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Development and application of eDNA-based tools for the conservation of white-clawed crayfish

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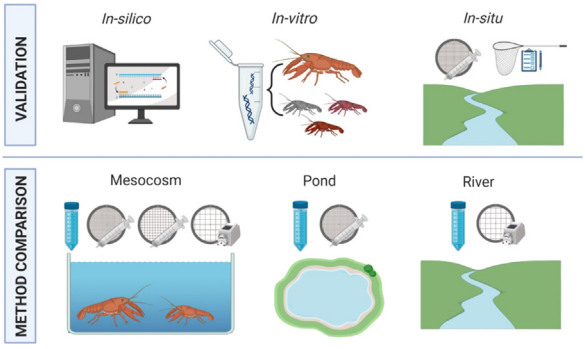
HIGHLIGHTS

- Development of an eDNA assay for the endangered European white-clawed crayfish
- Validation of this assay encompassing numerous *in-situ* and *ex-situ* experiments
- Optimal sampling method (filtration *vs.* precipitation) depended on target habitat.
- Demonstrate the application of eDNA for examining species interactions
- Highlights the importance of full methodological validation between environments

GRAPHICAL ABSTRACT



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ABSTRACT

eDNA-based methods represent non-invasive and cost-effective approaches for species monitoring and their application as a conservation tool has rapidly increased within the last decade. Currently, they are primarily used to determine the presence/absence of invasive, endangered or commercially important species, but they also hold potential to contribute to an improved understanding of the ecological interactions that drive species distributions. However, this next step of eDNA-based applications requires a thorough method development. We developed an eDNA assay for the white-clawed crayfish (*Austropotamobius pallipes*), a flagship species of conservation in the UK and Western Europe. Multiple subsequent *in-situ* and *ex-situ* validation tests aimed at improving method performance allowed us to apply eDNA-based surveys to evaluate interactions between white-clawed crayfish, crayfish plague and invasive signal crayfish. The assay performed well in terms of specificity (no detection of non-target DNA) and sensitivity, which was higher compared to traditional methods (in this case torching). The eDNA-based quantification of species biomass was, however, less reliable. Comparison of eDNA sampling methods (precipitation *vs.* various filtration approaches) revealed that optimal sampling method differed across environments and might depend on inhibitor concentrations. Finally, we applied our methodology together with established assays for crayfish plague and the invasive signal crayfish, demonstrating their significant interactions in a UK river system. Our analysis highlights the importance of thorough methodological

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development of eDNA-based assays. Only a critical evaluation of methodological strengths and weaknesses will allow us to capitalise on the full potential of eDNA-based methods and use them as decision support tools in environmental monitoring and conservation practice.

1. Introduction

Since its initial conception as a method for aquatic ecological surveys (Ficetola et al., 2008), the use of environmental DNA (eDNA) based methods are rapidly increasing in their application (Biggs et al., 2015; Harper et al., 2019; Jerde et al., 2013; Spear et al., 2015). Advantages such as higher cost effectiveness compared to established traditional species survey methods and non-invasive sampling are frequently emphasised (Goldberg et al., 2016; Huver et al., 2015; Rees et al., 2014; Takahara et al., 2012). Nevertheless, the true potential of eDNA-based methods is just starting to be realized. Currently, eDNA-based tools are mostly used for simple presence/absence surveys, whilst they could also be used to study ecological interactions that determine species distribution and the conservation status of target species. However, such advanced applications, require careful method evaluation and an improvement of sampling procedures to increase the robustness of species detection.

In the case of species-specific eDNA assays, the design and validation of the assay represents a critical first step (Geerts et al., 2018; Rees et al., 2014). During assay design, it is fundamental to ensure a high target-specificity (Bylemans et al., 2018) by selecting suitable amplicon lengths and performing extensive *in-silico* simulations to test against amplification of non-target DNA. *In-vitro* laboratory validation should then ascertain that effective limits of detection (LOD) and quantification (LOQ) are established (Klymus et al., 2019). Further, field comparisons with established traditional survey methods are recommended to complement reliability assessments (Smart et al., 2015). However, both traditional survey approaches and eDNA-based methods are affected by various error sources potentially creating inconsistencies that require careful interpretation (Hinlo et al., 2017a).

