District Level Licensing Monitoring

Extraction and analysis of Great Crested Newt eDNA samples via single species and metabarcoding 2024

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Foreword

This work was commissioned to build on a recent evidence review of great crested newt environmental DNA (eDNA) monitoring protocols (NECR476), and, on a small scale, compare results from two eDNA capture methods (ethanol precipitation and filtration) for the detection of the species following on from the April 2024 report for the same purpose (NECR534). The work here also seeks to identify whether metabarcoding is comparable with single-species detection of great crested newt. This report covers new areas in England to further the current understanding. The results from this project will contribute to an evaluation of the current methodology for monitoring great crested newt, and help support a shift towards a revised methodology, if the need for an updated protocol is identified.

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Executive summary

The aim of this ongoing surveillance project is to compare the results of great crested newt environmental DNA (eDNA) analysis from water that has been sampled by ethanol precipitation and filtration methods. This survey effort will deliver data to be used to monitor the success of habitat creation associated with District Licensing strategies for great crested newt in addition to evaluation of a filtration method for great crested newt eDNA monitoring. The monitoring will enable review and where necessary adjustment to ensure that schemes are contributing towards the favourable conservation status of the species. The analysis of filter samples was performed using the single species great crested newt assay following laboratory protocols in: Analytical and methodological development for improved surveillance of the great crested newt, and other pond vertebrates - WC1067 (Biggs and others 2014). This report also describes the analysis of a subset of water samples by DNA metabarcoding, to both determine whether great crested newt can be reliably detected using these techniques, and also to describe the 12S vertebrate community from which the samples came from. This data (year 2 of this survey effort) will add to the evidence base for any future update of the WC1067 protocol. This study was carried out across Natural England led District Level Licensing scheme areas in England.

To do this, Habitat Delivery Bodies surveyed 90 ponds for Natural England using the standard ethanol precipitation-based sampling kits (as per WC1067). At these same site visits a sample was also taken using a Sterivex-HV Pressure Filter Unit (0.45µm pore size) with the addition of an ethanol based preservative solution. In addition to the 90 pond samples, there were 10 additional field blanks taken; one sample was not returned, giving 99 results in total. The ethanol precipitation samples were analysed by Cellmark (a great crested newt eDNA analysis service provider) using the methods detailed in WC1067 and the results were shared with RSK ADAS to allow comparison with the results of the filter-based sample results for this report. After DNA was extracted from the filter samples, great crested newt analysis was performed using the methods in WC1067 followed by a sub-set of ten of the samples (several of which were positive for great crested newt with both sampling methods) being analysed by 12S metabarcoding.

Great crested newt results comparison of the two sample collection methods showed relatively good agreement with 30 of the samples being positive for great crested newt eDNA by both methods. However, 11 additional samples were positive for filtration collected samples (41 in total were positive) and 3 for ethanol precipitation collected samples (33 in total were positive). Metabarcoding of 12S DNA for vertebrate species resulted in 21 species being detected across the 10 samples with 6 of the 10 samples detecting great crested newt DNA.

Contents

Introduction

Natural England is the Government's advisor for the natural environment. It provides practical advice on how to safeguard England's natural wealth for the benefit of everyone. RSK ADAS is an environmental consultancy which exists to provide ideas, specialist knowledge and solutions to secure our food and enhance the environment.

Natural England wishes to deliver data to be used to monitor the success of habitat creation associated with District Licensing strategies for great crested newt. District Level Licensing (DLL) depends upon the creation of many new ponds for great crested newts and the restoration of existing ponds that are currently not suitable for use by great created newts. This monitoring will enable review and, where necessary, adjustment to ensure that schemes are contributing towards the Favourable Conservation Status (FCS) of the species.

The current methodology for collection of water samples for analysis for great crested newt eDNA uses ethanol precipitation of eDNA, one of the first methods described for eDNA recovery (Ficetola and others 2008, Biggs and others 2014). Since then, there have been extensive development and use of filter based eDNA capture for the sampling of eDNA from waterbodies (Turner and others 2014, Wilcox and others 2015, Hosler 2017, Deiner and others 2018, Sepulveeda and others 2019 etc.) and as such filtration has now largely replaced ethanol precipitation as the method of choice for capturing eDNA from water samples. This study will also provide additional evidence to Natural England to contribute to a future update of the WC1067 protocol.

Aims and Objectives

The overall aim of the project was for the resulting data to be used to monitor the success of habitat creation associated with District Licensing strategies for great crested newt to allow review and where necessary adjustment to ensure that schemes are contributing towards the FCS of the species. The main aim was to compare the use of ethanol precipitation-based sampling methodology with filtration-based sampling for great crested newt detection as described in WC1067. Additionally, a further aim was to evaluate the efficacy of metabarcoding for the detection of great crested newt from the filtration-based samples.

This report details the methodology employed, the results obtained and, discussion of the results. All data will be made available for further study.

Materials and Methods

Sample collection

Six Natural England DLL scheme areas were chosen to collect filtration samples in 2024, alongside standard precipitation surveys. Sample collection was completed by Habitat Delivery Bodies (HDBs) as follows: Greater Manchester Ecology Unit (GMEU); Tees Valley Wildlife Trust; Northumberland Wildlife Trust; Durham Wildlife Trust; Wildlife Trust for Bedfordshire, Cambridgeshire, and Northamptonshire (WT BCN); and Farming and Wildlife Advisory Group East (FWAG) (Table 1).

A subset of ponds within each area was chosen for sampling. This was a semi-random selection from ponds created or restored prior to 1st May 2023, ensuring a spread of ponds within each scheme area across the following criteria:

- Geographic spread across the scheme area based on the pond's district.
- Age of pond (ranging from the first to the fourth year of monitoring).
- Whether the pond was a creation or restoration.
- Previous monitoring results via precipitation eDNA testing (if carried out) – present, absent, or inconclusive (and if inconclusive, whether this was due to inhibition or degradation).
- Whether the pond is within a 'core', 'fringe' or 'outside' the modelled DLL Strategic Opportunity Areas for great crested newts.

Samples were taken using 0.45 µm sterivex filter kits prepared and supplied by RSK ADAS. Each kit contained the following:

a. One sterile 0.45 µM pore size PVDF Sterivex filter.
b. One sterile 40 mL sample dipper.
c. One sterile 50 mL luer-lock syringe.
d. Two sterile seals for filter.
e. One pair disposable gloves.
f. One sterile sampling bag.
g. One sterile 50 mL tube to house the filter unit after sampling during transport.
h. One 10 mL syringe containing ethanol-based preservative.

Those taking samples were supplied with written guidance (Appendix 1) and contact details for RSK ADAS for any queries. HDBs were advised to use bottled water for their control samples. HDBs returned their filtration samples to RSK ADAS via the Royal Mail, these were stored at 4°C in a fridge for up to ten weeks before processing.

Table 1. Number of samples collected in each area.

Region	Planned Number of Samples by Area
GMEU	30 ponds, 3 controls
Tees Valley WT	5 ponds, 1 control
Northumberland WT	13 ponds, 1 control
Durham WT	13 ponds, 1 control
WT BCN	15 ponds, 1 control
FWAG East	15 ponds, 2 controls
Total	100

Laboratory Standard and Specifications

All laboratory activities associated with DNA analysis are subject to errors if quality control is inadequate. DNA analysis in RSK ADAS laboratories follows a unidirectional workflow with separate laboratories and staff to act as a physical separation for the different aspects of the analysis work. This greatly reduces the potential for contamination of samples/reagents etcetera. 'Blank' PCRs (sterile water rather than DNA) are used to monitor for reagent/procedural contamination, and in addition positive control samples are used to increase confidence in the results and identify any cross-contamination issues, should they occur.

