Natural England Commissioned Report NECR326

Field testing of eDNA technologies to detect populations of non-native crayfish in Cumbria rivers

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Foreword

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.

Background

DNA – based methods offer a significant opportunity to change how we monitor and assess biodiversity. These techniques may provide cheaper alternatives to existing species monitoring or an ability to detect species that we cannot currently detect reliably.

However, for most species, there is still much development required before they can be used in routine monitoring. Natural England has been exploring the further use of these methods for environmental monitoring for several years, delivering a series of reports which focus on the development of DNA-based methods with potential in a particular area.

This report presents the development of a technique using eDNA from water samples to detect populations of non-native crayfish and crayfish plague across rivers in Cumbria. It compares the findings with records from traditional monitoring.

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Further information

This report can be downloaded from the Natural England Access to Evidence Catalogue: **http://publications.naturalengland.org.uk/**.

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Executive summary

Since the introduction of eDNA based surveys for great crested newts and freshwater fish in lentic environments, there has been an increasing amount of interest to apply eDNA based survey techniques to monitor freshwater invertebrate communities within lotic environments. There could be a number of far reaching applications and benefits of eDNA based survey methodologies, should they be proven as a reliable approach for screening lotic systems for the presence or absence of species of interest. This could include rare and endangered native species such as white-clawed crayfish (*Austropotamobius pallipes*), or invasive and non-native species including signal crayfish (*Pacifastacus leniusculus*) or the crayfish plague (*Aphanomyces astaci*).

This report aims to validate commercial eDNA assays for crayfish species detection, continuing on from the Natural England funded South West Peak Landscape Partnership eDNA sampling trials on known and unknown populations of white-clawed crayfish, signal crayfish and crayfish plague on the River Dove SSSI/SAC in Derbyshire (http://publications.naturalengland.org.uk/publication/6225382049316864). This report further assesses the application of two independently developed and publicly available eDNA assays for signal crayfish (Mauvisseau et al. 2017) and the crayfish plague (Vrålstad et al. 2009; Strand et al. 2011), for suitability as an eDNA based survey method in U.K. lotic systems.

Each of the assays were assessed for their detection sensitivity in a variety of upland river habitats within Cumbria (England), to validate the eDNA based survey approach for signal crayfish and crayfish plague. In addition, key challenges to their application as a commercial species presence/absence survey method were identified.

This report outlines the sample collection and analytical approaches used, providing key conclusions and recommendations associated with the application of eDNA based survey methods for signal crayfish and crayfish plague in lotic river systems.

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Glossary of terms

Amplicon	The piece of DNA which was the product of a PCR/qPCR reaction.
Ct	Cycle threshold. The number of qPCR cycles required for the fluorescent signal to cross the threshold (i.e. exceeds the general background level of fluorescence from non-amplified samples).
eDNA	Environmental DNA: intracellular or extracellular DNA that has been shed from an organism into a given environment such as water, soil or air. It can originate from sources such as shed skin cells, excretions, secretions, faeces, gametes, or deceased remains.
Haplotype	A collection of specific alleles (specific DNA sequences) that are likely to be inherited together, i.e. they are likely to be conserved as a sequence that survives the descent of many generations of reproduction originating from a single parent.
Isolate	A population of organisms that has little genetic mixing with other organisms within the same species.
NTC'S	Negative template controls. A control reaction that contains all essential components of the amplification reaction except the template to assess for the occurrence of contamination.
PCR	Polymerase chain reaction. A laboratory technique used to make multiple copies of a segment of DNA.
Primers	A short nucleic acid sequence which provides a starting point for DNA amplification in PCR, designed to target a particular DNA sequence.
Probe	A fluorescently labelled primer used in qPCR.
qPCR	Quantitative (real time) polymerase chain reaction. It is a quantitative method in contrast to conventional PCR, meaning that it can be used to determine the exact amount (relative or absolute) of amplified DNA in a sample.
Sanger sequencing	Also known as the "chain termination method", Sanger sequencing is a method for determining the nucleotide sequence of DNA.
Standard control	A positive sample of target DNA used to assess the assays efficiency.
Threshold	A measure of the general background level of fluorescence in a qPCR analysis run.

1. Introduction

Environmental DNA

Over the past decade, the emergence of molecular environmental DNA (eDNA) analysis has proven to be a valid and cost-effective method, for a number of species (Biggs et al. 2015, Davy et al. 2015). Now often applied within scientific research, eDNA shows promise as an additional option for species detection in the environment as in many of its applications the method has shown to be less invasive, more sensitive, more efficient and commercially viable (Smart et al. 2015; Wilcox et al. 2016).

Despite the increasing interest in using eDNA based techniques for species presence/absence surveys very few eDNA assays are currently available on a species-specific level. The most notable of which being the assay for the great crested newt, *Triturus cristatus*, which is now commercially available in the UK (Biggs et al. 2014), and commonly used to support presence/absence surveys of this species.

Before an eDNA assay can be applied on a national level for efficiency saving species monitoring, thorough validation and assessments of methodology applicability should be conducted. In particular, a focus on the sensitivity and reliability of the methods designed for use, in both laboratory and 'real-world' field test environments, is required (DNAqua-net, 2018)

Building on the approach of using eDNA to survey for great crested newts and freshwater fish in the lentic environment, there has been increasing interest in using eDNA techniques to monitor freshwater invertebrate communities in the lotic environment. Should this methodology prove reliable then this approach could have far-reaching conservation purposes for, in particular, threatened species such as the white-clawed crayfish *Austropotamobius pallipes*. Natural England is interested in the efficacy of DNA techniques and how they compare to traditional survey methods for detecting freshwater invertebrates and in particular non-native crayfish species.

Crayfish in the UK

The non-native and invasive American signal crayfish (*Pacifastacus leniusculus)* was introduced to UK rivers lakes and ponds for commercial purposes during the 1970s (Holdich & Rogers 1997). Over the last 40 years since its introduction, it has rapidly colonised a large proportion of rivers across the UK. This has been combined with the spread of the crayfish plague (*Aphanomyces astaci)*, a water mould which has little effect on signal crayfish, but can have a devastating impact on the UK's native crayfish species (Holdich et al. 2009), the white-clawed crayfish (*Austropotamobius pallipes)*. The spread of signal crayfish and crayfish plague across Europe has led to the decline of the white-clawed crayfish and its subsequent listing as endangered on the IUCN Red List (Füreder et al. 2010).

Crayfish, particularly at low abundance, can be notably difficult to find using existing survey efforts. This makes current survey techniques expensive and time exhaustive, often resulting in small pockets of isolated data with little large-scale implication. Existing efforts to monitor the spread and impact of both species are limited, with no early detection system in place for crayfish plague (detection is only confirmed after an outbreak has visibly caused damage to a population). For signal crayfish, only limited surveys are conducted as a result of the number of hours, licence requirements and resources required to conduct surveys at an appropriately sensitive level.

Plate 1. Non-native signal crayfish. Photo - GBNNSS

The development and application of novel species presence/absence survey methods using eDNA would therefore be suitably placed to provide a time- resource- and cost-effective approach for screening large areas for the presence of signal crayfish and crayfish plague. This could lead to potential efficiency savings if such a method was incorporated into existing monitoring programmes. However, before such approaches are implemented further examinations are required in to their efficiency and reliability.

eDNA-based surveys for crayfish

To date, eDNA has been applied (and as a result is available in peer-reviewed scientific journals) to a number of both native and non-native crayfish species which can be found within Europe. These include native species such as white-clawed crayfish (Robinson et al. 2018; Atkinson et al. 2019), narrow clawed crayfish - *Astacus leptodactylus* (Agersnap et al. 2017), and noble crayfish - *Astacus astacus* (Agersnap et al. 2017) and non-native species including marbled crayfish - *Procambarus virginalis* (Mauvisseau et al. 2019), red swamp crayfish - *Procambarus clarkii* (Tréguier et al. 2014), rusty crayfish - *Orconectes rusticus* (Dougherty et al. 2016), and the signal crayfish (Larson et al. 2017; Agersnap et al. 2017; Mauvisseau et al. 2017, Harper et al. 2018, Dunn et al. 2017). eDNA methodologies are now also available for the crayfish plague (Strand et al. 2014), based upon qPCR assays developed by Vrålstad et al. (2009).

The application of crayfish eDNA-based surveys in the UK

In 2018, Natural England funded the South West Peak Landscape Partnership to trial eDNA sampling on known populations of white-clawed crayfish and signal crayfish using different sampling techniques on the River Dove SSSI/SAC in Derbyshire (http://publications.naturalengland.org.uk/publication/6225382049316864), utilising qPCR assays outlined in Vrålstad et al. (2009) for crayfish plague and (Mauvisseau et al. 2017) for signal crayfish. Evidence of the presence of target crayfish species by eDNA techniques was found to be consistent with information obtained by non-eDNA methods. Crayfish plague was also screened for at the same time as the crayfish species, however it was concluded that crayfish plague detection, required further assessment and consideration, possibly as a result of the difficulty in obtaining clear comparable results using the traditional survey approach.

Using eDNA-based methods to detect non-native crayfish in Cumbria
Following the trials on the River Dove, further validation was recommended to continue field tests to validate commercial assays for crayfish species, to understand their applicability for detecting the presence and spread of signal crayfish and crayfish plague.

Cumbrian rivers and becks support some of the finest river habitats in England. The most important of these are the Rivers Eden, Derwent and Kent which are designated as Special Areas of Conservation for their habitats and supporting species. Cumbria is also vital in a UK context because it remains a stronghold for the native white-clawed crayfish. In 2005 signal crayfish were first recorded in the Derwent Catchment (St John's Beck) and have since been recorded at further sites across Cumbria. Natural England is committed to the conservation of the white-clawed crayfish in different catchments in Cumbria. This includes surveying and monitoring to assess the extent of resident native populations, and any non-indigenous crayfish populations.

Due to the recent and ongoing invasion of Cumbria, the region was selected as the most appropriate location for further validation of the crayfish eDNA service. The key aims of this report are:

(1) to assess signal crayfish and crayfish plague distribution across known populations in North Cumbria, whilst, contributing to a greater understanding on the levels of detection of specific primers for crayfish and;

(2) the key challenges that may arise when using eDNA methods in upland river catchments and the range of habitat types encountered.

2. Aims and objectives

Aims

i. To contribute to the field validation of crayfish assays in lotic environments to understand the key challenges of eDNA application.
ii. To use eDNA as a survey method to assess the extent of the spread of signal crayfish populations in the rivers of Cumbria.
iii. To use eDNA as a survey method to assess the extent of the spread of crayfish plague in the rivers of Cumbria.

Objectives

i. Devise and utilise a sampling strategy to meet the aims, for which 50 samples will be collected and screened for the presence or absence of signal crayfish and crayfish plague.
ii. Carry out eDNA tests on known (and unknown) populations of signal crayfish using established sampling techniques, mapping the extent and distribution of signal crayfish across the rivers in Cumbria.
iii. Perform quality control measures on the results to assure detection of target species, by sequencing resultant amplicon.

3. Study site

Natural England, the Environment Agency and the Eden Rivers Trust were responsible for the collection of water samples over summer/autumn 2019 from 41 sites across the River Eden and River Derwent catchments (Figure 1). Each of the sample collection sites were selected by Natural England on a site-by-site basis, with a focus on sites of important interest, sites with known signal crayfish and/or recorded crayfish plague outbreaks and sites with unknown presence of either species. The majority of sampling focussed around the River Caldew (Eden), St John's Beck (Derwent), River Greta and River Glenderamackin (Derwent) and the River Derwent (Cockermouth).

Sample collection was conducted in two waves, the first 27 samples were collected between 27/08/2019 and 29/08/2019. Once the analysis was completed for these samples an additional 23 samples were collected between 15/10/2019 and 29/10/2019. Samples which were collected at the same site on multiple occasions are represented by darker shading in Figure 1. Some sites were sampled in both phases of sample collection whilst others were sampled using multiple sample collection kits in order to obtain a sufficient volume of sampled water from each site (see Table 1 for individual site sample collection dates).