The reliability of eDNA-based tools is also strongly influenced by sampling methodology (Hinlo et al., 2017b). Currently, DNA precipitation and various filtration methods are applied to concentrate eDNA during field sampling. Filtration approaches have the advantage of collating eDNA from larger volumes of water compared to precipitation-based methods (Mächler et al., 2016). However, they can incorporate the risk of losing particles below the filter pore size (Minamoto et al., 2016). They may also lead to higher concentrations of inhibitors occurring, which would prevent the amplification of targeted eDNA (Mauvisseau et al., 2019a). Previous method comparisons have come to contrasting recommendations, a result which largely appears to be driven by the target species in question (Deiner et al., 2015; Dickie et al., 2018). Additionally, even for the same species the 'optimal' method for collecting eDNA may vary between lentic (*i.e.* ponds or lakes) and lotic (*i.e.* rivers and canals) systems (Geerts et al., 2018; Harper et al., 2019). Careful method comparisons are therefore urgently needed and recommended (Deiner et al., 2015).

In this study, we target the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), an endangered and important umbrella species in the United Kingdom and Western Europe (Füreder et al., 2010). Range reduction of white-clawed crayfish began in the 1860s, with declines rapidly accelerating in the UK after the introduction of invasive non-native signal crayfish *Pacifastacus leniusculus* (Dana 1852), which originated from North America in the 1970s (Holdich et al., 2009). Moreover, the spread of crayfish plague *Aphanomyces astaci* (Schikora 1906), an oomycete pathogen carried by the signal crayfish, has greatly exacerbated the negative impact of invasive competitors, pollution and habitat degradation (Holdich et al., 2009). Despite its legislative protection (EU Habitats Directive), white-clawed crayfish populations have continued to decline by as much as 50–80% between 2000 and 2010 (Füreder et al., 2010). Due (at least in part) to the rarity of the native species, traditional survey methods are having unsatisfactory success in monitoring populations (Gladman et al., 2010; Holdich and Reeve, 1991). This highlights the urgent need for the development of novel, more sensitive survey tools/methods to assess current states and trends of and in the remaining populations.

Consequently, the aim of this study was to develop a highly reliable, cost effective eDNA assay for the detection of white-clawed crayfish, one which allows for the assessment of interactions with competing species and pathogens, threatening the species survival. We designed a primer and probe set for the amplification of white-clawed crayfish mitochondrial DNA and critically evaluated the sensitivity and specificity of the assay through extensive *in-silico*, *in-vitro* and *in-situ* tests. Moreover, we evaluated the impact of different sampling methodologies on the reliability of the assay in mesocosm experiments and field tests implemented in different habitat types. This allowed us to assess in a UK river system the relationship between white-clawed crayfish, signal crayfish and crayfish plague, demonstrating the applicability of eDNA-based approaches for in-depth ecological investigations and ecosystem management.

2. Materials and methods

2.1. Primer design and in-silico tests

Crayfish are a priority conservation target and a number of eDNA-based assays have already been developed for species monitoring (Agersnap et al., 2017; Larson et al., 2017; Mauvisseau et al., 2018). However, for white-clawed crayfish, earlier studies either lack a thorough field evaluation or are based on high resolution melt-curve (HRM) approaches (Robinson et al., 2018). Whilst HRM is a promising technique, one which enables the simultaneous detection of multiple species (Yang et al., 2009), it does not yet reach the high robustness and specificity required for the monitoring of threatened species. Consequently, we developed in this study, a further qPCR assay for the detection of the white-clawed crayfish, which mirrors those available for the invasive signal crayfish and crayfish plague.