DNA extraction from filters

DNA was extracted from all filters using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions with minor modifications (below) and finally resuspended in 200 µL of elution buffer (Appendix 2). Extraction blanks (sterile water in place of filter or extraction buffers only) were included during each set of DNA extractions to monitor for any cross-contamination during this step.

- 540 µL ATL buffer, 300 µL AL buffer, and 50 µL proteinase K (all components of the kit) used instead of standard amounts.
- Addition of $5.7x10^{-5}$ µg/µL synthetic control DNA for DNA extraction efficiency monitoring
- 500 µL 100% ethanol used instead of standard amount.

All DNA extracts for metabarcoding analysis were quantified using a Qubit 3.0 Fluorometer (Invitrogen) following the manufacturer's instructions then stored at -20 ºC prior to PCR set up (Appendix 2).

DNA extraction was monitored by the addition of known concentration of a synthetic DNA control to the ATL added to the filter. The recovery of this synthetic DNA was measured by a qPCR assay specific for the sequence of the synthetic DNA control (Appendix 2) and was used as a proxy to monitor total eDNA extraction with acceptable limits being within two standard deviations of the average Cq value (95% of samples should be within this range).

eDNA assay

Great crested newt eDNA assay was performed in accordance with WC1067 which amplifies an 81 bp fragment of the cytochrome b gene (Table 2, Appendix 2). All samples were subjected to testing for eDNA recovery and sample extract inhibition.

Table 2. Great crested newt primer and probe sequences (Thomsen and others 2012).

Oligo Name	Sequence (5' - 3')
TCCBF	CGTAAACTACGGCTGACTAGTACGAA
TCCBR	CCGATGTGTATGTAGATGCAAACA
TCCB.probe	FAM-CATCCACGCTAACGGAGCCTCGC-BHQ1

Metabarcoding PCR

The primer combination used for the first round PCR amplification was 12S forward and 12S reverse (Riaz and others 2011). Overhang adapter sequences (Table 3) were included at the 5’ end of the primers to ensure compatibility with Illumina index and sequencing adapters (Illumina 2012). PCRs included one negative control (ddH_2O in place of DNA); two DNA extraction blanks; a positive control sample (*Scomber scombrus* Atlantic Mackerel); and the ten selected pond DNA samples. Pond samples were selected for metabarcoding analysis by Natural England upon completion of the great crested newt qPCR analysis.

Table 3. Primers used for metabarcoding first round PCR.

Primer Name	Oligonucleotides (5'-3')	Reference
12S forward (plus adapter)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TAGAACAGGCTCCTCTAG	Riaz (2011)
12S reverse (plus adapter)	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TTAGATACCCCACTATGC	Riaz (2011)
Index 1	CAAGCAGAAGACGGCATACGAGATXXXXXXXX**GTCTCGTGGGCTCGG**	Illumina (2011)
Index 2	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXX**TCGTCGGCAGCGTC**	Illumina (2011)

For the 12S primers, sequences marked in bold are the first round PCR primer adapter sequences, the remainder are the 12S locus-specific primer sequences. For the Index primers, sequences marked in bold are Illumina overhang adapter sequences, Index 1 and 2 sequences are marked with Xs as this sequence is variable for each different sample, those in normal text are the P5 and P7 sequences. Index 1 (i7) and Index 2 (i5) are examples of the type of primers used with the Index sequence itself being altered for different samples.

The first round PCR amplicons for each sample were pooled and run on a 1.5% agarose gel. Any bands of the correct size were excised and purified using NucleoSpin® Gel and PCR Clean-up purification columns (Macherey-Nagel) according to the manufacturers' instructions (Appendix 2).

Sequence Library Preparation and Sequencing

The second round of PCR or 'Index' PCR was performed by Source Bioscience to add molecular identification (MID) tags (unique 8-nucleotide sequences) and Illumina MiSeq sequencing adapters to the first round PCR products.

The indexed amplicons were quantified via a fluorometric method involving QuantiFluor dsDNA assay (Promega); and qualified using electrophoretic separation on the Agilent Fragment Analyzer 5300. This concentration and sizing information has been used to calculate the molarity of each sample. All samples passed QC checks carried out by Source Bioscience. Libraries were then pooled in equimolar amounts to create one library for Illumina sequencing. The amplicon library pool was spiked with 20 % PhiX Control v3 library (Illumina) and run on the Illumina MiSeq using a MiSeq Reagent Kit v2 500 cycle kit (Illumina), to generate 250-bp paired-end reads. PhiX DNA is derived from the small, well characterized bacteriophage PhiX genome, it is a concentrated Illumina library (10 nM in 10 µl) that has an average size of 500 bp and consists of balanced base composition at ~45% GC and ~55% AT and serves as an in-run QC for the Illumina sequencing.

Bioinformatic Processing

Data processing was performed on an Intel i7 PC running Ubuntu Linux 20.04.4 LTS. In an initial step, paired end reads were trimmed using trimmomatic 0.39 (Bolger, Lohse and Usadel, 2014) to remove Miseq adapters, to clip low quality and unpaired reads, and to truncate the sequence if the average phred score of a 5nt sliding window dropped below 25. An example command line to process a demultiplexed FASTQ sequence file was: java -jar trimmomatic-0.39.jar PE R1_001.fastq R2_001.fastq read1_paired_R1_001.fastq read1_unpaired_R1_001.fastq read2_paired_R2_001.fastq read2_unpaired_R2_001.fastq ILLUMINACLIP:NexteraPE-PE.fa:2:30:10:2:True LEADING:3 TRAILING:3 MINLEN:36 SLIDINGWINDOW:5:25

Paired reads were merged using FLASH 1.2.11 (Fast Length Adjustment of SHort reads, Magnoc and Salzberg, 2011) to convert paired end reads (R1 and R2 in the MiSeq platform) to a single merged read using a minimum overlap of 80 nucleotides and a maximum of 150 nucleotides.

After converting DNA sequences from FASTQ format to FASTA format using SeqKit (Shen and others 2016), template specific PCR primers at the 5' and 3' ends were removed using the "linked adapter" option of Cutadapt 3.5 (Martin, 2011) with a 10% error rate within the primer site i.e. 2 bp variants allowed per primer. Only trimmed sequences i.e. those containing both matching primer sequences were retained.

Before taxonomic assignment, standard Linux tools were used to identify 100% identical reads and condense them down to a single read to minimise time-consuming and repetitive BLAST searches, However, a record of the frequency of replicate sequences was maintained. Any reads with less than 5 replicates were excluded from the BLAST search as these were considered sequencing errors and omitted from further analyses (Harper and others 2018).

A custom 12S BLAST database was created on 9 October 2023 from the National Centre for Biotechnology Information (NCBI) database using the search terms 'vertebrata' AND '12S' before downloading the records in FASTA format. A total of 190K sequences downloaded from NCBI were included in the final database.

BLAST searching was performed using the "megablast" program which is optimised to identify alignments in highly similar sequences and returned the top hit for each query sequence in a custom tabulated format. An e-value of 1e-15 was set; higher values such as one or ten return a larger list of more low-scoring hits, and actual e-values returned were in the order of 1e-150 for a full-length alignment.

A custom perl script filtered the BLAST output, identifying hits sharing an accession number and passing a set of criteria covering the percentage similarity between the query sequence and the database sequence (≥97%), and having a query alignment length difference less than 6 bp. Read counts for each sequence passing the similarity and query

alignment length filters were pooled based on accession number to generate a final frequency count for each accession.

