Operationalisation of eDNA methods for Crayfish monitoring

Chief Scientist's Group report

December 2024

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Dr Robert Bradburne
Chief Scientist

Contents

Acknowledgements ... 5

Executive summary ... 6

1. Introduction ... 8

2. Aims and Objectives ... 9

3. Methods and experimental design ... 10

3.1 Study site selection ... 10

3.2 Sampling Protocol ... 11

3.3 Laboratory and analytical methods ... 14

4. Results ... 16

4.1 Limits of detection and limits of quantification of qPCR assays ... 16

4.2 Field testing ... 17

5. Discussion ... 21

5.1 Discussion of field-testing results ... 21

5.2 Thalinger Scale ... 23

5.3 Operationalisation ... 27

5.4 Future work ... 27

6. Case Study: Meanwood Beck ... 28

7. Conclusions ... 34

Appendix 1 Full sampling protocol ... 36

Appendix 2: Membrane filter size selection ... 59

Appendix 3: qPCR Conditions for target species ... 62

Appendix 4: LOD and LOQ results ... 67

References ... 73

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Executive summary

This research project tested and validated the use of a new species-specific monitoring method that analyses genetic material in the environment (eDNA) to detect white-clawed crayfish, signal crayfish, and crayfish plague in English waterbodies.

White-clawed crayfish are a native species found in English rivers. They play an important role in river ecosystems and the delivery of ecosystem services. However, their population has declined dramatically in recent decades, and they are now classified as 'endangered'. This is due to the introduction of invasive, non-native crayfish species which outcompete white-clawed crayfish, and disease, specifically crayfish plague, which is fatal to crayfish.

The Environment Agency is responsible for monitoring crayfish populations and their health in England. Currently, crayfish monitoring is reliant on trapping and visual field surveys, which can only be conducted in July, August, and September and is resource intensive. However, developments in technologies for analysing eDNA has led to the development of a new, more sensitive method for detecting white-clawed crayfish, signal crayfish, and crayfish plague in water bodies. This project aimed to validate and test the operationalisation of this method for crayfish monitoring in English waterbodies by the Environment Agency.

A sampling protocol was developed to collect eDNA from water bodies by filtration. Samples were collected from six waterbodies in northern England on a minimum of four occasions between November 2021 and February 2023. One or more target species were known to be present at four of the waterbodies, and were absent at two sites, which acted as control sites. This research showed that the protocol was effective in collecting eDNA and minimising contamination, and staff reported that it was easy to follow. The samples were analysed to determine whether white-clawed crayfish, signal crayfish, and crayfish plague eDNA was present in the samples. A framework for interpreting results from eDNA-based monitoring methods advises that given the work done to validate and test this method, we can interpret positive results as indicative that the target species is likely present.

The majority of results (94%) matched expectations regarding the presence or absence of the target species at the sampling location. This gives us confidence in the accuracy and specificity of this method. All three target species were detected at sites outside the traditional sampling window, meaning this method would enable more comprehensive, year-round monitoring. There were a couple of sampling instances where target species were not detected by the eDNA-based method, despite being expected to be present. Conversely, white-clawed crayfish and crayfish plague were unexpectedly detected on several occasions at one location; this is likely due to downstream transport of genetic material from white-clawed crayfish populations present in the river's tributaries. This highlights that future work to incorporate transport of genetic material into the interpretation of results through modelling would be valuable.

During the trial a crayfish plage outbreak at Meanwood Beck was detected by eDNA methods, which was later confirmed by visual surveying. This led to the declaration of a Category 1 incident in January 2023 and action to conserve healthy white-clawed crayfish was taken. The eDNA-based method meant the crayfish plague outbreak was detected much earlier and enabled early action to conserve the white-clawed crayfish population to be taken, compared to traditional methods.

This research supports the operationalisation of eDNA-based monitoring methods for crayfish monitoring and highlights the value of these novel methods to the Environment Agency.

1. Introduction

Crayfish play an important role in freshwater ecosystems; they are a keystone species in the trophic food web and act as ecological engineers (Reynolds and others, 2013). Populations of white-clawed crayfish (*Austropotamobius pallipes*), have declined dramatically over recent decades and are now classified by the International Union for Conservation and Nature as 'endangered' (Füreder and others, 2010). Some of the largest remaining populations of white-clawed crayfish in Europe are located in central and northern England (Holdich and Rogers, 2000), despite recent declines in population across England in recent decades (Holdich and others, 2009). The decline in white-clawed crayfish populations over the past c. 50 years can primarily be attributed to the establishment of invasive non-native species (INNS) of crayfish, such as signal crayfish (*Pacifastacus leniusculu)* which displace white-clawed crayfish from their habitat niches through predation and competition (Holdich and others, 2014). Signal crayfish also harbour a highly infectious fungal-like parasite, the crayfish plague (*Aphanomyces astaci)* that can cause up to 100% mortality in infected white-clawed crayfish populations (Yu and others, 2022).

The Environment Agency's monitoring programme of white-clawed crayfish populations aims to aid their conservation by identifying the location of native populations, and assessing the risk they face from INNS and crayfish plague. Furthermore, the programme identifies potential locations, known as ark sites, that are free from crayfish plague and INNS, where white-clawed crayfish populations could be translocated to help sustain populations. At present, the programme relies primarily on manual survey techniques to assess the location of crayfish populations.

Studies on the development, testing and refinement of eDNA-based methods for the detection and monitoring of crayfish species and outbreaks of crayfish plague, have been published and may be useful to the Environment Agency for crayfish monitoring (Strand and others, 2011, 2014, 2019). The method, which has also been refined by the Republic of Ireland Marine Institute, involves filtering a large volume of water using a specialised pump through a sterile glass fibre filter, from which eDNA is subsequently extracted and a qPCR test for the specific crayfish species or crayfish plague undertaken. This method has been deployed across freshwaters in Norway (Strand and others, 2014, 2019; Vrålstrad and others, 2017) as well a range of freshwater habitats in the Czech Republic (Rusch and others, 2020) and its comparability to trapping methods for the presence/absence of crayfish has been demonstrated. It was officially integrated into the Norwegian national crayfish plague monitoring programme in 2016. More recently, after some refinement, the method has successfully been incorporated into the Republic of Ireland (RoI) Marine Institute national crayfish surveillance programme (Mirimin and others, 2017, 2022; Brady and others, 2024).

The eDNA method has been shown to have several advantages over conventional methods. The method was shown to be able to detect crayfish plague in freshwaters 2.5 weeks earlier than conventional trapping methods (Strand and others, 2019). It was also able to detect crayfish plague over a longer sampling window (i.e., crayfish plague could

be detected during the winter), has greater reliability over conventional methods and is a relatively cheap monitoring tool (Wittwer and others, 2018). Furthermore, the method is less likely to spread crayfish plague than conventional methods and is advantageous over conventional methods in terms of animal welfare (Strand and others, 2019).

Given the advantages of eDNA-based monitoring over conventional monitoring methods, coupled with a decline in resource and associated surveying efforts in recent decades, there is a drive to implement eDNA-based monitoring methods into the Environment Agency's monitoring of crayfish and crayfish plague. Methods that make use of eDNA technologies to detect white-clawed crayfish, signal crayfish, and crayfish plague have previously been implemented by the Environment Agency, however, this has been on an ad-hoc basis and made use of sampling kits provided and subsequently analysed by commercial companies. These sampling kits deploy differing methodologies and methodologies are not always transparent or available in the public domain. As such, data generated by these methods through ad-hoc sampling operations are not easily compared, and the limitations of the methods are not understood.

This project tested and further developed species-specific crayfish assays for white-clawed crayfish, signal crayfish, crayfish plague, and field sampling approaches that are all in the public domain (Agersnapp and others, 2017; Atkinson and others, 2019; Strand and others, 2014, 2019). This work will provide the evidence to inform the suitability for integration of eDNA method into the Environment Agency's crayfish monitoring programme.

2. Aims and Objectives

This work aimed to test, validate and optimise the eDNA crayfish methods for detecting and monitoring crayfish and crayfish plague in English freshwaters for operational deployment by the Environment Agency.

Specifically, the objectives were to:

- Validate the method developed by Strand and others in our in-house laboratories.
- Determine the limit of detection (LOD) and limit of quantification (LOQ) for the qPCR assays.
- Develop a scientifically robust and practical sampling protocol for deployment as part of routine sampling by the Environment Agency.
- Test at a range of English freshwater catchments, including where white-clawed crayfish, signal crayfish and crayfish plague were both present and thought to be absent.
- Assess the temporal variability in eDNA detection to determine the potential to extend the monitoring window for crayfish and crayfish plague.

3. Methods and experimental design

3.1 Study site selection

Six catchments in the north of England, which comprised Boshaw Whams Reservoir (West Yorkshire), Sugley Dene (Tyne and Wear), River Blyth (Northumberland), River Ure (North Yorkshire), Wansbeck (Northumberland), and Meanwood Beck (West Yorkshire), were selected for testing and validating the eDNA-based method (Figure. 1). Sites were selected based on where one or more of the target species were known to be present in the upstream catchment, and two ‘control’ catchments where crayfish and crayfish plague were known to be absent (Sugley Dene and Boshaw Whams Reservoir). Availability of Environment Agency staff to conduct sampling also informed site selection. The expected presence or absence of each target species for each study catchment is outlined in Table 1.

Figure 1. Location of study sites across northern England.

Table 1. Expected presence or absence of target species at each catchment and sampling dates.

Catchment	Target Species			Sampling occassions
	White-clawed crayfish	Signal crayfish	Crayfish plague	
Boshaw Whams Reservoir	Absent	Absent	Absent	Nov ’21, Feb ’22, Jul ’22, Dec ‘22
Sugley Dene	Absent	Absent	Absent	Nov ’21, Jan ’22, May ’22, Jul ’22, Nov ‘22
River Blyth	Absent	Present	Absent	Nov ’21, Jan ’22, May ’22, Jul ’22, Nov ‘22
River Ure	Present	Absent	Present	Nov ’21, Jan ’22, May ’22, Jul ’22, Nov ‘22
Wansbeck	Present	Absent	Absent	Nov ’21, Jan ’22, May ’22, Jul ’22, Nov ‘22
Meanwood Beck	Present	Absent	Absent	Mar ’22, May ’22, Jul ’22, Dec ’22, Jan ’23, Feb ‘23

3.2 Sampling Protocol

Sites were sampled on several occasions between November 2021 and February 2023; this is outlined in Table 1.

Samples were collected from the Sugley Dene, the River Blyth, the River Ure, and Wansbeck study sites in November 2021 and in January, May, July, and November 2022. The Bosham Shaw Reservoir study site was also sampled on four of the five occasions. Meanwood Beck was sampled in March, May, July, and December 2022 and in January and February 2023.

The full sampling protocol is detailed in Appendix 1. Briefly, three 5L water samples were collected at each sampling locality. Each 5L sample was passed through a 2µm glass fibre filter membrane (Merck AP2504700) using a Masterflex L/S peristaltic pump (MK-07571-

02), illustrated in Figure 2. If filters clogged before 5L was collected, additional filters were used. Figure 3 outlines the process for deciding how much water and how many filters needed to be used in the sampling process. A small experiment was conducted at the time of the first sampling campaign to determine the most suitable membrane pore size to use in sampling, which concluded that use of a membrane with a pore size of 2µm was most appropriate. The methods and results of this experiment are outlined in Appendix 2. The volume of water filter was determined by measuring the outflow of the membrane into a graduated container.

Figure 2. Water filtering as part of the sampling protocol at River Ure, January 2022.

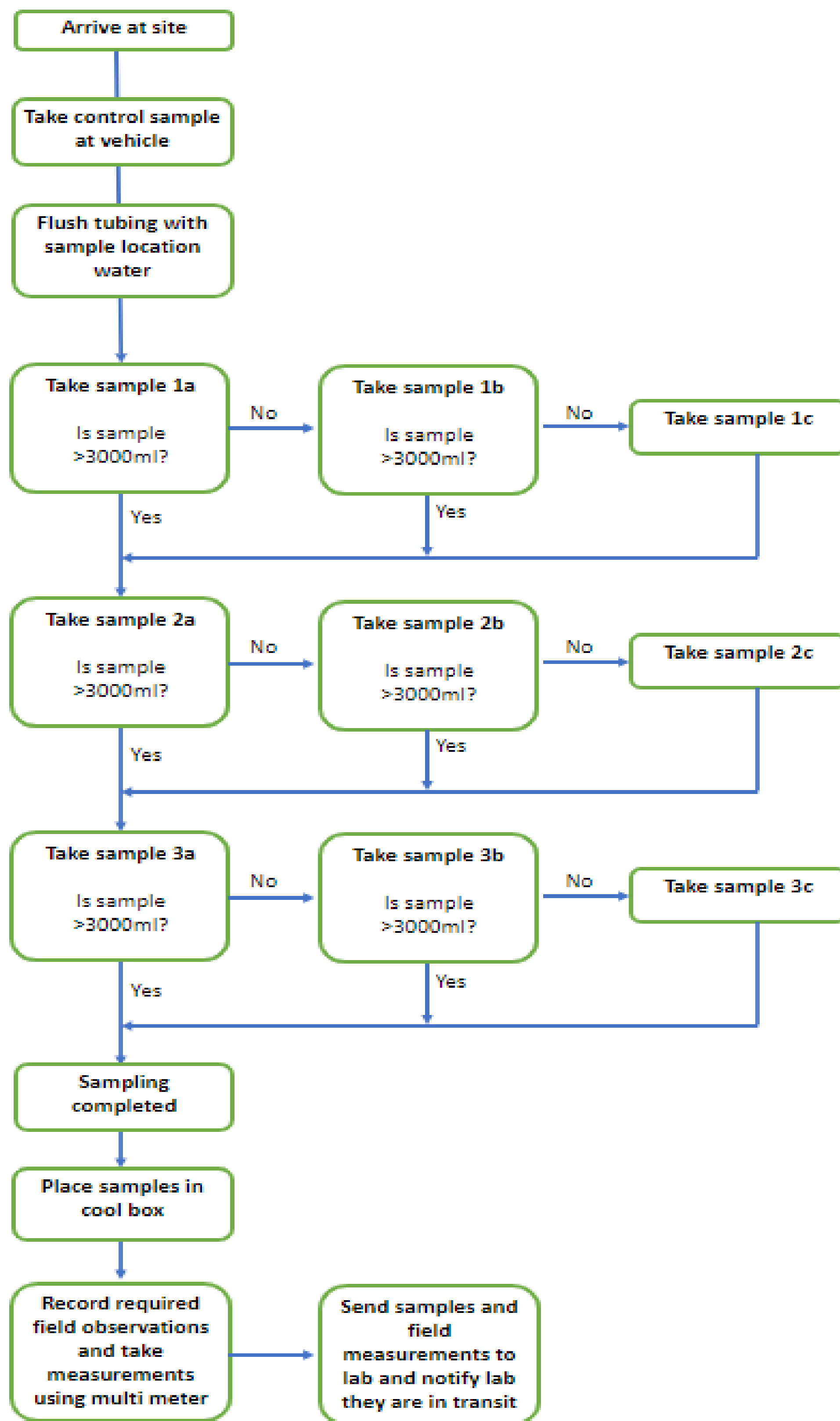

Figure 3. Flowchart for determining the number of samples and filters to use as part of the sampling protocol.