An overview of the method evaluation performed in this study is presented in the schematic (Fig. 1). This consisted of initial primer/probe design and validation, which followed the guidelines established by MacDonald and Sarre (2017). Here, primers and a probe, targeting the Cytochrome C Oxidase Subunit 1 (COI) mitochondrial gene of white-clawed crayfish, were designed *in-silico* using Geneious Pro R10 (Kearse et al., 2012). The forward primer WC2302F 5′-GCTGGGATAGT AGGGACTTCTTT-3′, reverse primer WC2302R 5′–CATGGGCGGTAACC ACTAC-3′ and probe WC2302P 5′-6-FAM-CTGCCCGGCTGCCCTAATTC-BHQ-1-3′ amplified a 109 bp fragment. To ensure specificity, the primers and probe were tested *in-silico* using the Basic Local Alignment Search Tool (BLASTn) and Primer-BLAST from the National Centre for Biotechnology Information (NCBI).

2.2. In-vitro validation

The specificity of the assay was further tested *in-vitro* against extracted DNA of both white-clawed crayfish and six taxonomically similar, or co-occurring crayfish species from Europe (see Supplementary information for details on samples and PCR primer validation). For qPCR, the reactions for both tissue extracts (above) and all eDNA

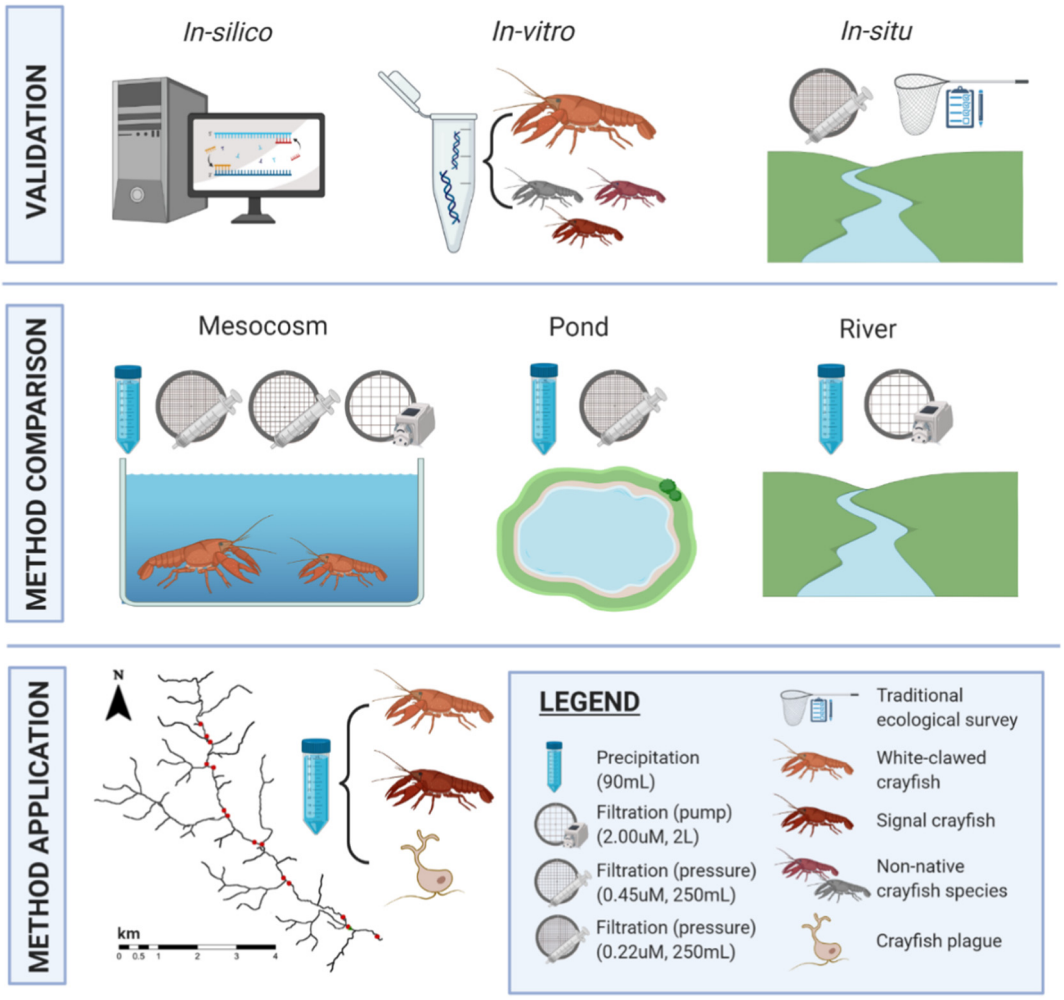

Fig. 1. Different steps of method development and application performed in this study. Method validation encompassed three key steps: *In-silico* (computer simulations of assay sensitivity), *in-vitro* (performance tests using target and non-target DNA) and *in-situ* assessments (quantitative comparison between eDNA and established field methods). Additionally, we examined possibilities to enhance assay performance in an extensive method comparison of different eDNA collating methods in controlled mesocosm experiments and field tests in lotic and lentic environments. The potential of the assay to evaluate interactions between different crayfish and crayfish plague was investigated in an extensive monitoring campaign of a target catchment. Water collation methods inherently differed in the water volumes collected, but for all method comparisons and applications, sampled water within each method was kept constant, facilitating site comparisons.

samples (see below) contained a standard mix of; 12.5 μL TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5 μL DH_20, 1 μL (10 μM) of each primer, 1 μL (2.5 μM) of probe and 3 μL of template DNA. qPCR's were performed with six replicates per sample (here after referred to as technical replicates). These were run on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). qPCR conditions were as follows: 50 °C for 5 min, denaturation at 95 °C for 8 min, followed by 50 cycles of 95 °C for 30 s and 55 °C for 1 min. Six no template controls (NTCs) were prepared using RT-PCR Grade Water (Ambion™) and run on each qPCR plate analysed. This was alongside a further 'control' consisting of duplicated standard curve serial dilutions of control white-clawed crayfish DNA (10^{-1}–10^{-3} ng μL^{-1}). In each subsequent experiment, negative PCR controls were included in this fashion.