Results

DNA Extraction

DNA was extracted from the 99 individual filter samples returned to RSK ADAS and the DNA quantified. Volumes of water sampled by the Habitat delivery bodies (Table 4) and the volume of recovered preservative were also recorded (~2 mL per filter). There was no recorded incidence of leakage of preservation solution from the filters either during transit or storage. Our DNA extraction control PCR suggest all samples were within acceptable limits except for three samples (01-017, W1-039, W1-148).

Table 4. Filter sample information. Some water volumes were not supplied by the samplers and are marked as 'unknown'. Samples marked 'Control' were not collected via ethanol precipitation and thus there were no corresponding precipitation results for these samples. DNA concentrations after 12S PCR amplicon clean up are only available for the 10 samples chosen for metabarcoding, all others are marked 'N/A'.

Kit barcode	Sample ID	Volume water filtered (mL)	great crested newt result filtration (positives out of 12)	great crested newt result ethanol precipitation (positives out of 12)	PCR amplicon DNA cocentration (ng/µL)
GCN006913	O1-001	250	0/12	0/12	N/A
GCN007501	O1-002	290	0/12	0/12	N/A
GCN007467	O1-017*	380	8/12	1/12	N/A
GCN007018	O1-022	Rubbed off tube	12/12	0/12	N/A
GCN007459	O1-024	192	10/12	2/12	N/A
N/A	O1-024 Control	480	0/12	N/A	N/A
GCN008095	O1-030	Rubbed off tube	0/12	0/12	N/A
GCN006890	O1-036	270	0/12	0/12	N/A
GCN006790	O1-037	Rubbed off tube	0/12	0/12	N/A
GCN008048	O1-046	240	10/12	12/12	N/A
GCN008078	O1-047	64	0/12	0/12	0.48
GCN008516	O1-049	60	0/12	1/12	N/A
GCN006932	O1-062	200	0/12	0/12	N/A
GCN006944	O1-063	270	9/12	0/12	N/A
GCN006716	O1-064	260	3/12	0/12	N/A
GCN006757	O1-065	200	3/12	1/12	N/A
GCN007122	O1-066	200	0/12	0/12	N/A
GCN007497	O1-067	200	2/12	6/12	0.55
GCN007152	O1-070	500	11/12	2/12	N/A
GCN006794	O1-071	450	0/12	0/12	N/A
GCN006957	O1-074	200	0/12	0/12	N/A
GCN006946	O1-078	500	0/12	0/12	N/A
GCN008137	O1-119	360	0/12	0/12	37.4
GCN006809	O1-129	500	12/12	3/12	N/A
GCN007107	O1-130	500	0/12	0/12	N/A
N/A	O1-130 Control	500	0/12	N/A	N/A
GCN007167	O1-140	200	12/12	11/12	N/A
GCN008321	O1-144	360	0/12	0/12	N/A
N/A	O1-144 Control	1500	0/12	N/A	N/A
GCN008068	O1-145	300	12/12	12/12	N/A
GCN008506	O1-146	270	12/12	12/12	N/A
GCN008782	O1-170	500	8/12	1/12	N/A
GCN008776	W1-003	500	0/12	0/12	N/A

Kit barcode	Sample ID	Volume water filtered (mL)	great crested newt result filtration (positives out of 12)	great crested newt result ethanol precipitation (positives out of 12)	PCR amplicon DNA cocentration (ng/µL)
GCN008794	W1-004	500	0/12	0/12	N/A
GCN008723	W1-005	500	0/12	0/12	N/A
GCN008905	W1-006	450	1/12	0/12	N/A
GCN007300	W1-007	750	0/12	0/12	N/A
GCN008666	W1-008	465	0/12	0/12	N/A
N/A	W1-008 Control	550	0/12	N/A	N/A
GCN007342	W1-009	300	2/12	0/12	N/A
GCN007325	W1-010	450	1/12	2/12	1.78
GCN008627	W1-019	120	0/12	0/12	N/A
GCN008629	W1-020	75	0/12	0/12	N/A
GCN008592	W1-037	Not supplied	0/12	0/12	N/A
GCN008710	W1-039*	Not supplied	0/12	0/12	N/A
GCN008631	W1-042	70	0/12	0/12	N/A
GCN006806	W1-045	250	0/12	0/12	N/A
GCN007060	W1-046	300	0/12	0/12	N/A
GCN008606	W1-057	Not supplied	0/12	0/12	N/A
GCN008906	W1-058	Not supplied	0/12	0/12	N/A
GCN006911	W1-060	60	0/12	0/12	N/A
N/A	W1-060 Control	90	0/12	N/A	N/A
GCN008595	W1-063	45	0/12	0/12	N/A
GCN008602	W1-088	200	0/12	0/12	N/A
N/A	W1-088 Control	200	0/12	N/A	N/A
GCN008698	W1-098	340	0/12	0/12	N/A
GCN008679	W1-099	300	0/12	0/12	N/A
GCN007287	W1-100	450	0/12	0/12	N/A
GCN008706	W1-101	500	0/12	0/12	N/A
GCN008671	W1-102	350	0/12	0/12	N/A
GCN008680	W1-120	200	8/12	1/12	1.66
GCN008726	W1-121	200	1/12	2/12	N/A
GCN008615	W1-122	200	12/12	8/12	N/A
GCN008681	W1-123	200	9/12	2/12	N/A
GCN007090	W1-148*	Not supplied	0/12	1/12	0.99
GCN007132	W1-166	100	0/12	0/12	N/A
GCN007394	Y1-007	500	2/12	2/12	N/A
GCN007416	Y1-010	250	0/12	0/12	N/A
N/A	Y1-010 Control	500	0/12	N/A	N/A
GCN007386	Y1-042	500	6/12	0/12	3.48
GCN007349	Y1-045	500	0/12	0/12	N/A
GCN008735	Y1-060	500	0/12	0/12	N/A
GCN008720	Y1-064	500	5/12	2/12	N/A
GCN008795	Y1-065	75	6/12	1/12	N/A
GCN007388	Y1-066	75	12/12	1/12	6.46
N/A	Y1-066 Control	300	0/12	N/A	N/A
GCN007374	Y1-069	500	5/12	0/12	N/A

Kit barcode	Sample ID	Volume water filtered (mL)	great crested newt result filtration (positives out of 12)	great crested newt result ethanol precipitation (positives out of 12)	PCR amplicon DNA cocentration (ng/µL)
N/A	Y1-069 Control	500	0/12	N/A	N/A
GCN007375	Y1-070	500	2/12	0/12	N/A
GCN008805	Y1-073	500	12/12	2/12	46.2
GCN008770	Y1-074	500	9/12	3/12	N/A
GCN008815	Y1-076	500	0/12	0/12	N/A
GCN007326	Y1-083	500	5/12	2/12	N/A
GCN007418	Y1-085	500	12/12	12/12	N/A
GCN007333	Y1-086	500	0/12	0/12	N/A
GCN007290	Y1-088	500	7/12	0/12	N/A
GCN007376	Y1-089	500	9/12	12/12	N/A
GCN007288	Y1-090	500	11/12	3/12	N/A
GCN007335	Y1-095	500	0/12	0/12	N/A
GCN007407	Y1-101	500	0/12	0/12	N/A
GCN007373	Y1-102	500	4/12	0/12	N/A
GCN007347	Y1-106	500	11/12	1/12	N/A
GCN008790	Y1-114	500	10/12	3/12	26.4
GCN008809	Y1-115	500	2/12	1/12	N/A
GCN008733	Y1-116	500	0/12	1/12	N/A
GCN008652	Y1-117	500	0/12	0/12	N/A
GCN008716	Y1-118	500	0/12	0/12	N/A
GCN008722	Y1-119	500	4/12	0/12	N/A
GCN007346	Y1-129	500	12/12	12/12	N/A

Dark grey shading represents samples that are positive for great crested newt by both sampling methods, and light green shading represents samples that are only positive for great crested newt with one sampling method.