A blank sample was also collected at the first sampling locality for each study site to detect any contamination of the equipment prior to sampling. This involved pumping and filtering 5L of tap water brought to the site along with the rest of the field equipment. All filters were stored in a cool box before returning to the laboratory where they were stored at -20°C prior to processing.

A detailed cleaning protocol was followed during fieldwork and in the laboratory to ensure that equipment was cleaned, disinfected, and dried prior to and after sampling. Cleaning is an important biosecurity step to ensure that crayfish plague spores and/or invasive species are not spread between sites and for ensuring there is no cross contamination of eDNA.

3.3 Laboratory and analytical methods

3.3.1 eDNA Extraction

Filters were stored at -20 °C until they were processed for DNA extraction. DNA extraction was undertaken in a separate PCR-free clean room, following the Qiagen Power Water protocol (Cat. No. 14900-100-NF), which had been modified by the RoI Marine Institute and adapted as follows.

The filters were cut in half. One half of each filter was used in the DNA extraction process and the other half was stored in a labelled 15mL centrifuge tube at -20 °C.

The half of the filter being processed was added to a 5ml bead tube along with 1ml of PW1 solution and vortexed using a SciQuip VariMix at 50% setting for 30 minutes to thoroughly homogenise the sample. The contents of the bead tube were then transferred to an open 5mL syringe nested within a 15mL centrifuge tube, which was then sealed with Parafilm and centrifuged at 4000 g for 3 minutes. This recovered a volume of solution of c. 1.5-2mL.

After the inhibitor removal, the supernatant was split and transferred into two separate 2mL tubes and 650µL of PW3 reagent added to both. Both tubes were then gradually filtered onto one spin column through repeated steps of centrifugation and waste disposal. The Qiagen Power Water protocol was then followed to the end of the extraction process.

Following DNA extraction, the eluted DNA was stored at -20 °C until analysed.

Where <3000mL water could not be passed through a single filter and multiple filters were used, DNA extractions were performed on individual filters and then extracts from the multiple filters were pooled.

3.3.2 Detection by qPCR

Previously developed primers and probes were used for qPCR reactions. Previous studies have shown that there is no cross-reactivity for closely related species (Vrålstad and others, 2009; Agersnap and others, 2017; Atkinson and others, 2019).

The PCR reaction mix and components were made up and aliquoted into a 96-well plate in a UV cabinet in a DNA-clean room which was free from any samples or positive controls. Extra wells were aliquoted and used as non-template controls (NTC) as a control for the contamination of reagents. Once aliquoted, the master mix was transferred to another clean room where artificial DNA of known amounts were added to quantify the target gene Samples and extraction controls were diluted 1:10 with PCR-clean molecular grade water and tested in triplicate at both neat and diluted concentrations. Once the reaction mix had been prepared, the PCR reactions were run using Agilent Aria Mx qPCR instruments (#G8830A). Full details of PCR conditions including master mix reagent concentrations and PCR cycling conditions are outlined in Appendix 3.

3.3.3. Determination of the limits of detection and quantification of the qPCR assay

To measure the limit of detection (LOD) and limit of the quantification (LOQ) of the qPCR assays, fresh artificial DNA controls were resuspended in manufacturer recommended volumes to give 10^{13} gene copies. Equal volumes of the forward and reverse controls were combined and diluted to make a working stock with a concentration of 10^5 gene copies/µl (gc/µl). Salmon sperm DNA was added to the diluent when making the working stock, which acts as background DNA above the concentration of the standard.

The working stock was subsequently diluted to a concentration of c. 30 gc/µL, which referred to herein as 'LOD neat'. The LOD neat stock was then diluted in a 1:2 ratio repeatedly until a final ratio of 1:128 was reached, with aliquots of the stock at each concentration reserved. Aliquots of the stock at each concentration were then tested in 10 qPCR wells.

The limit of detection at 95% certainty (LOD_{95}) was estimated following the PODLOD method (Wilrich and others, 2009), using the excel program 'PODLOD_ver11.xls' (available for download at https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html). Linearity for log10 mean values above the LOD_{95} were compared against anticipated values; those with a slope between 0.9 and 1.1 were deemed acceptable and were used to calculate the LOQ.

To determine the LOQ, well values were log10 transformed and the standard deviation of replicate wells values calculated. The LOQ was determined to be the concentration of gene copies from the highest dilution ratio where:

- the standard deviation between replicates was <0.33 log10 copies per well
- and all replicates had a value >0.33 log10 copies per well.

4. Results

4.1 Limits of detection and limits of quantification of qPCR assays

Mean and standard deviation of gene copies per well for white-clawed crayfish, crayfish plague, and signal crayfish for all dilution ratios analysed are summarised in Table 2 and detailed in full in Appendix 4. All assays performed within the acceptable limits for qPCR reactions (R^2>0.98, slope ranges 3.1 to -3.5, efficiency ranges 90-110%).

Table 2. Mean and standard deviation (std. dev.) gene copies per well for each dilution ratio of white-clawed crayfish, crayfish plague, and signal crayfish.

Dilution ratio	White-clawed crayfish		Crayfish plague		Signal crayfish	
	Mean (gc/well)	Std. dev. (gc/well)	Mean (gc/well)	Std. dev. (gc/well)	Mean (gc/well)	Std. dev. (gc/well)
Neat	82.04	23.21	248.92	17.49	114.75	19.09
1:2	35.20	14.58	118.03	31.64	71.02	15.58
1:4	22.06	14.36	49.63	11.08	33.52	9.67
1:8	13.44	8.38	21.45	5.74	12.79	2.87
1:16	5.49	3.00	14.09	4.64	7.64	3.42
1:32	4.22	3.33	5.61	2.82	1.85	1.07
1:64	-	-	4.04	2.49	3.29	2.12
1:128	5.71	2.42	1.97	0.97	2.80	1.80

The LOD_{95} and LOQ for the three target species are outlined in Table 3.

Table 3. Limit of detection (95% confidence interval; LOD_{95}) and limit of quantification (LOQ) for signal crayfish, crayfish plague, and white-clawed crayfish.

Species common name	LOD_{95} (copies/well)	LOQ (copies/well)	µL per well
Signal crayfish	3.8	7.2	2
Crayfish plague	12.8	15.6	5
White-clawed crayfish	21.2	41.0	5

4.2 Field testing

An outbreak of crayfish plague occurred at Meanwood Beck during the sampling period. These results are presented separately as a case study in Section 6.

4.2.1 Blank control samples

All blank control samples collected at the 6 study sites had negative qPCR test results for the three target species indicating that there was no contamination of the samples by target gene DNA.

4.2.2 Boshaw Whams Reservoir

None of the target species were expected to be present at Boshaw Whams Reservoir. The site was visited on 4 occasions between November 2021 and December 2022 and a total of 12 DNA extractions were analysed. No target species were detected in any of the DNA extractions analysed.

4.2.3 Sugley Dene

None of the target species were expected to be present at Sugely Dene. The site was visited on 5 occasions between November 2021 and November 2022 and a total of 13 DNA extractions were analysed. No target species were detected in any of the DNA extractions analysed.

4.2.4 River Blyth

Signal crayfish were expected to be present at the River Blyth and white-clawed crayfish and crayfish plague were not. The River Blyth was visited on 5 occasions between November 2021 and November 2022. Signal crayfish were detected on all sampling occasions; at least 1 sample replicate had a positive qPCR result on each sampling occasion (Figure 4). Crayfish plague was detected on a single sampling occasion (January 2022). White-clawed crayfish were also detected on this occasion (neat replicate only) and on one other (November 2022).

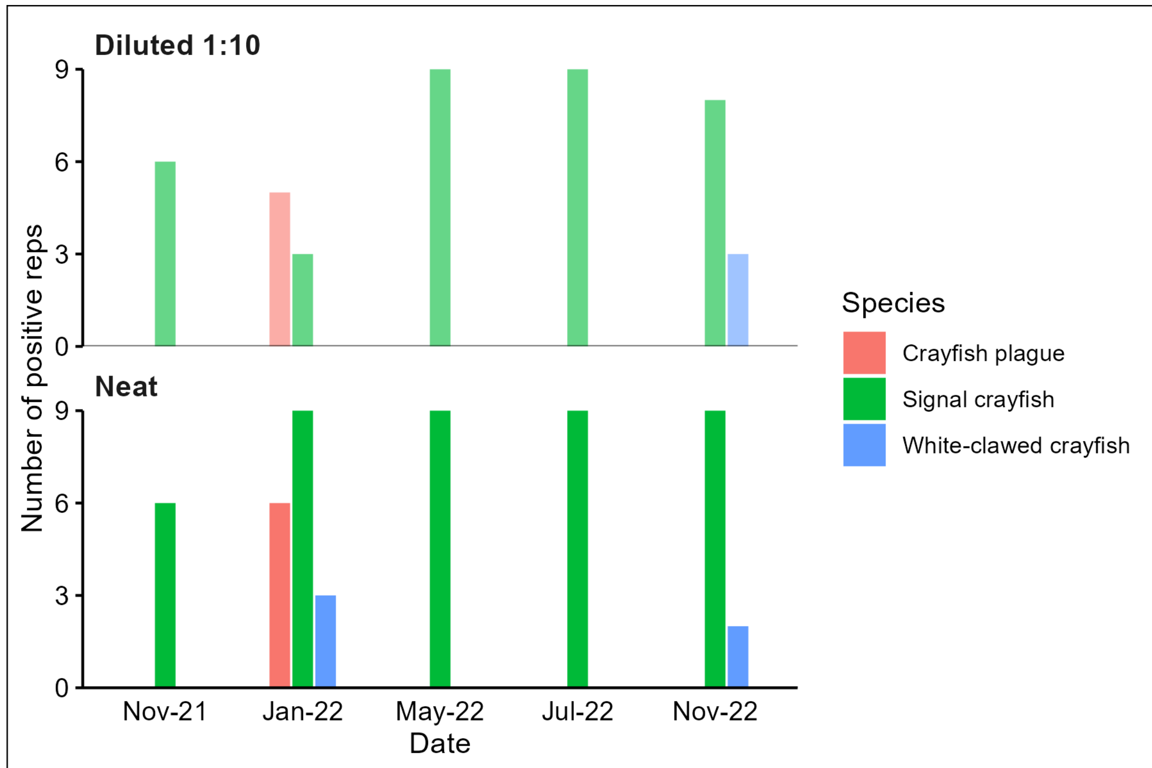

Figure 4. Number of sample replicates (neat and diluted crayfish plague, signal crayfish, and white-clawed crayfish) for each sampling occasion at the River Blyth.

4.2.5 River Ure

White-clawed crayfish and crayfish plague were expected to be present at the River Ure. Signal crayfish were not expected to be present. The site was sampled on 5 occasions between November 2021 and November 2022. Crayfish plague was detected on all sampling occasions, with ≥1 wells positive qPCR result for neat and diluted replicates on all occasions, except May 2022, which only had a positive qPCR result for the diluted replicate (Figure 5).

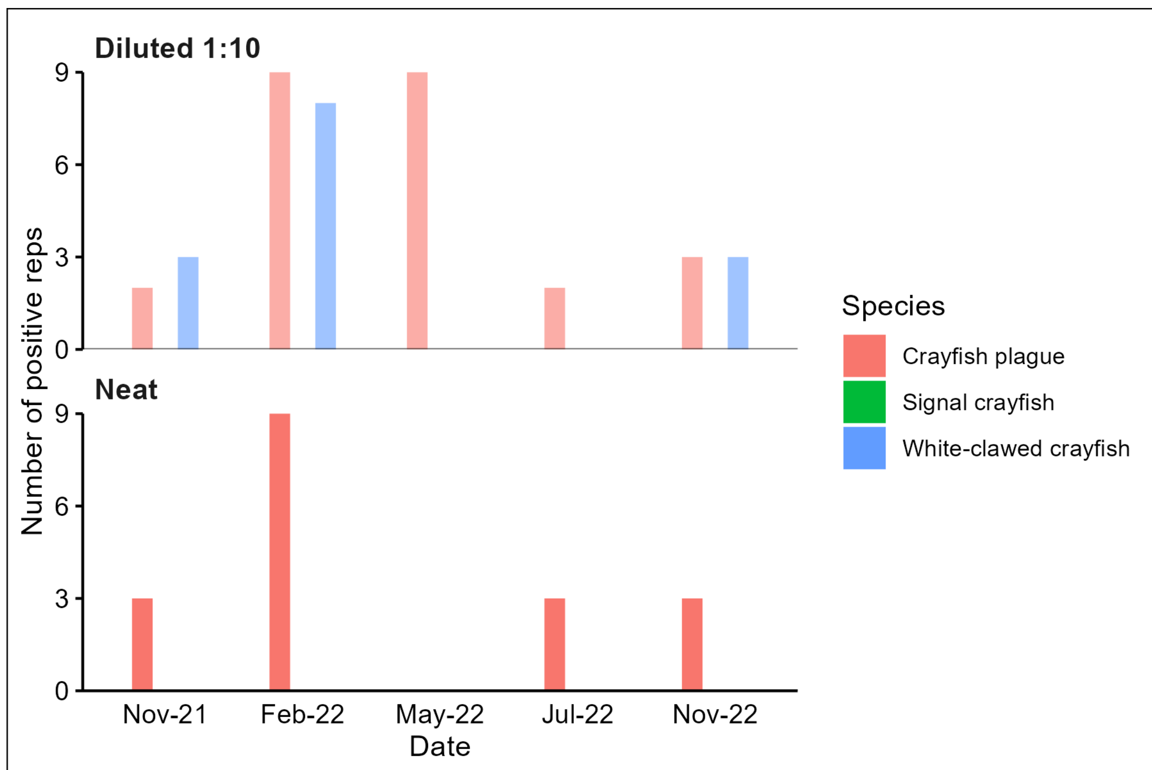

Figure 5. Number of sample replicates (neat and diluted at a 1:10 ratio) with a positive qPCR test result for the three target species (crayfish plague, signal crayfish, and white-clawed crayfish) for each sampling occasion at the River Ure.

White-clawed crayfish were detected on three sampling occasions (November 2021, February 2022, and November 2022). Only replicates which had been diluted had positive qPCR results. Signal crayfish were not detected at the site on any sampling occasion.

4.2.6 Wansbeck

White-clawed crayfish were expected to be present at Wansbeck. Signal crayfish and crayfish plague were not expected to be present at Wansbeck. The site was visited on 5 occasions between November 2021 and November 2022. White-clawed crayfish were detected on every sampling occasion (Figure 6). At least 3 replicates analysed at a dilution ratio had a positive qPCR result on each sampling occasions, whereas neat replicates only had ≥1 wells positive qPCR result on one occasion (January 2022). Signal crayfish and crayfish plague were not detected on any sampling occasion.

