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ORIGINAL ARTICLE

Environmental DNA can inform the trade-off between proactive and reactive strategies for crayfish conservation

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Abstract

The introduction of the signal crayfish *Pacifastacus leniusculus* to British rivers has led to ecological degradation and the decline of the native white-clawed crayfish *Austropotamobius pallipes*. To manage and mitigate the impact of the signal crayfish, conservation agencies and government bodies employ multiple conservation strategies. These take the form of proactive native crayfish breeding and stocking programs and reactive invasive crayfish control programs. Here, we used eDNA to assess the populations of native and invasive crayfish species across 50 sites in 10 river catchments in Norfolk, United Kingdom (UK). The sites were chosen to enable assessment of the potential of eDNA to inform proactive and reactive crayfish conservation strategies. Three of the catchments sampled were selected to assess the success of recent *A. pallipes* reintroduction, whereas the remaining seven were selected to better understand the distribution of each species at the landscape scale. Combining results of eDNA-based methods with net searches within an occupancy model enabled us to confidently determine the presence of *P. leniusculus* at eight sites, and *A. pallipes* at three sites, which was more than visual searches alone (five and two study sites, respectively). Neither eDNA nor net searches detected *A. pallipes* at sites where *A. pallipes* had been reintroduced. We recommend that practitioners using eDNA-based surveys for management and conservation of crayfish should consider: (1) designing eDNA surveys with an emphasis on large spatial scales to comprehensively describe the distributions of native and invasive crayfish in a region of interest; (2) work with local conservation organizations and/or government bodies to inform the selection of study sites to generate results that are meaningful to real-world conservation actions; and (3) use results from eDNA-based crayfish surveys to target limited conservation resources to appropriate proactive and/or reactive conservation actions.

KEYWORDS
Austropotamobius pallipes, conservation, environmental DNA, invasive species, management, *Pacifastacus leniusculus*

1 | INTRODUCTION

Ecologically harmful species introduced to terrestrial, marine, and freshwater environments outside of their native ranges cause widespread and far-reaching damage to ecosystems (Alidoost Salimi et al., 2021; Pyšek & Richardson, 2010). Once established, invasive species can alter biochemical and geomorphological processes, and modify ecosystem structure by disrupting food chain interactions and initiating trophic cascades (Paini et al., 2016).

Local authorities and conservation groups employ a combination of proactive and reactive conservation strategies to mitigate the effects of invasive species. Proactive strategies focus on managing an invasion by anticipating future trends and distributions rather than only responding after an invasion has occurred. Proactive conservation strategies include undertaking risk assessments to anticipate and categorize threats from invasive species, enforcing international trade regulations, and stocking of endangered native species to bolster local populations (Humair et al., 2015). In contrast, reactive conservation strategies focus on controlling an invasion after it has occurred by reacting to current trends and distributions, and they include the active management of invasive species (Dubreuil et al., 2022). Active management of invasive species populations can take many forms, including the reduction of individuals by trapping or hunting.

Globally, there are many examples of invasive crayfish species from multiple genera (*Cherax*, *Faxonius*, *Pacifastacus*, and *Procambarus*) that have invaded freshwater ecosystems (Baudry et al., 2021; Mauvisseau, Tönges, et al., 2019; Oficialdegui et al., 2020; Panteleit et al., 2019). In the United Kingdom, *P.leniusculus* was introduced in the 1970s and is now the most abundant crayfish species in rivers (Holdich et al., 2014). The proliferation of *P.leniusculus* has caused dramatic declines in benthic macroinvertebrate and fish populations (Galib et al., 2020). In addition, it has caused the destabilization of riverbanks and increased flood risk, costing an estimated £4,200,000 per year in UK riverbank restoration projects (Eschen et al., 2023). Notably, *P.leniusculus* is also a carrier of the crayfish plague (*Aphanomyces astaci*), a water-borne oomycete that exists in a balanced host–parasite relationship with *P.leniusculus* in North America but has decimated populations of native *A. pallipes* in Europe (Matthews & Reynolds, 1992). This has further impacted UK *A. pallipes* populations, already in decline due to pollution, overexploitation of water resources, and urbanization (Naura & Robinson, 1998). Recent estimates suggest that native *A. pallipes* have declined by ~95% in some parts of the UK, with an overall decline of 80% across Europe (Dunn et al., 2017).

The rapid decline of *A. pallipes* populations has prompted the adoption of multiple proactive and reactive crayfish conservation efforts in the UK, such as habitat management (Taylor et al., 2019), captive breeding programs (Rogers & Watson, 2007), population supplementation in rivers, creation of crayfish barriers (Krieg et al., 2021), and establishment of ark sites in sheltered areas free from invasive crayfish species (Nightingale et al., 2017) (Figure 1). Captive breeding programs in the UK, such as those at Bristol Zoo (Nightingale et al., 2017) and PBA Ecology in the Yorkshire Dales (Payne, 2012), have had particular success bolstering *A. pallipes* populations via the reintroduction of captive-bred adults back to the wild (Nightingale et al., 2017).