*marks those samples which were shown to have a poor DNA extraction from filters i.e. extraction control PCR was outside of acceptable limits.

Species-specific great crested newt results

A total of 30 of the samples were positive for great crested newt eDNA by both ethanol precipitation and filtration with 3 and 11 additional positive samples respectively by only one sampling method (Figure 1). The three additional positives observed when sampled using ethanol precipitation all showed very low levels of positivity (1/12). Of the 11 additional positives identified from the filtration samples five had low PCR scores: one had a PCR score of 1/12; two a PCR score of 2/12; and one a score of 3/12. Only three of the additional positives (O1-022 and O1-063) had high PCR scores of 12/12 or 9/12 respectively. The remaining five samples had medium PCR scores ranging from 4/12 to 7/12.

All field blanks were negative for great crested newt and all extraction blanks carried out by RSK ADAS were negative for great crested newt. All but one sample was within acceptable limits for inhibition (O1-030 was 1/2 replicates outside acceptable limits). As per the methods in WC1067, this sample was diluted 1 in 2 prior to great crested newt PCR analysis. The remaining 55 filter samples were negative for great crested newt eDNA, however, three of these samples (O1-017, W1-039, and W1-148) were found to be outside of acceptable limits for the DNA recovery control when defining acceptable limits as within two standard deviations of the average Cq value (95% of samples should be within this range) i.e. you would expect 5% of samples to be outside this range. When applying a nominal Ct cutoff of three cycles above the expected Ct if 100% of DNA was recovered the same three samples were found to be outside of acceptable limits and therefore indicated had poor DNA recovery.

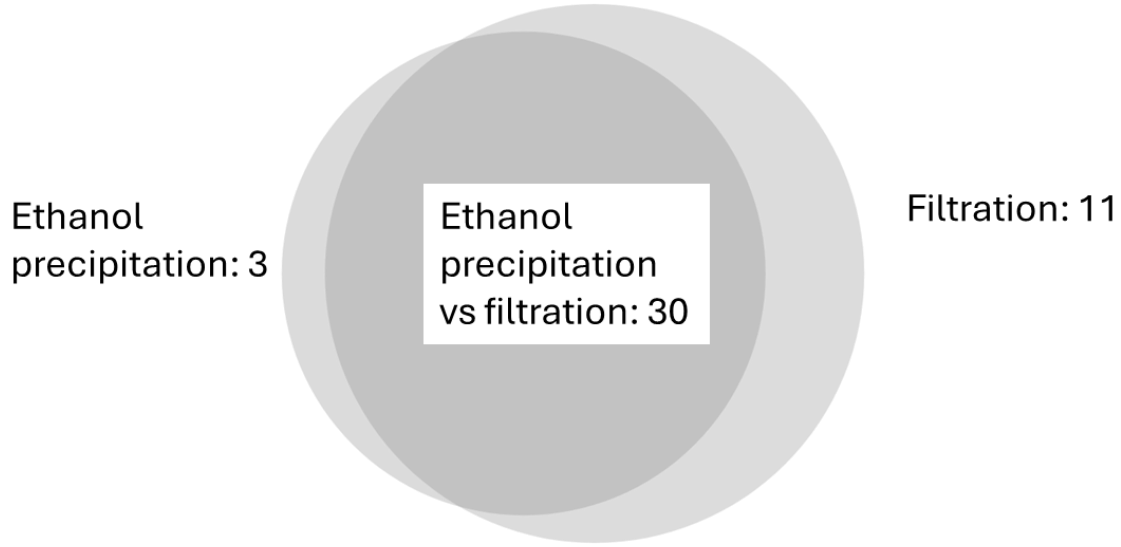

Figure 1. Venn diagram showing the number of great crested newt positive samples by one or both methods of sample collection.

Metabarcoding PCR and Library Production

All ten samples chosen for metabarcoding were successfully amplified, the amplicon DNA quantified and sent for indexing PCR and Illumina sequencing (Table 4). The ten samples were selected to represent six ponds from across the HDBs that were positive for great crested newt with both precipitation and filtration methods, two ponds that only tested positive with either the precipitation method or filtration method; and two ponds that were negative for great crested newt by both methods.

Bioinformatics and Data Analysis - Sequencing results

For the 12S analysis, of the sequences that passed all filters and went onto taxonomic assignment a total of 1.14M sequences (~97.7% of sequences) were assigned a taxonomic identification which represented 21 species including fish, birds, mammals, and amphibians. A total of 27K sequences (~2.3% of sequences) were unassigned. Unassigned sequences represented those with less than 98% sequence identity to sequences within reference libraries and those that did not match to anything in the database due to gaps in the reference libraries. The percentage of assigned and unassigned sequence reads per sample are shown in Figure 2. Results are shown in Table 5 and Appendix 3. Samples contained between five and ten species of vertebrate with an average of eight species per pond. The most common species were smooth newt, great crested newt and mallard duck. For the full list of 12S metabarcoding results, please see Appendix 3.

Six of the eight great crested newt PCR positive samples chosen for metabarcoding were found to contain great crested newt via metabarcoding (Table 6). One of the samples that was only great crested newt positive via one of the sampling methods (filtration) was found to be positive for great crested newt when using metabarcoding. Five of the samples which were positive using both sampling methods (O1-067, W1-120, Y1-066, Y1-073, and Y1-114) were also positive via metabarcoding. The remaining sample with low PCR scores via qPCR using both sampling methods (W1-010) was negative via metabarcoding. The two samples that were negative for great crested newt via qPCR using both methods were also negative for great crested newt via metabarcoding.

