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Environmental DNA-based methods detect the invasion front of an advancing signal crayfish population

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Abstract

Aquatic invasive species, such as the signal crayfish (*Pacifastacus leniusculus*), present a major threat to freshwater ecosystems. However, these species can be challenging to detect in recently invaded habitats. Environmental DNA (eDNA)–based methods are highly sensitive and capable of detecting just a few copies of target DNA from non-invasively collected samples. Therefore, they have considerable potential for broad-scale use in mapping and monitoring the spread of invasive species. In this study, we aimed to increase our understanding of the current distribution of signal crayfish in a headwater stream system in the United Kingdom (tributaries of the River Wharfe, Addingham, Yorkshire). Environmental DNA sampling, assessment of water chemistry variables, and conventional crayfish hand-searching were conducted across 19 study sites in five tributary streams. Using hand-searching, we detected signal crayfish at 26% of the sites (5/19 study sites). However, using eDNA-based methods, occupancy increased to 47% of study sites (9/19). Our sampling revealed previously unknown sites of crayfish occupancy, and using eDNA-based methods, we were able to define the geographical extent of the invasion front in each headwater stream sampled. This study highlights that eDNA-based methods are well-suited for detecting newly established signal crayfish populations in recently invaded habitats, even when the invasive species is at low abundance and, therefore, might otherwise be under-represented or undetected using conventional survey methods. Our study provides further evidence that headwater stream ecosystems are particularly vulnerable to signal crayfish invasion. However, their geomorphological features may make methods used to reduce or prevent invasive crayfish dispersal more effective than in other freshwater ecosystems.

KEYWORDS
eDNA, freshwater ecology, *Pacifastacus leniusculus*, upland streams

Genner and Battarbee joint senior co-authors.

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1 | INTRODUCTION

The introduction of invasive non-native species is a major threat to freshwater ecosystems globally and presents unique conservation and management problems (Dudgeon et al., 2006). Although aquatic invasive species can be abundant once established, they can be challenging to detect soon after an introduction event, or when invasion has occurred when individuals exist at low abundance (Jerde et al., 2011; Spear et al., 2021). Some conventional survey techniques currently used to detect aquatic invasive species, such as hand-searching for crustaceans, can yield a high capture probability (Chucholl & Schrimpf, 2016; Olarte et al., 2019). However, other traditional techniques, such as fyke netting and electrofishing for fish, can have a low capture probability per target organism and are only capable of detecting organisms when the population is at a medium or high density (Magnuson et al., 1994). In addition, trapping, the most widespread existing crayfish survey technique, can be ineffective at capturing juvenile crayfish (Chadwick et al., 2020; Dorn et al., 2005).

Environmental DNA (eDNA) has been shown to be an effective tool for detecting the presence of aquatic invasive species in freshwater ecosystems, such as Asian carp species (Jerde et al., 2013), Chinese mitten crab (*Eriocheir sinensis*) (Robinson et al., 2019), and crayfish species, including signal crayfish (*Pacifastacus leniusculus*), (Harper et al., 2018; Troth et al., 2020), Australian red claw crayfish (*Cherax quadricarinatus*) (Baudry et al., 2021), and Marmorkrebs (*Procambarus virginalis*) (Mauvisseau et al., 2019). The use of eDNA-based approaches have been heralded as highly sensitive non-invasive methods, with the ability to detect as little as 0.005 ng/µl of target DNA from environmental water samples (Robinson et al., 2019). eDNA is, therefore, well-suited for detecting populations of invasive species in recently invaded habitats when they are at low abundance and might otherwise be under-represented or undetected using conventional survey methods. Furthermore, eDNA is likely to be effective in small headwater streams as it can be assumed that they typically hold less water than other, larger freshwater ecosystems (Chucholl et al., 2021; Curtis et al., 2021). Small headwater streams are particularly vulnerable to invasion from species such as the signal crayfish as they often provide an optimum habitat of rocky substrate and soft riverbanks (Galib et al., 2020). Moreover, there is a pressing need to study and conserve small waterbodies, such as headwater streams, because they are more likely to be in a good ecological condition than larger downstream water bodies. However, surprisingly, headwaters are often the least studied freshwater habitats across Europe and commonly excluded from water management planning (Biggs et al., 2017).

Signal crayfish were introduced in the United Kingdom in 1976 and are now widely distributed across the country. In Yorkshire, small headwater streams act as natural laboratories for investigating the effects of signal crayfish invasion on native biota (Chadwick et al., 2020; Galib et al., 2020; Peay et al., 2009), and growing evidence shows that signal crayfish invasion can cause long-term ecological damage to headwater streams (Galib et al., 2020; Peay et al., 2009). In particular, significant reductions in macroinvertebrate species richness (Galib et al., 2020) have been observed following signal crayfish invasion along with declines in recruitment and densities of benthic fishes, including bullhead (*Cottus perifretum*) and brown trout (*Salmo trutta*; Galib et al., 2020; Peay et al., 2009). The presence of this invasive species has even been credited with the local extinction of bullhead and stone loach (*Barbatula barbatula*; Galib et al., 2020).

Due to the clear impact of signal crayfish on invaded freshwater habitats, it remains important to map their distribution, especially in the less-studied headwater streams where the information can be used to guide targeted crayfish control and eradication efforts. Therefore, here, we mapped the distribution of signal crayfish populations in five headwater stream tributaries of the River Wharfe, UK, using both eDNA and traditional survey methods (hand-searching). This system was chosen to investigate the hypothesis that signal crayfish invasive fronts can be accurately identified using these methods, thereby evaluating their prospects for the design and monitoring of future conservation interventions.