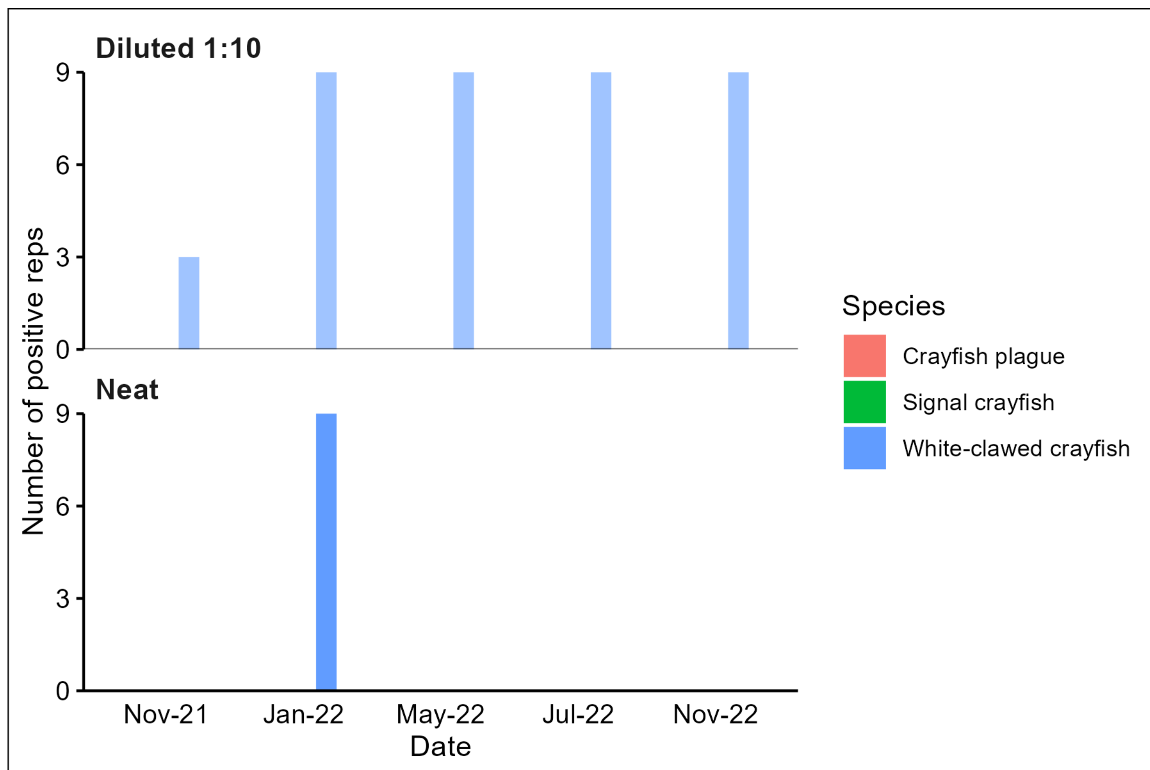

Figure 6. Number of sample replicates (neat and diluted at a 1:10 ratio) with a positive qPCR test result for the three target species (crayfish plague, signal crayfish, and white-clawed crayfish) for each sampling occasion at Wansbeck.

5. Discussion

In this section, the work undertaken thus far in developing and operationalising the white-clawed crayfish

White-clawed crayfish, signal crayfish, and crayfish plague assays were benchmarked against the Thalinger Scale, and the results from the field testing discussed. Practical considerations around the operationalisation of this method are also discussed. Finally, opportunities for future research are outlined.

5.1 Discussion of field-testing results

The analysis of all blank field samples yielded negative qPCR test results for all target species. This suggests that the control measures to minimise contamination that were implemented in the sampling protocol are effective and sufficient.

When the target species was not expected it was consistently not detected in the majority of sampling instances. Signal crayfish were not detected in any sampling instances where they were not expected to be present (Table 4). White-clawed crayfish and crayfish plague were detected when not expected in 14% and 5% of sampling instances.

Table 4. Summary of results from qPCR pilot testing. Results that did not match presence/absence prediction are highlighted in grey.

Site	White-clawed crayfish		Signal crayfish		Crayfish plague	
	Prediction	Occasions species detected	Prediction	Occasions species detected	Prediction	Occasions species detected
Boshaw Whams Reservoir	X	0/4	X	0/4	X	0/4
Sugley Dene	X	0/5	X	0/5	X	0/5
River Blyth	X	2/5	✓	5/5	X	1/5
River Ure	✓	3/5	X	0/5	✓	5/5
Wansbeck	✓	5/5	X	0/5	X	0/5

At the River Blyth, two target species, white-clawed crayfish, and crayfish plague, were unexpectedly detected by qPCR tests. White-clawed crayfish eDNA was detected during two sampling occasions in January 2022 and November 2022. Specifically, on each occasion, three out of a total of eleven replicate qPCR tests yielded positive results for white-clawed crayfish in January, and five out of eleven replicates tested positive in November. Crayfish plague eDNA was also detected in January 2022; 6 of a total of 11 replicate qPCR tests had a positive result.

It is most likely that the detection of white-clawed crayfish is a result of the detection of eDNA from white-clawed crayfish populations present in tributary rivers to the River Blythe which was transported downstream to the sampling locality; a study has shown that crayfish eDNA can be detected up to 7km downstream from the location of the source population (Chucholl and others, 2021). This highlights the value of incorporating eDNA transport into the interpretation of eDNA test results through occupancy modelling (Burian and others, 2021) and other modelling approaches (e.g. eDNA Integrating Transport and Hydrology; Carraro and others, 2020); further work to explore the potential value of these approaches to crayfish monitoring and the Environment Agency is discussed later in this report.

As crayfish plague was only detected on a single occasion at the River Blyth, and not detected on subsequent sampling occasions following its detection, the detection is not

indicative of a crayfish plague outbreak. Instead, this detection of crayfish plague DNA may be due to contamination of the sample material or a low-level detection of non-viable crayfish plague which was not able to establish itself at the site. It is also possible that it is a novel detection of the closely related *Aphanomyces fennicus*, which has been shown to interfere with the crayfish plague qPCR test method (Viljamaa-Dirks and Heinikainen, 2019). Further work to sequence the positive result would allow us to test this hypothesis. This highlights the value of repeated testing over time.

White-clawed crayfish were expected to be present at the River Ure sampling location but were not detected by qPCR on two occasions in May and July 2022. This may be due to the concentration of white-clawed crayfish eDNA in the water sample being lower than the LOD. This could be due to a higher river discharge diluting the eDNA or temporal variability in the life habit of crayfish which influences DNA shedding, although this is not seen at other sites during these months. Contaminants in water samples may also be higher and act to inhibit the PCR amplification process, thus leading to non-detection (e.g., Strand and others, 2014). Interestingly, white-clawed crayfish were not detected by qPCR during the traditional crayfish sampling window but were detected on sampling occasions that fell outside of the traditional sampling window. This highlights the value in repeated testing throughout the year at a locality, rather than one-off sampling and testing.

The qPCR tests conducted detected the presence of all three target species on sampling occasions throughout the year; white-clawed crayfish, signal crayfish, and crayfish plague were detected all year-round at Wansbeck, the River Blyth, and the River Ure respectively. Other studies have also demonstrated that qPCR testing is effective in detecting crayfish species throughout the year (e.g., Wittwer and others, 2018; Chucholl and others, 2021). The detection of the target species outside of the traditional crayfish monitoring period, which runs from July to September (inclusive), is beneficial for enabling more comprehensive monitoring of crayfish species, and in particular, monitoring crayfish plague outbreaks. This is demonstrated by the detection of a crayfish plague outbreak at Meanwood Beck in late 2022 (Section 6).

5.2 Thalinger Scale

The ‘Thalinger’ scale is a descriptive validation framework for the classification of qPCR assays based on their accuracy and sensitivity for single-species detection (Thalinger and others, 2021). The framework was developed to articulate the reliability of the assays and inform how qPCR results should be interpreted, based on the extent to which the assay has been validated. An overview of the levels of the validation scale and the appropriate interpretation of results are outline in Figure 7.

Level 1 Incomplete	Level 2 Partial	Level 3 Essential	Level 4 Substantial	Level 5 Operational
Assay designed tested on target tissue	Level 1 + **assay optimized** tested on closely related non-target species	Level 2 + **assay tested on eDNA samples** positive detections obtained all samples processing steps reported in detail	Level 3 + **Limit of Detection (LOD) established** extensive field testing and in vitro testing on co-occurring non-target species	Level 4 + **detection probabilities estimated by statistical modelling** comprehensive specificity testing and investigating environmental influences

Interpretation of Results

Levels 1 and 2	Level 3	Levels 4 and 5
impossible to tell if target species is present or absent	not detected: impossible to tell if target is present or absent detected: target species is likely present if field controls return of negative, lab is appropriate for eDNA, and positive detections are sequences	not detected: target likely absent, assuming appropriate timing and replication in sampling. Level 5 provides probability of species presence despite negative result. detected: target very likely present

Figure 7. Overview of the 'Thalinger' scale levels and the appropriate interpretation of results for each level (Thalinger and others, 2021).

Each level of the 'Thalinger' scale has a number of 'thematic variable blocks' which comprise the aspects of work which underpins the validation of a qPCR assays. More basic validation processes are associated with the lower levels of scale, and more comprehensive and advanced work is associated with higher levels. The 'Thalinger' scale

also has minimum criteria that should be met for each variable block. It should be noted that the scale is additive (i.e., all of the variables for level 1 and level 2 must be met in order to classify at level 2).

The variable blocks and minimum criteria for each level, and those met for the crayfish assays tested in this work and by previous work (Strand and others, 2011, 2014, 2019; Mirimin, 2017; Rusch and others., 2020; Mirimin and others., 2022; Brady and others, 2024) are outlined by Table 5. As illustrated, all the requirements for Level 3 have been met and some of the requirements for Level 4 (specifically the determination of the limit of detection and extensive field testing) were met through this research project. We are therefore able to interpret positive qPCR test results using the assays outlined in this project as indicative of the target species being likely present (if stipulated requirements outlined in Figure 7 are met). However, we are not yet meeting sufficient criteria to interpret negative results as indicative of the absence of the target species. Future work to meet all the criteria for level 4 and subsequently level 5 would be advantageous as it would increase confidence in the interpretation of positive qPCR test results and facilitation of null qPCR test results.

Table 5. Thalinger scale criteria and criteria fulfilled in the validation of crayfish assays by this study and previous work by Strand and others, (2011, 2014) and the RoI Marine Institute.

Validation level	Variable blocks	Minimum criteria	Fulfilled by this study/previous work	Fulfilled by previous work
Level 1	In silico analysis	Target species		√
	Target tissue testing	Target tissue		√
	Target tissue PCR	Primer (and probe) sequence		√
Level 2	Comprehensive reporting of PCR conditions	DNA extract volume in PCR	√	√
	In vitro testing on closely related species	Any *in vitro* non-target testing	√	√
Level 3	Extraction method performed on eDNA samples	Method of extraction	√	√
	Concentration of eDNA from environmental sample	Filter type or precipitation chemicals	√	√
	Detection obtained from environmental samples	Detection from an environmental sample (artificial or natural)	√	√
Level 4	Limit of Detection (LOD)	LOD determined	√	√
	Extensive field testing of environmental samples	Multiple locations or multiple samples	√	√
	In vitro testing on co-occurring non-target species	Any advanced in vitro testing		
Level 5	Comprehensive specificity testing	Non-co-occurring/closely related species checked from in silico		

Validation level	Variable blocks	Minimum criteria	Fulfilled by this study/previous work	Fulfilled by previous work
	Detection probability estimation from statistical modelling	Any effort made towards detection probability estimation		
	Understanding ecological and physical factors influencing eDNA in the environment	Any factor influencing eDNA in the environment tested.		

5.3 Operationalisation

The sampling protocol developed through this work was effective in collecting sufficient DNA from the target species while minimizing contamination, as evidenced by the results of this work. The protocol was developed alongside Environment Agency field operations staff (for example, the selection of filter size; Appendix 2) and found to be a feasible protocol to follow in the field. Staff were confident that this sampling protocol could be introduced as part of routine monitoring more widely.

5.4 Future work

There are several areas for future research which could bolster the use and operationalisation of qPCR testing for crayfish species and crayfish plague.

Firstly, it would be beneficial to improve our understanding of the temporal variability of crayfish eDNA concentration, and thus the likelihood of detection by qPCR, over the course of the year. As demonstrated by this report, the target species are not always detected throughout the year by qPCR test (e.g., the detection of white-clawed crayfish at the River Ure). Similarly, other studies have shown that the concentration of crayfish DNA and the subsequent detection of crayfish species varies throughout the year. This can be attributed to a number of factors, including DNA shedding which can be linked to the life stage of the crayfish, the size of the crayfish population, eDNA degradation and river discharge (Chucholl and others, 2021). A more comprehensive understanding of the temporal variability of crayfish species and plague detection by qPCR would help to identify whether there are optimal times during the year to undertake qPCR testing.

Secondly, since this project began, Strand and others (2023) have developed and validated an improved species-specific assay for the detection of crayfish plague. This

newer assay has a similar sensitivity to the assay tested in this report, however, it does not amplify *Aphanomyces fennicus*, a new species, which has been shown to interfere with eDNA methods of crayfish plague (Viljamaa-Dirks and others, 2019), including the assay tested here. Field testing this assay would allow us to ensure that we are utilising assays that do not co-amplify co-occurring similar species, therefore avoiding spurious results.

It would also be advantageous to undertake work to fully meet the criteria for levels 4 and 5 of the 'Thalinger' scale, as this would allow us to interpret negative test results as indicative of the absence of the target species, which we are currently unable to do. In particular, applying statistical modelling and occupancy modelling techniques, such as the eDITH model which integrates eDNA shedding, decay, and transport in the catchment (Carraro and others, 2020), and/or the eDNAplus model which accounts for all sources of error and variation in the eDNA data generation process (Diana and others, in press). The adoption of these approaches would allow us to understand the probability that the target species would be detected at any location on the river network in a specific catchment. Furthermore, the eDITH model can be used to inform and optimise (eDNA) sampling design (Carraro and others, 2021), which could be used by the Environment Agency to inform future monitoring efforts.

Finally, field testing and the operationalisation of species-specific assays for the detection of a range of other target species would allow us to build on and improve the Environment Agency's capacity for using eDNA-based methods for species monitoring.

6. Case Study: Meanwood Beck

There are known native and non-native crayfish populations across Yorkshire, which have been confirmed through annual traditional survey methods; the distribution of these populations across Yorkshire's main rivers is illustrated by Figure 8. White-clawed crayfish are known to be present at Meanwood Beck, however, the white-clawed crayfish population in the adjacent Wyke Beck was lost in 2017. White-clawed crayfish populations at Meanwood Beck were confirmed healthy by the annual traditional survey in July 2022 and by further trapping in September 2022.