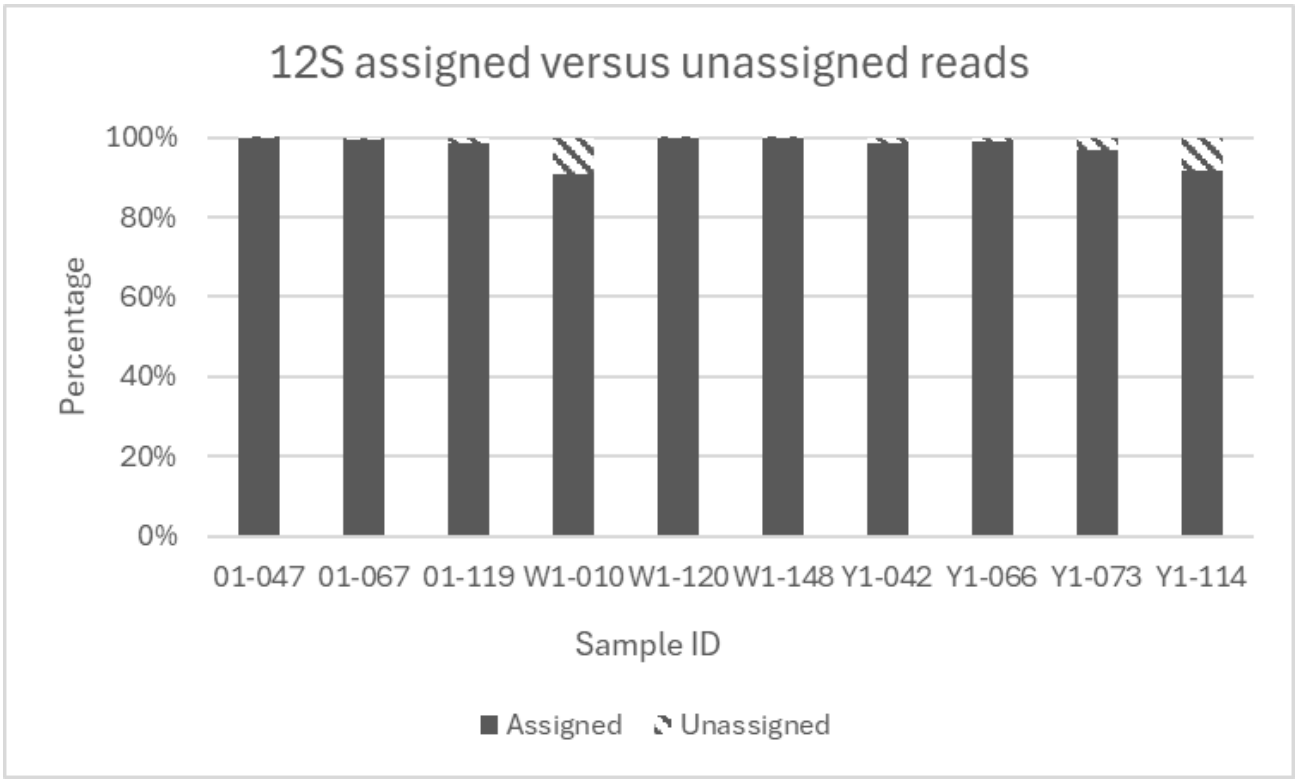

Figure 2. 12S proportion of assigned and unassigned sequence reads.

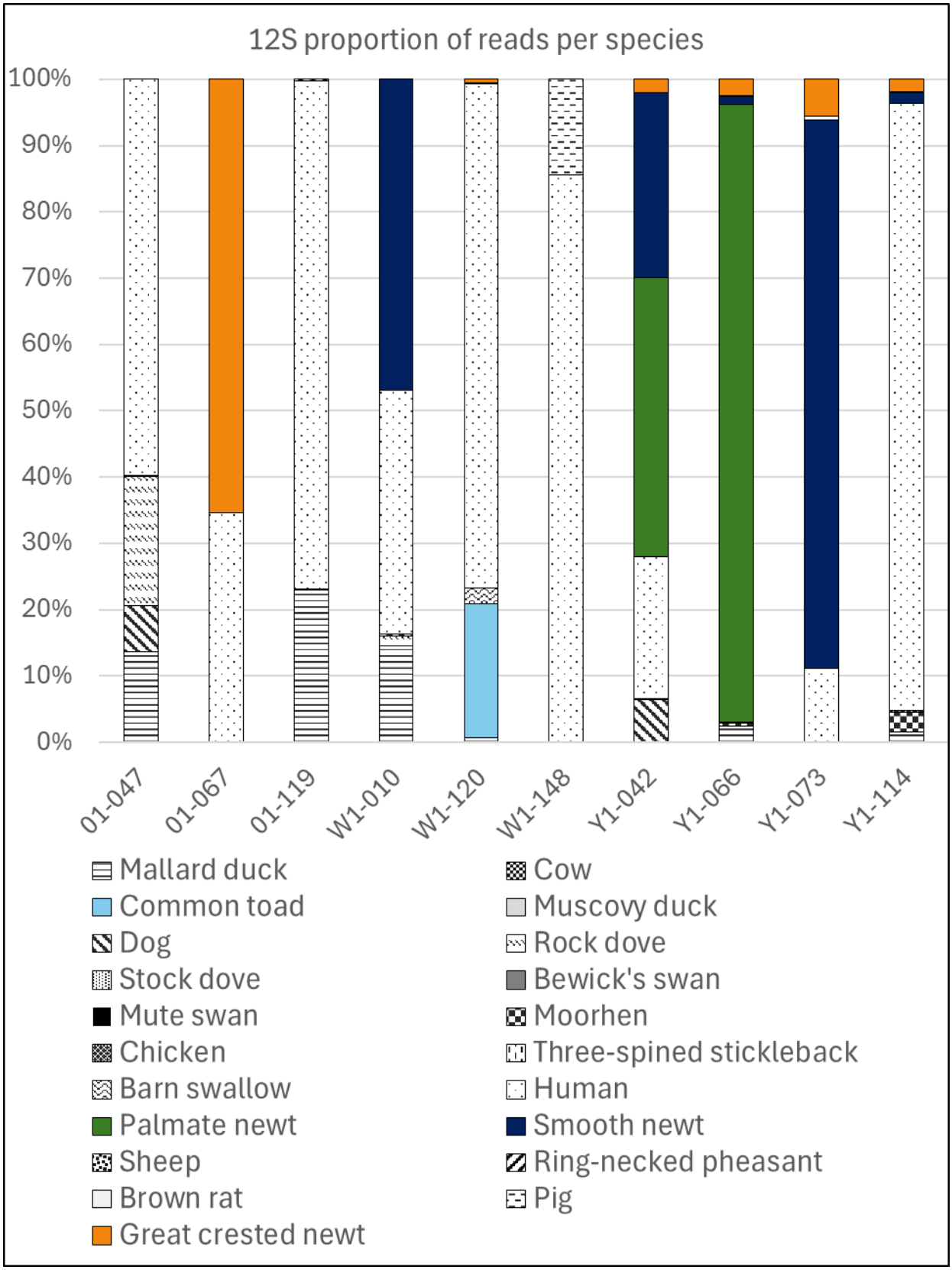

Figure 3. 12S proportion of assigned reads per species

Table 5. Number of ponds containing species (vertebrates) using 12S primers.

Scientific name	Common name	no. of ponds
Anas platyrhynchos	Mallard duck	7
Bos taurus	Cow	1
Bufo bufo	Common toad	2
Cairina moshata	Muscovy duck	2
Canis lupus familiaris	Dog	2
Columba livia	Rock dove	3
Columba oenas	Stock dove	1
Cygnus columbianus bewickii	Bewick's swan	1
Cygnus olor	Mute swan	2
Gallinula chloropus	Moorhen	3
Gallus gallus	Chicken	1
Gasterosteus aculeatus	Three-spined stickleback	1
Hirundo rustica	Barn swallow	1
Homo sapiens	Human	10
Lissotriton helveticus	Palmate newt	2
Lissotriton vulgaris	Smooth newt	7
Ovis aries	Sheep	1
Phasianus colchicus	Ring-necked pheasant	1
Rattus norvegicus	Brown rat	1
Sus scrofa domesticus	Pig	2
Triturus cristatus	Great crested newt	6

Table 6. qPCR and metabarcoding results comparison

Sample name	great crested newt result filtration (number of positives out of 12)	great crested newt result ethanol precipitation (number of positives out of 12)	Metabarcoding result for great crested newt (numbers indicate read count where relevant)
01-047	**0/12**	0/12	Negative
01-067	**2/12**	6/12	Positive; 20,398
01-119	**0/12**	0/12	Negative
W1-010	**1/12**	2/12	Negative
W1-120	**8/12**	1/12	Positive; 1,725

Sample name	great crested newt result filtration (number of positives out of 12)	great crested newt result ethanol precipitation (number of positives out of 12)	Metabarcoding result for great crested newt (numbers indicate read count where relevant)
W1-148	**0/12**	1/12	Negative
Y1-042	**6/12**	0/12	Positive; 2,380
Y1-066	**12/12**	1/12	Positive; 3,090
Y1-073	**12/12**	2/12	Positive; 6,786
Y1-114	**10/12**	3/12	Positive; 1,919

Dark grey shading represents samples that are positive for great crested newt by qPCR using both sampling methods and metabarcoding. Light green shading represents samples that are positive for great crested newt by qPCR using one sampling method and metabarcoding.