Advances in molecular techniques have enabled the detection of crayfish species using environmental DNA (eDNA) from water samples (Dunn et al., 2017; Ficetola et al., 2008; Greenhalgh et al., 2022; Harper et al., 2018; Robinson et al., 2018; Tréguier et al., 2014; Troth et al., 2020, 2021). With careful design of sampling and stringent analysis of data, eDNA-based surveys can map the distributions of invasive crayfish and provide a valuable framework for

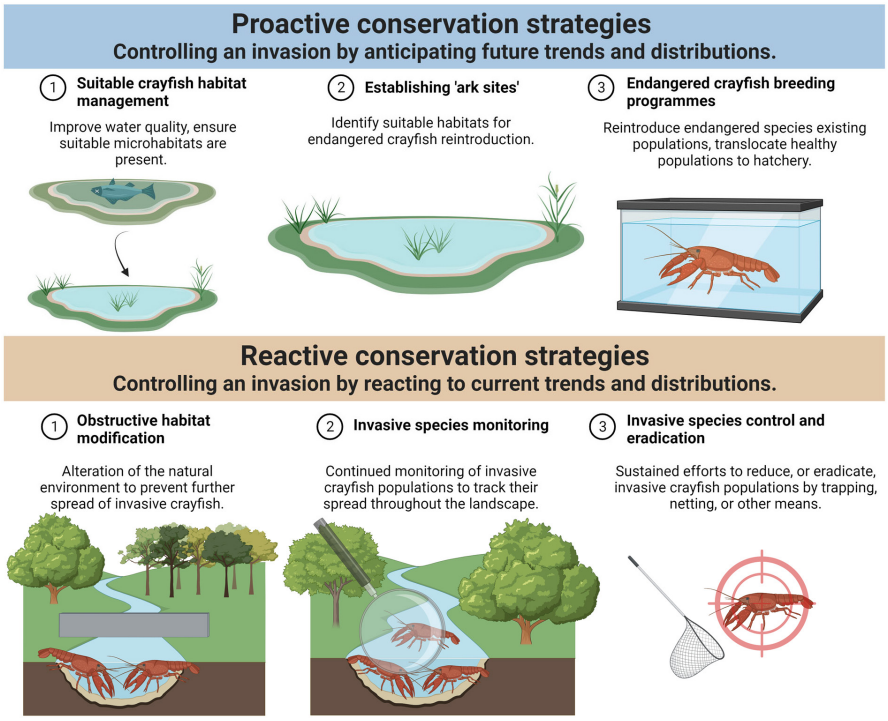

FIGURE 1 A comparison between proactive and reactive crayfish conservation strategies.

evidence-based conservation efforts (Burian et al., 2021; Cowart et al., 2018). Here, we used eDNA-based methods to survey both native and introduced crayfish at 50 study sites in 10 river catchments in Norfolk, UK. We aimed to answer the following questions: (1) Can eDNA-based surveys assess success of proactive crayfish conservation strategies by determining presence of recently restocked *A. pallipes* populations in ark sites, and (2) Can eDNA surveys identify river reaches vulnerable to crayfish invasion to help inform reactive crayfish conservation strategies?

2 | MATERIALS AND METHODS

2.1 | Study location and survey design

This study was conducted in Norfolk in the east of England (Figure 2). The underlying geology of the region is Cretaceous chalk (Jay & Holdich, 1981), which has generated chalk streams, of which the UK contains 85% of these globally rare freshwater habitats (Mondon et al., 2021). Historically *A. pallipes* was abundant across Norfolk (Baker, 1983; Jay & Holdich, 1981). By the 1990s, however, *P. leniusculus* was recorded as present in the county, which coincided with dramatic declines in *A. pallipes* populations (Holdich et al., 2014).

In total 10 river catchments located across Norfolk were selected for sampling, based on their use within ongoing monitoring and conservation efforts. The catchments were divided into two groups. Group 1 included catchments with suspected *A. pallipes* populations (Tas and Beeston Beck) and ark sites with recently supplemented *A. pallipes* populations (Ingol, Cong, and Stiffkey; stocked in 2018). Group 2 included catchments with suspected *P. leniusculus* populations (Tud, Wensum, Wissey, Bure, and Glaven). Five sampling points were assigned to each study catchment to provide broad spatial coverage (Figure 2). Sampling points in each catchment included one 'headwater' site, three 'midwater/tributary' sites, and one 'downstream' site.

2.2 | Environmental DNA sampling

Samples were collected between 2nd and 13th August 2021. At each sampling point, surface water was collected (one sterile bottle per replicate). Using a 50 mL sterile syringe, a total of 250 mL of this water was passed through a 0.22 μm pore Sterivex filter (Merck Millipore, Burlington, USA). This process was repeated three times, providing three replicates that filtered a total of 750 mL of water at each site. A sample volume of 250 mL for each replicate enabled the survey team to maximize spatial variation in the limited time available. Each filter was preserved on site using 0.33 mL of ATL tissue lysis buffer (Qiagen, Hilden, Germany) before being 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 drinking water for sample water (treated otherwise identically). In total, 150 water samples were collected alongside 11 negative field controls. Centrifuge tubes containing Sterivex filters were sealed in sterile plastic bags before storage at −20°C prior to DNA extraction. The environmental DNA sampling protocol is described in Collins (2021).