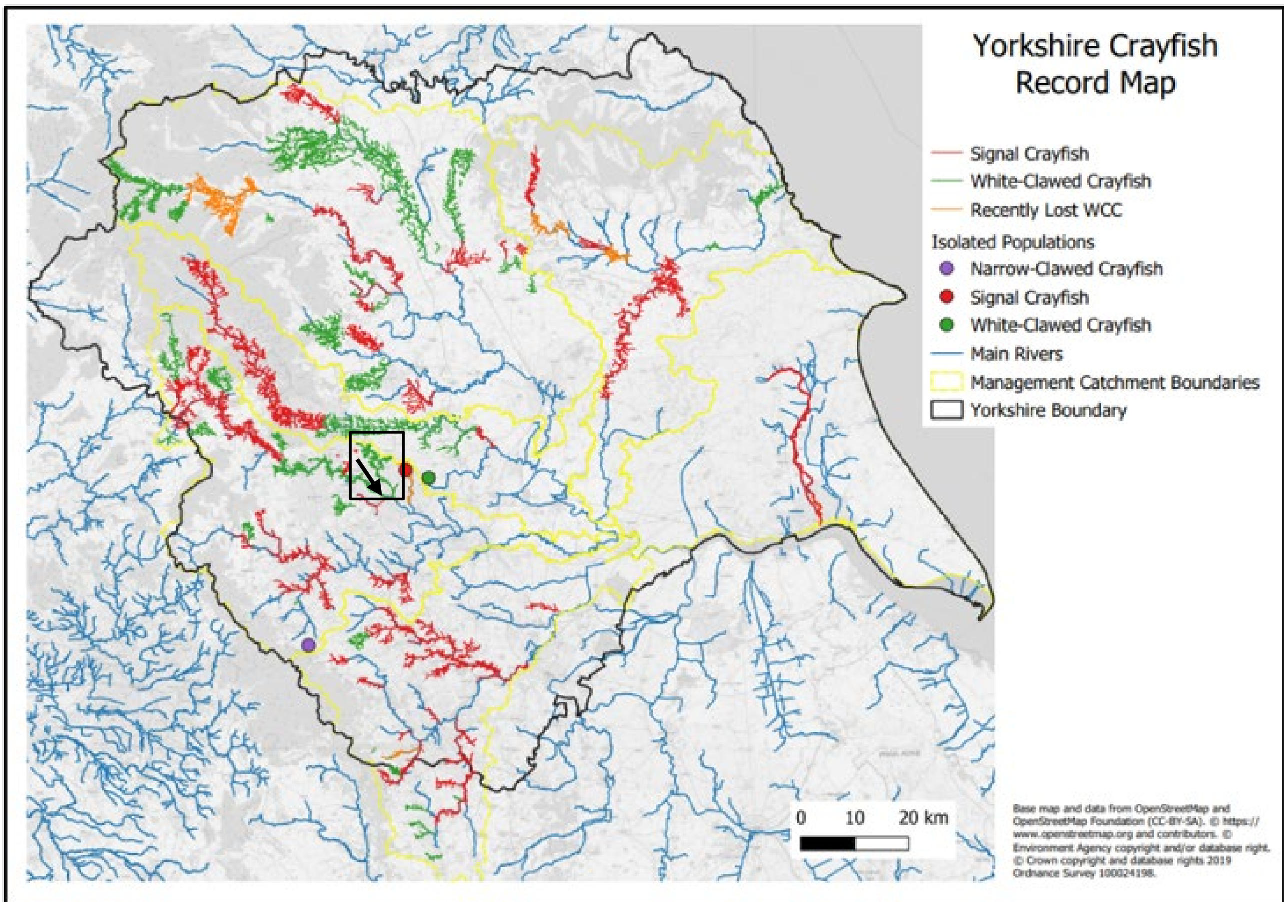

Figure 8. Map showing the main rivers of Yorkshire and the location of native (white-clawed crayfish) and non-native (signal and narrow-clawed crayfish). Meanwood Beck is highlighted by the black box and flow direction is illustrated by the arrow.

The eDNA qPCR tests for detecting white-clawed crayfish, signal crayfish, and crayfish plague were tested at Meanwood Beck, North Yorkshire on seven occasions between March 2022 and February 2023. White-clawed crayfish were detected on all sampling occasions; qPCR results from replicates that had been diluted at a 1:10 ratio were more consistent than those from neat replicates (Figure 9).

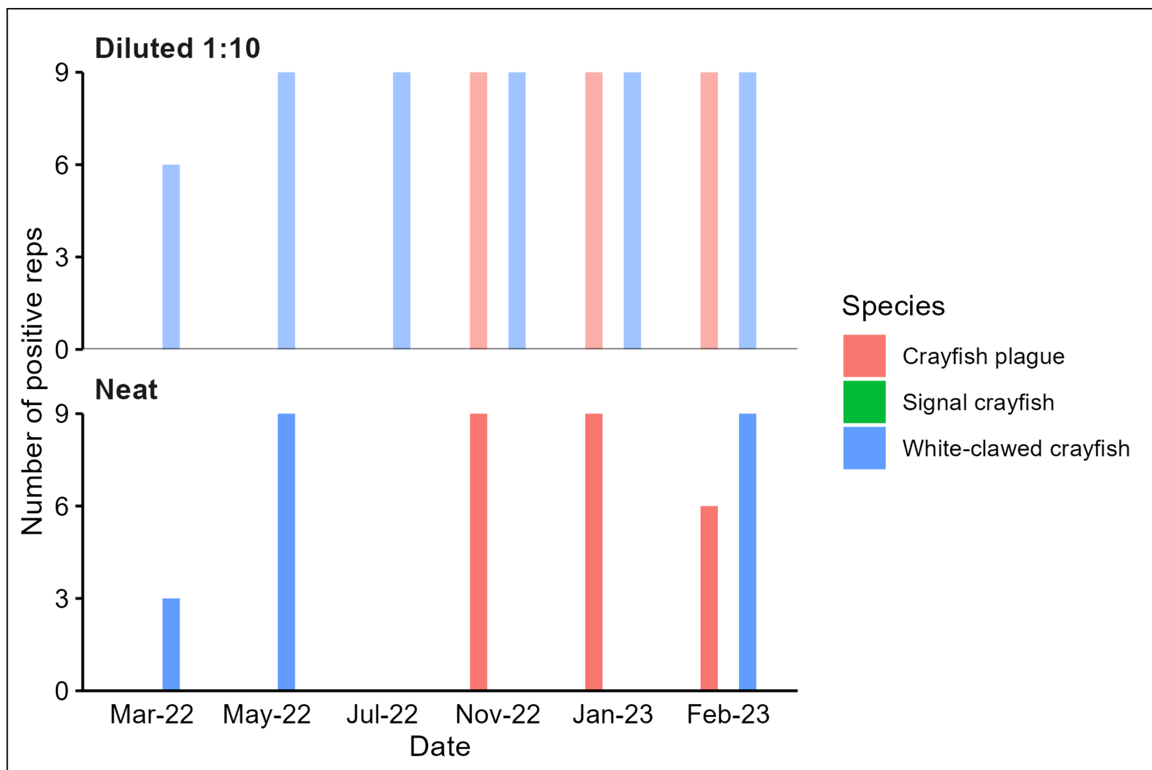

Figure 9. Number of sample replicates (neat and diluted at a 1:10 ratio) with a positive qPCR test result for the three target species (crayfish plague, signal crayfish, and white-clawed crayfish) for each sampling occasion at Meanwood Beck.

No other target species were detected on sampling occasions in March, May, and November 2022. However, the qPCR test undertaken in late November 2022 had a positive result for crayfish plague (Figure 9). This was suggestive of a possible outbreak of crayfish plague at Meanwood Beck. To investigate the possibility of a crayfish plague outbreak, further Environment Agency and commercial (NatureMetrics NM) eDNA tests were undertaken at the initial study site (Site 1; Figure 10) and further upstream (NM test only) at Site 5 on 5th January 2023. All eDNA tests results were positive for crayfish plague, corroborating the qPCR test results of November 2022. High river flows meant that hand surveying was unable to be undertaken at this time.

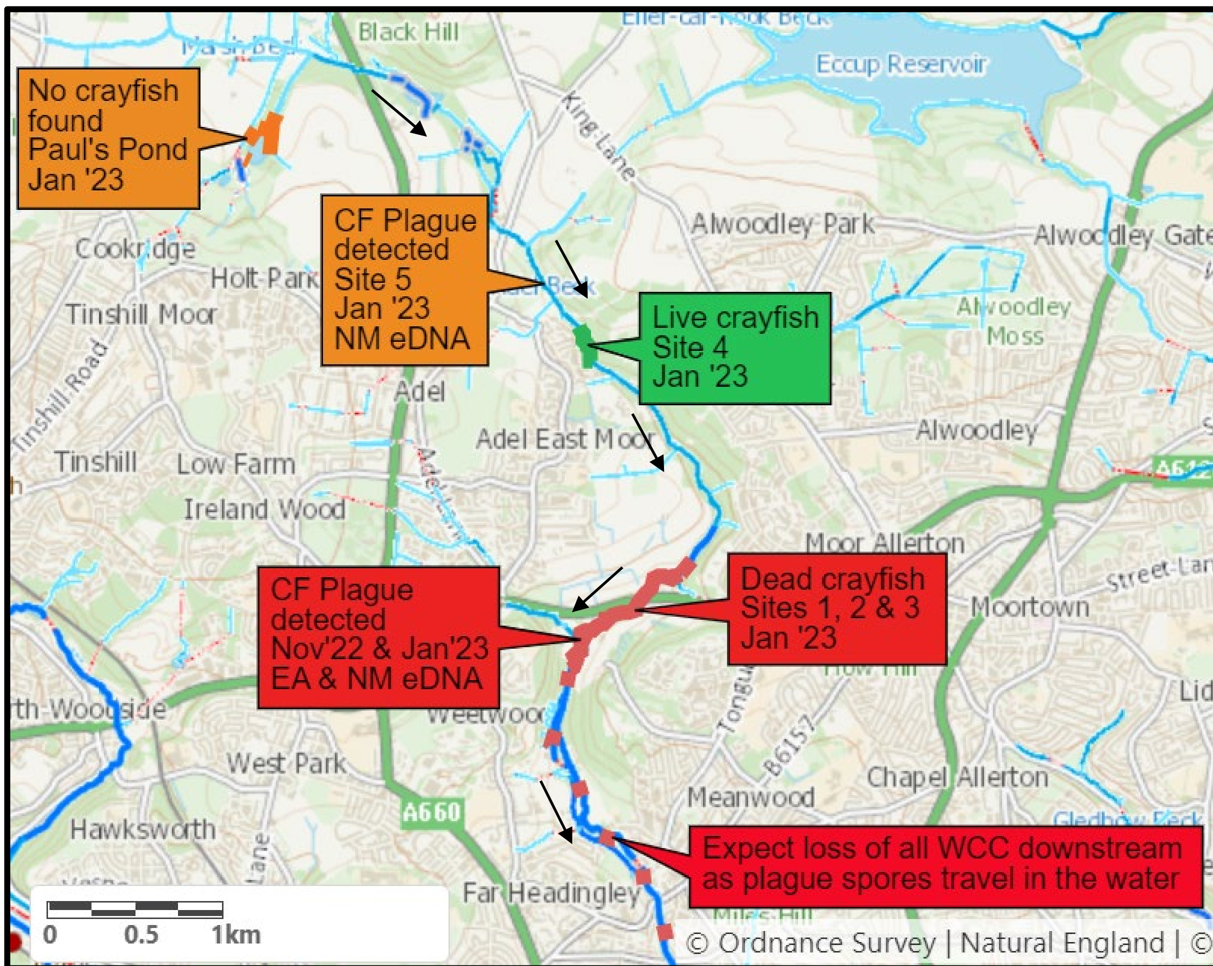

Figure 10. Map of Meanwood Beck, and the localities and outcomes of eDNA testing, surveying, and trapping undertaken to assess the extent of the crayfish plague outbreak. Flow direction is indicated by black arrows.

Following the positive qPCR test results for crayfish plague in November 2022 and early January 2023, traditional surveying was undertaken around the initial sampling location and further upstream on 25th and 26th January 2023 (Figures 10 and 11). Dead crayfish were found at Sites 1, 2, and 3 (Figures 10 and 12). These specimens were sent to Cefas (Centre for Environment, Fisheries and Aquaculture Science) for confirmatory testing, which confirmed that they were infected with crayfish plague. Sadly it was anticipated that white-clawed crayfish populations downstream of these sites would be lost due to the downstream spread of crayfish plague. Live crayfish were found upstream at Site 4 and specimen material was also sent to Cefas for testing which confirmed that these individuals were not infected with crayfish plague. white-clawed crayfish were not found at Paul's pond, which was the furthest upstream site surveyed (Figure 10). However, this may be the result of surveying taking place out of the regular survey window.

Figure 11. Hand surveying (a) and trapping (b) undertaken at Meanwood Beck 2023 to confirm the outbreak of crayfish plague.

Figure 12. Remains of dead crayfish found during survey downstream of initial **location where crayfish plague was initially detected.**

Due to the detection of crayfish plague eDNA at Meanwood Beck and confirmation of the crayfish plague outbreak by traditional sampling and analysis of white-clawed crayfish specimen material by Cefas, a category 1 incident was declared (in line with Environment Agency Common Incident Classification System guidance) in January 2023.

Native crayfish were assessed and rescued by Environment Agency staff and volunteers, kept in quarantine at York Gate Garden to ensure they were free of crayfish plague, and

subsequently released into Bodington Pond at the University of Leeds (Rescued endangered native crayfish moved to safe haven - GOV.UK (www.gov.uk). Bodington Pond is an ark site, which is a site known to be safe from crayfish plague and other INNS and where native crayfish are re-located to maintain and breed to sustain their populations. There is a possibility white-clawed crayfish could be re-introduced to Meanwood Beck if and when the crayfish plague has run its course and if no signal crayfish are detected. eDNA will be used as a tool to assess the suitability of Meanwood Beck for reintroduction of the native crayfish populations.

Interestingly, no signal crayfish were detected by DNA methods or observed during surveying or trapping (Figure 9), suggesting that signal crayfish were not the disease vector.

This case study demonstrates how eDNA-based methods for detecting and monitoring crayfish plague and crayfish populations can be advantageous, especially when used alongside conventional methods. In this instance, crayfish plague was able to be detected much earlier than had only conventional annual surveying been conducted. Uninfected white-clawed crayfish were able to be translocated to an ark site because the plague outbreak was caught early. Had the plague outbreak not been detected, it is probable that the translocation interventions to help protect native crayfish populations, would have been less successful.

7. Conclusions

The laboratory and field work undertaken as part of this research, building on work undertaken by Strand and others (2011, 2014, 2019) and the RoI Marine Institute has validated the assays used in qPCR testing to detect white-clawed crayfish, signal crayfish, and crayfish plague to Level 3 on the 'Thalinger' Scale (Thalinger and others, 2021). This means that, subject to field control samples yielding negative results, samples being processed in an 'eDNA appropriate' laboratory, and sequencing of positive detections, qPCR test results can be interpreted as indicative of the presence of the target species. However, conclusions about the presence of absence of the target species cannot be made on the basis of a negative qPCR test result. Future work to meet the criteria for Level 4 (and 5) would enable us to infer absence of the target species from negative qPCR test results, as well as increase confidence in conclusions drawn from positive test results.

The field-testing results mostly matched our expectations as to the presence or absence of target species, which gives us confidence in the accuracy and specificity of the assays and thus the use of these assays in field operations. Crayfish species and crayfish plague were detected by positive qPCR test beyond the traditional crayfish monitoring period (July-September); all three target species were detected year-round at, at least one sampling location. The ability of qPCR to detect target species beyond the extent of the traditional monitoring period will allow more comprehensive monitoring of crayfish species, and most beneficially, crayfish plague.

On a few sampling occasions at the River Blyth, white-clawed crayfish and crayfish plague were detected, despite not being expected to be present. The detection of white-clawed crayfish eDNA is likely due to the downstream transport of eDNA from white-clawed crayfish populations established in the tributaries of the River Blyth. The detection of crayfish plague on one sampling occasion could be attributed to several reasons including contamination of the sample, a low-level detection of non-viable crayfish plague which was unable to establish itself at the site, or a novel detection of the species *Aphanomyces fennicus.* On the other hand, white-clawed crayfish were expected to be present at the River Ure catchment but were only detected on three of five sampling occasions. This may be due to the concentration of DNA being below the LOD or a higher concentration of contaminants in the water sample which acted to inhibit the PCR amplification process. Both instances highlight the value of repeated testing over time at a locality.

The sampling protocol co-developed with operational field staff through this research has been shown to be fit for purpose, as demonstrated by the success of the field testing which resulted in all control samples yielded negative test results, and positive feedback from staff about the ease of undertaking the protocol in the field.