Discussion

For single-species testing of great crested newt, the use of filtration and ethanol precipitation for sample collection were reasonably comparable with filtration giving an additional 11 great crested newt positive results than ethanol precipitation, and ethanol precipitation giving an additional three great crested newt positive results than filtration. For those three samples which were positive for great crested newt when collected using ethanol precipitation (O1-049, W1-148, and Y1-116), all had a PCR score of 1 of 12 replicates being positive for great crested newt i.e. all three had a very low PCR score. One of these samples (O1-049) only achieved 60 mL of filtered water (i.e. 2/3 of the volume of water collected for ethanol precipitation). One had 500 mL water filtered (Y1-116) which was more than the average volume filtered (450 mL, all samples; 377 mL, great crested newt positive samples), and the third filtration volume was not supplied (W1-148). Of the 11 additional samples positive for great crested newt when collected via filtration (O1-022, O1-063, O1-064, W1-006, W1-009, Y1-042, Y1-069, Y1-070, Y1-088, Y1-102, and Y1-119) there was a range of PCR scores (12, 9, 3, 1, 2, 6, 5, 2, 7, 4, and 4 out of 12 respectively) and a range of water volumes filtered (rubbed off tube, 270, 260, 450, 300, 500, 500, 500, 500, 500, and 500 mL respectively). Ten of the samples were from volumes of water of between 3 and 5.6 times the volume collected via ethanol precipitation (11th sample volume was unknown) and the larger sample volumes could explain why these samples were positive for great crested newt when collected via filtration and negative when collected via ethanol precipitation.

For the metabarcoding, a sub-set of ten filtered samples were chosen by Natural England; where possible this followed previous examples of similar metabarcoding work, instead of designing and trialling new PCR primers, which was beyond the scope of this project.

Primers that had previously been described and are in widespread use (additionally used in Rees and others 2023a), were used to generate PCR amplicons from each sample (Riaz and others 2011). These primers were chosen as they can amplify a range of classes of Chordata including Amphibia, Aves, and Mammalia and have been successfully used in other Natural England studies (Rees and others 2023b). In carrying out the first round PCR the aim was to capture as much of the sequence diversity as possible that is contained within the samples. Metabarcoding identified great crested newt in six of the eight great crested newt positive samples (two were negative for great crested newt via qPCR) and a total of 21 vertebrate species across all samples analysed. It has been suggested that the eDNA score could be a surrogate for target eDNA within a sample (Biggs and others 2014). Here the fact that all but one (O1-067, 2 out of 12) of these six positives were from samples with high PCR scores and likely containing high amounts of great crested newt eDNA, would suggest that metabarcoding is not sensitive enough to detect great crested newt in samples that have levels of target eDNA that are associated with low PCR scores. Metabarcoding has been shown to be less sensitive than qPCR for multiple species (Harper and others 2018, Hikaru and others 2018, Schenekar and others 2020, Yu and others 2022) and is a known drawback of metabarcoding. This explanation would also fit for the samples which were only great crested newt positive by one of the sampling methods or the samples with lower PCR scores not having great crested newt detected by metabarcoding.

The number of species found in some of the ponds was low with an average of eight species per pond however, this was similar to the four species found on average (one to 17 species per pond) during the citizen science Genepools project (Rees and others 2023b) and the average of five species found during the 2023 study (Rees and others 2023a). All the ponds were constructed relatively recently, ranging from one to four years of age, which could affect the number of species that you would expect to find in them. Several species of bird were found consistent with visits to ponds to drink and bathe. There are relatively few 12S sequences for birds within Genbank and this could, in part, be an explanation for some expected species not being found. The finding of Bewick’s swan in one of the ponds (O1-047) would appear to be unusual given that this species tends to be a winter visitor in Eastern England and the Severn Estuary. This, coupled with the low read count (55 reads, 0.08%) suggests that this could be a sequencing error (mute swan was also found in the same sample).

Human DNA contamination of samples was an issue despite all samples being processed within a laminar flow cabinet equipped with UV decontamination. This is not unexpected as the primers used can detect human DNA as well as other vertebrates. Human DNA accounted for ~52% of all assigned reads, and in the worst case accounted for 91% of the sequence reads in the sample. Interestingly, one sample (Y1-066) had very little human DNA contamination (0.03% of reads) suggesting that the precautions taken in the laboratory were sufficient to prevent human DNA contamination and that the human DNA contamination may have come from the sample itself or during sample collection. The sample was taken from within a patch of woodland that was likely not to be accessible to

the public which could account for the low proportion of human DNA reads. Two of the samples with the highest proportion of human sequence reads (W1-148 and Y1-114), were from a country park known to be popular with the public and a farm next to a public footpath respectively again possibly accounting for the high proportion of human sequence reads. To reduce this human DNA contamination, it is possible to use blocking primers (Seyama and others 1992; Vestheim and Jarman, 2008; Craig and others 2014) which effectively prevent human DNA from being amplified during the first round of the metabarcoding PCR. This should allow for more efficient amplification of the other species eDNA within the sample.

DNA from certain species can be misrepresented in the pool of eDNA - either DNA from species that are much smaller in size than others within the sample pool, or DNA from species present in much smaller numbers than the dominant species. Therefore, sequence read number does not necessarily correlate with species abundance. Additionally, DNA may have been inefficiently extracted from different species and/or there was differential degradation of the DNA. DNA is liable to degradation by factors such as nucleases, UV light, microbial action and the temperature and humidity of storage conditions all of which will affect DNA quality after sample collection.

PCR amplification biases must also be considered in any metabarcoding study, and these affect the ability of metabarcoding to give information on abundance of species. The primers that are used in the initial PCR may have missed some species due to biases and/or the primers used may simply not work efficiently for some species (Preston and others 2022). The success of metabarcoding is dependent upon the primer set chosen for use and its target loci and can be informed by in silico analysis. Ideally primers should target a hypervariable region (for high resolution taxonomic discrimination) and thus will determine the efficiency and accuracy of species detection and identification. DNA is in constant competition to bind to the primers during PCR amplification, and this competition can prevent the effective amplification of all species present as only the more common template DNAs are likely to be amplified (Kelly and others 2014). This can mean that low abundance species are not detected termed ‘species masking’ (Brandon-Mong and others 2015; Evans and others 2016; Kelly and others 2014). Metabarcoding may therefore be less capable of identifying the DNA of less abundant species within a community than a species-specific qPCR. It is possible that in this study, the high human content results in ‘species masking’ of great crested newt (and other species) within some of the samples.

The number of unassigned reads corresponded to ~2.3% of the total number of reads (ranging from 0.1 to 10.1% for individual samples). Unassigned reads are primarily due to a lack of available sequence data termed ‘gaps’ in the sequence databases which is a known problem (reviewed in Macadam and others 2020) and it is inevitable that there will be unassigned reads in any metabarcoding study. Sequencing efforts such as the Darwin Tree of Life project which aims to generate DNA barcodes and full genomes for all UK species will help to alleviate this problem over the next several years and data generated can be reassessed as more sequence data becomes available.