2.3 | Net-searching for crayfish

At each of the 50 sites, manual searches of suitable crayfish habitat (e.g., under macrophytes, submerged tree roots) were conducted by trained individuals using a long-handled standard pond net (mesh = 1 mm). A ~25 m stretch of river upstream and downstream of the eDNA sampling location was defined as the search area. Net searches at each site were undertaken by multiple individuals simultaneously for ~30 min in a habitat of submerged macrophytes and tree roots. Net searches followed eDNA sampling, and were conducted by different personnel to reduce contamination risk.

2.4 | Environmental variables

Five environmental variables (Table 1) were measured nine times at each of the 50 study sites [pH, temperature (°C), conductivity (μS/cm), flow (m/s), and depth (m)]. Measurements comprised three replicates in an 'upper,' 'middle', and 'lower' section of each study site. The 'middle' section was defined as the location where the eDNA sample was collected, and the 'upper' and 'lower' sections were defined as ~5 m upstream and downstream of the 'middle' section, respectively. Water pH, temperature and conductivity were measured using a Hach water chemistry probe (Hach, Loveland, USA). The depth of each stream was measured using a metre rule. Water flow rate was measured using a standard flowmeter (GeoPacks, Hatherleigh, UK) with a moveable impeller connected to a resettable liquid crystal display counter. Flow data were not available for six sites, and those data were interpolated using the knnImputation function and data from the four other environmental variables using the package DMwR2 v.0.0.2 (Torgo, 2016) in R v4.3.1 (R Core Team, 2023).

2.5 | Environmental DNA extraction and purification

DNA was extracted from field samples within five months of collection. Extractions were in batches of 24 samples, including at least one extraction negative control containing ATL buffer in each extraction batch. Laboratory equipment and surfaces were sterilized using 70% ethanol, then 10% bleach solution, and then 70% ethanol. A two-hour exposure to UV light was used to further sterilize the laboratory prior to extractions. Gloves were worn continuously and changed between each extraction step, and between handling samples from different sites. First, 20 μL of proteinase K (Qiagen, Hilden, Germany) was added to each Sterivex filter, which was then incubated at 56°C for 2 h while being shaken continuously. The