2 | MATERIALS AND METHODS

2.1 | Study location and survey design

The study area is in the catchment of the River Wharfe in West Yorkshire, UK, which has an underlying geology comprising

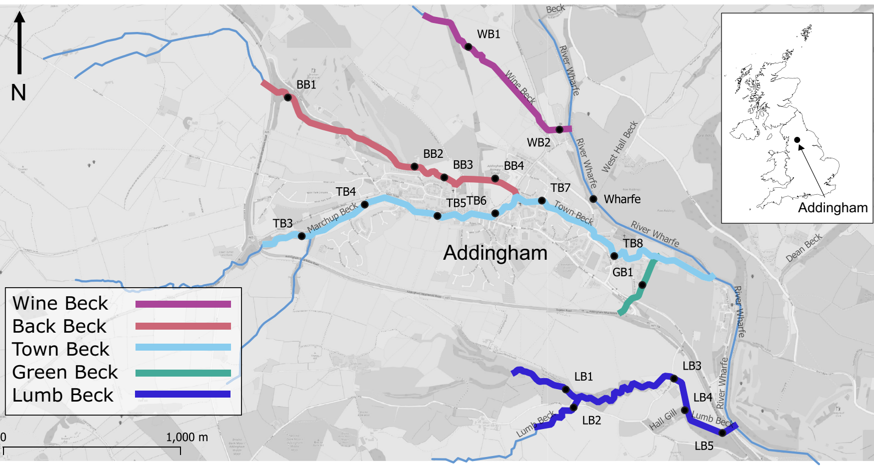

FIGURE 1 Survey locations (black circles) of tributaries of the River Wharfe in and around the village of Addingham, West Yorkshire, UK. BB, Back Beck; GB, Green Beck; LB, Lumb Beck; TB, Town Beck; WB, Wine Beck; Wharfe, River Wharfe sample point. Numbers refer to sample points

primarily of Millstone Grit of the Carboniferous age (Walling et al., 1999). All study sites are located in streams and around the village of Addingham. Some of the streams rise on the moorland above the village. All flow through agricultural land used for livestock farming and two (Town Beck and Back Beck) in their lower reaches also flow through the village itself before entering the Wharfe (Figure 1).

Signal crayfish are believed to have escaped from a trout farm next to the River Wharfe ~43 km upstream of Addingham in 1983 and have since advanced downstream to and beyond the study area (Peay et al., 2009), being first seen in Town Beck in Addingham in 2007 (D. Law, personal communication, September 2, 2020). A few native white-clawed crayfish (*Austropotamobius pallipes*) populations persist in Yorkshire, notably in North Yorkshire (Dalton Beck) and West Yorkshire (Malham Tarn and Eller Beck, which is ~7 km upstream of the River Wharfe; Bubb et al., 2008; Holdich & Reeve, 1991; Robinson et al., 2000). However, white-clawed crayfish has been locally extinct from Addingham for at least 20 years following the development of a housing estate in the village due to changes in the hydrology of the stream caused by rapid surface water runoff (R. Battarbee, personal communication, September 1, 2020). The signal crayfish is also a carrier of the crayfish plague (*Aphanomyces astaci*), which has been shown to infect and decimate populations of white-clawed crayfish (Holdich & Rogers, 1997). However, since the introduction of signal crayfish to Kilnsey trout farm, PCR tests have shown no evidence of the crayfish plague (*A. astaci*) in the River Wharfe (Peay et al., 2009).

2.2 | Crayfish hand-searching

Crayfish hand-searches were conducted by turning over ~100 rocks over a stretch of ~100 m at each site while placing a hand net downstream of each rock as they were turned to capture any escaping crayfish underneath. Conventional and eDNA methods were conducted by different people to reduce the risk of contamination. Once captured, the carapace length of each crayfish was measured using a pair of forceps and a ruler, and the sex and presence/absence of claws were noted. The number of signal crayfish found at each study site by hand-searching is, henceforth, referred to as 'relative signal crayfish abundance'. Crayfish that were observed escaping, but not caught (one at Town Beck 8), and crayfish carcasses (one at Town Beck 7 and one at Back Beck 4) were also counted. The signal crayfish carcass at Town Beck 7 was spotted upon arrival at the site, and its exact origin is unknown. A crayfish hand-search was not conducted at the River Wharfe study site due to the river's large size and heavy flow. The River Wharfe study site was excluded from statistical analysis due to the lack of signal crayfish detection using either method, in addition to its lack of relevance in the hypotheses being tested. In addition, the study site Lumb Beck 3 was also excluded from statistical analysis due to potential contamination. However, both sampling sites are included in Figure 1 and 2 for context.

2.3 | Environmental DNA sampling

Samples were collected between the 31st of August and 4th of September, 2020. We did not expect signal crayfish to be present in the uppermost reaches of the streams; therefore, we started our survey in the study sites further downstream, but well above the point they were known to occur. We worked in a downstream direction to minimize contamination in the field. Environmental DNA samples were collected by first taking a bulk surface-water sample at each site by rinsing a new sterile bottle at each site three times with gloved hands in river water. Three replicates of 250 ml were then subsampled from the bulk water sample by passing water through a Sterivex filter (0.22 μm pore diameter) (Merck Millipore) with a 50-ml sterile syringe. A battery-powered pump was not available to facilitate the filtration of larger volumes of stream water; therefore, all stream water was hand pumped. A sample volume of 250 ml was chosen to maximize the number of sites visited within the finite period of fieldwork time available. Each filter was preserved on-site using 0.33 ml of ATL tissue lysis buffer (Qiagen) and sealed with a combi-stopper and placed in a sterile 50-ml centrifuge tube. Negative field controls were collected on-site by substituting 250 ml of supermarket mineral water for sample water (treated otherwise identically). All centrifuge tubes containing Sterivex filters were then sealed in sterile plastic bags. Samples in plastic bags were then stored in a cool bag while in the field before being transferred to a −20°C freezer in the laboratory until they were required for DNA extraction.