Conclusions

The collection of pond water samples using filtration allowed a larger volume of water to be sampled, for most ponds, which resulted in the detection of great crested newt in 11 additional samples when compared with ethanol precipitation as a sample collection method. However, it should be noted that ethanol precipitation also resulted in three samples where great crested newt was detected, and these were not in the corresponding filtration samples.

As a method for the detection of great crested newt, metabarcoding was not as sensitive as qPCR which was as expected as in general only samples containing higher amounts of great crested newt eDNA were positive for great crested newt via metabarcoding. If human DNA reads were lower, then it is possible that great crested newt may have been found in more samples as on average 52% of the read counts were human. It is also worth noting that this metabarcoding study was carried out on a small scale which may require a larger scale repetition in the future. The metabarcoding study carried out by Harper and others (2018) which looked to determine if metabarcoding could be used to detect great crested newt in eDNA samples should also be taken into consideration as this analysed far larger numbers of samples and could help to inform a better understanding of the results.

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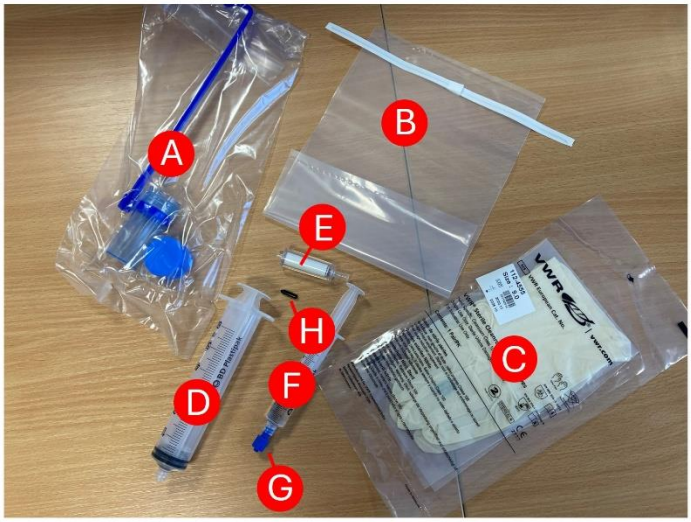
Appendix 1 Filtration Guidance

Taking a sample

Pro-tip: Pushing the water through the filter can be difficult. Use a sealant gun to make pushing the syringe easier (nothing fancy, just a cheap one from B&Q will work). As with usual protocols, the sealant gun will need to be cleaned between sites to prevent cross contamination (e.g. wiping with bleach).

What's in your kit?

A Sampling ladle
B Sampling bag
C 1 pair sterile gloves
D 50mL syringe
E eDNA filter capsule
F Preservation solution in 10mL syringe
G Filter inlet cap
H Filter outlet cap



- Samples should be taken on the same site visit as usual ethanol precipitation sampling.
- If a pond selected for filtration is dry, please select another pond on site or, if not available, the next available pond to sample.
- Each kit will be labelled with a unique identification number and barcode and will contain a field data form for recording pond ID and project details
- In addition, the kit contains: gloves, a sampling bag, a sampling syringe, 1x Sterivex capsule filter, a syringe with preservative solution, caps, resealable bags and a sampling ladle. The water sample collection methodology involves:

1. Open your kit and put on the pair of the gloves, minimise contact with the water to avoid introducing your own DNA into the water.
2. Open one of the sterile sampling bags by tearing off the plastic strip along the perforated line, then pull the tabs.
3. Use the collection ladles to collect 20 subsamples spaced out around the pond perimeter, empty each subsample into the collection bag, then fold the end of the bag several times and close off by folding in the tabs.
4. Mix the sample by inverting the collection bag 10 times.
5. Filter the water using the syringe to draw up water from the sampling bag. Attach the syringe to the filter inlet and press the plunger to push the water through the filter. Repeat until the filter is clogged- this can happen anywhere above c. 0.2L of water passed through the filter so do not be alarmed if you can't get any more water through the filter.
6. Record the volume of water filtered.
7. Dry the filter by injecting air with the syringe until all the water has been expelled. You can also tap on a tissue, or give a few hard flicks.
8. Take the pre-filled 10mL syringe, twist off the cap (keep for future use) and attach the syringe to the filter unit. Hold the filter unit upside-down and slowly push the liquid into the filter until it just starts to come out of the top. STOP injecting preservative as soon as this happens. Note from ADAS: I now find it easier to put the black sealing cap onto the end of the filter unit before very gently pushing the ethanol into the filter unit (the pressure can be released by unscrewing the syringe and reattaching periodically whilst injecting the ethanol). If you don't do this the ethanol can leak out of the filter and be lost. I would now also hold the syringe above the filter unit to watch it fill up. This year the caps will be one black push on cap and one red luer lock cap.
9. Cap the filter and place into the small bag/tube.

Taking a control sample

You will have been given additional kits on top of the number of ponds we have asked you to survey. You can choose which pond the control is taken at, but we'd recommend if you have more than one control, you take these at different sites.

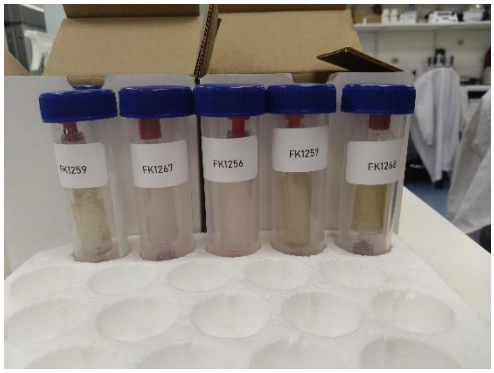
Whilst you are taking your sample, open a fresh bottle of water (at least 500ml) at the side of the pond – leave open by the side of the pond whilst you are taking other samples.

Once you have finished taking your main sample follow steps 1 to 7 above using the bottled water with a new filtration kit for your control sample. Label this appropriately (see below).

Sample labelling

Please make sure that samples are labelled clearly with the site name and pond ID. If you are taking a control, please add the suffix '-C' to your pond ID (i.e. 'B2-035-C') in order for the lab to distinguish which result is the control.

Please also record the volume of water filtered. The volume can be recorded on the tube that the filter unit goes back into to return to ADAS along with the pond ID.



Storage and return of samples

The samples do not need to be refrigerated, but please store them in a cool dry place.

ADAS will provide an addressed envelope for you to return your samples. These can be returned via Royal Mail. These can be sent back weekly or fortnightly throughout the survey season (or, if you're doing all of the ponds in one week, you can send them back all together).

Appendix 2

DNA extraction from sterivex filters

All surfaces were cleaned with bleach solution prior to commencing DNA extraction and then periodically during DNA extraction process.