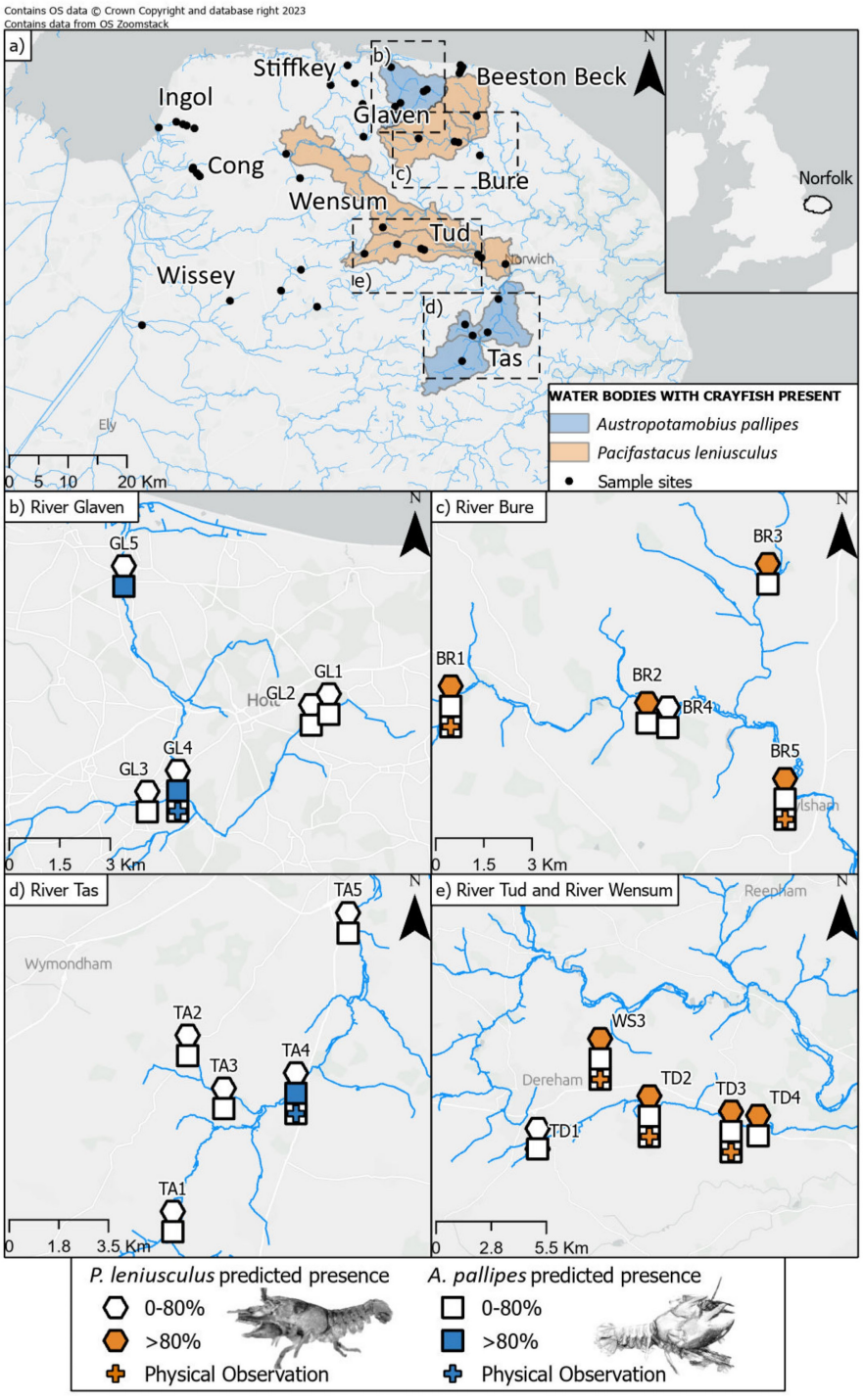

FIGURE 2 Probability of presence of *Pacifastacus leniusculus* (orange hexagons) and *Austropotamobius pallipes* (blue circles) from occupancy modeling of eDNA detections and net search detection (colored crosses, orange, and blue, respectively) in 10 waterbodies and 50 sites. (a) Norfolk, UK. (b) Glaven (GL 1–5), (c) Bure (BR 1–5), (d) Tas (TD 1–5), (e) Tud and Wensum (WS 3, TD 1–4).

sample was centrifuged for 90 s at 13,000 *g* to remove debris, and the DNA was then extracted from the supernatant using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) with reagent volumes scaled up to account for the increased ATL buffer volume. Extracted DNA was eluted into 105 μL pre-warmed AE elution buffer and stored in 1.5 mL LoBind microcentrifuge tubes at −20°C. Extracted eDNA samples were subjected to a final clean using a OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, USA), following the manufacturer's protocol. The extraction protocol is described in Collins (2021).

2.6 | *Pacifastacus leniusculus* DNA sequencing

It is considered best practice to evaluate eDNA assays against target populations, especially when target populations possess high mitochondrial DNA diversity (Taberlet et al., 2018). *P. leniusculus* in Europe possess multiple mitochondrial DNA haplotypes, across regions and populations (Petrusek et al., 2017). We extracted DNA from the gill tissue of one *P. leniusculus* individual from the Bure catchment using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Extracted DNA

TABLE 1 Description of water quality and environmental variables sampled.

Variable	Mean	Range	*n*
pH	7.49	6.20–8.20	50
Conductivity (mS/cm)	785.85	550.00–950.00	50
Water temperature (°C)	15.01	11.00–21.00 50	50
Flow rate (rpm)	151.24	0.11–561.33	44
Water depth (cm)	310.18	0.00–>2000.00	50

Note: Values presented are derived from study site means (*n* = number of sites sampled). A full description of all covariates can be found in the Supporting Information (Table S3).

was purified using magnetic beads (Qiagen, Hilden, Germany). We PCR-amplified the mitochondrial cytochrome c oxidase I (COI) region using the primers CO1-PI-02-F and CO1-PI-02-R (Mauvisseau, Tönges, et al., 2019). The PCR reaction contained 10 μL GoTaq Green mastermix (Promega, Madison, USA), 2 μL forward primer (2 nmol), 2 μL of reverse primer (2 nmol), 5 μL distilled water, and 1 μL of DNA template. Thermocycling was as follows: 50°C for 5 min, denaturation at 95°C for 8 min, followed by 42 cycles of 95°C for 30 s and 55°C for 1 min. PCR products were cleaned using magnetic beads, and sequenced using the Mix2Seq service (Eurofins Genomics, Wolverhampton, UK). The resulting sequence confirmed our selection of primers and probes as appropriate, with complementary probe and primer binding sites.

2.6.1 | Quantitative PCR assays

We used synthetic oligonucleotide standards for each crayfish species (Table S1). A sequential dilution series produced a set of standards ranging from 1,000,000 copies to 10 copies per μL.

For *P. leniusculus*, each 5 μL qPCR reaction comprised the following: 2.5 μL of GoTaq mastermix (Promega, Madison, USA), 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. Thermocycling was as follows: an initial denaturation at 95°C for 3 min, followed by 42 cycles of denaturation at 95°C for 5 s and annealing-extension for 30 s at 60°C. We used the primers (CO1-PI-02-F; CO1-PI-02-R) and associated probe (Table S1) reported by Mauvisseau et al. (2018).