The application of eDNA monitoring methods at the Meanwood Beck catchment which detected a crayfish plague outbreak in late 2022, which would have not been exposed on a comparable timescale by field testing, and which informed interventions to protect native white-clawed crayfish populations, demonstrates the value of eDNA-based monitoring

methods for field operations. This work has provided the evidence and information to support the operationalisation of this protocol on a wider basis, and thus aid and inform the protection of native crayfish species in England.

Appendix

Appendix 1: Full sampling protocol

Introduction

This field protocol has been developed to collect environmental DNA (eDNA) to confirm the presence of white-clawed crayfish, crayfish plague and other INNS.

This protocol builds upon the work of Fiona Swords and Bogna Griffin of the Marine Institute, Republic of Ireland as part of the national Irish crayfish plague monitoring programme. It also utilises the methods developed by David Strand from his surveillance programme for crayfish plague *Aphanomyces astaci* in Norway 2018.

The protocol is intended to be utilised by staff in Analysis and Reporting or Fisheries, Biodiversity and Geomorphology teams within the Environment Agency.

Facility requirements

A clean, tidy, defined working area is required for the disinfection and collating of kit used as part of the sampling procedure. These two activities should ideally utilise different areas and different work benches and sinks to reduce the risk of contamination.

Access to a laboratory with an agreed and defined working area for storage of materials and equipment is strongly recommended. This will reduce the risk of unintentional contamination through activities undertaken by the Environment Agency.

Couriering samples

Completed site samples must be placed and kept in a cool box with ice packs. Samples must be received at the National Laboratory Service (NLS) next day to enable freezing to preserve the DNA on the filters. For samples being sent to the NLS, access to the sample fridge of your chosen office must be secured and appropriate paperwork completed prior to samples being left for collection. Samples to be sent to the NLS are collected by courier each day. Collection times should be confirmed with facilities to ensure they are not missed.

If alternative laboratories or couriers are to be utilised, a clear agreed method for collection and receipt must be agreed prior to sampling being undertaken to ensure samples can reach the laboratory in good condition.

Labels for sending samples should clearly show the details of which laboratory the samples are being sent to, the name and department of the receiver as well as full contact details of the sender.

Vehicle requirements

The eDNA sampling method requires a large amount of equipment which needs to be separated into different work areas to minimise the opportunity for DNA contamination between samples. Therefore, a van or large estate vehicle is required.

There are three defined areas within the vehicle (Figure 1).

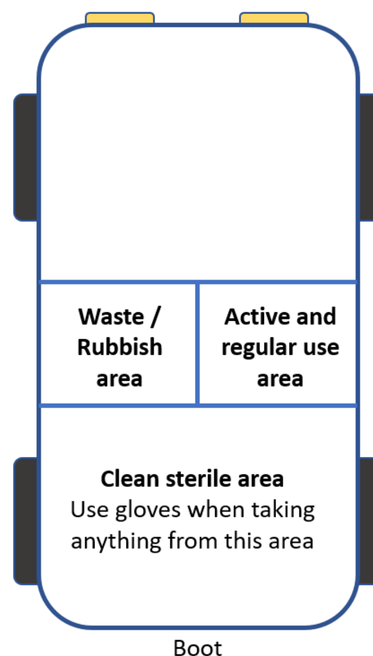

Figure 1. General layout of vehicle. All areas should be lined with tarpaulin and separated by raising tarpaulin up to provide a physical barrier.

Clean kit area

This area contains all sterile site sampling kit and washdown equipment. The area must be lined with tarpaulin to ensure spill containment and to reduce contamination.

Gloves must be put on before touching anything from this area. If you are to use multiple items as part of washdown or sampling, these should be removed in groups to avoid the need for excessive glove changes.

Contents of large sundries container to be used at sites (Figure 2):

- Large plastic container (ideally with lid)
- 1 x 0.5L spray bottle of 10% bleach
- 1 x 0.5L spray bottle of 10% sodium thiosulphate (125g per litre)

- 1 x 20L of tap water container for control samples
- 1 x 5L of tap water (spare)
- 1 x 5L pump sprayer filled with tap water
- 1 x 1.5L Pump sprayer containing virkon
- 1 x Zip lock bag containing mole grips, zip ties and grease
- 1 x roll of large bin liners
- 1 x roll of absorbent paper towel
- 1 x box of nitrile powder free gloves
- 1 x disinfection wipes

Figure 2. (a) Large container with all sundries. Note placement of 20L jerry can for ease of access. (b) Large container with lid. Note wheels for ease of transport.

Single site sample kit contents:

- One small container (10L recommended) containing all equipment required for a site sample (Figure 3a, b)
- 4 x sample bags containing forceps, gloves, parafilm and a 2µm membrane in a petri dish (Figure 3c)
- A small container, containing the following (Figure 3d):
    - 1 x 2 metre Masterflex size 24 tubing – for outlet from filter holder
    - 1 x 7.6m (25ft) Masterflex size 24 tubing – for inlet to sample holder
    - 3 x zip ties
    - 1 x 4 oz fishing weight
    - 1 x peg for holding tubing during pumping

Note: For sampling particularly turbid sites, additional sample bags will be required.

Figure 3. (a) Single site sample kit, (b) Filter, weight, zip ties and peg within small container, (c) Sample bag containing forceps, gloves, parafilm and a 2µm membrane in a petri dish -four of these are within the Single site sample kit, (d) Single site sample kit contents.

Spare membrane sample bags container contents

- One small container (3L+ recommended) containing eight spare membrane sample bags for sampling (Figure 4).
- 20 x sample bags containing forceps, gloves, parafilm and a 2µm membrane in a petri dish.

Note: For sampling particularly turbid watercourses, additional sample bags are recommended.

Note: This must be restocked after each sample run.

Figure 4. Spare membrane sample bags container.

Active site bucket contents (Figure 5)

- One lidded flexi bucket (30L recommended)
- 1 x 5L jug
- 1 x small flexi tub with handles
- 1 x permanent marker
- 1 x stopwatch
- 1 x notebook and pencil

Figure 5. Contents of Active site kit bucket

Waste/Rubbish area

This area is used for all waste, rubbish and used equipment. This should be lined with a tarpaulin. This should be gathered to form a physical barrier from the other areas of the vehicle.

Rubbish, used kit, and waste containers

- 1 x 30L lidded flexi bucket for rubbish and used kit (Figure 6a). Plastic bin bags can also be used but are not as efficient for later sorting of consumables and equipment to disinfect for repeat use.

- 5L screw top container for holding waste liquids prior to disposal at lab/office (Figure 6b).

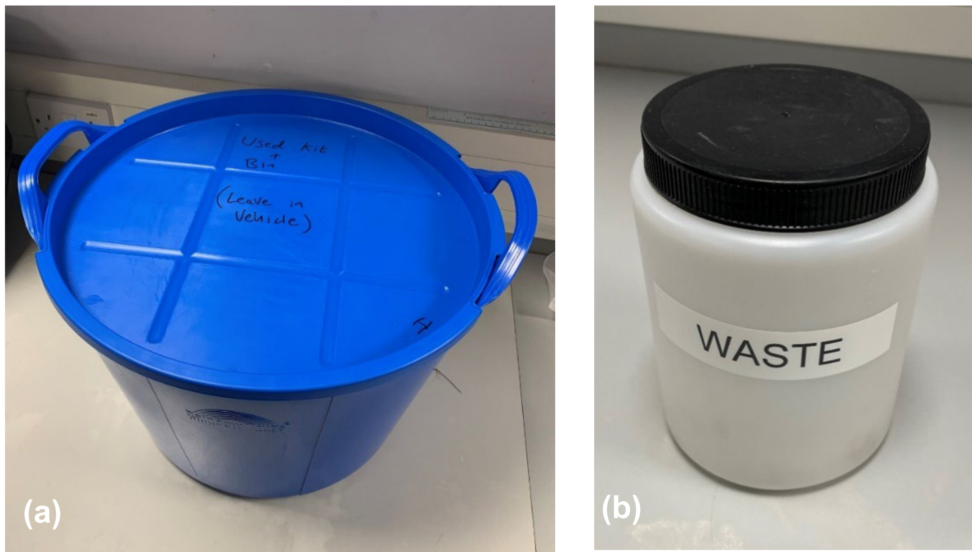

Figure 6. (a) 30L tub for holding used kit for later disinfection and for use as bin, (b) Waste container for waste liquids.

__Active and regular use area__

This area is for equipment that does not require stringent sterilisation and is used semi-regularly. This should be lined with a tarpaulin. This should be gathered to form a physical barrier from the other areas of the vehicle.

Equipment:

- Cool box (Figure 7a).
  - 10L minimum with ice blocks. A minimum one ice block is recommended. Petri dishes must be placed carefully to avoid fracturing.
- A correctly calibrated field meter and aluminium sampling can (Figure 7b).
  - Must be able to undertake measurements for water temperature, DO (%), conductivity, LDO, turbidity, velocity, substrate type, pH
- Sample can on pole (made of stainless steel; Figure 7c).
- Biosecurity kit.
  - Wire or stiff plastic brush and hand sprayer or small pressure washer to clean mud of PPE (7d).
- 20L Jerry can for still water site (Figure 7e).
  - Used for still water sampling only.
- Peristaltic pump (Figure 7f).
  - In hard case with instruction manual. Must be fully charged and in good working order.

Note: If pressure washer is to be used, eye protection must be worn

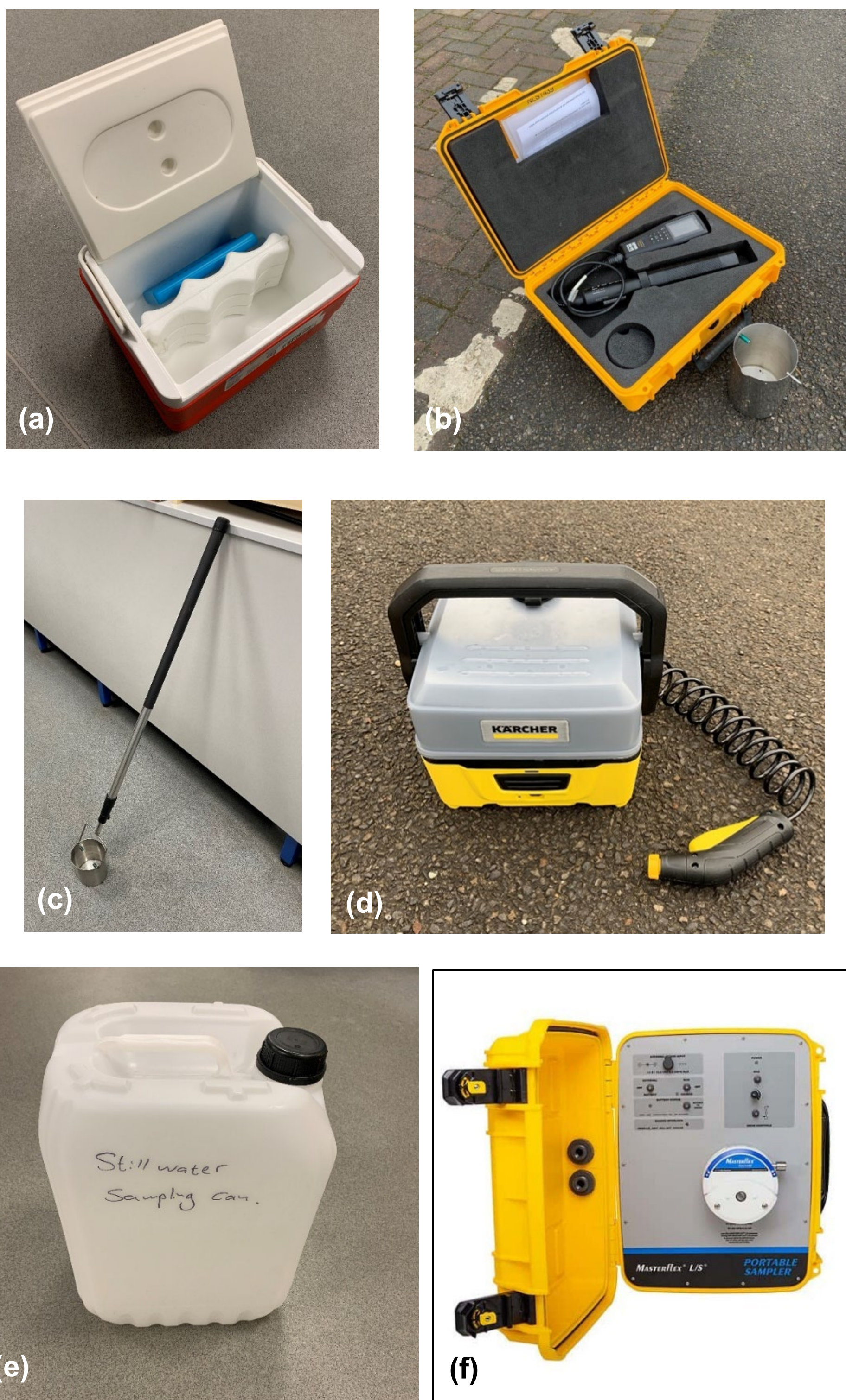

Figure 7. (a) Cool box with minimum of two ice blocks. Note circular areas for holding drinks area ideal for holding stacked Petri dishes, (b) Multi meter in case, (c) Sample pole, (d) Battery operated 4L pressure washer, (e) 20L Jerry can for still water sampling, (f) Masterflex portable sampler.

Small backpack, to carry:

- Stopwatch
- Note pad – waterproof recommended
- Weather writer
- Permanent marker – multiple recommended due to potential for nib to be wetted through sampling process
- Pen
- Pencil
- Small roll of paper towels to wipe dry surfaces to write on with permanent pen

Sampling protocol

The information below offers a detailed step-by-step guide on undertaking samples at both flowing and still water sites. Read through the protocol and consider access, permissions, lone worker systems, secure working areas and all relevant H&S concerns prior to undertaking a site visit.

Prior to site visit

The following actions must be undertaken prior to planned site visit. Instructions for charging must be carefully read.

- An appropriate number of single site sample kits should be disinfected, prepared and ready to be used in the field.
- Charge pump – Overnight charge of 12 hours required for full charge.
- Freeze blocks – Recommended overnight.
- Ensure multi meter is charged.

Notes on operation and maintenance of pump

The Masterflex E/S Portable Sampler instruction manual and data sheet can be accessed at the following links:

https://pim-resources.coleparmer.com/instruction-manual/07571-02-manual.pdf

https://pim-resources.coleparmer.com/literature/4579-mflex-portable-sampling-pump-ss.pdf

Pump must be charged for a recommended minimum of 12 hours. Follow operation manual to check battery is charged.

A mains adapter or new power lead will be required if operating the pump in England as it is supplied with an American two pin plug socket. These should be of high quality.

Pump must be maintained to manufacturers specifications and portable appliance testing (PAT) must be undertaken at the recommended interval. Usually annually after initial purchase.

Faults should be reported immediately to the manufacturer to enable rapid resolution of issues and prevent damage.

Pump control panel is not waterproof and may malfunction if water ingress occurs. Take care to keep the control panel face and edges dry. If sample collection cannot be rearranged, care should be taken to keep lid closed as much as possible if rain is forecast.

To preserve battery the pump should be left in the off position and flow direction set to neutral when not in use.