1. ATL and AL Buffers were pre-warmed at 56°C.
2. An appropriate amount of ATL and AL buffers were pre-mixed with each filter requiring: 540 µL ATL and 300 µL AL. Additionally, a piece of synthetic control DNA was added at a known concentration to allow monitoring of DNA extraction.
3. An individual filter sample was removed from its container and the outside wiped down with bleach solution.
4. Preservative was removed from the filter (after removal of the inlet and outlet caps) into 1. 5 mL microcentrifuge tube/s using a fresh sterile 10 mL luer lock syringe for every filter. The volume recovered was recorded and retained for future use.
5. The outlet cap was replaced and 840 µL of pre-warmed ATL/AL solution and 50 µL proteinase K was added to the filter before replacing the inlet cap.
6. Steps 3 to 5 were repeated on each filter sample, changing gloves between each filter sample.
7. An extraction blank was set up on each DNA extraction day by adding 840 µL of the pre-warmed ATL/AL solution and 50 µL Proteinase-K to a clean 1.5 mL microcentrifuge tube.
8. All filters (and extraction blank) were briefly vortexed before being placed into 50 mL centrifuge tubes and incubated at 56°C in a water bath for 1 hour.
9. All filters were briefly vortexed every 10 minutes to ensure even and thorough digestion of material on all parts of the filter.
10. Whilst incubating filters, the microcentrifuge tube tubes containing the expelled preservative were centrifuged at 13,000 x*g* for 30 min at room temperature to pellet any DNA or material present.
11. The supernatant was removed using a pipette (avoiding pellet) and tubes retained for step 12.
12. Using the appropriate luer lock syringes from step 4 the digestion mixture was expelled from the filter (after removal of inlet and outlet caps) into the microcentrifuge tube with corresponding preservative pellet before briefly vortexing to resuspend the pelleted material.
13. The outlet cap was replaced and 500 µL 100% ethanol to each filter before replacing the inlet cap and briefly vortexing the filters.
14. The ethanol was expelled into the corresponding microcentrifuge tube containing the digest solution using the appropriate luer lock syringes from step 4/12 (after removal of inlet and outlet caps) and the microcentrifuge tube briefly vortexed to thoroughly mix.
15. The digest mixture was added onto a DNeasy spin column in 650 µL volumes (repeated until the entire extract has been passed through the spin column) and centrifuged at 6,000 xg for 1 minute.

16. The spin column was placed into a new 2 mL collection tube and the flow-through discarded.
17. 500 µL buffer AW1 was added and centrifuged for 1 minutes at 6,000 xg.
18. The spin column was placed into a new 2 mL collection tube and the flow-through discarded.
19. 500 µL buffer AW2 was added and centrifuged for 3 minutes at 20,000 xg. The flow-through was discarded and spin columns were re-centrifuged for 1 minute to dry the column membrane.
20. The spin columns were transferred to pre-labelled 1.5 m microcentrifuge tubes.
21. DNA was eluted by the addition of 200 µL AE buffer before incubating at room temperature for 1 minute and centrifugation for 1 minute at 6,000 xg.
22. The DNA samples were aliquoted in 4 equal amounts before storage at -20 °C.

DNA Quantification

DNA extracts were quantified using the Qubit® dsDNA BR assay kit and Qubit 3.0 fluorimeter as follows:

1. The Qubit® working solution was prepared by diluting the Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer.
2. Make up two standards by adding 190 µL Qubit® working solution into each of two tubes before adding 10 µL of each Qubit® standard to the appropriate tube. Mix by vortexing.

For each extract make up a tube with a final volume of 200 µL containing 1-20 µL extract and 180-199 µL Qubit® working solution

DNA extraction control PCR

PCRs were set up in a total volume of 25 µL consisting of:

a. 3 µL of extracted template DNA,
b. 1 µL of each primer/probe (0.4 µmol/L DegL; 0.4 µmol/L DegR; 0.1 µmol/L Deg.probe),
c. 12.5 µL of TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase),
d. 6.5 µL ddH2O.

Each sample was run in duplicate and each plate included 8 positive controls (4 replicates each at $1x10^{-3}$ and $1x10^{-4}$ ng/µl synthetic control DNA) and 4 negative controls (ultrapure water in place of DNA) on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 5 minutes at 56.3ºC then 10 minutes at 95°C; followed by 35 cycles with a melting temperature of 95°C for 30 seconds and an annealing temperature of 52ºC for 1 minute.

Species-specific great crested newt qPCR

PCRs were set up in a total volume of 25 µL consisting of:

e. 3 µL of extracted template DNA at 1 ng/µL,
f. 1 µL of each primer/probe (0.4 µmol/L TCCBL; 0.4 µmol/L TCCBR; 0.1 µmol/L TCCB.probe),
g. 12.5 µL of TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase),
h. 6.5 µL ddH2O.

Each sample was run as 12 replicates and each plate included 8 positive controls (4 replicates each at $1x10^{-3}$ and $1x10^{-4}$ ng/µl great crested newt DNA) and 4 negative controls (ultrapure water in place of DNA) on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 5 minutes at 56.3ºC then 10 minutes at 95°C; followed by 55 cycles with a melting temperature of 95°C for 30 seconds and an annealing temperature of 52ºC for 1 minute.

Nucleospin® gel and PCR cleanup

For DNA extraction from agarose gels:

1. Excise DNA fragment from gel with a fresh sterile scalpel blade for each sample.
2. Determine the weight of the gel slice and add 200µl buffer NTI for every 100mg of agarose gel
3. Incubate for 5-10 minutes at 50°C vortexing every 2-3 minutes until the gel slice is completely dissolved.
4. Place a NucleoSpin® Gel and PCR clean-up column into a collection tube and load 700µl of sample onto the spin column and centrifuge for 30 seconds at 11,000 xg.
5. Wash the silica membrane by adding 700 µL Buffer NT3 to the column and centrifuge for 30 seconds at 11,000 xg.
6. Discard the flow-through and place the column back into the collection tube before repeating this wash step.
7. Dry the silica membrane for one minute at 11,000 xg to remove Buffer NT3 completely.

Elute the DNA by placing the column into a fresh 1.5 mL microcentrifuge tube and add 20 µL Buffer NE and incubate at room temperature for one minute before centrifuging for one minute at 11,000 xg

Index PCR

PCRs were set up in a total volume of 50 µL consisting of:

a. 25 µl 2x KAPA HotStart ReadyMix
b. 5 µl Nextera XT Index 1 Primers
c. 5 µL Nextera XT Index 2 Primers
d. 10 µL PCR grade water
e. 5 µL DNA

PCR cycling was as follows: an initial incubation for 3 minutes at 95°C; followed by 8 cycles with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds; and a final extension step at 72°C for 5 minutes before holding at 4°C until collection of PCR products for analysis.

Appendix 3 12S metabarcoding results Note: some cells have been left blank

Scientific name	Common name	01-047	01-067	01-119	W1-010	W1-120	W1-148	Y1-042	Y1-066	Y1-073	Y1-114
Anas platyrhynchos	Mallard duck	9022	12	14004	14416	2289			3083		1579
Bos taurus	Cow			9							
Bufo bufo	Common toad					66934	5				
Cairina moshata	Muscovy duck			11	11						
Canis lupus familiaris	Dog	4540						7479			
Columba livia	Rock dove	12920		88	1461						
Columba oenas	Stock dove	19									
Cygnus columbianus bewickii	Bewick's swan	55									
Cygnus olor	Mute swan	8	6								
Gallinula chloropus	Moorhen				187				539		2857
Gallus gallus	Chicken										297
Gasterosteus aculeatus	Three-spined stickleback							209			
Hirundo rustica	Barn swallow					8031					
Homo sapiens	Human	39365	10785	46910	36398	252961	78692	25056	38	13502	91518
Lissotriton helveticus	Palmate newt							49208	114649		
Lissotriton vulgaris	Smooth newt	5			46341	416		32673	1570	99616	1664
Ovis aries	Sheep										48
Phasianus colchicus	Ring-necked pheasant			129							
Rattus norvegicus	Brown rat									562	
Sus scrofa domesticus	Pig						13256		54		
Triturus cristatus	Great crested newt		20398			1725		2380	3090	6786	1919
	Total read counts	65934	31201	61151	98814	332356	91953	117005	123023	120466	99882

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