For *A. pallipes*, each 5 μL qPCR reaction comprised the following: 2.5 μL of GoTaq master mix (Promega, Madison, WI), 0.25 μL of primer-probe mix (400 nM primer and 200 nM probe concentrations), 0.75 μL of distilled water, 0.5 μL of Bovine Serum Albumin (New England BioLabs, Ipswich, USA) to enhance qPCR yield given the lower sensitivity of the assay, and 1 μL of DNA template. Thermocycling was as follows: an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing for 30 s at 52°C, and extension for 30 s at 72°C. We used the primers (WC2302F; WC2302R) and associated probe (Table S1) reported by Troth et al. (2020).

The limits of detection and quantification for each assay were calculated using the amplification results of the serial dilution template. Each concentration was amplified in triplicate (18 qPCRs in total). The modeled limit of detection (LOD.rep1) was defined as the lowest concentration at which 95% detection was achieved using a single qPCR of a sample, while the metric (LOD.rep3) was defined as the lowest concentration at which 95% detection was achieved using three qPCRs of a sample (Klymus et al., 2020). The limit of quantification (LOQ) was defined as the modeled lowest standard concentration with an amplification coefficient of variation (CV) value below 35% (Klymus et al., 2020). Both LOD and LOQ statistics were calculated using the R code from Merkes et al. (2019).

Field samples were run in plates of 48 wells. Each plate included three positive controls (*P. leniusculus* standards at 100 copies/μL or *A. pallipes* standards at 1000 copies/μL), 12 field samples (each in triplicate), a field control (in triplicate), and a laboratory control (in triplicate), and three no-template controls which contained 1 μL of sterile distilled water instead of template.

All reactions were run on an Eco48 thermal cycler machine (PCRMax, Stafford, UK). Spurious amplifications (e.g., very early or of atypical shape) were removed after visual inspection of the amplification curves and Cq values were generated in EcoStudy v5.2.11.0 (PCRmax) using default settings. DNA copy number was calculated by inputting Cq values generated by the standard curves and samples into an NEBio online calculator (https://nebiocalculator.neb.com/#!/qPCRGen).

2.7 | Modeling

We determined the probability of species presence at each sampling site using the occupancy modeling approach and associated Rshiny app of Diana et al. (2021). The method accounts for both true positive and false positive observations at two stages of analysis; stage 1 when determining whether the DNA of the target species is present at the site, and stage 2 when determining whether a qPCR on a sample yields a successful amplification. We included confirmed presence information from the manual net searches. We also included five environmental variables (pH, conductivity, temperature, flow and depth) as predictor co-variables for species presence. Each analysis included 2000 burn-in iterations, 2000 iterations, 4 chains, and 20 thinned iterations. All other parameters were retained as the default.

We used a general linear mixed model in lme4 v.1.1.34 (Bates et al., 2015) to test for an association between crayfish detection by pond net searching and eDNA copy number at each site. Here, crayfish eDNA copy number was used as the response variable in each model, with species identity and crayfish detection by pond net search (1,0) as the predictor variables, and site as a random factor. The model was summarized using the tab_model function in sjPlot v2.8.14 (Lüdecke, 2023).

3 | RESULTS

3.1 | Limit of detection and quantification

The *P. leniusculus* qPCR assay had the efficiency of 93.4% and R^2 of 99.1. The assay amplified all standards between 1,000,000 and 100 copies/μL (of template in the qPCR reaction), while only two of the three qPCR replicates were amplified at 10 copies/μL. The limit of detection for a single qPCR of a sample (*LOD.rep1*) was 27.2 copies/μL, the limit of detection for three qPCRs of a sample (*LOD.rep3*) was 9.1 copies/μL. The limit of quantification (LOQ) was estimated as 29 copies/μL.

The *A. pallipes* qPCR assay had the efficiency of 85.7% and R^2 of 99.8. The assay consistently amplified all standards between 1,000,000 and 1000 copies/μL (of template in the qPCR reaction), while only two of the three qPCR replicates were amplified at 100 copies/μL, and there were no amplifications at 10 copies/μL. The limit of detection for a single qPCR of a sample (*LOD.rep1*) was 223.2 copies/μL, the limit of detection for three qPCRs of a sample (*LOD.rep3*) was 94.7 copies/μL. The limit of quantification (LOQ) was estimated as 223.2 copies/μL.

3.2 | Net-searching for crayfish

Pacifastacus leniusculus were detected by net searching at five out of 50 of the sites surveyed (10%), whereas *A. pallipes* were only detected by net searches at two out of the 50 sites surveyed (4%) (Table S2).

Austropotamobius pallipes was detected by net searching in the Tas and Glaven catchments. *Pacifastacus leniusculus* was detected by net searching in the Tud, Wensum and Bure catchments. No crayfish were detected by net searching in the Tas, Cong, Stiffkey, Beaston Beck or Wissey catchments.