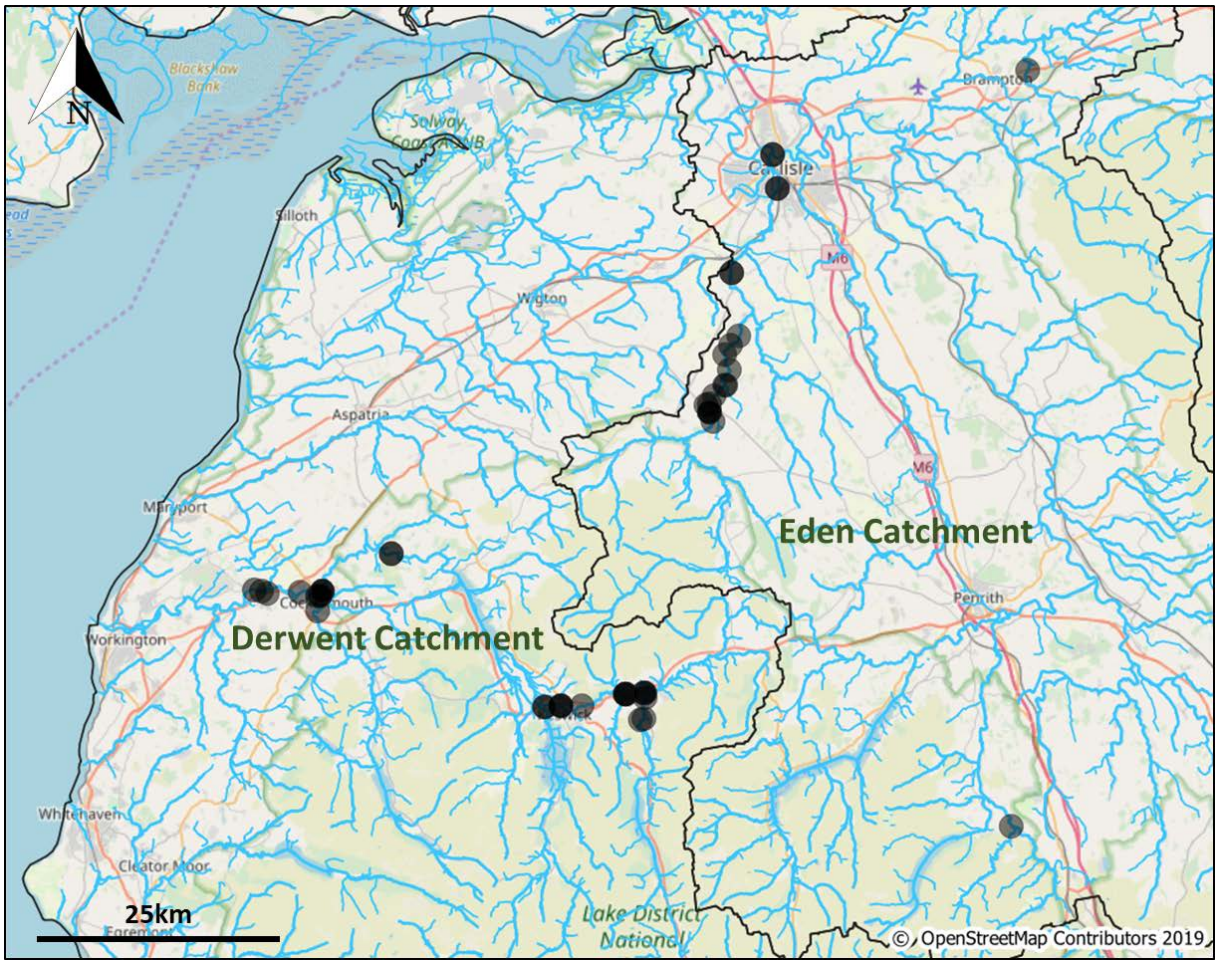

Figure 1. Indicative locations of each site sampled for the project, darker site markers are indicative of multiple samples collected at the same site, and/or samples taken in close proximity.

4. Methodology

Sample collection

The sample collection approach was based upon Spens et al. (2017), following the Sterivex filter preserved with ethanol approach. At each of the sample collection sites (as chosen by Natural England) a filtered water sample was collected following the protocol outlined within the sample collection form (Appendix 1) and further detailed sample instructions (Appendix 2) provided with each sample collection kit.

In brief, 20 subsamples of river water (50ml) were collected and pooled from evenly spaced locations within each site (total volume 1L) in order to obtain a representative eDNA sample. Sampling was conducted working from a downstream to upstream direction to avoid the disruption of sediment into the sample. Sample collection was focussed around areas within each site deemed likely to be habitable to signal crayfish. The pooled sample was homogenized by shaking for 10sec, then, 50ml was taken using a syringe and manually pressure filtered through the enclosed filter unit (Sterivex, 0.45µm Polyvinylidene fluoride membrane). Additional volumes of 50ml were passed through the filter until 1L of water sample was filtered, or the filter became clogged or saturated with filtrate. The volume of sample filtered was recorded for each sample. Once all water had passed through the filter unit, the filter casing was filled 2ml absolute ethanol to preserve the filter which contained a DNA spike (to assess for sample inhibition and post sampling degradation of samples). Samples were stored at room temperature prior to analysis.

Plate 2. Manual filtration of water sample with syringe. Photo – Gavin Measures

DNA extraction

All samples were extracted using the Qiagen Blood and Tissue DNA Extraction Kit following the methods outlined in Spens et al. (2017), for the extraction of DNA from a Sterivex filter. All samples were incubated on a shaking incubator for a minimum of 4 hours. With each batch of extractions, a field negative control was processed, consisting of an unused sample collection kit which should be absent of target DNA. Laboratory negative extraction controls were also included with each batch. Samples were diluted with 200µl of buffer AE (from Qiagen Blood and Tissue DNA Extraction Kit) and then stored at -20°C prior to analysis.

Analysis by quantitative PCR

The detection of signal crayfish and crayfish plague was conducted using two separate quantitative PCR (qPCR) protocols, each one specific to the intended target species.

A further assay was used in multiplex with these assays to asses for the presence and quantity of DNA spike within each sample. Lower than expected quantities or absence of spike DNA could infer degradation or inhibition of sample.

- Signal crayfish

  Analysis for signal crayfish eDNA was conducted using a qPCR assay designed to amplify a 114bp fragment of the mitochondrial cytochrome oxidase subunit (COI) gene of signal crayfish (Mauvisseau et al. 2017).

  qPCR was performed in a final volume of 25µl containing 12.5µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 6.5µl ddH2O, 1µl of each primer (10 µM), 1µl probe (2.5 µM) and 3µl of template DNA. Thermal cycling conditions were set as recommended in Mauvisseau et al. (2017) with 50°C for 5 min and 95°C for 10 min followed by 55 cycles of 95°C for 30 s and 56°C for 1 min. Each sample was run in 12 replicates (increased from 6 to improve accuracy and reliability) in an ABI 7500 FAST qPCR System (Applied Biosystems). A three-step, 2 replicate 10x dilution series of positive signal crayfish DNA standard control (10^{-3} to 10^{-5}) was run with each qPCR assay. 6 x negative template control samples (NTC's) comprised of ddH2O were also run on each qPCR plate.

  Primers (Mauvisseau et al. 2017):

Forward:	CO1-Pl-02-F	TGAGCTGGTATAGTGGGAACT
Reverse:	CO1-Pl-02-R	AGCATGTGCCGTGACTACAA
Probe:		FAM-CGGGTTGAATTAGGTCAACCTGGAAG-BHQ1

- Crayfish plague

  Analysis for the crayfish plague was conducted using primers and conditions designed by Vrålstad et al. (2009) to amplify a 59bp DNA fragment of the ITS1 (internal transcribed spacer 1 region) nrDNA (nuclear ribosomal DNA) of crayfish plague.

  A 25µl reaction was set up containing: 12.5µl TaqMan Environmental Master Mix 2.0, 3µl ddH2O, 1.25µl (10µM) of each primer, 2µl (2.5µM) of probe with the addition of 5µl template. qPCRs were performed with 12 qPCR replicates of each eDNA sample on the ABI 7500 FAST qPCR System under the conditions: 50°C for 5 min, denaturation at 95°C for 8 min, followed by 50 cycles of 95°C for 30s and 58°C for 1 min. A three-step, 2 replicate 10x dilution series of positive crayfish plague DNA standard control (10^{-3} to

10^{-5}) was run with each qPCR assay. 6 x negative template control samples (NTC's) comprised of ddH2O were also run on each qPCR plate.

Primers (Vrålstad et al. 2009):

Forward:	AphAstITS-39F	AAGGCTTGTGCTGGGATGTT
Reverse:	AphAstITS-97R	CTTCTTGCGAAACCTTCTGCTA
Probe:	AphAstITS-60T	FAM-TTCGGGACGACCC-MGBNFQ

Interpretation of qPCR results

Species presence within a site was inferred by the positive amplification of target species eDNA within at least one of the twelve qPCR replicates for each sample. Ct (cycle threshold) values were recorded for each positive amplification and reported alongside the number of positive replicates for each species at each site.

Sequencing of positive results

To assess the specificity of each assay and to ensure positive qPCR detections are representative of the target species (i.e. no non-specific amplification on non-target DNA), a proportion of positive qPCR samples were selected at random and sequenced using Sanger sequencing. 15µl of each selected PCR product was combined with 2µl of forward qPCR primer and sent to Eurofins (Germany) for sequencing. Resulting DNA sequences were then analysed by uploading them to NCBI BLAST database and comparing them to publicly available DNA sequences of all species to infer the similarity of the sequence reads to the target species.

5. Results

No inhibition or degradation of samples was observed throughout the study, with spike DNA present in each sample tested. As a result, no further analysis or clean-up of samples was required. Throughout the sample collection the volume of river water which was filtered was variable, ranging from 450ml in more turbid conditions to up to 850ml in clearer, less turbid river sites (Table 1 – complete dataset listed in Appendix 3).

Both signal crayfish and crayfish plague were detected in a number of the study sites across Cumbria (Figure 1). As qPCR is a quantitative technique, the number of qPCR replicates positive for each species in each site can be used as a proxy for the amount of eDNA present within each sample/site. Across the study, the number of positive qPCR replicates was varied and is therefore likely to be representative of differing signal crayfish population sizes or crayfish plague expression at each respective site.

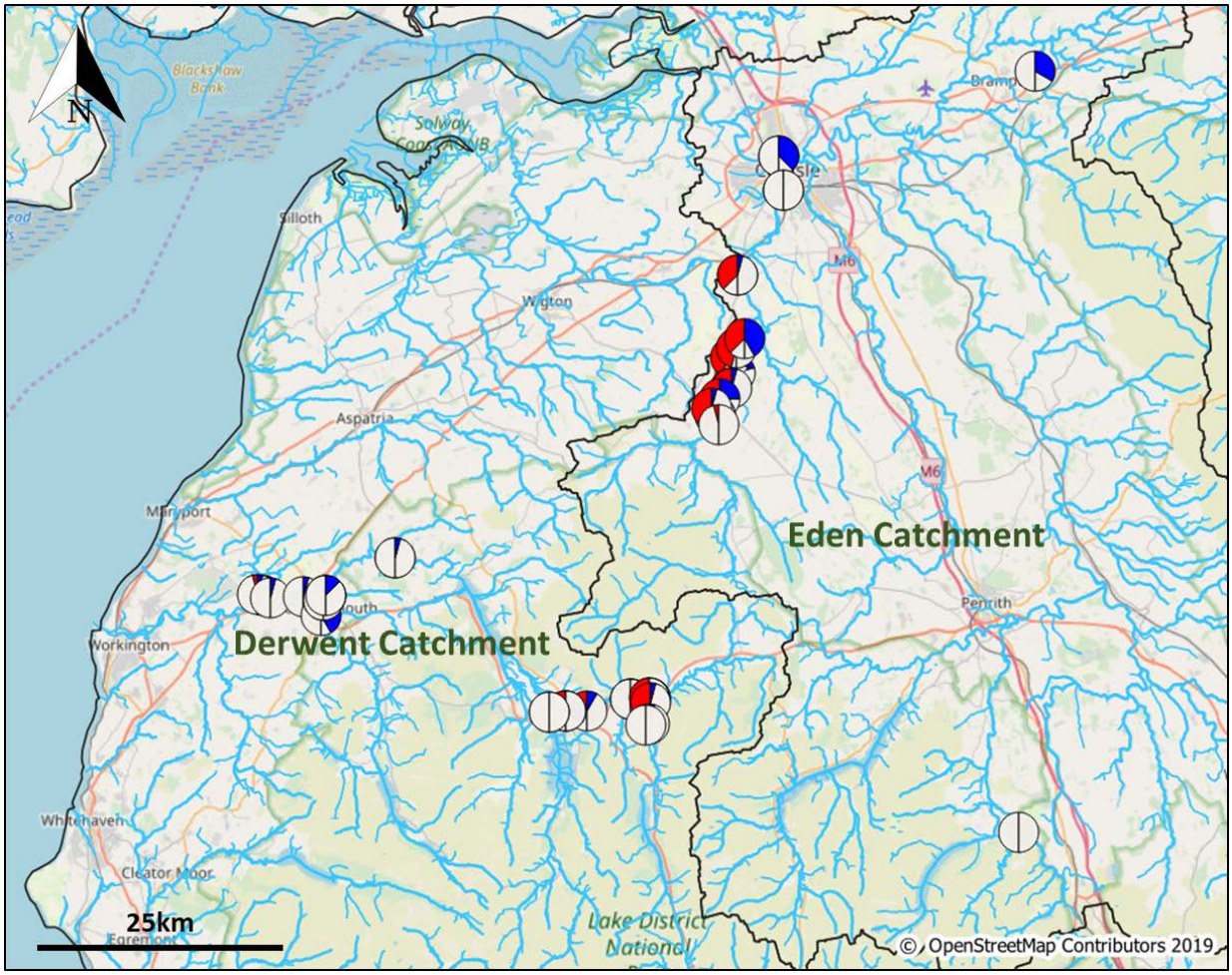

Figure 2. Overview of species detection within each area surveyed using eDNA for signal crayfish. Where signal crayfish eDNA was detected, left-hand semi-circles are red [% of qPCR replicates /12 positive]) and right-hand semi-circles are blue in the case of positive crayfish plague eDNA detection [% of qPCR replicates /12 positive].