2.4 | Environmental variables

All environmental variables were measured nine times at each study site, consisting of three replicates in an 'upper', 'middle', and 'lower' section of the study site. Water physico-chemistry variables, namely, pH, water temperature (°C), total dissolved solids (mg/L), and conductivity (mS/cm) were measured using a CDS107 water chemistry probe (Omega Engineering). In addition, the depth (cm) of each stream was measured using a metre rule. Water flow was measured using a standard flowmeter (Geopacks) with a moveable impeller connected to a resettable liquid crystal display counter. The distance of each study site from the River Wharfe (m) was also obtained using the measurement tool in QGIS v 3.14.16.

2.5 | Environmental DNA extraction

We extracted the DNA from all field samples within one month of collection in extraction batches of 12 samples at a time. Additionally,

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we added one extraction control for each extraction batch containing absolute ethanol instead of the sample buffer. Prior to each batch of extractions, all laboratory equipment and surfaces were sterilized using 70% ethanol, then 10% bleach solution, and then 70% ethanol once more, before a 2-h period of UV light that covered the entire laboratory. Gloves were worn continuously and changed between each extraction step, and between handling samples from different sites. First, 20 μl of proteinase K (Qiagen) was added to each Sterivex filter, which was then incubated at 56°C for 1.5 h while being shaken continuously. DNA extractions were then conducted using a DNeasy Blood and Tissue Kit (Qiagen), after centrifuging the sample for 1.5 min at 13,000*g* to remove debris. Extracted DNA was eluted into 105 μl of pre-warmed AE elution buffer and stored in 1.5-ml lobind microcentrifuge tubes at −20°C. Before use, extracted eDNA samples were treated with a OneStep PCR Inhibitor Removal Kit (Zymo Research), following the manufacturer's protocol. The eDNA sampling protocol used in this study can be found in full in Collins (2021).

2.6 | Signal crayfish eDNA assay

The assay targeted a 114 base pair fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, using the primers CO1-PI-02-F (5′-TGAGCTGGTATAGTGGGAACT-3′), CO1-PI-02-R (5′-AGCATGTGCCGTGACTACAA-3′) and the associated probe incorporating 5′ FAM and 3′ Black Hole Quencher-1 modifications (5′-FAM-CGGGTTGAATTAGGTCAACCTGGAAG-BHQ1-3′), based on the study by Mauvisseau et al. (2018).

2.7 | Environmental DNA purification and amplification

The DNA from a signal crayfish tissue sample was extracted, purified, and amplified using end-point PCR and quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) to create a set of qPCR standards. Next, we converted the quantified signal crayfish tissue sample mass to copy number using the Thermo Fisher Scientific DNA Copy Number and Dilution Calculator. The quantified signal crayfish tissue PCR product was then diluted sequentially by a factor of 10, creating a set of qPCR standards ranging from 1,000,000 template copies to 1 template copy per 1 ml of water. These standards served as positive controls on each qPCR plate and were used to generate a standard curve for quantification of eDNA abundance (copies/L of stream water).

Each qPCR plate was loaded with the set of seven signal crayfish tissue standards (each in triplicate) and six field samples (each in triplicate) and three no-template controls, which contained 1 μl of sterile distilled water instead of the template. Each 5-μl qPCR reaction consisted of the following reagents: 2.5 μl of GoTaqGreen Master Mix (PCR Biosystems), 0.25 μl of primer-probe mix (400 nM primer and 200 nM probe concentrations), 1.25 μl of distilled water, and 1 μl of DNA template. The reactions were run on an Eco48 thermal cycler machine (PCRMax) in 48-well plates with ROX normalisation. Thermocycling parameters were as follows: an initial denaturation at 95°C for 3 min, followed by 42 cycles consisting of a denaturing period at 95°C for 5 s, and an annealing-extension period for 30 s at 60°C. Spurious amplifications were removed after careful visual inspection of the amplification curves, and C_q values were generated in EcoStudy v5.2.11.0 (PCRmax) using the default settings.

2.8 | Limit of detection and probability

Three qPCR replicates were run on each plate for all ten-fold standard dilutions, in addition to three negative non-template controls. The limit of detection within a single qPCR reaction (LOD_i) was defined as the lowest concentration at which there is a 95% chance of successful amplification in any individual qPCR reaction. In addition, we calculated the LOD_{iii} to determine the lowest concentration with a 95% chance of successfully amplifying in any one of three technical qPCR replicates of the same sample. The limits of detection were calculated by fitting sigmoidal logistic models using CurveExpert v2.7.3 (Hyams Development), as in Alzaylaee et al. (2020). The limit of quantification was defined as the lowest concentration at which 90% of all ten-fold standard dilutions run were successfully amplified.

2.9 | Correlation coefficients

We tested for significance between signal crayfish eDNA copy number and distance upstream from the River Wharfe and signal crayfish eDNA copy number and relative signal crayfish abundance. First, a Shapiro–Wilk test for normality was calculated using the variables ‘distance upstream from the River Wharfe’ ($p = 0.57$) and ‘relative signal crayfish abundance’ ($p < 0.005$) to check that the data were normally distributed. A Shapiro–Wilk test p value >0.05 indicated that the data were normally distributed. However, Spearman's test was selected for the analysis of both variables due to suspected lack of bivariate normality in the model with signal crayfish eDNA copy number.