Before operating the pump, care must be taken to confirm all flow connections to filter holders are firm, flow direction is correct and that the speed is set to minimal. This will prevent filter blow out, strain to the pump and potential contamination from liquid being released under pressure from faulty flow connections.

Control sample protocol

Preparing equipment for control sample

1. Arrive at site and park at suitable location away from watercourse on level ground.
2. Open boot.
3. Put on clean nitrile gloves from Large sundries container.
4. Open one 20L tap water container.
5. Take sample bag from a site kit from Single site sample kit.
6. Take Active site kit bucket from car and place on floor near boot of car.
7. Take Peristaltic pump and place on ground near to active site bucket.
8. Feed long length of Masterflex tubing through peristaltic pump, close the mechanism ensuring tubing is not pinched and place outflow end in small flexi tub.
9. Place intake end of Masterflex tubing into 20L tap water container. It can be useful to feed the tubing through the handle prior to placing in the 20L bottle to prevent removal through pump vibration.
10. Take out filter holder from individual site kit - assemble if required (nylon hose barbs, filter support, O-ring and luer-lock vent plug). Ensure all connections are snug.
11. Slide tubing from outflow of peristaltic pump and slide tubing over nylon hose barbs on inflow side (side with airlock).
12. Slide zip tie over nylon hose barb on filter inlet and tighten (Figure 8a). Avoid excessive force to prevent splitting tubing or damaging hose barbs.
13. Slide short length (2 metres) of Masterflex tubing over nylon hose barbs on outflow of filter holder (zip tie not required).
14. Place connected filter holder into small flexi tub, ensure end of 2 metre tubing is sufficient distance from working area and will not result in flooding of work area once pumping begins (Figure 8b).

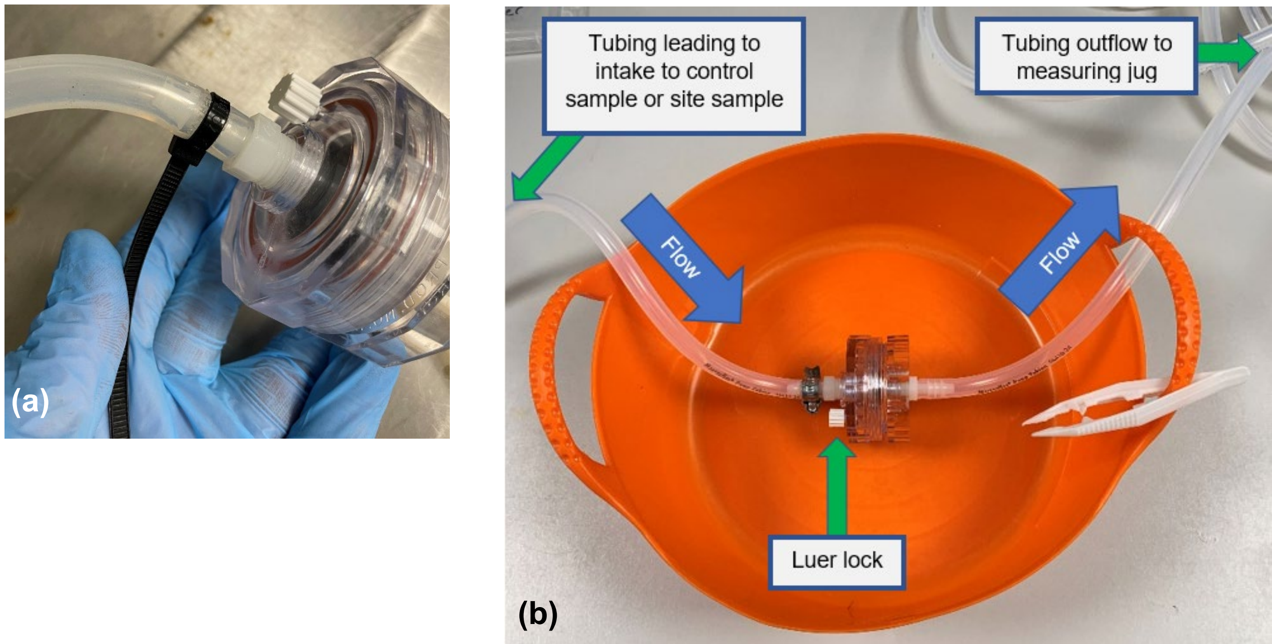

Figure 8. (a) Zip tie properly secured over barb [Step 12], (b) Filter holder and tubing seated in small flexi tub with arrows denoting flow of water. Note: luer lock is on intake site side [Step 14].

Figure 9. Final set up ready for control sample.

Control sample collection procedure:

1. Open Sample bag and put on gloves.
2. Open filter holder and using sterilised forceps, ensure O ring is seated correctly on male side of filter holder. Utilise handle of tub to hold forceps whilst undertaking following steps.
3. Take Petri dish containing 2µm filter and place in filter holder on top of filter support.

4. Ensure filter is central, not folded or damaged (Note: filter may fray at edges due to use of forceps. If this is at the edge of the filter this is not a concern).
5. Tighten filter holder, visually check to ensure the filter and O ring is not deformed prior to pumping.
6. Ensure luer-lock vent plug is tightened (Figure 10a).
7. Place filter holder in small flexi tub.
8. Place outlet end into 5L jug, using peg to secure tubing and prevent vibration moving tubing from the jug (Figure 10b).
9. Ensure all elements of pump are off and minimise pump speed. Check the tubing is seated correctly in the pump and it is not pinched.
10. Turn pump to battery, on, and forward clockwise rotation. You may need to toggle between clockwise and anti-clockwise direction to enable pump to engage the tubing correctly.
11. Start timing of sample using stopwatch.
12. Increase pump speed slowly over approximately 20 seconds until it reaches maximum.
13. Run pump until 5L of tap water has passed through.
14. Turn off pump and stop stopwatch – record the time.
15. Take Petri dish from sample bag, take off lid so it is ready to receive filter
16. Carefully unscrew filter holder, lay both sides in small flexi tub.
17. Use forceps used to initially place filter in the filter holder to detach the edge of the filter from the filter support (Figure 10c). You may need to take O ring from filter, if so, place this in the base of the small flexi tub.
18. Carefully place the membrane into the Petri dish.
19. Place the forceps in the rubbish container and pick up the Petri dish lid, and place on Petri dish.
20. Use a permanent marker to write control, and the following on the Petri dish lid (Figure 10d).
    - Date
    - Site name
    - Total number of litres filtered
    - Sample number (e.g. Control, Sample 1a, Sample 1b etc)
    - Time taken to filter

21. Take strip of Parafilm M and carefully stretch and wrap the tape around the Petri dish to form a seal.
22. Place the Petri dish in the same zip lock bag it was removed from and place in the cool box.
23. Place gloves used equipment from rubbish container in bin in the car.
24. Put on new pair of nitrile gloves from Car boot.
25. Remove tubing from 20L tap water container and replace lid.
26. Remove tubing from peristaltic pump and place filter holder and all tubing in Active site kit bucket. 2μm control sample is now complete.

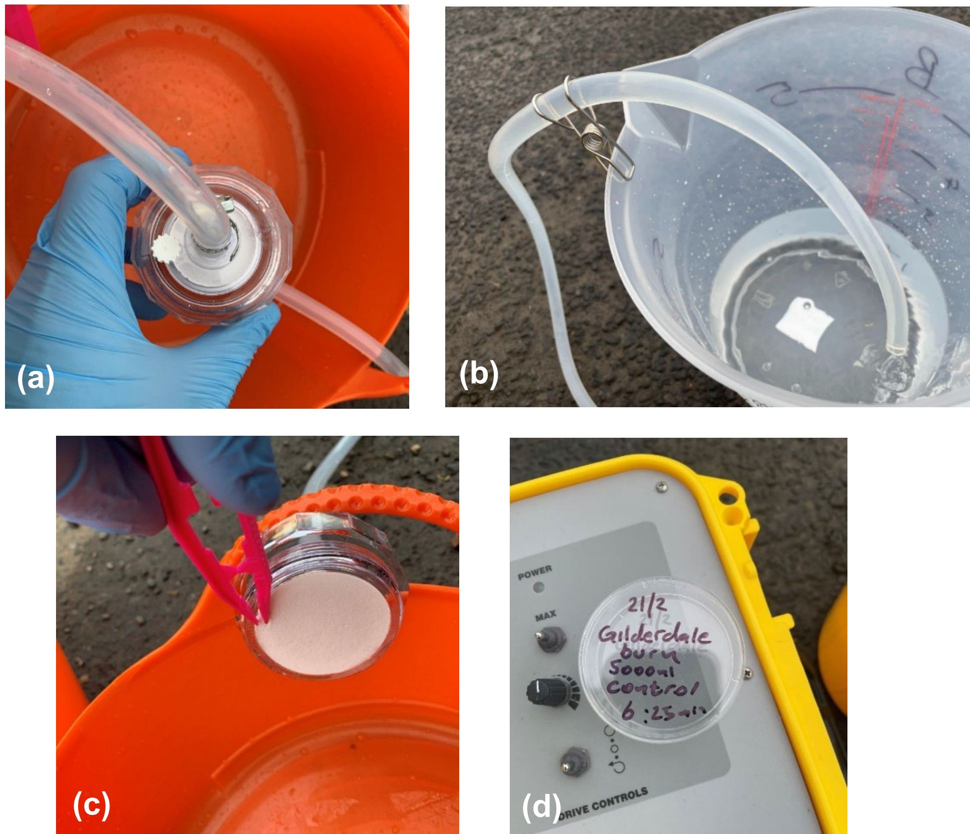

Figure 10. (a) A properly seated O ring and filter [Step 6], (b) peg used to secure tubing to jug during pumping [Step 8], (c) filter being removed from filter holder using sterile forceps [Step 17], (d) example of labelled Petri dish showing site name [Step 20].

<u>River site sample protocol</u>

Preparing for river site sample after control sample

1. Carry the following to the site sampling point
    - Peristaltic pump
    - Single site sample kit box
    - small flexi tub with handles
    - Active site kit bucket (still in bin liner) containing: 5L jug, filter holder with tubing still attached
    - Backpack
2. Upon arrival at sampling point, place all kit away from water to prevent splashing or immersion in water.

River site sample set up

Note: Inside lid of in use site tub can be utilised as a working area for placing petri dish etc on while sample is being taken.

1. Put on new pair of gloves from sample bag.
2. Open filter holder and using sterilised forceps, ensure O ring is seated correctly on male side of filter holder. Utilise handle of tub to hold forceps whilst undertaking following steps.
3. Tighten filter holder, visually check to ensure the filter and o ring is not deformed prior to pumping.
4. Ensure luer-lock vent plug is tightened.
5. Place filter holder in small flexi tub.
6. Place intake end into 5L jug, using peg to secure tubing and prevent vibration moving tubing from the jug (Figure 8b).
7. Attach a zip tie to the weight and using a second zip tie, attach this to the end of Masterflex tubing sufficiently tight so it does not move freely. Distance from jubilee clip to end of tubing will depend upon the depth of the water and the depth of the sediment. The inlet end of the tubing should be above sediment (Figure 11).
8. Place intake end of tubing into watercourse in central location. If watercourse is too wide to reach centre, place in area of obvious flow. Backwaters, pools and areas of low flow should be avoided (Figure 12).
9. Ensure all elements of pump are off and minimise pump speed. Check the tubing is seated correctly in the pump and it is not pinched.
10. Turn pump to battery, on, and forward clockwise rotation. You may need to toggle between clockwise and anti-clockwise direction to enable pump to engage the tubing correctly.
11. Start timing of sample using stopwatch.
12. Increase pump speed slowly over 20 seconds until it reaches max.
13. Run pump for one minute. This will ensure any activated sediment will pass through and no remnants of bleach or sodium thiosulphate remain in the system. Visually check to ensure excessive material is not being collected. Change location of the tubing intake if required.
14. Site sample is now ready to be taken.

Figure 11. Sediment being pumped through tubing. This should be avoided. There may be a need to reposition the intake location [Step 7].

Figure 12. When sampling, areas similar to those shaded in red should be avoided due to lack of flow and potential lack of representative DNA available [Step 8].

River site sample collection procedure

The flow diagram below sets out the sampling process for river sites (flowing water; Figure 13). In some cases, multiple membranes will be required. The sample sheet should be referred to and completed as the samples are taken.

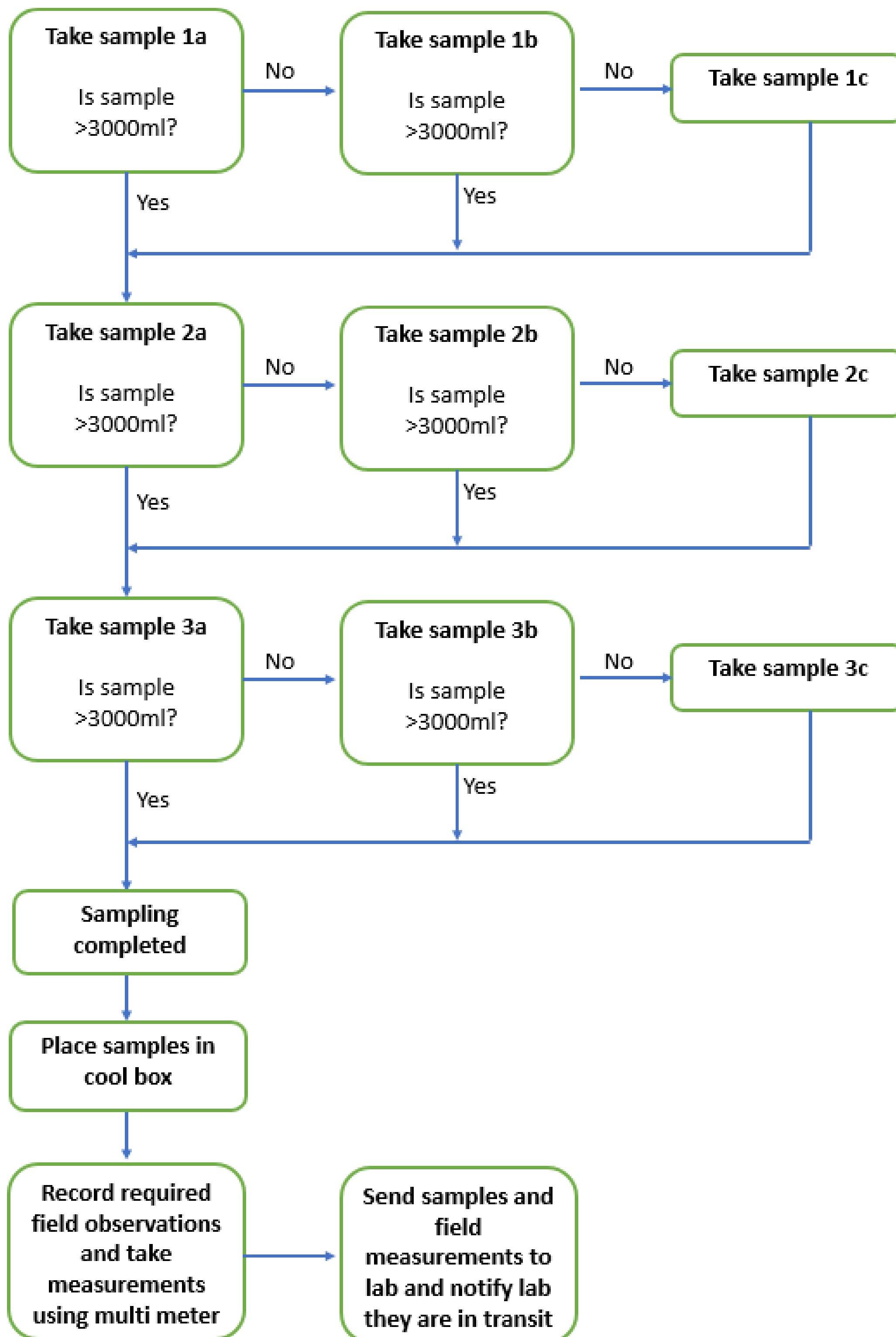

Figure 13. On site sampling flow diagram after control sample has been taken.