Table 1. Sample site details and qPCR results [/12] for the presence or absence of both signal crayfish and crayfish plague eDNA within the rivers of Cumbria assessed within this project.

Lab ID	Sample ID	Site location description	Collection date	OS Ref	Volume filtered (ml)	RESULTS	
						Signal crayfish [/12]	Crayfish plague [/12]
Eden Catchment							
C0103	ROSGILL1	Rosgill 1	29/08/2019	NY 53491 16385	540	0	0
C0099	CLD1	Caldew - Upstream 2	29/08/2019	NY 35824 40847	800	**1**	0
C0096	CLD2	Caldew - Upstream 1	28/08/2019	NY 35639 41426	790	0	**9**
C0143	CLD2.2	Confluence 2 - Upstream	15/10/2019	NY 35639 41426	850	0	0
C0148	CLD2.1	Confluence 2 - Upstream	23/10/2019	NY 35639 41426	850	0	**2**
C0092	WNB1	Warnell Beck - Downstream Next to Road	28/08/2019	NY 35662 41918	500	**12**	**2**
C0093	WNB2	Warnell Beck - Upper	28/08/2019	NY 35415 41870	550	**12**	**1**
C0098	CLD3	Collingwood Bank	29/08/2019	NY 35902 42404	850	**10**	**6**
C0091	CLD4	Bell Bridge Welton	28/08/2019	NY 36587 42992	750	**9**	**12**
C0097	CLD5	Ellery Wood Beck	29/08/2019	NY 36638 42996	800	**9**	**1**
C0089	CLD6	Crookholme Mill - Downstream	28/08/2019	NY 36869 43893	800	**5**	**5**
C0090	CLD7	Holm House	28/08/2019	NY 36536 44802	800	**10**	**8**
C0094	CLD8	Parkhouse Wood	28/08/2019	NY 36941 45359	750	**11**	**7**
C0095	CLD9	Rose Bridge	28/08/2019	NY 37470 45921	800	**9**	**10**
C0146	CLDALSTON01	Dalston Footbridge	23/10/2019	NY 37062 49738	700	**9**	**9**
C0147	CLDALSTON02	Dalston Footbridge	23/10/2019	NY 37062 49738	700	**9**	**1**
C0154	DENTONHOLME01	Denton Holme	23/10/2019	NY 39908 54668	650	0	0
C0153	DENTONHOLME02	Denton Holme	23/10/2019	NY 39908 54668	650	**2**	**2**
C0142	EDN1	Caldew Confluence - Upstream	15/10/2019	NY 39620 56723	650	0	**10**
C0144	EDN2	Caldew Confluence - Upstream	15/10/2019	NY 39620 56723	600	0	**9**
C0145	IRTH1	Mill Beck Bridge	15/10/2019	NY 54877 61571	500	0	**8**

Table continued…

Table 1. Continued.

Lab ID	Sample ID	Site location description	Collection date	OS Ref	Volume filtered (ml)	RESULTS	
						Signal crayfish [/12]	Crayfish plague [/12]
Derwent Catchment							
C0159	SJB4	St John's Beck at Wanthwaite Bridge - Upstream	23/10/2019	NY 31229 23046	700	0	0
C0114	SJB4	St John's Beck Wanthwaite	27/08/2019	NY 31405 23146	500	0	**1**
C0113	SJB2	St John's Tributary	27/08/2019	NY 31486 24377	450	**10**	**1**
C0112	SJB1	St John's Beck Bridge	27/08/2019	NY 31443 24670	500	**8**	0
C0160	GMK1	Glenderamackin St John's Confluence - Upstream	23/10/2019	NY 31497 24683	700	0	**1**
C0107	GMK1	Glenderamackin Confluence	29/08/2019	NY 31468 24681	500	0	**1**
C0108	GRT1	Greta	27/08/2019	NY 30373 24613	550	**4**	**1**
C0109	NDB1	Naddle Beck at Greta	27/08/2019	NY 30348 24621	500	0	0
C0150	GRT1A	Greta at Naddle Confluence - Upstream	15/10/2019	NY 30345 24618	700	**7**	0
C0155	GRT1	Greta at Naddle Confluence - Downstream	23/10/2019	NY 30345 24618	700	0	0
C0115	GCRT2	Greta at Keswick	27/08/2019	NY 27708 23952	700	**2**	**2**
C0111	GRT3	Greta Fitz Park	27/08/2019	NY 26449 23976	700	0	**5**
C0151	GRT3	Greta Fitz Park	23/10/2019	NY 26449 23976	700	**5**	0
C0149	GRT3A	Greta Fitz Park	23/10/2019	NY 26449 23976	700	**1**	**9**
C0156	DW8	Derwent at Portinscale Footbridge	23/10/2019	NY 25440 23895	700	0	**1**
C0162	DW8A	Derwent of Portinscale Footbridge	23/10/2019	NY 25440 23895	700	0	0
C0158	DW10	Cocker at Cockermouth YHA	23/10/2019	NY 11935 29921	700	0	**10**
C0161	DW5	Cocker of Derwent Confluence	23/10/2019	NY 12082 30822	700	0	**3**
C0105	DW5	Cocker at Derwent Confluence	27/08/2019	NY 12082 30822	600	**1**	**11**
C0152	DW9	Derwent at Isel Bridge	23/10/2019	NY 16404 33295	700	0	**2**
C0163	DW9A	Derwent at Isel Bridge	23/10/2019	NY 16404 33295	700	0	**1**
C0157	DW6	Derwent Memorial Gardens - Upstream	23/10/2019	NY 12210 31113	700	0	0
C0102	DW6	Derwent Memorial Gardens - Upstream	29/08/2019	NY 12210 31113	600	0	**1**
C0164	DW6A	Derwent Memorial Gardens - Upstream	23/10/2019	NY 12210 31113	700	0	**3**
C0104	DW1	Cockermouth Memorial Gardens	27/08/2019	NY 11947 30844	500	0	**9**
C0110	DW2	Derwent at Papcastle	27/08/2019	NY 10912 31049	600	0	**1**
C0106	DW7	Ellerbeck at A66 - Upstream	27/08/2019	NY 08903 31033	500	0	**1**
C0100	DW4	Broughton Beck at Derwent Confluence	29/08/2019	NY 08542 31185	600	**1**	**5**
C0101	DW3	Derwent at Broughton Bridge	29/08/2019	NY 08189 31275	600	**1**	**1**

River Caldew (Eden)

The original site where signal crayfish were first identified (through traditional ecological survey methods) within the Eden catchment was reported at Warnell Beck (C0092 and C0093). To determine the spread of signal crayfish from this introduction site the sampling strategy was designed to target areas both upstream and downstream of Warnell Beck.

Plate 3. Warnell Beck – upper. Photo – Gavin Measures

A significant number of positive signal crayfish eDNA detections were observed along the river Caldew upstream of Carlisle within the Eden catchment (Figure 3, 4). This indicates the presence of a more widely spread signal crayfish population than previously reported along the entire river, in particular downstream of Warnell Beck. A small amount of signal crayfish eDNA was detected upstream of Warnell Beck (C0099), however no crayfish eDNA was detected within three independent samples (C0096, C0143 and C0148) collected from the site immediately upstream of Warnell Beck. The detection at site C0099 may be indicative of a small population upstream or of DNA transfer to the site via wildlife or human-based activities (fishing gear, animal activity etc.). The sites immediately downstream from the introduction site contained the highest concentrations of signal crayfish eDNA. At sites further downstream within the urban area of Carlisle (Figure 3), only small residual traces of signal crayfish eDNA at one sample location time point were detected and is therefore likely a result of downstream flow of crayfish eDNA from the upstream sites.

In terms of crayfish plague, eDNA-based detection was observed at each site at least once throughout the duration of the study, indicating its presence along the entire study area within the River Caldew.

St John's Beck and River Greta and Glenderamackin (Derwent)

In St John's Beck, signal crayfish were not detected at the upstream sites, however further downstream relatively high indications of eDNA were observed. Across this region there was found to be persistent but low levels of crayfish plague eDNA (Figure 5, 6). Signal crayfish were detected using eDNA-based surveys at a number of sites along the River Greta, in

varying concentrations, suggesting the existence of an evenly spread population of signal crayfish within the catchment. Downstream at the confluence with the River Derwent no signal crayfish were detected, indicating the potential of signal crayfish absence at this site.

Plate 4. River Greta. Photo Gavin Measures

River Derwent at Cockermouth

At the River Derwent at Cockermouth signal crayfish eDNA was also detected, however at very low levels of just one replicate in each of three sites out of 13 sites studied within the region (Figure 7). Despite the low prevalence of signal crayfish as inferred by the eDNA-based survey results, crayfish plague was identified in varying concentrations across the catchment. At two sites, C0105 (Derwent confluence) and C0158 (YHA) in Cockermouth there was a notably high recorded presence of crayfish plague.

Rosgill and Quarry Beck (Eden)

In addition to these study regions, two further sites were assessed. Rosgill on the River Lowther had a previous unconfirmed record of signal crayfish presence, however, neither signal crayfish nor crayfish plague was detected using the eDNA-based method. The second site was Mill Beck Bridge (Quarry Beck) on the river Irthing, where only crayfish plague was identified as present, mirroring results from previous eDNA survey-based detection of crayfish plague at this site from 2018.

