisational, data collection and data use issues (Conrad and Hilchey, 2011). A number of projects based on traditional survey techniques have already overcome many of these problems indicating that there is great potential and scientific credibility in the use of volunteers to deliver well designed monitoring programmes (Botham et al., 2013; Risely et al., 2012). However, in freshwater habitats in particular, we expect eDNA to substantially increase the range of species and community monitoring work that could be undertaken by 'citizen scientists'.

We anticipate that eDNA surveys will play a valuable role in generating interest in biological recording in freshwaters, particularly by allowing non-specialists to make quick initial detections of cryptic or scarce species (e.g. Herder et al., 2013) which would normally require considerable time in the field or expertise. Traditional citizen surveys in freshwater are currently mainly confined to taxonomic groups which are well-known taxonomically such as birds, amphibians, plants, mammals and dragonflies. Groups which are more taxonomically challenging can often only be identified at higher taxonomic levels which, although potentially correlated with metrics of species richness or rarity (Fore et al., 2001), does substantially limit the use of such data for species based conservation programmes. Apart from taxonomy, aquatic surveys generally present methodological challenges because of the inherent complexity and levels of training involved in surveying aquatic environments. The use of eDNA techniques could greatly extend the range of aquatic biota that can be regularly, and with high taxonomic fidelity, be surveyed by non-specialists. eDNA can also help overcome the data use issues because, with appropriate genetic protocols, the risks of false positive records (the main concern with species identifications by non-specialists) is largely eliminated. However, although eDNA is attractive in cost terms it must still be borne in mind that the most effective volunteer programmes typically require an element of centrally-funded professional co-ordination to make the best use of the volunteer resource. As Roy et al. (2012) note: 'Citizen science can be cost effective but it is not free'.

Conflict of Interest

None of the authors have financial conflicts of interest.

Role of the funding source

The authors were responsible for the study design, which was then approved by the project steering group, and for the collection, analysis, and interpretation of data. The funding agencies supported the decision to submit the paper for publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.11.029.

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