2.10 | Detection and occupancy probability modeling

To investigate the relationship between environmental variables and signal crayfish DNA detection probability, we calculated hierarchical occupancy estimation models as described by MacKenzie et al. (2002). The likelihood of an organism or its eDNA to occupy a study site (psi) is a function of both the presence of the signal crayfish and, therefore, its DNA and the ability to detect it (p). The

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resulting estimation of detection probability, however, is often imperfect (Rice et al., 2018). To account for imperfect detection, hierarchical occupancy estimation models infer occupancy and detection probability with the sampling that is replicated in either space or time (MacKenzie et al., 2002). In our study, however, replicates were taken from a bulk sample and, therefore, were not replicated sufficiently in space or time, and the occupancy modeling requirements were, therefore, not all fulfilled. As a result, the results of the occupancy modeling presented below should be interpreted with caution.

Candidate occupancy probability models were fit using the R package *unmarked* v2.12.0 (Fiske & Chandler, 2011), using R v 1.2.1335. We used study sites as our unit of occupancy and eDNA field replicates (n = 3 at each study site) as our replicated units used for estimating detection probability. Signal crayfish presence was determined at each site by a positive detection using crayfish hand-searching and or the successful amplification of at least one technical replicate from field samples. We included relative signal crayfish abundance as the only predictor of eDNA occupancy and stream depth (cm), in-stream water temperature (°C), pH, distance upstream from the River Wharfe (m), and relative signal crayfish abundance as predictors of signal crayfish eDNA detection probability. Conductivity was not included due to collinearity with total dissolved solids ($\rho_s = 1$). All predictor covariates used in modeling were standardized by Z-transformation to have a mean of 0 and a standard deviation of 1. Flow and total dissolved solids were also removed from further statistical analysis because they resulted in the global model failing to converge.

We hypothesized that as distance upstream from the River Wharfe (the location of the signal crayfish source population) increased, the detection probability of signal crayfish eDNA would decrease. We assumed that study sites further upstream from the River Wharfe would be more recently invaded by signal crayfish than sites closer to the Wharfe and, therefore, possess a smaller signal crayfish population, which in turn would release less eDNA into the water column.

We used Pearson's chi-square statistic to examine model fit and calculated the overdispersion statistic, c-hat, using the R package *AICCModavg* v2.3-1 (Mazerolle, 2015). Well-supported models were defined as those having ΔQAICc <2. A likelihood ratio-based pseudo-r-squared value was calculated for the only well-performing model to assess model fit.

3 | RESULTS

3.1 | Crayfish hand-searching

Signal crayfish were detected by hand-searching at 5/18 tributary study sites (28% of sites). We found the highest abundance of signal crayfish (nine individuals per search) at Town Beck 7, one of which was a carcass, and the fewest crayfish (4) at Back Beck 4, one of which was also a carcass (Table 1).

3.2 | Environmental variables

Mean values of environmental variables measured at each study site are listed in Table 2.

3.3 | Limit of detection and probability

The *P. leniusculus* qPCR assay varied in efficiencies from 91.30% to 100.97%, while R^2 ranged from 0.995 to 0.997. The *P. leniusculus* qPCR assay amplified all standards between 1,000,000 and 10 copies/μl. Amplification success became slightly inconsistent at 1 copies/μl (22/24 qPCR reactions). Therefore, the resolved limit of quantification was 10 copies/μl. The 95% probability of successful individual PCR amplification (LOD_i) was 1.48 copies/μl, and the 95% probability of successful amplification in at least one of the three qPCR replicates (LOD_{iii}) was 1.31 copy/μl.

3.4 | Environmental DNA sampling

We detected signal crayfish eDNA at 9/19 study sites (47% of field replicates; Figure 2). Signal crayfish eDNA copies/L ranged from 0 to 3217.51, with a median of 0, an average of 312.56, and a standard error of ±175.2 (Table 1). We detected signal crayfish eDNA at all study sites where we found signal crayfish by hand-searching, and in addition four where we did not find signal crayfish by hand-searching (Table 1). We detected no signal crayfish eDNA at 10/19 study sites (52% of field replicates). No successful amplification was detected for any of the no-template controls (0/24), or the negative field controls Lumb Beck 2, Lumb Beck 4, Lumb Beck 5, Back Beck 1, Back Beck 3, and Town Beck 6 (0/3). However, 1/3 qPCR replicates of the Lumb Beck 3 negative field control were successfully amplified.

We found a positive relationship between signal crayfish eDNA copy number (L^{-1} of stream water) and relative signal crayfish abundance (Figure 3a). By contrast, we found no significant relationship between signal crayfish eDNA copy number (L^{-1}) and distance upstream from the Wharfe (Figure 3b).