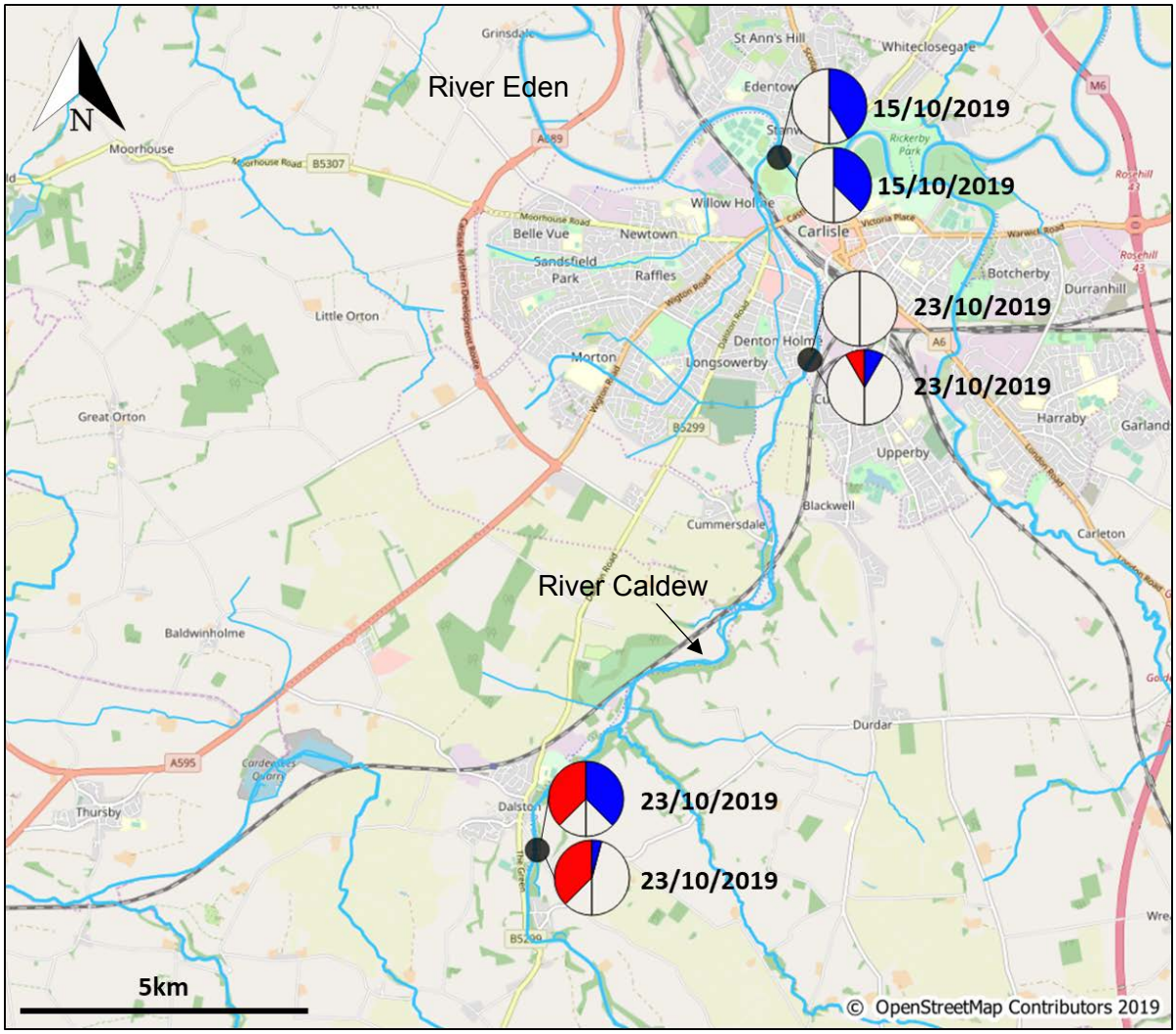

Figure 3. Presence/absence of signal crayfish eDNA (red [% of qPCR replicates /12 positive]) and crayfish plague eDNA (blue [% of qPCR replicates /12 positive]) on the **River Caldew (downstream sites)**, (River Eden, Cumbria).

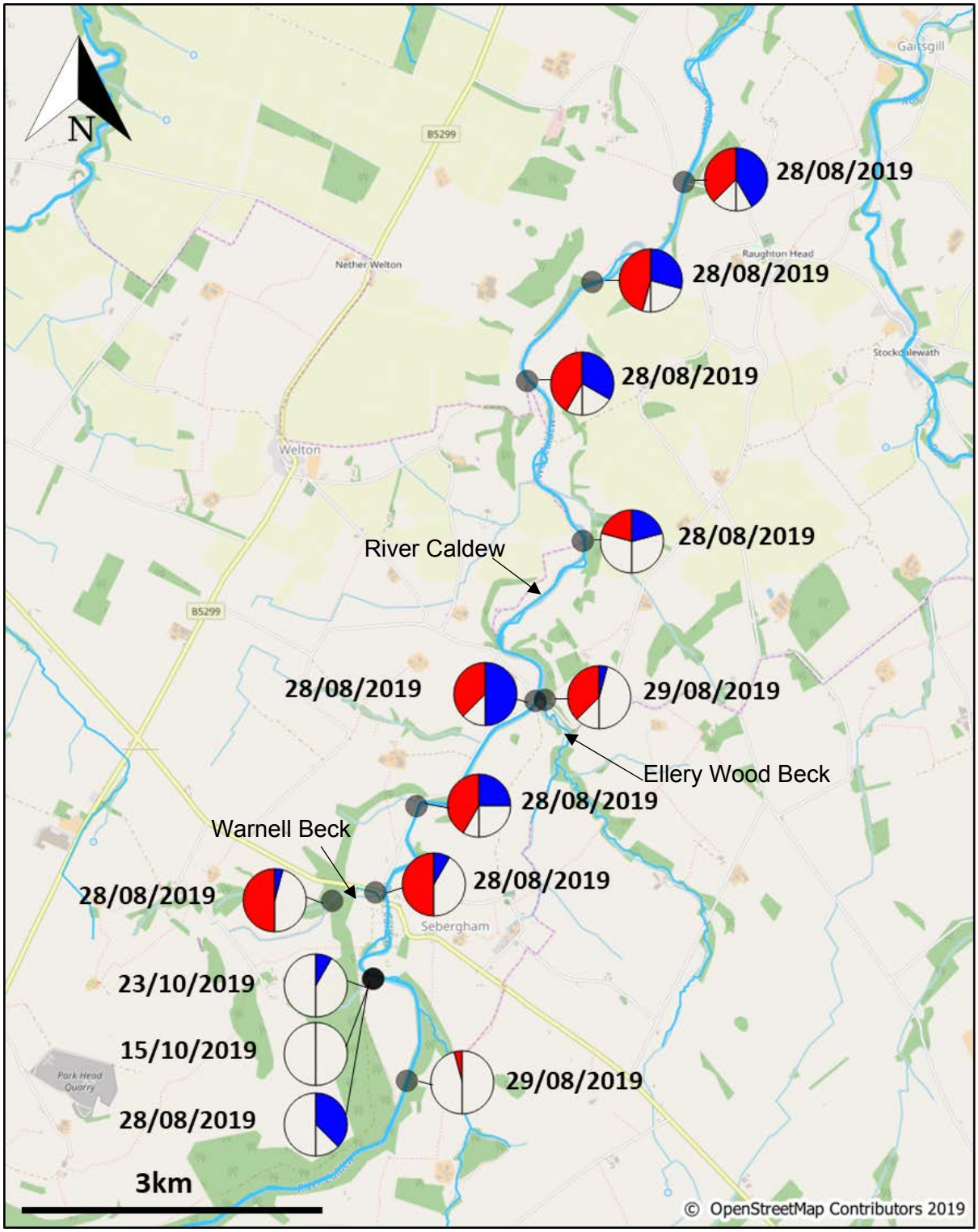

Figure 4. Presence/absence of signal crayfish eDNA (red [% of qPCR replicates /12 positive]) and crayfish plague eDNA (blue [% of qPCR replicates /12 positive]) on the **River Caldew (upstream sites)**, (River Eden, Cumbria).

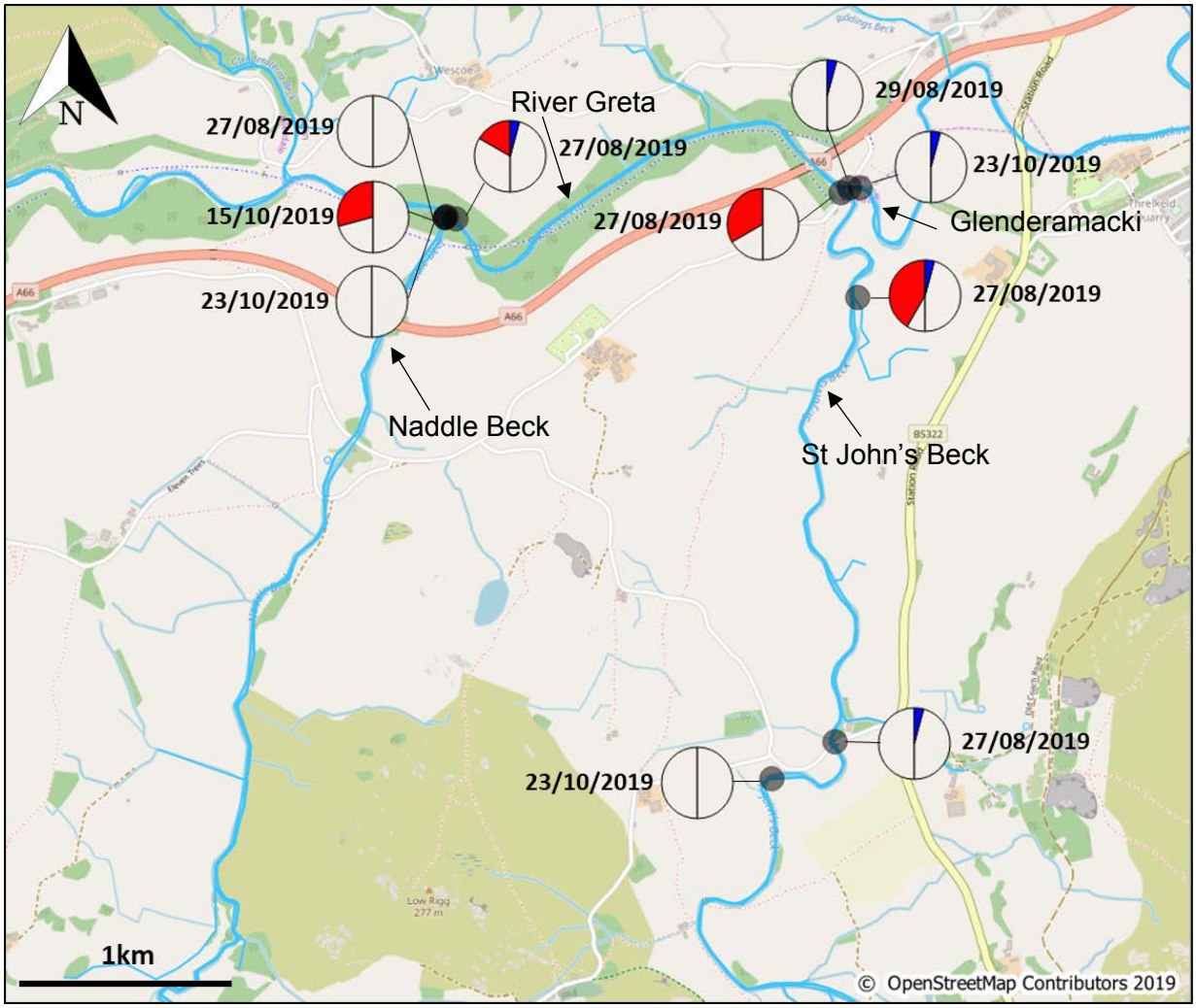

Figure 5. Presence/absence of signal crayfish eDNA (red [% of qPCR replicates /12 positive]) and crayfish plague eDNA (blue [% of qPCR replicates /12 positive]) in **the River Glenderamackin, River Greta** and **St John's Beck** (River Derwent, Cumbria).