Sampling procedure

1. Open Sample bag and put on gloves.
2. Open filter holder and using new sterilised forceps, ensure O ring is seated correctly on male side of filter holder.

3. Take Petri dish containing 2µm filter and place filter holder. Utilise handle of tub to hold forceps.
4. Ensure filter is central, not folded or damaged (Note: filter may fray at edges due to use of forceps. If this is at the edge of the filter this is not a concern)
5. Tighten filter holder, visually check to ensure the filter and O ring is not deformed prior to pumping.
6. Ensure luer-lock vent plug is tightened.
7. Record the time the pump is started.
8. Place filter holder in small flexi tub.
9. Place outlet end into 5L jug, using peg to secure tubing and prevent vibration moving tubing from the jug.
10. Ensure all elements of pump are off and minimise pump speed. Check the tubing is seated correctly in the pump and it is not pinched.
11. Turn pump to battery, on, and forward clockwise rotation. You may need to toggle between clockwise and anti-clockwise direction to enable pump to engage the tubing correctly.
12. Start timing of sample using stopwatch.
13. Increase pump speed slowly over approximately 20 seconds until it reaches maximum.
14. Observe measuring jug to determine amount of water filtered.
    Note:
    - During pumping, observe tubing between pump head and filter holder. If tubing becomes overly expanded or pump appears to strain, slow pump, turn off, inspect set up and re-seat tubing in pump head.
    - If flow suddenly increases – Visually check that membrane has not blown. If membrane has blown, membrane should be removed, and sample will need to be repeated
    - In particularly turbid sites, determining when filter has reached maximum capacity can be difficult. If pump is struggling and amount filtered is not increasing, reduce pump rate to half to avoid filter blow out and to maximise amount filtered. Observe measuring jug carefully. If amount filtered is not increasing for a minute or more, stop pumping.
    - Maximum pump time for any one filter is 15 minutes.
    - A sample of >3L is deemed acceptable. If <3L of water is capable of being sampled, this filter must be stored in a Petri dish and an additional sample must be utilised. If the second filter is also <3L, a third must be used. Multiple filters will be required. The maximum number of site samples possible (excluding the control sample) per site is nine.

15. Once maximum amount of sample has been pumped, turn off pump and stop stopwatch – record the time.
16. Take Petri dish from sample bag, take off lid so it is ready to receive filter.
17. Carefully unscrew filter holder, lay both sides in small flexi tub.
18. Use forceps used to initially place filter in the filter holder to detach the edge of the filter from the filter support. You may need to take O ring from filter, if so, place this in the base of the small flexi tub.
19. Carefully place the membrane into the Petri dish.
20. Place lid on Petri dish.

21. Use a permanent marker to write the following on the Petri dish lid:

    - Site name
    - Sampler name
    - Sample number (e.g. Sample 1 - Filter 1, sample 1 – filter 2 etc)
    - Date
    - Time taken to filter
    - Total number of litres filtered

22. Take strip of Parafilm M and carefully stretch and wrap the tape around the Petri dish to form a seal. One piece of Parafilm M will stretch around a single Petri dish (Figure 14).
23. Place the Petri dish in the same zip lock bag it was removed from and place in the cool box. Label zip lock bag with sample number and date in case of damage to Petri dish.
24. Repeat until adequate number of samples are taken.

Figure 14. Applying Parafilm M to Petri dish [Step 22].

Packing up procedure

1. Once all samples are taken, reconstruct filter holder and reverse pump direction to empty as much water from system as possible.
2. Carry all equipment back to vehicle.
3. At vehicle:
    - Place zip lock bag containing all samples in cool box.
    - Place all used equipment back into the single site sample kit box: filter holder and all related components, masterflex tubing, gloves, forceps, used paper

towels etc and other pieces of equipment used on site. This will be sorted and disinfected in the lab.

Still water sample collection protocol

Prep for site sample after control

1. Put on new pair of nitrile gloves.
2. Carry the following to the site sampling point:
    - 20L still water sampling container
    - Sampling can on pole
3. Upon arrival at sampling point, place all kit away from water to prevent splashing or immersion in water.

Still water site sample collection set up

Note: For this method, it is recommended that samples are taken approximately every 10 metres focussing upon areas of optimal habitat. Therefore, for a 20L container, samples covering 200m of shoreline with optimal habitat can be collected. In some waterbodies there will be areas of shoreline with little or non-optimal habitat, these should be avoided. These areas should be identified prior to sampling beginning. The areas of shoreline sampled should be noted in the comments section.

Note: for large waterbodies, a small trolley may be required to move the 20L container to prevent manual handling related injuries.

1. Identify first location for sampling on shore of the site. To reduce risks posed by manual handling. The first sampling location should be the furthest point from the area where sample processing will be undertaken.
2. Areas of optimal habitat for crayfish should be identified and preferentially sampled. Samples should be taken on average every 10m of the shore.
3. Use disinfected sampling can on pole to take 1L sample from water column. Crayfish are benthic and therefore sample should be taken from the base of the waterbody. Limit disturbance to sediment at the bottom of waterbody.
4. Move approximately 10 metres to the next sample point, repeat procedure until 20l has been collected. Make notes of rough sample coverage of the shoreline.
5. Carry 20L container to vehicle to undertake filtering procedure.

Still water sample processing procedure

1. Open Sample bag and put on gloves
2. Open filter holder and using new sterilised forceps, ensure O ring is seated correctly on male side of filter holder.
3. Take Petri dish containing 2µm filter and place filter holder. Utilise handle of tub to hold forceps.
4. Ensure filter is central, not folded or damaged (Note: filter may fray at edges due to use of forceps. If this is at the edge of the filter this is not a concern)
5. Tighten filter holder, visually check to ensure the filter and O ring is not deformed prior to pumping.

6. Ensure luer-lock vent plug is tightened…
7. Record the time the pump is started
8. Place filter holder in small flexi tub
9. Place outlet end into 5L jug, using peg to secure tubing and prevent vibration moving tubing from the jug.
10. Place inlet end of tubing into 20L container.
11. Ensure all elements of pump are off and minimise pump speed. Check the tubing is seated correctly in the pump and it is not pinched.
12. Turn pump to battery, on, and forward clockwise rotation. You may need to toggle between clockwise and anti-clockwise direction to enable pump to engage the tubing correctly.
13. Start timing of sample using stopwatch.
14. Increase pump speed slowly over approximately 20 seconds until it reaches maximum.
15. Observe measuring jug to determine amount of water filtered.
    Note:
    - During pumping, observe tubing between pump head and filter holder. If tubing becomes overly expanded or pump appears to strain, slow pump, turn off, inspect set up and re-seat tubing in pump head.
    - If flow suddenly increases – Visually check that membrane has not blown. If membrane has blown, membrane should be removed and sample will need to be repeated
    - In particularly turbid sites, determining when filter has reached maximum capacity can be difficult. If pump is struggling and amount filtered is not increasing, reduce pump rate to half to avoid filter blow out and to maximise amount filtered. Observe measuring jug carefully. If amount filtered is not increasing for a minute or more, stop pumping.
    - Maximum pump time for any one filter is 15 minutes.
    - A sample of >3L is deemed acceptable. If <3L of water is capable of being sampled, this filter must be stored in a Petri dish and an additional sample must be utilised. If the second filter is also <3L, a third must be used. Multiple filters will be required. The maximum number of site samples possible (excluding the control sample) per site is nine.

16. Once maximum amount of sample has been pumped, turn off pump and stop stopwatch – record the time.
17. Take Petri dish from sample bag, take off lid so it is ready to receive filter.
18. Carefully unscrew filter holder, lay both sides in small flexi tub.
19. Use forceps used to initially place filter in the filter holder to detach the edge of the filter from the filter support. You may need to take O ring from filter, if so, place this in the base of the small flexi tub.
20. Carefully place the membrane into the Petri dish.
21. Place lid on Petri dish.
22. Use a permanent marker to write the following on the Petri dish lid.

    - Site name
    - Sampler name
    - Sample number (e.g. Sample 1 - Filter 1, sample 1 – filter 2 etc)

- Date
- Time taken to filter
- Total number of litres filtered

23. Take strip of Parafilm M and carefully stretch and wrap the tape around the Petri dish to form a seal (Figure 15).
24. Place the Petri dish in the same zip lock bag it was removed from and place in the cool box. Label zip lock bag with sample number and date in case of damage to Petri dish.
25. Repeat until adequate number of samples are taken.

Figure 15. Peristaltic pump being cleaned with 10% bleach solution

Multiple Stillwater site disinfection procedure

Note: If multiple still water samples are to be taken at a single site or across multiple sites using the same 20L jerry container, this must be disinfected between each homogenised sample collection.

1. Pour approximately 300ml of 10% bleach solution into sample container.
2. Screw lid on tightly and shake, ensuring all internal surfaces have come into contact with the solution. Leave for 10 minutes.
3. Rinse thoroughly with tap water from pump sprayer.
4. Pour rinsed water into waste container.
5. Pour approximately 300ml of 10% sodium thiosulphate solution into sample container. Screw lid on tightly and shake, ensuring all surfaces have come into contact with the solution. Leave for 10 minutes.
6. Rinse thoroughly with water from pump sprayer.
7. Pour rinsed water into waste container.
8. If water appears sudsy, repeat treatment with sodium thiosulphate and spray with tap water until no suds are evident.

9. Ensuring lid is placed back on jerry can. Lightly wipe outside of jerry can with paper towel sprayed with 10% bleach solution. Avoid spraying around lid area.

Field measurement protocol

Biosecurity note: multi meter probe must not be placed in the waterbody. A sample can should be used to collect an adequate volume of water and the reading taken from that can. Water is then disposed of to land, visually inspected for biological material, disinfected using Virkon and rinsed with tap water after five minutes contact. This will ensure a reduced risk of the transfer of aquatic diseases.

1. It is recommended that the meter readings are undertaken after sampling is complete and all equipment has been returned to vehicle. However, if sample point is some distance from vehicle, the meter may need to be taken with all sampling kit. In this instance the meter and sample can should be carried in a separate bag to prevent contamination
2. Observe water on site and make note of the following using pocket notebook or eDNA site survey form.

    - Flow
    - Turbidity
    - Weather conditions
    - Any additional comments

3. Follow procedures for sampling using multi meter and record.

    - Water temp:
    - DO (%):
    - Conductivity:
    - LDO
    - Turbidity:
    - Velocity:
    - Substrate type:
    - PH

Washdown procedure – Prior to travelling to next site

1. Put on new set of gloves from vehicle.
2. Remove from vehicle ready to be used the following:

    - 5L pump sprayer containing tap water
    - Hand sprayer containing 10% bleach solution
    - Hand sprayer containing 10% sodium thiosulphate
    - Hand sprayer containing Virkon
    - Paper towels
    - Bin bag

3. Spray the following with 10% bleach solution whilst in active site kit bucket:

    - Small flexi tub
    - 5L jug
    - Active site bucket lid - top and bottom
    - The active site bucket -sprayed inside and out whilst still in the bin liner.

    Ensure sprayer is operated below the rim of the container to prevent bleach from entering the environment. Leave for 15 minutes. Spray paper towel with 10% bleach solution and gently wipe operating surface of peristaltic pump.

4. Spray all items in previous step with water from pump sprayer. Pour this into the Waste container.
5. Spray all equipment previously mentioned with 10% sodium thiosulphate – leave for five minutes.
6. Spray all items in previous step with tap water from pump sprayer. Empty this into waste container. If water appears sudsy, spray again with sodium thiosulphate, leave for five minutes and then spray again with water.
7. Remove as much water as possible from all the kit through gentle shaking and place in the active site bucket.
8. Remove old bin liner from around active site bucket and place in bin
9. Place bucket in new large bin liner, twist top of bin liner and place in car ready for next site.
10. Return all other equipment to vehicle.
11. Spray sampling can and pole with Virkon.
12. Wash all debris and mud off footwear and other PPE using biosecurity pump sprayer and other and treat with Virkon.

General kit photos for reference

(i) 5L pump sprayer holding tap water, (ii) Tools and spares containers. Sealed container with spare parafilm and forceps. Grease for pump and shears and locking pliers. (iii) 20L Site control jerry can and spare 5L water jerry can, (iv) still water sampling can for collecting and homogenising sample, (v) 5L jerry cans and hand sprayers containing 10% solutions of bleach and sodium thiosulphate, (vi) virkon spray bottle for disinfection between sites.

Photo - Full site kit excluding peristaltic pump and multi meter

Appendix 2: Membrane filter size selection

Water samples collected as part of a sampling strategy to analyse eDNA must be filtered to capture the eDNA suspended in the water sample. The volumes of water filtered and composition of filter material and pore size varies between studies (Bruce and others, 2021) and unfortunately there is no "one-size-fits-all" consensus on what is best. The advantage of a larger pore size allows bigger volumes of water to be filtered, however, this can be at the expense of capturing smaller particles. The downside is that smaller pore sizes lead to the filter becoming clogged quicker in turbid water, restricting the amount of water that can be filtered as a greater proportion of suspended material is trapped by the filter (Natural England, 2020). Therefore, there is a need to balance the capture of adequate DNA material to maximise detection of target species with the time and effort of the filtration approach.

The sampling protocol used in this study involves the collection of 5L of water, which is filtered using a 2µm pore size glass fibre filter. This approach has been shown to be effective in detecting crayfish species and crayfish plague. We decided to conduct a small experiment to compare the use of the 2µm glass fibre filter with a commonly used 0.45µm pore size filter used to capture eDNA from microbial and macrobial organisms.

Two separate water samples were collected in November 2021 from each of five study sites (Boshaw Whams Reservoir, River Blyth, River Ure, Sugley Dene, and Wansbeck) and filtered through membranes with a pore size of 0.45µm or 2µm. For both membrane sizes, if the filter pores clogged prior to 3L of water being filtered, more membranes were used until 3L of water had been filtered or 3 membranes had clogged.

Water samples taken from all sites except Boshaw Wham Reservoir only required one 2µm membrane to be used to filter sufficient water, whereas three 0.45µm membranes were needed to filter sufficient water at all sites (Table i). eDNA copy numbers of white-clawed crayfish, signal crayfish, and crayfish plague per 100ml of water when filtered using membranes with 0.45µm and 2µm pore size are reported in Table ii. Similar copy numbers per 100ml of white-clawed crayfish and crayfish plague were determined from the analysis of membranes with 0.45µm and 2µm pore sizes. Copy numbers of white-clawed crayfish were slightly higher for the 2µm filters (78/100ml and 183/100ml at the River Ure and Wansbeck respectively) compared to the 0.45µm filters (78/100ml and 183/100ml at the River Ure and Wansbeck respectively), whereas copy numbers of signal crayfish in water samples from the River Ure were slightly higher for the 0.45µm filter than the 2µm filter (34/100ml and 21/100ml respectively). The crayfish plague copy number was an order of magnitude higher for the 2µm filter than the 0.45µm filter (2,253/100ml and 124/100ml respectively).