3.5 | Detection and occupancy probability modeling

Our global occupancy probability exhibited some overdispersion (c-hat = 1.81). Therefore, the global model AIC was converted into QAIC using the overdispersion parameter, c-hat. Model selection produced one well-performing model (ΔQAIC <2.0) for signal crayfish eDNA detection probability (Table 3), which included two predictor variables: distance from the River Wharfe and relative signal crayfish abundance. Specifically, there was a positive relationship between relative signal crayfish abundance and predicted detection probability (p) (Figure 4a) and a negative relationship between

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TABLE 1 Signal crayfish detections using hand-searches and eDNA (quantitative PCR)

Study site	Decimal latitude, longitude	Sampling date (dd/mm/yy)	Physical observation (no. of crayfish found *via* hand-searching)	eDNA detection (no. of +ve qPCR replicates)	Mean signal crayfish DNA copies (L^{-1})
Back Beck 1	53.950352, −1.900563	31/08/20	0	0/9	0.00
Back Beck 2	53.946835, −1.889679	31/08/20	0	0/9	0.00
Back Beck 3	53.946279 −1.887121	01/09/20	0	0/9	0.00
Back Beck 4	53.946221, −1.882745	04/09/20	4	2/9	300.19
Town Beck 3	53.943327, −1.898768	02/09/20	0	0/9	0.00
Town Beck 4	53.944907, −1.893965	02/09/20	0	0/9	0.00
Town Beck 5	53.944326, 1.887713	02/09/20	6	7/9	93.37
Town Beck 6	53.944452, −1.882743	02/09/20	5	9/9	965.01
Town Beck 7	53.945096, −1.878728	04/09/20	9	9/9	949.40
Town Beck 8	53.942271, −1.872511	04/09/20	5	9/9	3217.51
Lumb Beck 1	53.935094, −1.875855	01/09/20	0	0/9	0.00
Lumb Beck 2	53.934877, −1.875775	01/09/20	0	0/9	0.00
Lumb Beck 3	53.936042, −1.867393	01/09/20	0	1/9	19.72
Lumb Beck 4	53.934436, −1.866467	01/09/20	0	6/9	189.02
Lumb Beck 5	53.933275, −1.863243	01/09/20	0	0/9	0.00
Wine Beck 1	53.952911, −1.885032	02/09/20	0	0/9	0.00
Wine Beck 2	53.948684, −1.877205	02/09/20	0	2/9	32.51
Green Beck 1	53.940202, −1.870540	01/09/20	0	9/9	172.01
Wharfe	53.945503, −1.874638	03/09/20	N/A*	0/9	0.00

Note: N/A = no crayfish hand search was conducted and * indicates known record. Mean signal crayfish DNA copies are scaled to (L^{-1}) of stream water.

TABLE 2 Averaged values of water chemistry parameters for each study site

Study site	pH	Water temperature (°C)	Total dissolved solids (mg/L)	Conductivity (mS/cm)	Depth (cm)	Stream flow (rpm)
Back Beck 1	9.27	12.37	173.78	263.22	21.78	43.11
Back Beck 2	9.59	11.98	176.22	266.33	11.72	82.89
Back Beck 3	9.26	12.18	185.33	282.22	14.11	93.11
Back Beck 4	8.98	13.11	154.51	237.00	25.00	103.44
Town Beck 3	9.26	11.72	146.67	219.78	18.67	3.11
Town Beck 4	9.27	11.80	134.36	202.81	12.44	112.00
Town Beck 5	9.49	12.31	152.11	229.78	12.44	112.22
Town Beck 6	9.53	12.60	152.22	230.33	9.56	146.67
Town Beck 7	9.39	13.10	122.62	186.50	13.78	155.89
Town Beck 8	9.27	13.50	128.24	196.54	31.67	54.67
Lumb Beck 1	9.34	10.71	131.42	195.41	22.44	90.89
Lumb Beck 2	9.54	10.80	101.56	153.11	11.89	168.22
Lumb Beck 3	9.48	11.88	108.08	163.00	13.33	200.33
Lumb Beck 4	9.40	12.20	109.26	163.67	18.44	85.89
Lumb Beck 5	9.55	12.50	112.53	170.17	14.22	359.44
Wine Beck 1	9.29	13.60	141.22	216.22	12.22	83.78
Wine Beck 2	9.27	12.66	163.44	248.22	15.00	102.33
Green Beck 1	8.46	14.57	168.89	257.44	13.00	1.33
Wharfe	9.53	13.50	129.64	196.78	N/A	N/A

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FIGURE 2 Signal crayfish eDNA detections in Addingham village, Yorkshire, UK