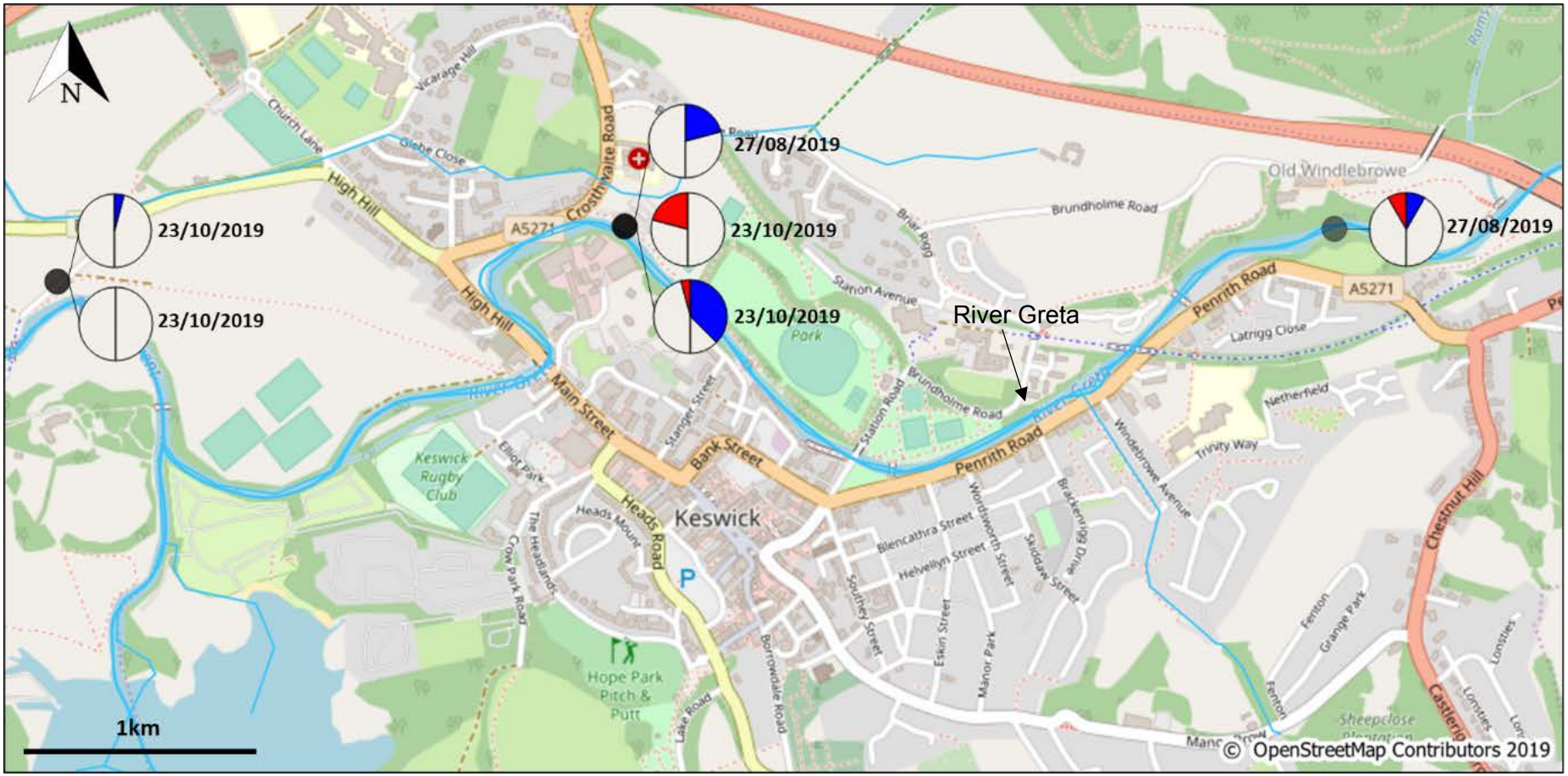

Figure 6. Presence/absence of signal crayfish eDNA (red [% of qPCR replicates /12 positive]) and crayfish plague eDNA (blue [% of qPCR replicates /12 positive]) in the **River Greta** at Keswick (River Derwent, Cumbria).

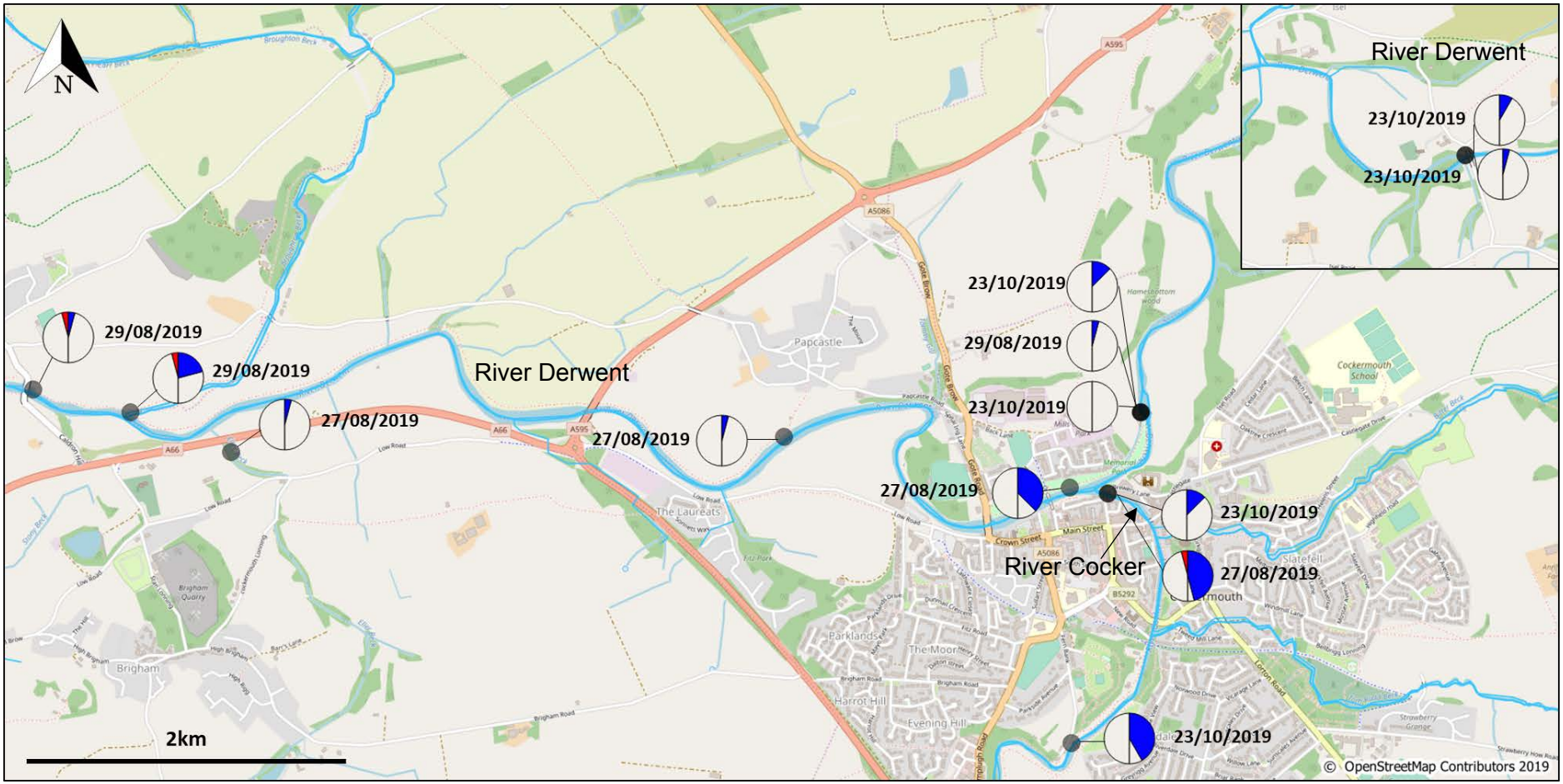

Figure 7. Presence/absence of signal crayfish eDNA (red [% of qPCR replicates /12 positive]) and crayfish plague eDNA (blue [% of qPCR replicates /12 positive]) on the **River Derwent** at Cockermouth, Cumbria. Insert – upstream sites studied on River Derwent.

Comparison of additional signal crayfish eDNA assay

Although signal crayfish were successfully detected in multiple sites across the study, in some instances the efficiency of the qPCR analysis was sub-optimal indicating potential poor efficiencies of the qPCR assay used. Poor qPCR efficiency can lead to lower levels of DNA detection and amplification, which in cases could lead to potential false-negative results, if not appropriately accounted for. In some qPCR analyses of signal crayfish, amplification was observed with poor efficiencies, leading to the requirement of careful interpretation when differentiating positive and negative results (Figure 8A/B). A number of variable factors can lead to poor efficiencies, such as: poor assay design, primer/probe/reagent issues, faulty or uncalibrated analytical equipment.

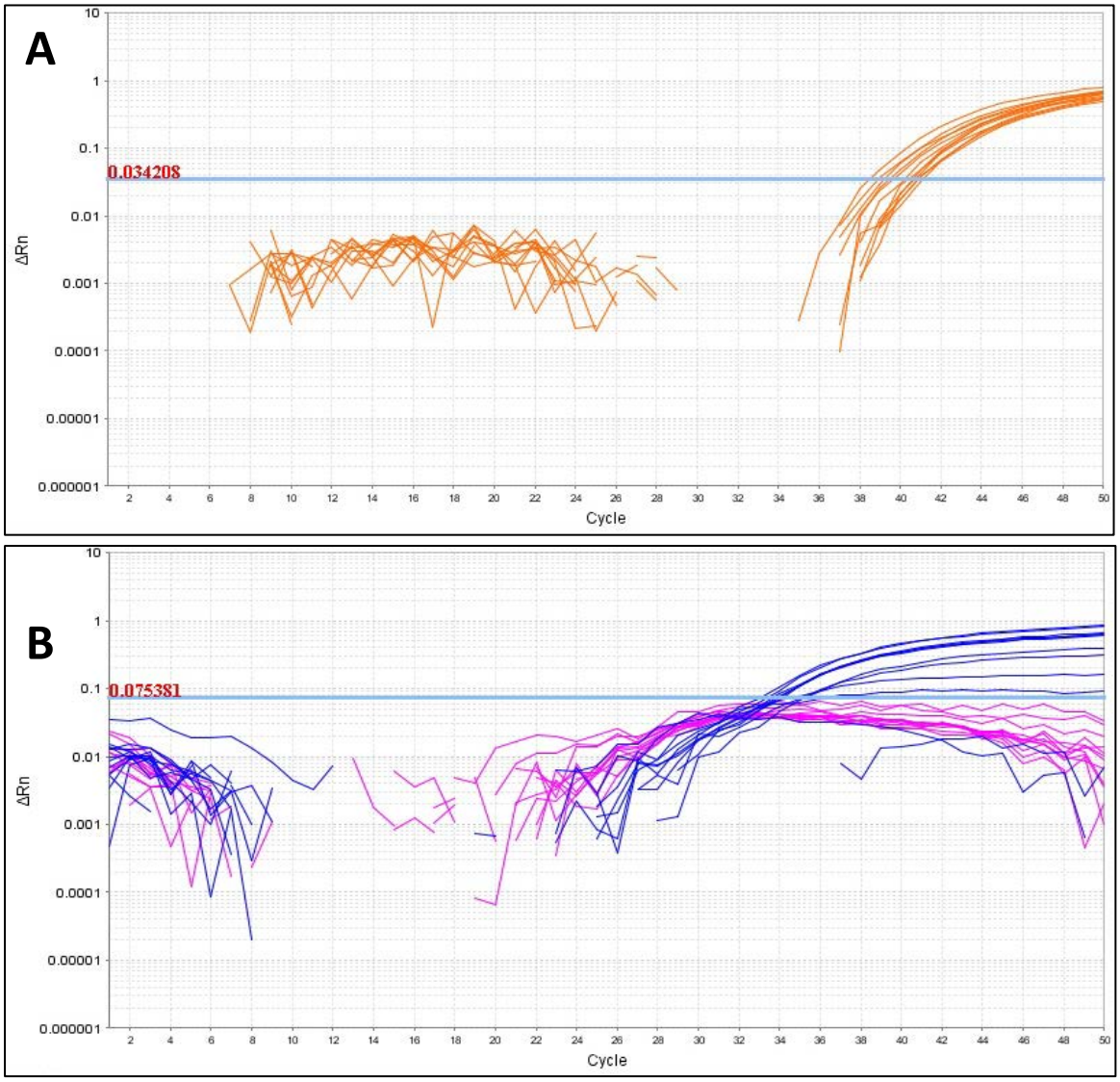

Figure 8. (A) An example of a clear qPCR amplification curve for crayfish plague eDNA detection where positive qPCR replicates (orange curve) can clearly be differentiated above the threshold (blue horizontal line) background fluorescence. **(B)** An example of a poor amplification curve with low efficiency for the signal crayfish assay, demonstrating the difficulty in determining if a given sample is truly positive or just as a result of background fluorescence due to the amplification of positive DNA relative to the background fluorescence threshold. Blue sample here demonstrated positive amplification of signal crayfish DNA and the pink line demonstrates no amplification.

Upon the use and re-dilution of new reagents and primers the efficiency did improve, however perfect amplification conditions were still observed to be impeded. As a result of these poor efficiencies in early qPCR results for signal crayfish it was recommended that an additional primer set be tested to assess alternative approaches for suitability. The qPCR primer and probe set from Harper et al. 2018 were assessed for this purpose. A random selection of 14 negative and positive (to varying degrees) samples (as detected using the Mauvisseau et al. 2017 primers) were analysed in a 25µl qPCR reaction. This contained: 12.5µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 2µl ddH2O, 1µl of each primer (10 µM), 1µl probe (2.5 µM) and 7.5µl of template DNA.