2.3. Limits of detection (LOD) and quantification (LOQ)

The reliability of our assay was also assessed, following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines, which recommend the establishment of a calibration curve to determine LOD and LOQ (Klymus et al., 2019). We prepared a serial dilution of DNA extracted from white-clawed crayfish starting from 0.79 ng μL^{-1} to 7.9×10^{-8} ng μL^{-1} with 10 qPCR replicates per dilution analysed. The LOD was defined as the last standard dilution that resulted in a detection of target DNA with at least one qPCR replicate at a threshold cycle (Ct) of <45. The LOQ was defined as the last standard dilution in which targeted DNA was detected and quantified in a minimum of 90% of qPCR replicates of the calibration curve under a Ct of 45 (Mauvisseau et al., 2019c).

2.4. In-situ validation

The reliability of the assay was further field tested by comparing eDNA-based and traditional capture-mark-recapture sampling techniques at six sites of confirmed white-clawed crayfish presence (2017) in the Centre-Val de Loire region, France. Each site was visited at least twice in subsequent nights between 22nd June and 1st of August 2018 (see Supplementary information, Table S1). Individual white-clawed crayfish were surveyed using a torching approach, counted and marked using a white waterproof marker stain. In the second night the survey was repeated and marked, and non-marked crayfish were differentiated. Additionally, eDNA samples (two environmental replicates) were collected at each site using the 0.22 μM Sterivex filters (see below for detailed description). eDNA samples were collected

between the 22nd and 29th June 2018, at both time points, one eDNA sample was collected from the same site (totalling 2 replicates per site). Unfortunately, the water volume filtered vaired between sites, a result due to cases of high turbidity. That said, we ensured a consistent minimum volume of 150 mL, was always filtered (see Table S2 for list of site details, sample volumes and water temperature measurements). To facilitate the extended transport times between sample collection and storage on ice, eDNA filters were fixed with 2 mL of ethanol. It should be noted that all sampled locations for this part of the study are part of an extensive monitoring programme for white-clawed crayfish populations, therefore site locations are not reported for conservation reasons.