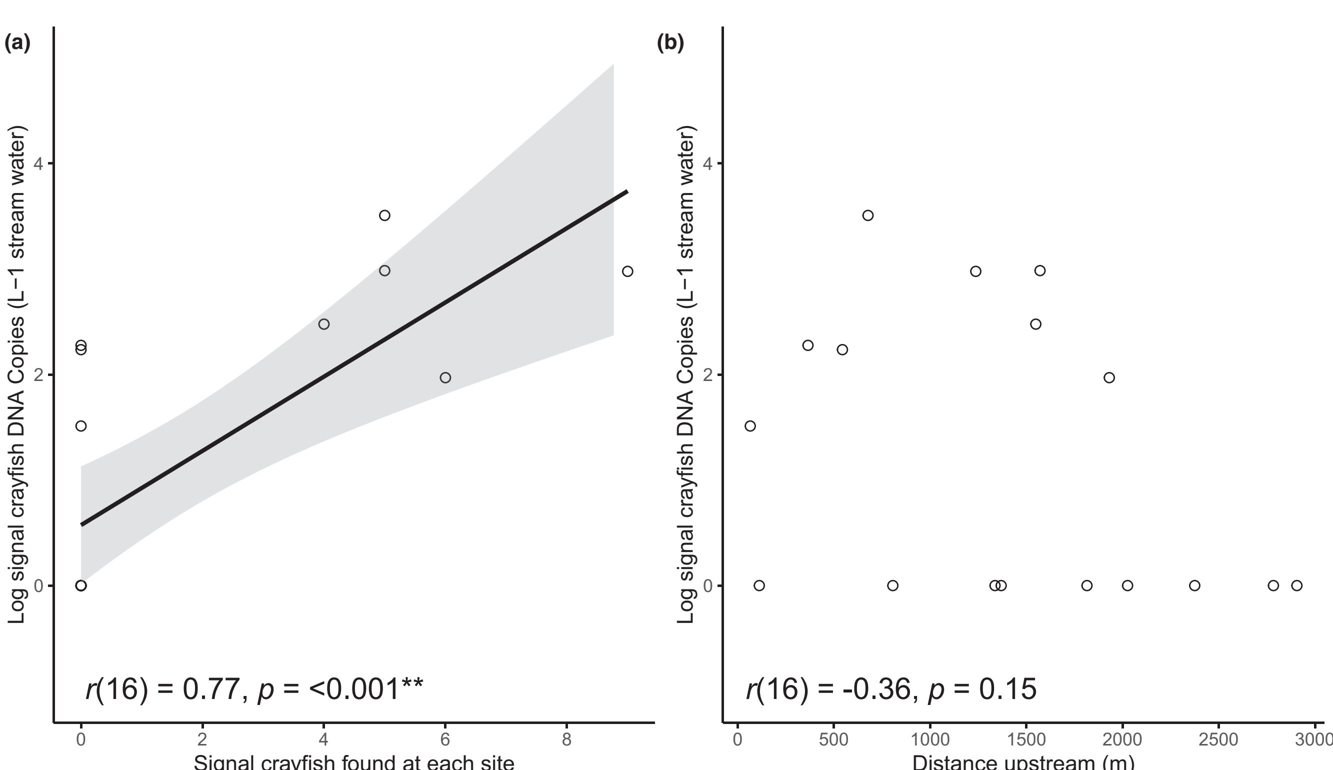

FIGURE 3 Relationship between the $\log_e$ transformed number of signal crayfish DNA copies detected and: (a) number of signal crayfish found by hand-searching at each site, and (b) the distance upstream of each site from its connection with the River Wharfe. Gray shaded areas represent 95% confidence intervals

distance from the River Wharfe and predicted detection probability (*p*) (Figure 4b).

4 | DISCUSSION

Our results demonstrate that eDNA-based methods are an effective tool for detecting the presence of signal crayfish in headwater streams in the UK. We successfully detected the presence of eDNA from the species at all study sites where we found crayfish by hand-searching (Table 1). Furthermore, we detected signal crayfish eDNA at four study sites where no crayfish were found by traditional survey methods, resulting in the recording of two previously undiscovered signal crayfish populations in Wine Beck and Lumb Beck (Figure 2). We were also able to define the geographical upstream range of signal crayfish invasion in each of the studied streams. The data gathered here, therefore, support previous work that successfully detected the presence of crayfish in lotic systems (Ikeda et al., 2016; Rice et al., 2018), and in particular signal crayfish in British rivers (Harper et al., 2018; Robinson et al., 2018, 2019; Troth et al., 2020). Detection probability modeling indicated that the variables 'distance upstream from the River Wharfe' and 'relative signal crayfish abundance' produced the best model for detecting signal crayfish eDNA.

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TABLE 3 Candidate models for signal crayfish eDNA occupancy (psi) and detection probability (*p*) ranked by QAICc

Model	QAICc	ΔQAICc	R^2
psi(.), *p*(Crayfish Abundance + Distance)	30.77	0.00	0.73
psi(.), *p*(Crayfish Abundance + Depth + Distance)	36.18	5.41	N/A
psi(Crayfish Abundance), *p*(Crayfish Abundance + Distance)	36.34	5.57	N/A
psi(.), *p*(Crayfish Abundance + Distance + pH + Temp)	36.94	6.17	N/A
psi(.), *p*(Crayfish Abundance + Distance + pH)	38.41	7.64	N/A
psi(Crayfish Abundance), *p*(Crayfish Abundance + Distance + pH)	39.50	8.73	N/A
psi(.), *p*(Crayfish Abundance + Distance + Temp)	40.12	9.35	N/A
psi(Crayfish Abundance), *p*(Crayfish Abundance + Distance + Depth)	41.27	10.50	N/A

Notes: Well-performing models (ΔQAICc <2) are shaded in gray. A likelihood ratio-based pseudo-*r*-squared (R^2) value was calculated for the best performing model to assess model fit. Environmental variables were imported as means of nine replicates at each study site ($n = 17$). Environmental variables: pH, in-stream water temperature (°C), and depth (cm).