Initial assessment of results suggests that at higher concentrations of signal crayfish eDNA, Harper et al. (2018) may provide greater levels of detection cross the 12 qPCR replicates (Table 2). Despite this, the results throughout both assays were consistent, often presenting results with the exact same or similar (within 1 qPCR replicate) number of qPCR replicates.
As a result of the similar results and level of efficiency between both assays, the remainder of the qPCR's were conducted using the original primer set, with care taken in the interpretation of results. Despite these apparent poor efficiencies, the assay was still capable of reliable species detection across a large number of sites, indicating that is sufficiently sensitive for use in the detection of signal crayfish populations. However, it is recommended that further investigations are conducted on signal crayfish eDNA assays to improve this sensitivity and efficiency, there are currently a large number of signal crayfish assays available within published literature (Larson et al. 2017; Agersnap et al. 2017; Mauvisseau et al. 2017, Harper et al. 2018, Dunn et al. 2017). A comparative assessment of each would be particularly useful and would likely allow for an improvement in detection sensitivity and efficiency of signal crayfish eDNA-based detection.

Table 2. Comparison of Mauvisseau et al. (2017) and Harper et al. (2018) eDNA assays for the amplification of signal crayfish DNA from 14 samples collected from rivers within Cumbria. Results indicate the number of positive replicates observed at each site out of a total of 12.

Sample ID	Mauvisseau et al. (2017) [/12]	Harper et al. (2018) [/12]
C0089	5	11
C0091	9	12
C0092	12	12
C0105	1	0
C0106	0	0
C0109	0	0
C0115	2	2
C0144	0	0
C0146	9	8
C0149	1	2
C0150	7	6
C0153	2	3
C0159	0	0
C0164	0	0

Sequencing of qPCR products

To confirm if the amplified products which were detected using qPCR were from the intended original target species (i.e. to confirm the absence of non-specific amplification of a non-target species) a number of positive qPCR technical replicates for both the signal crayfish and crayfish plague assays were randomly selected and sequenced. The sequences amplified during qPCR were identified and compared against known sequences from the target species. By comparing each of the sequenced qPCR amplicons to publicly available databases of DNA sequences (from all known sequenced species) on BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), we were able to determine that in each case signal crayfish was identified as the amplified product, confirming that each assumed qPCR positive replicate is likely signal crayfish and not as a result of non-specific amplification (Table 3). Sequence similarities (an indication of the confidence of the assigned identification) ranged from 93% to 100%, indicating with high likelihood that signal crayfish were correctly detected within the samples (i.e. results were not as a result of false species identification through qPCR).

Sequencing results of signal crayfish eDNA taken from the sites revealed three different isolates/haplotypes of signal crayfish across the catchments. These were GenBank accession numbers: MK439898.1, KY947324.1 and KU603496.1, all of which were found within the River Caldew. These distinct populations were similar in sequence to three different populations of crayfish (one from USA, and two from Czech Republic). A number of different haplotypes of signal crayfish are known to exist and due to the numerous primary and secondary introductions of individuals across the continent since their introduction in the last century has made it difficult to track their original introduction and subsequent distributions (Petrusek et al. 2017). The distributions of the haplotypes within the River Caldew mirrored the patterns identified by Petrusek et al. (2017) with signal crayfish of all three haplotypes found along the length of the river, within the same populations. This could be indicative of a minimum of three separate introductions of signal crayfish to the River Caldew.

In the case of crayfish plague, as a result of the short fragment length of the amplicon (59bp), sequencing of qPCR products was unsuccessful. When samples were run on a gel for visualisation there were indications of multiple product bands in some of the samples at around 150-400bp in length (Figure 9), additionally to the expected bands at 59bp. Further recommendations for additional sequencing optimisation for these amplicons are noted within the discussion.

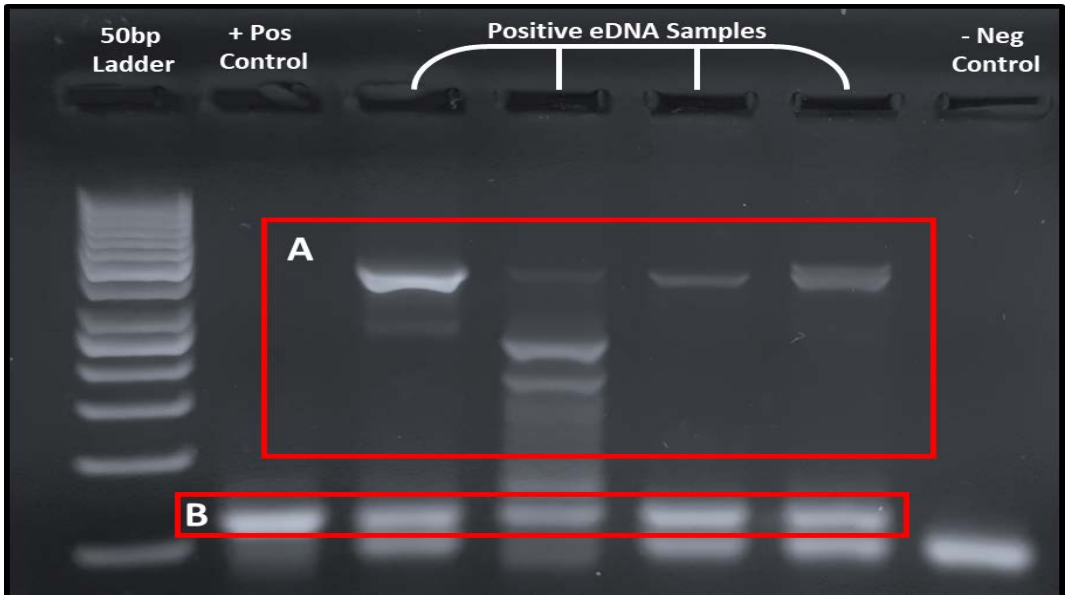

Figure 9. Gel image of qPCR product from crayfish plague eDNA detection demonstrating non-specific amplification indicated by additional bands of amplified DNA (A) in the region of 150-400bp to the expected bands (B) at 59bp.

Table 3. DNA sequences obtained by sequencing amplified PCR product from a selection of positive qPCR replicates from the signal crayfish assay.

Sample ID	River	qPCR Product Sequence	Similarity score [%]	Closest Database Sequence Match	GenBank Accession
C0089	Caldew	AGGTCNCCTGGAAGATTAATTGGAGATGATCAAATTTAT AATGTTGTAGTCACGGCACATGCTAANNGTCCTGGGGA GGAACATCGGAGTGAGATGTCTTGTTTAGTTTGCTGAC	96.88	*Pacifastacus leniusculus* isolate KO03 cytochrome oxidase subunit I (COI) gene	MK439898.1
C0090	Caldew	ACCTGGAGATTAATTGGAGACGACCAAATTTATAATGTT GTAGTCACGGNA	98.00	*Pacifastacus leniusculus* haplotype E13 cytochrome c oxidase subunit I (COI) gene	KY947324.1
C0091	Caldew	NNNTTAGGTCACCTGGAAGATTAATTGGAGACGACCAAA TTTATAATGTTGTAGTCACGGCACATGCNCNAN	98.46	*Pacifastacus leniusculus* haplotype E13 cytochrome c oxidase subunit I (COI) gene	KY947324.1
C0093	Caldew	AGANGANCAAATTTATAATGTTGTAGTCACGGCACATGN NNCNNN	94.74	*Pacifastacus leniusculus* isolate KO03 cytochrome oxidase subunit I (COI) gene	MK439898.1
C0094	Caldew	NNNNNTAGGTCACCTGGAAGATTAATTGGAGACGACCA AATTTATAATGTTGTAGTCACGGCACATGCTAANNNCCG	98.46	*Pacifastacus leniusculus* haplotype E13 cytochrome c oxidase subunit I (COI) gene	KY947324.1
C0095	Caldew	NTAGGTCACCTGGAAGATTAATTGGAGACGACCAAATTT ATAATGTTGTAGTCACGGCACATGCNNA	98.44	*Pacifastacus leniusculus* haplotype E13 cytochrome c oxidase subunit I (COI) gene	KY947324.1
C0097	Caldew	NNNNNGATTAGGTCACCTGGAAGATTAATTGGAGATGAT CAAATTTATAATGTTGTAGTCACGGCACATGCTCNCCCT	98.51	*Pacifastacus leniusculus* isolate KO03 cytochrome oxidase subunit I (COI) gene	MK439898.1
C0098	Caldew	AGGTCNCCTGGAAGATTAATTGGAGACGACCAAATTTAT AATGTTGTAGTCACGGCACATGCNCNNGN	96.83	*Pacifastacus leniusculus* haplotype E13 cytochrome c oxidase subunit I (COI) gene	KY947324.1
C0146	Caldew	NNNNTGAGTAGGTCANCCTGGAGANTAATTGGAGANGA TCAAATTTATAATGTTGTAGTCACGGCACAT	93.54	*Pacifastacus leniusculus* isolate PLL_572_Cla cytochrome oxidase subunit I (COI) gene	KU603496.1
C0147	Caldew	NNCTGGNGATTAATTGGAGANGANCAAATTTATAATGTT GTAGTCACGGCACATNNN	95.74	*Pacifastacus leniusculus* isolate KO03 cytochrome oxidase subunit I (COI) gene	MK439898.1
C0151	Greta	CCTGGAAANTAATCGGANNNATCAAATTTATAATGTTGTA GTCACGGCACAAGCTAAC	100	*Pacifastacus leniusculus* isolate KO03 cytochrome oxidase subunit I (COI) gene	MK439898.1
C0154	Caldew	NNCNAAATTTATAATGTTGTAGTCACGGCACATGCTAGC ATGCNCC	100	*Pacifastacus leniusculus* isolate KO03 cytochrome oxidase subunit I (COI) gene	MK439898.1

6. Discussion

The use of eDNA-based species detection methods for decision making and conservation management decisions is becoming increasingly popular (Bohman et al. 2014; Lacoursière-Roussel et al. 2016), however, to date the only target species for which eDNA detection is reliably available on such as scale is the great crested newt (Biggs et al. 2014). This project was designed to further investigate the potential adoption of eDNA based survey techniques to species of national monitoring programme interest. Here, we explored the application of eDNA survey technology to assess its suitability in monitoring freshwater biodiversity with the potential to lead to efficiencies in Natural England's monitoring programmes of freshwater invertebrates in lotic environments, namely the signal crayfish and the associated crayfish plague fungal pathogen.

Interpretation of results

The analysis of eDNA samples collected from the River Eden and River Derwent catchments in Cumbria highlight that both signal crayfish and crayfish plague are present within both rivers.

To assess the sensitivity of each assay (both signal crayfish (Mauvisseau et al. 2017) and crayfish plague (Vrålstad et al. 2009)), the target gene standard control was diluted to very low concentrations and analysed as a small standard curve, with dilution step concentrations at 300 copies, 30 copies and 3 copies of the respective target genes (Figure 10). Sensitivity was much higher in the crayfish plague assay, where successful detection of all three dilution steps was observed throughout the investigation. However, for signal crayfish there was no detection of the 3 copies and only limited detection of the 30 copies DNA standard (Figure 10), indicating that this assay is less sensitive and has a lower limit of detection than the assay for crayfish plague. Lower sensitivities could prove problematic in some instances, particularly within eDNA studies where the purpose of the study is to detect population of low abundance (which will have a proportionately low DNA release rate). This could result in false negative detection of the species, particularly when conditions are sub-optimal (i.e. extreme weather, or sampling is conducted 'out of season'). For this reason, it is essential that the sensitivity of the assay is investigated and reported alongside any result and, if required, measures should be put in place to increase detection sensitivity (i.e. additional/multiple sample replicates, repeat sampling on alternative dates).

Throughout the investigation the crayfish plague was identified as present within a high proportion of sites, despite, in many occurrences the absence of signal crayfish. The high sensitivity of the crayfish plague assay coupled with the short fragment length of DNA that this assay targets allows it to detect much lower DNA concentrations than the assay for signal crayfish. Despite this, it is important to note that this increased detection not necessarily indicates the presence of active virulent or pathogenic spores of crayfish plague. eDNA detection of crayfish plague relies on the detection of a small amount of DNA, which could survive for longer periods within the environment than the time for which a spore can remain virulent (Unestam 1969). Likewise, the presence of plague cannot in every case be directly linked to the presence of signal crayfish, due to the risk of DNA transfer from animal movement between sites or due to anthropogenic activity such as through fishing gear movement, water sports or species translocation from infected to non-infected sites for example.