2.5. Comparison of eDNA sampling methodologies in mesocosms

Once we were sure of the assays practical application in the field, we then aimed to assess the impact of eDNA sampling methodology on the probability of eDNA detection and the signal strength (*i.e.* Ct) of its detection. Here, we tested differences between the most commonly utilised eDNA sampling methods (Fig. 1). These included (i) ethanol precipitation (Biggs et al., 2015), (ii) 2 μM pump-based filtration (Strand et al., 2014), (iii) 0.45 μM pressure filtration and (iv) 0.22 μM pressure filtration (Spens et al., 2017). All methods were compared in two mesocosms, housed at Bristol Zoological Gardens, Bristol, UK during autumn 2018. Both mesocosms were designed the same yet contained different water volumes and crayfish numbers (see Table S3 for detail on design and stocking densities). For this part of the study, six 'environmental' replicates per method were sampled (see below) from both mesocosms (totalling $n = 12$ samples per method assessed).

In brief, eDNA samples classified hereafter as 'precipitation' samples were collected following the protocol outlined in Biggs et al. (2014). 1 L of water (20 × 50 mL subsamples) was collected from ~15-20 cm below the surface of the water and at least 20 cm above the bottom, in order to avoid disruption and collection of any sediment. After homogenization, a subsample of 90 mL (6 × 15 mL) was aliquoted into sterile tubes containing a pre-mixed buffer solution (Biggs et al., 2014). Samples were stored at -20 °C prior to extraction. Extraction followed the procedure outlined in Tréguier et al. (2014). When batches of samples were analysed, two negative extraction controls were included and processed analogously with each batch of samples.

eDNA samples taken with a 2 μM pump-based filtration consisted of 2 L of water (40 × 50 mL, homogenized) and were filtered through a Millipore Glass fibre filter AP25, 47 mm (2 μM pore size) using a peristaltic pump (Masterflex E/S Portable Sampler, Cole-Parmer, USA). The filter was housed in an In-Line Filter Holder 47 mm (Merck) connected by silicone tubing. The combined use of a peristaltic pump and a larger filter pore size allowed us to substantially increase the amount of water filtered. The filter was then removed from the pump system and stored at −20 °C before extraction. Equipment was soaked and cleaned with 10% bleach between samples. eDNA sample collections for 0.22 μM and 0.45 μM pressure filtration were undertaken in the same manner. 20 sub-samples were collected and a 50 mL syringe (BD Plastipak™, Ireland) was then used to pressure filter 250 mL of water through a sterile enclosed filter (Sterivex™, Merck®, Germany) with either a pore size of 0.22 μM (polyethersulfone membrane) or 0.45 μM (polyvinylidene fluoride membrane). All filters were stored at -20 °C, and extracted following Spens et al. (2017). Sample volumes across methods were not standardised, this was done intentionally in order to facilitate effective comparisons of the 'typical' application of each methodology by end-users in a field setting.

2.6. In-situ comparison of eDNA sampling methodologies

Complementary to the tests in the mesocosm experiment, we also evaluated sampling methodologies under natural conditions. However, we performed only pairwise method comparisons between precipitation (the standard method used in commercial application of eDNA for *Triturus cristatus* in the UK) and one 'additional' method in order to contain sampling and processing effort. As a test in a lentic system, eDNA samples were collected from a 1000 m^2 pond in the South West of England after the release of 40 white-clawed crayfish individuals (equal juvenile-adult and male-female ratios, total biomass of 436 g; prior to release, absence of signal and white-clawed crayfish were confirmed by torching and eDNA-based surveys). Here, precipitation (sample volume: 90 mL) was compared against 0.22 μM pressure filtration (sample volume: 250 mL). Prior to sampling, the pond was confirmed absent of crayfish through trapping and eDNA-based surveys. Sampling started on the 20th April 2018 and was repeated 2 h, seven days, 14 days and 35 days after crayfish release. At each sampling time, three field replicate eDNA samples were taken from four different sites around the perimeter of the pond. This was undertaken for both methods. Additionally, 20 × 50 mL sub-samples (taken from the entire pond perimeter) were pooled, homogenized and sampled, again in triplicate and again for each method (this gave us $n = 60$ environmental samples of eDNA per method for across comparison analysis).