3.3 | Environmental DNA sampling

Pacifastacus leniusculus eDNA was amplified from 12 of the 50 sites surveyed (24%; Table S2), with quantities of between 2.2 and 743.3 copies/μL (median 202.4 copies/μL; mean 311.5 copies/μL; standard error ± 73.0). The negative field controls, DNA extraction controls and qPCR controls did not amplify. Occupancy modeling of these *P. leniusculus* data showed a very high probability of true positives (>0.98) and a very low probability of false positives (<0.02) at both the sample site and qPCR replicate levels (Table 2). Overall, the modeling of species presence showed a high confidence (>80%) of *P. leniusculus* presence at eight sampling sites, in the Tud (3/5 sites), Wensum (1/5 sites), and Bure (4/5 sites) (Figure 2). No environmental variables were shown to be strong predictors of *P. leniusculus* presence, with all 95% posterior credible intervals of covariate coefficients (ψ values) encompassing zero (Table 2).

Austropotamobius pallipes eDNA was detected at three of the 50 sites surveyed (6%; Table S2) with quantities of between 50.3 and 500.6 copies/μL (median 153.24 copies/μL; mean of 254.74 copies/μL; standard error ± 127.2). The negative field controls, DNA extraction controls and qPCR controls did not amplify. Occupancy modeling of these *A. pallipes* data showed a moderately high probability of true positives (~0.6) and very low probability of false positives (<0.01) at both the sample site, and qPCR replicate levels (Table 2). Overall, the modeling of species presence showed a high confidence (>80%) of *A. pallipes* presence at three sampling sites, in the Tas (1/5 sites) and Glaven (2/5 sites). We were unable to detect any crayfish eDNA in catchments where *A. pallipes* were suspected to be present (Ingol, Tas, Cong, Stiffkey, Beeston Beck), which included the three recently supplemented *A. pallipes* ark sites (Ingol, Cong, Stiffkey). No environmental variables were shown to be strong predictors of *A. pallipes* species presence, with all 95% posterior credible intervals of covariate coefficients (ψ values) encompassing zero (Table 2).

TABLE 2 Results of occupancy modeling for the two focal species, including the probability of true positive and false positive observations within samples and within qPCR replicates, and estimated contributions of environmental variables to species presence.

Parameter	*Pacifastacus leniusculus* mean (± 95% credible interval)	*Austropotamobius pallipes* mean (± 95% credible interval)
Overall presence probability across sites (ψ)	0.155 (0.071 to 0.268)	0.075 (0.018 to 0.178)
Site true-positive probability (θ_{11})	0.985 (0.922 to 1.000)	0.608 (0.178 to 0.997)
Site false-positive probability (θ_{10})	0.004 (0 to 0.021)	0.006 (0 to 0.028)
qPCR replicate true-positive probability (p_{11})	0.993 (0.966 to 1.000)	0.591 (0.262 to 0.928)
qPCR replicate false-positive probability (p_{10})	0.016 (0.005 to 0.031)	0.003 (0 to 0.012)
pH (ψ)	−0.206 (−0.848 to 0.428)	0.076 (−0.748 to 0.916)
Conductivity (ψ)	0.133 (−0.515 to 0.788)	0.078 (−0.668 to 0.846)
Water temperature (ψ)	0.291 (−0.390 to 0.976)	0.152 (−0.606 to 0.900)
River flow (ψ)	0.353 (−0.245 to 0.945)	0.307 (−0.438 to 1.019)
River depth (ψ)	−0.455 (−1.178 to 0.227)	0.246 (−0.515 to 0.985)

Overall, sites where crayfish were discovered during the manual pond net searching had significantly more eDNA copies of that species (mean 459.62 copies μL^{-1}) than sites where a species was absent (mean = 10.49 copies μL^{-1}) (presence-absence fixed effect estimate = 443.02, 95% CI 381.12–504.91, $t = 14.210$, $p < 0.001$), with no detectable difference in the pattern between the species (species fixed effect estimate = 26.72, 95% CI −4.72 to 58.17, $t = 1.687$, $p = 0.095$).

4 | DISCUSSION

Our results indicate that eDNA-based surveys can be effective tools for detecting and mapping invasive and endangered crayfish species, supporting other studies with similar results (Baudry et al., 2021; Cowart et al., 2018; Greenhalgh et al., 2022; Harper et al., 2018; Robinson et al., 2018; Tréguier et al., 2014; Troth et al., 2020, 2021). In this case, combining manual searches and eDNA-based information using an occupancy model enabled us to confidently determine the presence of *P. leniusculus* at eight sites, and *A. pallipes* at three sites, which was more than visual searches alone (five and two study sites, respectively). This model indicated the presence of *P. leniusculus* in three of the 10 river catchments sampled. In contrast, we were only able to confidently determine the presence of *A. pallipes* eDNA in two river catchments, the Glaven and Tas (Figure 2). Interestingly, but not surprisingly, the sites where crayfish were manually collected had more eDNA copies detected than the sites where the species was not detected in the manual net searches. This result supports previous observations that abundance and assay sensitivity play key roles in the success (and failure) of eDNA surveys (Burian et al., 2021).