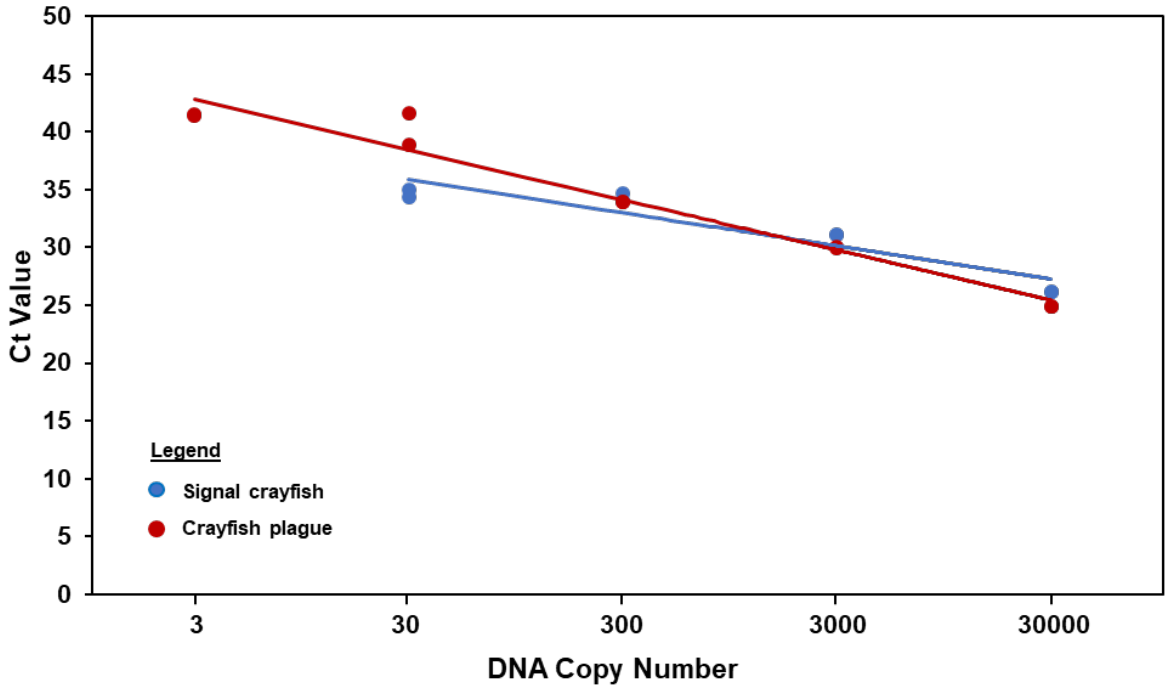

Figure 10. Standard curves of crayfish plague and signal crayfish.

For reliable interpretation of eDNA survey results, it is key to consider any potential influences from variable factors or methodology limitations, particularly where these factors can have an impact on the occurrence of false negative or false positive detection and species presence/absence results. These include: weather, water flow rate and time of year (particularly in areas of high seasonal environmental variability and for species with different levels of activity depending on a yearly cycle). The second batch of samples (C0142 to C0164) in this project were collected towards the end of the autumn season (mid-late October 2019) where increased flow rates, turbidity and rain showers posed a difficulty to sample collection on a number of days, severely restricting the days on which sample collection was possible.

Throughout the study, detection of both signal and crayfish plague ranged from lower amounts of 1/12 or 2/12 qPCR replicates positive to up to 12/12 positive qPCR replicates. When analysing eDNA results it is important to consider lower detection sensitives alongside the limitations to the techniques used and environments surveyed and incorporate such information into any interpretations. Currently, the approach adopted by the great crested newt eDNA protocol is to accept any sample as positive should it have 1/12 or higher positive qPCR amplification replicates (Biggs et al. 2014). Due to the sensitivity of eDNA-based methods, and the likelihood of small numbered populations of crayfish present in many sites it is important to interpret a positive result as a likely indicator for species presence somewhere within the vicinity of sample collection. For these conclusions to be reliably made it is essential that negative controls are conducted to ensure samples are not biased by contamination during any stage of sample collection and analysis. Within the project, no contamination was observed across all negative controls leading to the conclusion that the presence of 1/12 qPCR replicates are likely to relate to species presence. However, without full understanding of the abiotic factors of eDNA transport and persistence, eDNA based methods for signal crayfish and crayfish plague can only indicate species presence within the region upstream of the sampling site, and not pin-point individual populations with high accuracy.

Comparison of eDNA with previous site population data

The majority of eDNA-based detection results for the Derwent catchment confirm/support what was known from previous conventional sampling (signals present in the lower stretch of St John's and within some of the River Derwent below Cockermouth), or suspected (absent from the River Glenderamackin and upstream St John's Beck; likely to be relatively low numbers through the River Greta and likely to be widespread in the lower Derwent). Some results e.g. plague positive in the Cocker and Eden, and negative at Isel, has provided new information at sites which have not previously been surveyed. See Appendix 4 for further details.

Sample collection approach

During sample collection the volume of water filtered through each of the Sterivex filters varied within the region of 450ml to 850ml (total volume collected) between sites. This was noted to be as a result of various environmental factors such as turbidity and water purity leading to premature filter clogging. In sites with a high turbidity, it became difficult to filter larger volumes of water and as such lower volumes of sample were filtered at such sites, this was particularly evident towards the end of sampling the second batch of samples in late October, where there were more turbid conditions as a result of seasonal weather changes. To allow for simplicity in comparing datapoints in an ideal situation a standard volume of sample would be collected at each site, however due to filters clogging up quickly, this was not possible within this study. Instead, in sites where sample collection was impeded by the inability to filter a suitable volume of water during the second batch of sample collection, duplicate samples were collected. Whilst this approach would increase the chances of species detection, it also increases the cost of sample collection at each site, involving the use of two collection kits and two DNA extractions and analyses. The non-standardised variable nature of natural river systems makes collecting standardised volumes across sites difficult to achieve, particularly with changes in weather conditions leading to greater flow and turbidity between different sampling sites. Alternative filters could be utilised, with larger surface areas or pore size which would enable the filtration of a greater sample volume leading to an increase in the reliability and accuracy of detection, however, would require thorough assessment and validation before its use. A seasonal recommended survey period should also be proposed and investigated in order to allow for the collection of a sample at the most appropriate time of year, targeting conditions which would increase DNA concentrations within a given site - to match around crayfish activity (i.e. breeding/mating) and/or periods of less extreme water flow conditions (i.e. summer).

Physical and hydrological characteristics of a river can change significantly between sites only a few miles apart (i.e. between headwaters and downstream near confluences with larger rivers or estuaries). This is even more so the case with upland rivers, such as those studied within Cumbria, as a result of their fast flowing and changeable nature. Due to these changes, adaptations may often be required to sampling strategies, which may result in a more or less representative sample collected from any given site. During this project sample collection was generally applied following the guidance from Appendix 1, however, in some cases due to access constraints, river size, flow rate and limitations in place for health and safety purposes it was not always possible to collect samples in such a manner. Therefore, some samples during this investigation were collected from one or both sides of the river bank, whilst other samples were collected across the entire river span, and others were collected only on areas near to the river bank, focussing on areas which represent more typical crayfish habitat. Further investigations are recommended in order to ascertain the effect that such variations in sampling strategy can have on the success of eDNA-based applications. The development

of an efficient, reproduceable and consistent sampling strategy design is instrumental in achieving accurate and reliable repeatable results, particularly when important conservation and management decisions may rely on the accuracy of the data obtained.

Plate 5. Sample collection on the River Caldew (Eden) – Photo Gavin Measures

Assay suitability

Limited differences in the sensitivity of both signal crayfish qPCR assays (Mauvisseau et al. 2017; Harper et al. 2018) were observed within the comparative study. In most samples analysed the detection was reported at similar proportions of positive qPCR replicates. The assay developed by Harper et al. (2018) also proportionately requires over twice the volume of sample (compared to mastermix) to be loaded into each qPCR reaction which may offer an explanation for the slightly increased percentage of qPCR replicates presenting as positive for signal crayfish in a small number of samples.

For presence/absence detection of signal crayfish, either assay could be recommended as both are sensitive to a comparative level of detection, with a number of the eDNA-based detections reporting results which matched known or expected records. However, for reliable interpretation of results traditional ecological surveys are recommended in order to confirm the observed eDNA results and rule out false positive or false negative detections.

The detection and amplification of three different isolates/haplotypes of signal crayfish revealed through the sequencing of qPCR products across the catchments reveal that both sets of primers are suitably sensitive to amplify DNA from different populations of signal crayfish to those originally examined in the original research papers.

Sequencing of crayfish plague

It is important that before an eDNA assay is applied as a species detection method that it is validated and tested to ensure that it is both sensitive and target-species specific.

Signal crayfish are reported to have multiple genotypes, with at least five recently identified (Minardi et al. 2018). This variation may lead to some differences between populations of crayfish plague, although, the short fragment length (only 59bp) used in this study limits differences to ensure that each of the crayfish plague genotype variants are detected. It could also be highlighted that potential variation between genotypes hypothetically could lead to increased or decreased virulence across populations of different genotypes.

Sequencing of the crayfish plague PCR product (amplicon) has been identified as a challenging hurdle to determining the specificity of the qPCR assay used to detect the crayfish plague (i.e. does the assay amplify the intended target species or does it amplify an alternative species). The main reason for this is that the qPCR assay produces a quite short amplification product, with its size being only 59 base pairs (bp) in length. It is important to note here that for effective and sensitive eDNA-qPCR analysis it is important to have a short amplicon sequence (ideally below 150bp), with the shortest fragments most preferable due to their increased resistance to DNA degradation and hence increased likelihood of detection.

To attempt direct PCR fragment sequencing the recommended length is 150 - 200bp (this is a requirement from several companies providing sequencing service). Two approaches are possible to undertake in order to sequence the product. The first one includes the extension of the fragment length. Technically this will include the extension of the plague PCR primers with additional sequences (M13 universal primers) and performing amplification with them. With this approach the length can be increased up to 100bp. Our experience with sequencing short PCR products around this size (recent example: signal crayfish product is 114bp) demonstrates that this is possible. So far, the PCRs performed with the extended primers produced the band of expected size, however its sequencing results were not satisfactory and, most likely, contained some unspecific products. The protocol is being improved now to produce better results in further attempts.

The second plausible approach involves the cloning of the fragment into a plasmid (artificial DNA construct). This protocol is more time consuming, expensive and labour intensive. It includes several stages: PCR amplification reaction, PCR product purification, ligation reaction, bacterial transformation with subsequent DNA extraction and sequencing. Preliminary results show that the method requires optimisation. The plague PCR reactions are not very specific producing several products in addition to the target fragment. This implies that for cloning the fragment must be extracted and purified from the gel before cloning. Also, the quantity of the target fragment is quite low after PCR. All this reduces ligation/cloning efficiency. So far, this strategy has not produced results and requires further optimisation.

7. Recommendations

As a result of this study, a number of recommendations can be made in order to improve the efficiency and reliability of eDNA testing for signal crayfish and crayfish plague. This includes the necessity for further development and standardisation of the eDNA industry as a whole, with a particular focus on sample collection. Recommendations are summarised below:

Sample collection and analyses:

- A follow up investigation is recommended using conventional survey methods such as trapping and hand searching to compare and confirm results from the eDNA analysis.
- Establishment of guidelines for minimum water quantities to be filtered for each sample to ensure a sufficient amount of sample is collected.
- Assessment of different filter sizes and approaches to determine the effect of these on the success of species detection.
- Investigation of the effect that variances in water flow, turbidity, temperature and other site-based variations and constraints can have on eDNA-based surveys so that these can be factored into analysis or result in the formation of sample collection guidelines. For the success of any eDNA method on an informed conservation management level, standardisation is a key factor which needs to be factored into sample collection design wherever possible.
- Assessment of the appropriate crayfish eDNA sample collection season, to determine the effect of changing weather and environmental conditions through the year and crayfish/crayfish plague life/seasonal cycle events on the DNA concentrations within a river system and the resulting effect on detection.
- Further work to determine the distance downstream from a site of species inhabitation which eDNA for that species can be detected, taking into account numerous factors which could influence detection. This would enable a much more accurate interpretation of site-specific results within each catchment.
- To interpret the crayfish plague results effectively, further research into plague dynamics (i.e. how long can it persist within an environment, alternate carrier species, concentrations of plague present within a population, any closely related species which have not been described/sequenced etc.) are required.

Assay verification:

- Further investigations are conducted on signal crayfish assays to improve their sensitivity and efficiency, there are currently a number of signal crayfish assays published in the literature. A comparative assessment of each would likely allow for an improvement in detection sensitivity and efficiency of signal crayfish eDNA-based detection.
- An assessment on the number of replicates (both qPCR replicates per sample, and number of replicate samples) that is required per site to increase assay sensitivity in order to reliably and accurately infer the true presence/absence result for each site.
- Sequencing of the non-specific crayfish plague amplicons in (Figure 9) is needed to determine their origin.