Our second field test was conducted in a lotic system. In this instance we sampled 10 sites (situated approx. 1 km apart) along a river in Dorset (UK), with a further four sites along a stretch of river in Derbyshire (UK). All sampling was conducted during September 2017. Here, we compared pump-based filtration (with a pore size of 2 μM and a sample volume of 2 L) against the ethanol precipitation method. Similarly, three environmental replicates were taken at each site, per method (giving us $n = 42$ environmental samples of eDNA per method for across comparison analysis). The pump-based filtration system, with its larger filter pore size was chosen in this case due to a higher risk of increased turbidity commonly encountered in many lotic sites. Samples collected in the river system ($n = 20$ pooled 50 mL sub-samples totalling 1 L) were taken at 1-2 m intervals along a diagonal downstream-to-upstream transect across the river. In this field test, we also assessed the ability to screen for crayfish plague using both sampling methods. qPCRs for plague detection were operated using the primers and probe developed by Strand et al. (2014). For all the field method comparisons, bottled distilled water was sampled on site and processed as field negative controls.

2.7. Field test of white-clawed crayfish, signal crayfish and crayfish plague co-existence

Finally, we assessed the distribution of white-clawed crayfish, signal crayfish and crayfish plague in a river-system in Derbyshire (UK). This was undertaken in order to assess the potential of eDNA-based methods to capture complex interactions among multiple species. Here, we collected two environmental replicates, at eight different sites along the river during November 2017 (giving us a total of $n = 16$ environmental samples of eDNA). Six of these sites were in proximity to the inflow of tributaries and the two site replicates were taken upstream and downstream of their confluence to capture the influence of populations potentially present within tributaries (Supplementary information, Fig. S1). The others consisted of the two replicates being taken side by side. Sampling was conducted using the precipitation method outlined above and water samples were tested for the occurrence of all three species. Protocols of Mauvisseau et al. (2018) for signal crayfish and of Strand et al. (2014) for crayfish plague were applied.

2.8. Statistical analysis

Samples measured for the establishment of a standard curve were analysed using a linear regression to evaluate the relationship between DNA dilution and Ct. A log-log data transformation decreased the models Akaike Information Criterion (AIC; reduction by 23 units compared to second best model) and was therefore used for downstream analyses. Residuals were tested for autocorrelation, normal distribution and any remaining patterns (same procedure applied in all regression analyses). A logistic regression analysis was also applied to test the

relationship between DNA concentration and binomial detection data assessing the change of detection probability with DNA concentrations. For *in-situ* method validation, the relationship between the population density established by traditional sampling methods and (i) the Ct values and (ii) detection probability (calculated as the fraction of technical replicates that resulted in positive detection) of eDNA measurements were examined in a linear regression model. Due to low population densities and relative low recapture rates associated with the traditional sampling methods, we used average individual counts of repeated field visits as predictors, instead of estimates of true population sizes. Differences in sample volumes between locations (due to turbidity) were accounted for by including sample volume as a predictor in regression models, and log-log and untransformed models were compared using AIC. Further, Ct and detection probability of different sampling methods were compared using ANOVA analyses followed by Tukey's HSD post-hoc tests, and *t*-tests or nested ANOVA's (lotic and lentic systems, where only two methods were compared). Although we acknowledge sample volume differed between sampling methods, it was kept constant within samples of each method within the methodology comparison and so we have not incorporated volume as a random effect in this instance. Prior to ANOVAs, heteroscedasticity was evaluated, and data transformed if necessary. Finally, the co-existence of white-clawed crayfish with signal crayfish and crayfish plague was tested in regression models using detection probability of all three species. Parsimonious models were evaluated using Akaike Information Criterion (AIC). All described statistical analyses were performed using R version 3.4.1 (R Core Team, 2017).

3. Results

3.1. Assay development and in-silico and in-vitro validation

The primers and probe were highly species-specific as *in-silico* and *in-vitro* tests did not reveal any matches with non-target species (Table S4). Analysis of the standard curve (Fig. 2A) revealed a strong dependency of Ct values on DNA concentrations ($y = -1.73x + 20.8$, $p < 0.001$, $r^2 = 0.996$). Likewise, the detection probability was also positively related to DNA concentration in the sample ($y = -0.18x + 1.39$, $p = 0.0016$, $r^2 = 0.804$; Fig. 2B). This highlights the possibility of a quantifiable assay being developed. Method sensitivity analyses revealed a LOD of 7.9×10^{-5} ng and a LOQ of 7.9×10^{-4} ng crayfish DNA extract per μL^{-1}.