Table i. Number of membranes used to filter 3L of water, up to a maximum of 3 membranes.

Study Site	0.45μm	2μm
Boshaw Whams Reservoir	3	3
Sugley Dene	3	1
River Blyth	3	1
River Ure	3	1
Wansbeck	3	1

Table ii. Membrane pore size comparison showing the highest result (neat or 1:10 dilution) for each DNA extract for each marker per 100ml of water analysed.

Site	White-clawed crayfish		Crayfish Plague		Signal crayfish	
	0.45μm	2μm	0.45μm	2μm	0.45μm	2μm
Boshaw Whams Reservoir	0	0	0	0	0	0
Sugley Dene	0	0	0	0	0	0
River Blyth	0	0	124	2,253	0	0
River Ure	65	78	0	0	34	21
Wansbeck	23	183	0	0	0	0

While there were differences in the copy numbers when water samples were filtered through 2μm and 0.45μm membranes, the copy count of white-clawed crayfish, signal crayfish, and crayfish plague was not consistently higher for water samples filtered using membranes with the smaller pore size. Use of membranes with larger pore sizes resulted in, on average, fewer membranes being needed to filter sufficient water for analysis, which

also increases cost of analysis as previously reported (Natural England, 2020). Staff undertaking the sampling also reported that it was quicker to filter the water using membranes with a larger pore size and expressed preference for using the filters with the larger membrane size because the faster filtering time and lower number of membranes needed (on average) made sampling quicker and easier. Therefore, filters with a 2μm pore size were chosen and used in the sampling protocol adopted for the rest of this study, on the grounds that it made the overall work quicker and cheaper, with no loss of detection.

Appendix 3: qPCR Conditions for target species

Signal Crayfish

As previously described in Agersnap and others (2017) and Strand and others (2019).

Table iii - DNA sequence for each component of the qPCR reaction for signal crayfish

Component	DNA sequence (5' – 3')
F Primer 'Paclen_COI_F0336'	AACTAGAGGAATAGTTGAAAG
R Primer 'Paclen_COI_R0397'	CCGCTGCTAGAGGAGGATAA
Probe 'Paclen_COI_P0357'	FAM-AGGAGTGGGTACTGGATGAACT-MGB

Table iv - Volume (per well) of each component used in the qPCR reaction for signal crayfish

Component	Volume per well (µl)
Molecular Biology grade water	1.55
Paclen_COI_F0336 (6 µM)	2.70
Paclen_COI_R0397 (6 µM)	2.70
Paclen_COI_P0357 (5 µM)	0.70
BSA (4%)	0.35
Taqman Univ. MM	10.0
eDNA extract	2.00

PCR cycling conditions

- 50°C for 5 minutes
- 95°C for 10 minutes

- 45 cycles of:
    - 95°C for 30 seconds
    - 60°C for 60 seconds
- 72°C for 10 minutes

White-clawed Crayfish

As previously described by Atkinson and others (2019).

Table v - DNA sequence for each component of the qPCR reaction for white-clawed crayfish

Component	DNA sequence (5’ – 3’)
F Primer ‘Auspal_COI_F’	GGGTTAGTGGAGAGAGGGGT
R Primer ‘Auspal_COI_R’	AATCCCCAGATCCACAGACG
Probe ‘Auspal_COI_P’	FAM-TCAGCTATTGCCCACGCA-MGB

Table vi - Volume (per well) of each component used in the qPCR reaction for white-clawed crayfish

Component	Volume per well (µl)
Molecular Biology grade water	4.50
F Primer (10 µM)	1.25
R Primer (10 µM)	1.25
Probe (10 µM)	0.50
Taqman Univ. MM	12.5
eDNA extract	5.0

PCR cycling conditions

- 50°C for 2 minutes
- 95°C for 10 minutes
- 45 cycles of:
    - 95°C for 15 seconds
    - 60°C for 60 seconds

Crayfish plague

As previously described by Vrålstad and others (2009).

Table vii - DNA sequence for each component of the qPCR reaction for crayfish plague

Component	DNA sequence (5' – 3')
F Primer 'AphAstITS-39F'	AAGGCTTGTGCTGGGATGTT
R Primer 'AphAstITS-97R'	CTTCTTGCGAAACCTTCTGCTA
Probe 'AphAstITS-60T'	FAM-TTCGGGACGACCC-MGB

Table viii - Volume (per well) of each component used in the qPCR reaction for crayfish plague

Component	Volume per well (µl)
Molecular Biology grade water	4.50
F Primer (10 µM)	1.25
R Primer (10 µM)	1.25
Probe (10 µM)	0.50
Taqman Univ. MM	12.5
eDNA extract	5.0

PCR cycling conditions

- 50°C for 2 minutes
- 95°C for 10 minutes
- 45 cycles of:
    - 95°C for 15 seconds
    - 60°C for 60 seconds

White-claw Crayfish and Crayfish Plague Duplex qPCR conditions

As previously described in Vrålstad and others, 2009 (crayfish plague) and Atkinson and others, 2019 (white-clawed crayfish).

Table ix - DNA sequence for each component of the qPCR reaction for white-clawed crayfish and crayfish plague

Component	DNA sequence (5' – 3')
F Primer 'AphAstITS-39F'	AAGGCTTGTGCTGGGATGTT
R Primer 'AphAstITS-97R'	CTTCTTGCGAAACCTTCTGCTA
Probe 'AphAstITS-60T'	FAM-TTCGGGACGACCC-MGB
F Primer 'Auspal_COI_F'	GGGTTAGTGGAGAGAGGGGT
R Primer 'Auspal_COI_R'	AATCCCCAGATCCACAGACG
Probe 'Auspal_COI_P'	VIC-TCAGCTATTGCCCACGCA-MGB

Table x - Volume (per well) of each component used in the qPCR reaction for signal crayfish

Component	Volume per well (µl)
Molecular Biology grade water	2.7
AphAstITS-39F (10µM)	1.25
AphAstITS-97R (10µM)	1.25
AphAstITS-60T (10µM)	0.5
Auspal_COI_F (10µM)	0.6
Auspal_COI_R (10µM)	0.6
Auspal_COI_P (10µM)	0.6
Taqman Univ. MM	12.5
eDNA extract	5.0

PCR cycling conditions

- 50°C for 2 minutes
- 95°C for 10 minutes
- 45 cycles of:
  - 95°C for 15 seconds
  - 60°C for 60 seconds

Appendix 4: LOD and LOQ results

Crayfish Plague

Table xi - Values for calculating LOD_{95} and LOQ for Crayfish plague assay

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD neat	248.92	243.05
LOD neat	248.92	273.30
LOD neat	248.92	263.27
LOD neat	248.92	223.79
LOD neat	248.92	249.75
LOD neat	248.92	225.60
LOD neat	248.92	275.36
LOD neat	248.92	250.37
LOD neat	248.92	253.51
LOD neat	248.92	231.17
LOD 1:2	124.46	81.18
LOD 1:2	124.46	90.72
LOD 1:2	124.46	141.43
LOD 1:2	124.46	93.53
LOD 1:2	124.46	137.95
LOD 1:2	124.46	103.99
LOD 1:2	124.46	127.08
LOD 1:2	124.46	177.62
LOD 1:2	124.46	78.15
LOD 1:2	124.46	148.67
LOD 1:4	62.23	52.57
LOD 1:4	62.23	41.86
LOD 1:4	62.23	46.47
LOD 1:4	62.23	33.31
LOD 1:4	62.23	39.64
LOD 1:4	62.23	67.62
LOD 1:4	62.23	59.56
LOD 1:4	62.23	43.99
LOD 1:4	62.23	66.95
LOD 1:4	62.23	44.32
LOD 1:8	31.11	21.53
LOD 1:8	31.11	21.15
LOD 1:8	31.11	28.23
LOD 1:8	31.11	13.17
LOD 1:8	31.11	13.37

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:16	15.56	19.38
LOD 1:16	15.56	19.87
LOD 1:16	15.56	16.61
LOD 1:16	15.56	9.69
LOD 1:16	15.56	9.27
LOD 1:16	15.56	17.61
LOD 1:16	15.56	17.97
LOD 1:16	15.56	7.03
LOD 1:16	15.56	14.56
LOD 1:16	15.56	8.90
LOD 1:32	7.78	4.83
LOD 1:32	7.78	5.27
LOD 1:32	7.78	-
LOD 1:32	7.78	4.95
LOD 1:32	7.78	5.09
LOD 1:32	7.78	-
LOD 1:32	7.78	3.73
LOD 1:32	7.78	7.57
LOD 1:32	7.78	7.82
LOD 1:32	7.78	-
LOD 1:64	3.89	-
LOD 1:64	3.89	4.31
LOD 1:64	3.89	4.08
LOD 1:64	3.89	1.95
LOD 1:64	3.89	-
LOD 1:64	3.89	-
LOD 1:64	3.89	2.24
LOD 1:64	3.89	7.82
LOD 1:64	3.89	2.26
LOD 1:64	3.89	5.61
LOD 1:128	1.94	2.10
LOD 1:128	1.94	2.10
LOD 1:128	1.94	-
LOD 1:128	1.94	1.75
LOD 1:128	1.94	-

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:8	31.11	17.79
LOD 1:8	31.11	23.57
LOD 1:8	31.11	17.69
LOD 1:8	31.11	27.52
LOD 1:8	31.11	30.44

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:128	1.94	-
LOD 1:128	1.94	-
LOD 1:128	1.94	1.92
LOD 1:128	1.94	-
LOD 1:128	1.94	-

White-clawed crayfish

Table xii - Values for calculating LOD_{95} and LOQ for white-clawed crayfish assay

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD neat	82.04	119.50
LOD neat	82.04	80.84
LOD neat	82.04	100.21
LOD neat	82.04	98.00
LOD neat	82.04	87.03
LOD neat	82.04	43.45
LOD neat	82.04	80.73
LOD neat	82.04	59.34
LOD neat	82.04	50.29
LOD neat	82.04	100.99
LOD 1:2	41.02	21.33
LOD 1:2	41.02	28.22
LOD 1:2	41.02	39.40
LOD 1:2	41.02	70.12
LOD 1:2	41.02	37.09
LOD 1:2	41.02	24.19
LOD 1:2	41.02	24.04
LOD 1:2	41.02	26.18
LOD 1:2	41.02	52.01
LOD 1:2	41.02	29.41
LOD 1:4	20.51	42.47
LOD 1:4	20.51	25.75
LOD 1:4	20.51	16.43
LOD 1:4	20.51	7.15
LOD 1:4	20.51	21.44
LOD 1:4	20.51	12.05
LOD 1:4	20.51	16.96
LOD 1:4	20.51	13.88
LOD 1:4	20.51	54.33
LOD 1:4	20.51	10.16
LOD 1:8	10.25	-
LOD 1:8	10.25	-
LOD 1:8	10.25	-
LOD 1:8	10.25	-
LOD 1:8	10.25	3.67
LOD 1:8	10.25	11.51
LOD 1:8	10.25	20.18
LOD 1:16	5.13	-
LOD 1:16	5.13	-
LOD 1:16	5.13	-
LOD 1:16	5.13	-
LOD 1:16	5.13	4.46
LOD 1:16	5.13	4.82
LOD 1:16	5.13	9.02
LOD 1:16	5.13	-
LOD 1:16	5.13	3.68
LOD 1:16	5.13	-
LOD 1:32	2.56	-
LOD 1:32	2.56	2.55
LOD 1:32	2.56	-
LOD 1:32	2.56	0.28
LOD 1:32	2.56	-
LOD 1:32	2.56	7.91
LOD 1:32	2.56	0.27
LOD 1:32	2.56	5.75
LOD 1:32	2.56	8.54
LOD 1:32	2.56	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:64	1.28	-
LOD 1:128	0.64	-
LOD 1:128	0.64	3.89
LOD 1:128	0.64	-
LOD 1:128	0.64	-
LOD 1:128	0.64	-
LOD 1:128	0.64	-
LOD 1:128	0.64	-

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:8	10.25	14.00
LOD 1:8	10.25	8.09
LOD 1:8	10.25	23.20

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:128	0.64	-
LOD 1:128	0.64	-
LOD 1:128	0.64	7.52

Signal crayfish

Table xiii - Values for calculating LOD_{95} and LOQ for Signal crayfish assay

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)	Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD neat	114.75	123.93	LOD 1:16	7.17	7.86
LOD neat	114.75	98.79	LOD 1:16	7.17	11.08
LOD neat	114.75	137.86	LOD 1:16	7.17	6.41
LOD neat	114.75	117.77	LOD 1:16	7.17	1.65
LOD neat	114.75	115.22	LOD 1:16	7.17	8.06
LOD neat	114.75	144.53	LOD 1:16	7.17	8.23
LOD neat	114.75	92.61	LOD 1:16	7.17	13.70
LOD neat	114.75	129.71	LOD 1:16	7.17	4.85
LOD neat	114.75	104.93	LOD 1:16	7.17	3.93
LOD neat	114.75	82.12	LOD 1:16	7.17	10.58
LOD 1:2	57.37	81.54	LOD 1:32	3.59	0.55
LOD 1:2	57.37	74.39	LOD 1:32	3.59	2.25
LOD 1:2	57.37	56.24	LOD 1:32	3.59	2.25
LOD 1:2	57.37	41.86	LOD 1:32	3.59	1.71
LOD 1:2	57.37	68.21	LOD 1:32	3.59	1.19
LOD 1:2	57.37	83.90	LOD 1:32	3.59	1.60
LOD 1:2	57.37	67.79	LOD 1:32	3.59	-
LOD 1:2	57.37	97.63	LOD 1:32	3.59	1.58
LOD 1:2	57.37	56.30	LOD 1:32	3.59	3.67
LOD 1:2	57.37	82.35	LOD 1:32	3.59	-
LOD 1:4	28.69	38.98	LOD 1:64	1.79	-
LOD 1:4	28.69	31.92	LOD 1:64	1.79	3.15
LOD 1:4	28.69	43.32	LOD 1:64	1.79	2.92
LOD 1:4	28.69	27.38	LOD 1:64	1.79	4.55
LOD 1:4	28.69	34.61	LOD 1:64	1.79	1.34
LOD 1:4	28.69	29.60	LOD 1:64	1.79	2.11
LOD 1:4	28.69	55.52	LOD 1:64	1.79	2.37
LOD 1:4	28.69	28.57	LOD 1:64	1.79	2.46
LOD 1:4	28.69	24.78	LOD 1:64	1.79	2.30
LOD 1:4	28.69	20.57	LOD 1:64	1.79	8.38
LOD 1:8	14.34	10.11	LOD 1:128	0.90	1.32
LOD 1:8	14.34	10.79	LOD 1:128	0.90	5.14
LOD 1:8	14.34	11.63	LOD 1:128	0.90	-
LOD 1:8	14.34	16.87	LOD 1:128	0.90	-
LOD 1:8	14.34	13.82	LOD 1:128	0.90	4.36
LOD 1:8	14.34	8.57	LOD 1:128	0.90	-
LOD 1:8	14.34	12.68	LOD 1:128	0.90	1.57

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:8	14.34	10.65
LOD 1:8	14.34	17.83
LOD 1:8	14.34	14.94

Dilution	Anticipated Value (gc/well)	Obtained Value (gc/well)
LOD 1:128	0.90	3.03
LOD 1:128	0.90	-
LOD 1:128	0.90	1.39

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