Acknowledgements

All sample collection was conducted by staff and volunteers from Natural England, Environment Agency and Eden Rivers Trust. Sample analysis was conducted by SureScreen Scientifics.

8. References

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9. Appendices

i) Sample collection form and instructions

SureScreen Scientifics

SureScreen Scientifics
Morley Retreat, Church Lane, Morley,
Derbyshire, DE7 6DE
+44 (0)1332 292003
edna@surescreen.com
www.surescreenscientifics.com

Crayfish eDNA - Sample Collection Form

*Required Fields

*NAME: ..... *COMPANY: .....

*EMAIL: .....

*INVOICE ADDRESS: .....

*TEL: ..... *PURCHASE ORDER .....

Note: Please include a PO for this work, analysis will NOT be started until a valid PO has been received. When samples are sent in batches, each individual PO will be its own report and invoice. The report can be sent to multiple emails if required.

*SAMPLE NAME: .....

*VOLUME OF WATER FILTERED: .....

Site Name: .....

O/S Reference: .....

County: ..... Sampling Date: .....

SPECIES TO DETECT (Please Tick)

White Clawed Crayfish ☐

Signal Crayfish ☐

Crayfish Plague ☐

Note: There are different costs associated with analysing for one, two or three target species.

We currently only offer a standard turnaround time for this service of up to 10 working days.

RELEVANT NOTES

INSTRUCTIONS FOR SAMPLE COLLECTION

1. Identify where 20 sub-samples will be taken from the pond/river. The location of these should be spaced as evenly as possible around the site. In rivers, samples should be taken against the flow of the stream, working upstream in a diagonal pattern where possible to ensure that any disturbed ancient DNA is not collected and to avoid contamination, should it be necessary to enter the watercourse.
2. Wearing gloves, open the sterile Whirl-Pak bag and collect 20 samples of water from around the site using the ladle. Once collected close the bag securely using the top tabs and shake for 10 seconds. This mixes DNA across the water sample.
3. Using the large sterile syringe, take 50ml of sample and then attach the syringe using a half twist action to the filter unit (the syringe will only fit to one end of the filter). Apply pressure to the syringe until all liquid has passed into and through the filter unit. Remove the filter unit from the syringe and repeat this step until 500ml is filtered/the filter becomes clogged/you are no longer able to push any liquid through. The more liquid passed through, the better. Record the amount of liquid which has been filtered on this sheet.
4. Empty the syringe and fill with air, attach this to the filter and push air through the syringe until it is completely free of water.
5. Screw one red cap onto the thick end of the filter unit. Place to one side.
6. Using the small syringe, collect 2ml of preservative solution, connect to the open end of the filter unit and apply gentle pressure until all 2ml of solution is stored within the filter casing. Repeat if necessary until filter casing is full of preservative.
7. Screw the red-caps to secure both ends of the filter and then place the filter into the 50ml tube provided. Sample can be stored in a cool dry place for a maximum of 3 weeks, longer if chilled, however for best results it is recommended to return to the laboratory as soon as possible.

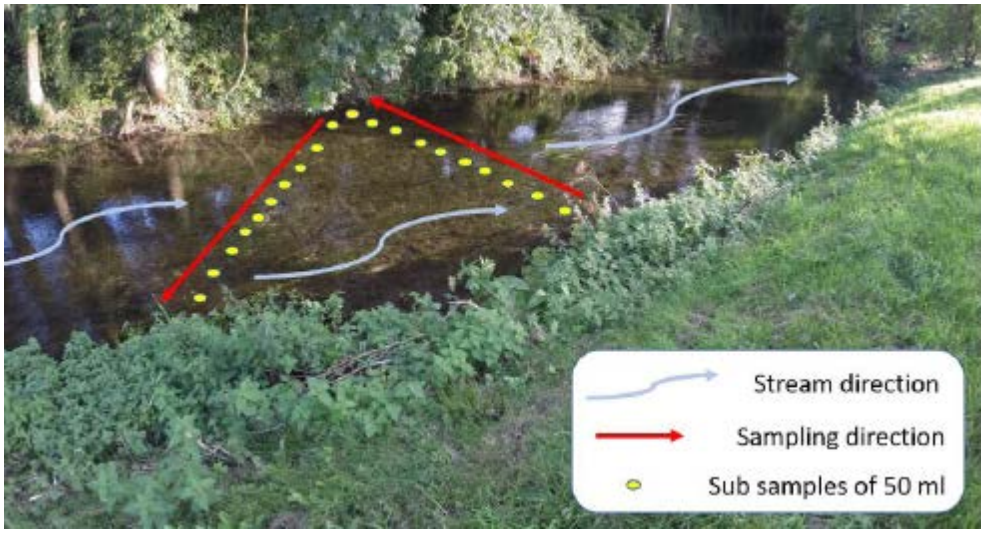
Kit components are single use only and must not be reused for any other samples. Components can be returned to the laboratory for recycling. FAQ's and a more detailed explanation as to how to collect a sample in different scenarios in available on our website.

LABORATORY SAMPLE ID

This form is available to download and edit as a word document at: www.surescreenscientifics.com/edna/crayfish

ii) **Detailed Sample Collection Method for Crayfish eDNA.**

1. Identify where 20 sub-samples will be taken from the pond/river. The location of these should be spaced as evenly as possible around the site. In rivers, samples should be taken against the flow of the stream, working upstream in a diagonal pattern where possible to ensure that any disturbed ancient DNA is not collected, should it be necessary for the collector to enter the watercourse.



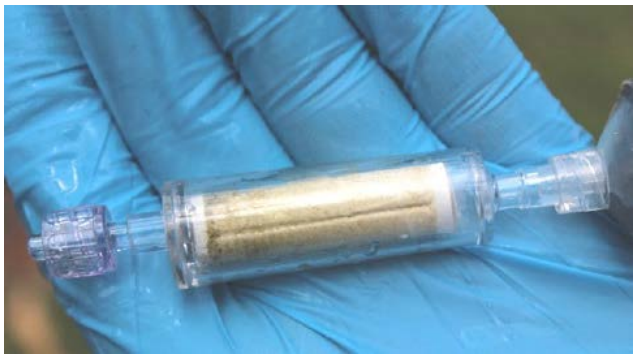
2. Wearing gloves, open the sterile Whirl-Pak bag and **collect 20 samples of around 50 mL of water** from around the site using the ladle (fill the ladle) into the Whirl-Pak. The water sample should be taken from the middle of the water column. Where possible, avoid any disruption of sediment as this can not only clog the filter quicker, but introduce ancient DNA into the sample. In larger sites it may be necessary to use a telescopic pole.

Once 20 samples have been taken close the bag securely using the top tabs and shake the Whirl-Pak for 10 seconds. This mixes DNA across the water sample.

3. Using the sterile syringe, take up 50mL of sample from the Whirl-Pak and then attach the syringe using a half twist action to the Sterivex™ Filter (The syringe will only fit to one end of the filter and twisting too far can damage the luer lock connection on the filter.). Apply pressure to the syringe until all liquid has passed into and through the filter.

4. Remove the Sterivex™ Filter from the syringe and repeat until you have filtered 1L OR you are no longer able to push any liquid through. Record the amount of liquid which has been filtered on the sample collection form.

5. Empty the syringe and fill with air, attach this to the filter and push air through the syringe until it is completely free of water.



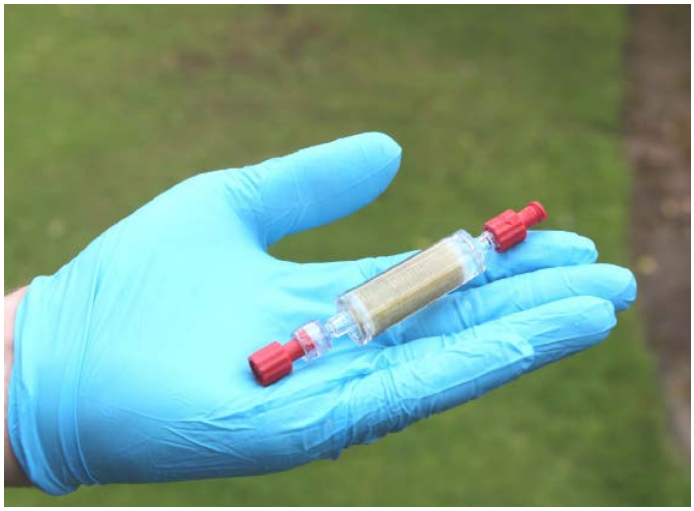
6. Screw one red cap tightly on to the thick end of the filter unit. Place the filter unit to one side. Using the small blue syringe, collect up 2ml of preservative solution from the preservative tube. An excess of preservative solution is provided. It is important to add preservative solution into the filter unit to prevent sample degradation during transport to the laboratory.

7. Attach the syringe to the open end of the filter unit. Apply light pressure until all 2ml of preservative solution is within the filter casing. Repeat process if necessary until internal casing is filled by preservative solution.

8. Finally, screw the second red cap on to the filter inlet. Ensure that both caps are secured tightly to avoid leakage of preservative solution during transport to the laboratory.

Place the sample into the 50ml tube provided and return to laboratory.

Samples can be stored at room temperature for up to 3 weeks. Longer if chilled.



iii) **Complete sample collection and analysis dataset for each site studied**

Attached as a separate spreadsheet (Excel).

iv) **Comparisons with previous site population data**

St John's Beck
After the initial report of signal crayfish in St John's in 2005 and removal of 40+ individuals from the area immediately upstream of the bridge, surveys have always suggested a population at relatively low densities compared to lowland productive rivers.

Trapping catch rates from conventional traps in the main river have been of the order of 0.05 crayfish per trap night or lower. The small tributary appears to function as a nursery area with relatively large numbers of juveniles taken from open "refuge traps" over the last 2-3 years. There has been no clear decline in catch from the tributary to date.

The eDNA results back up our conclusions from trapping, other than one sample upstream of what we believe from trapping and searching to be the current upper range limit giving a low positive for plague.

River Glenderamackin
No signal crayfish have been found by conventional survey in the Glenderamackin to date, but both Glenderamackin samples gave low positive results for crayfish plague.

River Greta
There has been relatively little conventional survey on the River Greta given its size, accessibility and nature, with only one crayfish found on the river in 2006. There is no obvious reason for signal crayfish not to have colonised the river, but it is very upland in nature so unlikely to provide suitable habitat throughout.

The eDNA results suggest signal presence down to Keswick, although possible that positives relate to washed down eDNA from further upstream. We hope to undertake or contribute to further conventional survey there based on this report.

River Derwent
No conventional surveys have been done below Keswick since 2006: two weeks of trapping then produced a nil result, matched by the eDNA results.

Very little is known about the Cockermouth population as only limited trapping has been undertaken, with that latterly focussed only on areas already known to have signals. There have been reports of sightings over the last seven years from the river through Cockermouth and downstream at Papcastle Bridge, but the size of the river does not lend itself easily to hand searching.

Both eDNA and conventional survey results from Cockermouth and downstream on the Derwent suggest the presence of a relatively low density population.

As with the other low positive reults for plague, the results at Isel (in between Bassenthwaite Lake and Cockermouth) give some uncertainty. There has been no conventional survey undertaken there and it would again be difficult to do so given the size of the river. Further refinement of eDNA surveying would be of great interest for such sites where there may be signal crayfish present at very low density, and where conventional survey is difficult and potentially unreliable.

River Cocker (Derwent)
No conventional survey has been done in the River Cocker to our knowledge so the eDNA results there are new, the high plague positive making the river a priority for survey effort or repeat eDNA sampling in the immediate future.

River Caldew (Eden)

Signal crayfish were found for the first time in the River Eden system in 2012 at Warnel Beck, a tributary of the River Caldew. Surveys have since found crayfish many miles downstream of the source beck on the main stem of the Caldew towards Dalston. In 2013 signal crayfish were collected and destroyed from one short stretch of Warnel Beck over a 6 week period, although the overall numbers did not show enough of a depletion to warrant continuing the process.

The eDNA results back up our conclusions from previous trapping and indicates that crayfish are more widely spread than previously reported along the entire river, in particular downstream of Warnell Beck.

Very little is known about the population downstream at Carlisle as only limited trapping has been undertaken. The small residual traces of signal crayfish eDNA at one sample location is likely a result of downstream flow of crayfish eDNA from the upstream sites. The high plague positive results makes the river a priority for survey effort or repeat eDNA sampling in the immediate future.