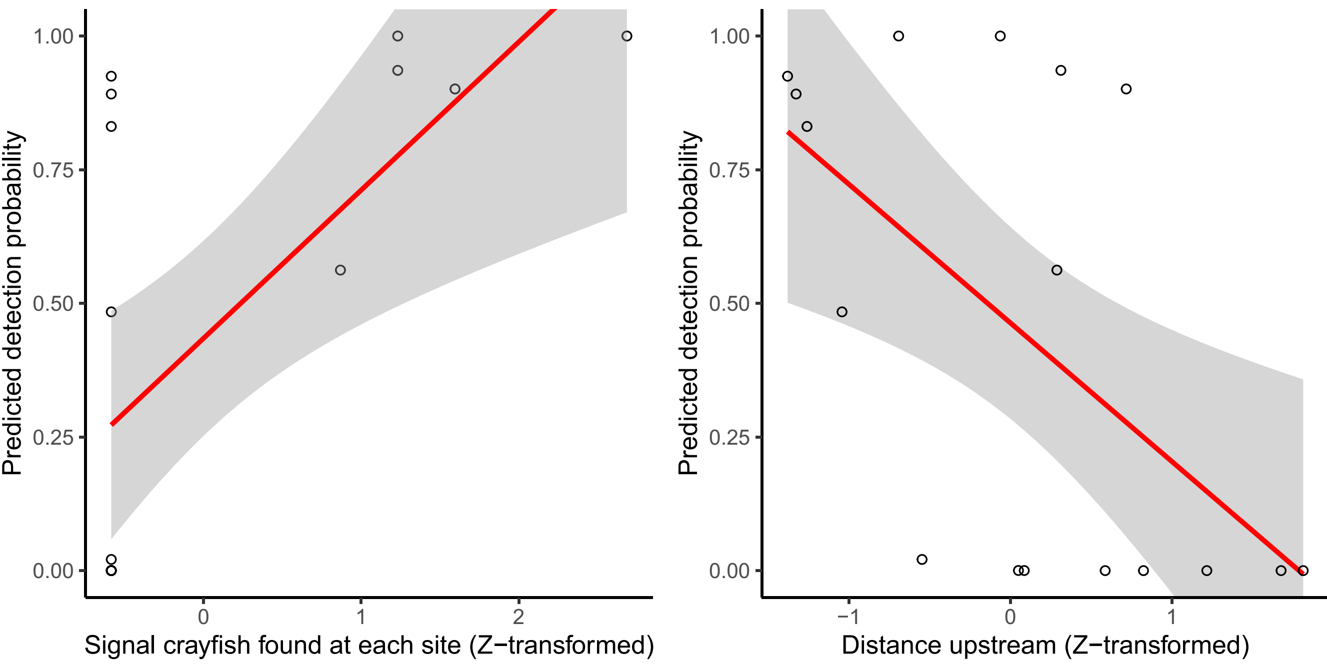

FIGURE 4 Fitted lines show the trends for the key variables selected in the optimum model, as selected using the ΔQAICc criterion (Table 2). Each variable has been standardized by *Z*-transformation to ensure a mean of 0 and a standard deviation of 1. Gray shaded areas show 95% confidence intervals of the fitted linear models

Many eDNA field studies conducted in freshwater ecosystems have reported positive relationships between the abundance of eDNA and the abundance of source target organism, for example in fish (Lacoursière-Roussel et al., 2016; Spear et al., 2021; Takahara et al., 2012; Yates et al., 2019) and amphibians (Pilliod et al., 2013). However, crayfish eDNA field studies have typically reported weak relationships with relative crayfish abundance and eDNA concentration (Cai et al., 2017; Dougherty et al., 2016; Larson et al., 2017; Rice et al., 2018). Some studies conducted in lentic systems (Dougherty et al., 2016; Larson et al., 2017) reported weak correlations with crayfish abundance due to the dilution of crayfish eDNA or differing life histories of target species in large waterbodies. Here, we illustrate a positive relationship between signal crayfish eDNA copy number and relative crayfish abundance (Figure 3). The time of year in which this survey was conducted may have played a part in generating a relatively strong relationship between eDNA concentration and relative crayfish abundance. Warmer water during summer months increases signal crayfish activity levels as they seek to reproduce before winter, potentially releasing more eDNA into the stream water and increasing detection probability (Dunn et al., 2017; Wright & Williams, 2000).

Detection probability was shown to increase further downstream, potentially suggesting that crayfish eDNA from upstream populations accumulated downstream (Burian et al., 2021). The likely patchy distributions of crayfish, along with the downstream transportation of eDNA, may very well explain the relatively poor relationships found in other eDNA-based studies conducted in lotic systems with regard to relative crayfish abundance and detection probability. However, it should be noted that in our models, we utilized 'upstream distance' from a known signal crayfish source

population as a variable factor, rather than mapping or trying to predict downstream transportation of eDNA. Indeed, previous studies have shown that eDNA can be detected at distances >10 km and potentially up to 100 km downstream from the source populations (Pont et al., 2018), highlighting an issue with reliability unless multiple sites are factored into the sampling design to account for this.

Regardless of downstream transportation of eDNA, we illustrate that (a) as distance from the River Wharfe increases, predicted occupancy of crayfish decreases and (b) a negative relationship between eDNA detection probability and distance upstream from the River Wharfe. Taken together, this suggests that the signal crayfish are advancing upstream in each of the study streams from the likely origin site of the River Wharfe. Therefore, eDNA-based methods are likely to be identifying the position of the invasion front in each study stream. For example, for Town Beck, the invasion front would be between sites Town Beck 5 and Town Beck 4; and for Back Beck, this would be between Back Beck 4 and Back Beck 3. However, Lumb Beck site 5 does not fit this pattern and was negative for signal crayfish eDNA. Lumb Beck 5 was downstream of two study sites with positive eDNA detections (Lumb Beck 3 and Lumb Beck 4) and closest to the proposed source population of crayfish in the River Wharfe. One explanation for this negative could be that it is a false-negative, driven by increased suspension of river sediments. At Lumb Beck 5, cattle were seen to wade through the stream, and the Sterivex filters were noticeably dirty with sediment after filtering stream water from the site. Disruption of sediment has been shown to increase the amount of PCR inhibition for eDNA assays often resulting in false-negatives (Conroy et al., 2016). Alternatively, the disturbance of the sediment by the cattle might make the site less favorable for crayfish; however, this does not explain the lack of downstream transport from Lumb Beck 4, for example. It is important to note that one of the Lumb Beck 3 negative field control qPCR replicates successfully amplified, indicating contamination. It is possible that this amplification is the result of field contamination; however, it is very unlikely as result of field contamination from any other study streams as all sites located in the other tributaries were not visited or surveyed until after all Lumb Beck sites were sampled. Rather, it is much more likely that the contamination occurred in the field while sampling other Lumb Beck study sites or in the laboratory while conducting DNA extraction or qPCR procedures. Despite this, the evidence for the presence of signal crayfish DNA in Lumb Beck is notable, with 1/9 and 6/9 qPCR replicates successfully amplifying from study sites Lumb Beck 3 and 4, respectively. It is also important to address the lack of detection of signal crayfish in the proposed origin site, the River Wharfe, given that the species has been detected at this location historically (Peay et al., 2009). It is possible that the increased volume of water in the River Wharfe (relative to its tributaries) has diluted the eDNA below the limit of detection, resulting in a false-negative result at this locale (Curtis et al., 2021). In addition, the relatively low filter volume (250 ml) of stream water sampled in this study for each replicate will have limited signal crayfish eDNA detection probability and may also have generated false-negative results.