4.1 | Proactive strategies: assessing the status of *A. pallipes* ark sites and reintroductions

Captive *A. pallipes* breeding programs in the UK collectively function as a proactive conservation strategy at the local level by anticipating the need to reintroduce individuals at carefully selected ark sites to bolster native populations ahead of the arrival of invasive species. Here we surveyed three catchments where *A. pallipes* were reintroduced by the Norfolk Rivers Trust in 2018, the Ingol, Cong, and Stiffkey (U. Juta, pers. obs., 21st July 2022). However, our surveys suggest the reintroduced crayfish are now absent or below detectable abundances, as we were unable to find crayfish during manual searches or via the use of our eDNA assay. Improvements to the sensitivity of the *A. pallipes* eDNA assay may yield detections at these sites. Notably, Cowart et al. (2018) did successfully detect native crayfish (*Pacifastacus fortis*) in locations equivalent to ark sites in California.

Despite many efforts to reintroduce *A. pallipes* across Europe, surprisingly few studies have reported the details of restocking failures or successes (Manenti et al., 2021; Troth et al., 2021). This is despite a general understanding of the predictors of successful *A. pallipes* reintroductions required to maximize reintroduction success. Specifically, previous management plans for the reintroduction of *A. pallipes* in Spain (Diéguez-Uribeondo et al., 1997) and the UK (Rogers & Watson, 2007) have cited high water quality and the absence of crayfish plague or other invasive crayfish species as key factors in successful *A. pallipes* reintroduction (Manenti et al., 2021). It is possible that pollution from agricultural sources and the dumping of untreated wastewater from storm overflow drains adjacent to the reintroduction sites is responsible for destabilizing nascent *A. pallipes* populations, preventing them from establishing.

4.2 | Reactive strategies: identifying expanding *P. leniusculus* populations and areas to focus conservation resources

Once established, *P. leniusculus* can achieve high densities, for example, 310 g/m^2 in Lake Tahoe, USA (Flint & Goldman, 1977). As a result, it can be challenging and expensive for local conservation organizations and government agencies to employ reactive strategies to control established populations (Holdich et al., 2014). The construction of barriers can help prevent the spread of invasive crayfish, but they also reduce ecological connectivity. However, it might be possible to construct barriers that also permit the movement of fish (Krieg et al., 2021).

In this study, occupancy modeling combining eDNA detections with physical net search detections, suggests the presence of *A. pallipes* in the Glaven and Tas and *P. leniusculus* in the Wensum, Bure, and Tud catchments (Figure 2). *Austropotamobius pallipes* was detected (via eDNA) at two sites in the Glaven catchment, and a small 'signal' of *P. leniusculus* eDNA was also noted (Table S2). While the positive amplification of a *P. leniusculus* in a sample from the lower reaches of the Glaven may represent a false positive, it may indicate that *P. leniusculus* is present in the lower reaches of the catchment and may be advancing upstream. Notably, we found the largest concentration of *A. pallipes* eDNA of the whole survey directly upstream of this potential *P. leniusculus* population and found multiple adult *A. pallipes* individuals after a net search. This suggests that if the *P. leniusculus* population in the Glaven exists, it does so at a very low abundance, but it has the potential to threaten an established *A. pallipes* population further upstream. If the presence of *P. leniusculus* in the Glaven can be confirmed, then the information can be used for targeted reactive conservation strategies. These may include actions to control *P. leniusculus* while it exists at a low abundance.

A positive amplification of *Austropotamobius pallipes* eDNA was present from a sample in the upper reaches of the River Wissey catchment, while a positive amplification of *P. leniusculus* eDNA was present from a sample in the lower reaches of the catchment (Table S2), but in both cases, the probability of presence was resolved as very low (<0.03) by occupancy modeling. Both of these

results are therefore plausibly false positives, but if shown to be genuine positives through further research then the results would indicate that *P. leniusculus* has invaded the lower reaches of the catchment and is likely to advance upstream. In this case then implementation of a fish-passable crayfish barrier would potentially be an effective proactive conservation action to slow the spread of *P. leniusculus* upstream (Krieg et al., 2021).

Expanding *P. leniusculus* populations are increasingly coming into conflict with *A. pallipes* populations in Norfolk and across the UK. Although there is some evidence of *P. leniusculus* and *A. pallipes* coexisting in large lakes (Filipova et al., 2013), it is evident that the introduction of *P. leniusculus* has many negative effects for native crayfish and riverine biota (Dunn et al., 2009; Galib et al., 2020). For example, *A. pallipes* tend to be smaller in mixed populations and are evicted from their burrows, resulting in increased rates of predation and susceptibility to disease (Dunn et al., 2009).

4.3 | The influence of environmental variables on crayfish detectability

Multiple environmental variables in riverine systems are responsible for the degradation of eDNA molecules, such as UV light intensity, water temperature, and pH (Sansom & Sassoubre, 2017; Strickler et al., 2015). In this study, we found no association between eDNA detectability of either crayfish species and environmental variables (Table 2). Closer associations between species presence and measured environmental variables may be apparent at other times of the year. For example, although detection is possible year-round, breeding behaviors appear to increase the eDNA detectability of crayfish (Chucholl et al., 2021; Dunn et al., 2017; Troth et al., 2021), while hibernation (Chucholl et al., 2021; Flint, 1977) reduces its scope/detection. The breeding season of both our focal species takes place around October, around two months later than the August sampling period of our study (Grandjean et al., 2000; Stebbing et al., 2003). Additionally, we did not measure multiple habitat features that provide spatial microhabitat heterogeneity, including the density of submerged root structures, presence or channel vegetation, or the complexity of riverbank structure. Such variation may take a significant role in determining crayfish presence as resolved through both eDNA assays (Troth et al., 2021) and manual surveying (Holdich et al., 2014).

4.4 | Limitations

Improving the reliability of eDNA-based studies and data interpretation is currently an important focus of research activity (Burian et al., 2021). For example, studies have shown that sampling greater volumes of water, especially in large lakes, leads to 'more' detections (Schabacker et al., 2020). Here we favored spatial coverage over sampling larger volumes (>250 mL) with the goal of gaining insights into the distribution of endangered and invasive crayfish at sites in mostly small, shallow (median depth of 162 cm) riverine environments across Norfolk in a restricted time period. Increasing the pore size of the filters used to sample the river water might have allowed for the filtration of a larger volume of water and increasing detection probability. It is also worth noting that only one *P. leniusculus* individual from the study location was sampled in order to validate primer selection, which is not as effective as in silico testing against COI haplotypes known from European or UK individuals (Petrusek et al., 2017). In addition, the *A. pallipes* assay used here was not as sensitive as the *P. leniusculus* assay. Although *A. pallipes* individuals were found by net searches in the Tas catchment at study site TA4, we did not detect any *A. pallipes* eDNA there, resulting in at least one false negative result for the eDNA due to low assay sensitivity. The use of droplet digital PCR (ddPCR) is one potential method of improving sensitivity of the *A. pallipes* assay, as demonstrated for the endangered stone fly (*Isogenus nubecula*) in the River Dee (UK), where it is found at low abundance (Mauvisseau, Davy-Bowker, et al., 2019). It is also possible that some eDNA positive detections reported here are false positives derived from the downstream transportation of target species eDNA from populations upstream (Burian et al., 2021; Rice et al., 2018).

4.5 | Implications for the use of eDNA-based surveys to inform crayfish conservation strategies

We have shown that eDNA-based sampling is a sensitive method for detecting crayfish and an effective tool for informing crayfish conservation strategies, in this case, for the management and control of endangered and invasive crayfish species. In particular, eDNA-based monitoring can be used to quickly and accurately survey large spatial areas to identify specific vulnerable river reaches that could benefit from further monitoring and targeted conservation interventions (Rice et al., 2018). Based on the data presented here, we recommend that reactive crayfish conservation interventions, such as the control of *P. leniusculus*, should be concentrated in areas identified by eDNA sampling to contain newly established invaders. This will maximize the likelihood of success and the effectiveness of resources deployed. The construction of fish passable barriers could contain, or slow down the spread of newly established populations (Krieg et al., 2021).

Proactive crayfish conservation interventions, such as efforts to restock native populations with captive-bred individuals, must be undertaken before the opportunity to do so has passed. Suitable *A. pallipes* ark sites require the absence of invasive crayfish from the catchment selected for reintroductions, and also from the neighboring catchments. As a result, the number of suitable *A. pallipes* ark sites are likely to decrease as *P. leniusculus* populations spread. In contrast, reactive conservation strategies to control *P. leniusculus* populations should, in this case, focus on the Glaven catchment, where *P. leniusculus* threatens one of the few remaining *A. pallipes* populations in Norfolk. The Glaven catchment is also located next to the Stiffkey catchment, which has potential as a suitable *A. pallipes* ark site.

5 | CONCLUSIONS

We showed that eDNA-based surveys can inform both the success of proactive and reactive crayfish conservation strategies by quantifying the success of restocking *A. pallipes* ark sites, and identifying reaches of rivers vulnerable to the rapid expansion of an invasive crayfish species. *Pacifastacus leniusculus* is rapidly spreading across regions of the UK, threatening endangered *A. pallipes* populations. We recommend that conservation practitioners consider the following while using eDNA-based surveys in the management and conservation of crayfish: (1) design eDNA-based surveys with an emphasis on covering large spatial scales to quickly estimate the extent of native and invasive crayfish populations in a region of interest; (2) work with local conservation organizations and/or government bodies to inform the selection of study sites to produce results that are meaningful to real-world conservation actions; and (3) use the results obtained from eDNA surveys to target limited conservation resources to implement appropriate proactive and/or reactive conservation actions as determined by the results of the survey.

AUTHOR CONTRIBUTIONS

JG conceived the idea, and led the methodological design, data collection and analysis, and wrote the manuscript. UJ assisted in the selection of study sites, providing access to study sites, and data collection. BS created the site map, RB, SR, JT, KW, and GW assisted in providing access to study sites and data collection. RC, AS, and MG assisted in the laboratory work. MS provided the primer and probes used in the qPCR analysis. GJ, MG, and MS provided access to laboratory facilities, and obtained funding.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data and code associated with this study are publicly available on Zenodo: https://doi.org/10.5281/zenodo.8345029.

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SUPPORTING INFORMATION

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