Signal crayfish are spreading across the UK, and this invasion is being increasingly well-documented using traditional survey methods (Holdich et al., 2014), as well as novel approaches, such as the triple drawdown method (Chadwick et al., 2020) and now the use of eDNA (Harper et al., 2018; Robinson et al., 2019; Troth et al., 2020). However, understanding what can be done to stop the spread, and, therefore, preserve native species such as the white clawed crayfish is less well-understood. We suggest that eDNA-based surveys can shed light on the subject. Signal crayfish can advance through any river system once present but can also move overland, therefore increasing the chance of new, previously uncolonized systems being invaded (Thomas et al., 2019). This advance in invasion is often exacerbated during flood events which, in turn, are increasing in recent years due to climate change and change in land use policies (Arnell et al., 2021). It has been shown that the presence of natural or manmade barriers can be key in moderating signal crayfish dispersal, especially in the case of flooding events (Hudina et al., 2017; Light, 2003). In this study, a long culvert was located directly upstream of one of our survey sites (study site Town Beck 5), and a high step was present in another (Back Beck 4). These barriers coincide with lack of detection of the signal crayfish upstream of these sites. However, the presence of barriers in such river systems also reduces ecological connectivity and impacts biodiversity, in particular impeding the migration of fish such as salmonids and the European eel (*Anguilla Anguilla*; White & Knights, 1997). In fact, the removal of such barriers has become a key component of many river restoration programs across Europe (Magilligan et al., 2016; O'Hanley, 2011). However, Krieg et al. (2021) show that a fish-passable crayfish barrier can be constructed by building a smooth overhanging lip which creates a central area of laminar flow for fish to utilize. The combination of the high current velocity (at least ≥0.65 m/s) and its smooth surface appear enough to prevent upstream crayfish dispersal while permitting fish migration (Krieg et al., 2021). That said, given the ability of signal crayfish to walk over land (Thomas et al., 2019), any barrier is likely to only slow the spread rather than stop the invasion in its tracks. Indeed, Cowart et al. (2018) showed using eDNA-based methods that barriers are seemingly ineffective at preventing signal crayfish dispersal in California. Other, more extreme methods of prevention would, therefore, also need to be explored at these sites to stem the spread of the signal crayfish, for example, extended land barriers constructed using stainless steel plates (Krieg et al., 2021).

Regardless of the management strategies employed once a detailed assessment of the current state of signal crayfish presence or absence has been conducted, our results demonstrate the effectiveness of eDNA-based methods as tools for detecting new signal crayfish populations in recently invaded headwater streams and allow for the definition of geographic invasion fronts of those populations. On the basis of this study, we recommend the need for the creation of distribution maps illustrating current and advancing signal crayfish populations in headwater stream ecosystems. Such maps can be rapidly produced using eDNA-based methods as highlighted here, and these methods could also be utilized to identify suitable areas for the establishment of native white-clawed crayfish ark sites,

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where the signal crayfish are unlikely to reach. In our study area, the white-clawed crayfish has been extirpated for at least 20 years following the development of a housing estate that released a substantial amount of sediment into the streams (R. Battarbee, personal communication, September 1, 2020). Continued monitoring at sites, Wine Beck 1, Back Beck 1–3, Town Beck 3–4, and Lumb Beck 1–2 would indicate if the signal crayfish are advancing or stopped by barriers. Reintroduction of white-clawed crayfish could then begin at these sites to re-establish natural biodiversity. The high sensitivity of the eDNA survey methods would be particularly valuable for detecting undiscovered signal crayfish populations in recently invaded areas when they are still at low abundance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JAG made major contributions to (i) the conception or design of the study, (ii) the acquisition, analysis, or interpretation of the data; and (iii) writing of the manuscript. RAC made major contributions to (i) analysis and interpretation of the data and (ii) provided a supporting role in manuscript editing. DEE made major contributions to (i) data acquisition. MJG made major contributions to (i) providing laboratory facilities required for data analysis and interpretation and (ii) provided a supporting role in manuscript editing. JH made major contributions to (i) data acquisition. GJ made major contributions to (i) providing laboratory facilities required for data analysis and interpretation, and (ii) provided a supporting role in manuscript editing. LL made major contributions to (i) data acquisition. MO made major contributions to (i) data acquisition. MJS made major contributions to (i) data analysis by providing the primer and probes required and (ii) provided a supporting role in manuscript editing. RWB made major contributions to (i) the conception and design of the study and (ii) provided a supporting role in manuscript editing.

DATA AVAILABILITY STATEMENT

The raw data underlying the main results of this study will be archived in a Google Drive that can be freely accessed using this link: https://drive.google.com/drive/folders/16j_3FincRBMLztp9ykXCsQgBq_ALQDio?usp=sharing.

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