3.2. In-situ validation

Populations of white-clawed crayfish were found in five out of the six surveyed sites using traditional survey methods. eDNA-based measurements indicated the presence of white-clawed crayfish in all six sites, although the site with no visual white-clawed crayfish sightings was characterised by a very low detection probability. The Ct values from the six river sites were converted into DNA concentrations using the calibration curve, which allowed us to compare the relationship between detection probability and DNA concentration in laboratory and field samples (Fig. 2B). Four out of the six field sites lay outside of the 95% confidence interval of the standard curve, indicating systematic differences between *in-vitro* validation and field samples. The relationship of the mean number of crayfish detected using conventional species survey methods (torching), and detection probability of eDNA measurements (Fig. 2D) was significant, but only when water temperature was included ($y = 0.0118x_1 - 0.117x_2 + 1.77$; x_1 = mean survey count, x_2 = temperature, $p = 0.035$, $r^2 = 0.82$, best model resulted in reduction of AIC by 10 units). The relationship between Ct and the mean number of crayfish detected using torching was marginally non-significant but showed a reasonable model fit (Fig. 2C; $y = -0.00067\log(x) + 3.76$, $p = 0.079$, $r^2 = 0.47$, best model resulted in reduction of AIC by 5 units). Differences in filtered sample volume did not significantly influence results.

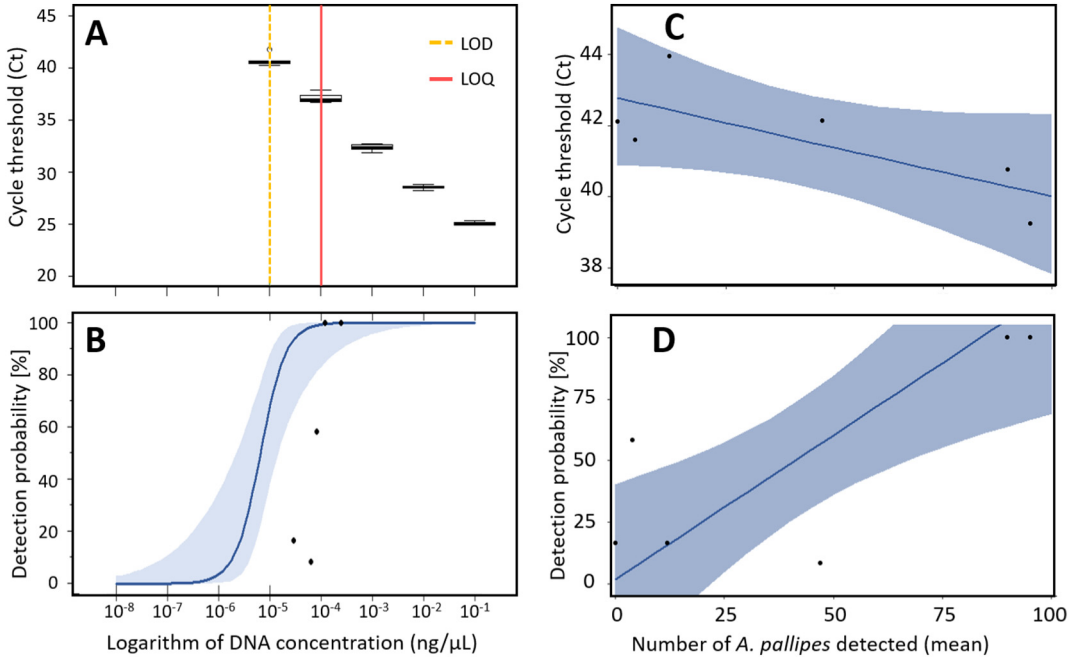

Fig. 2. (A) Relationship between cycle threshold (Ct) and DNA concentration from white-clawed crayfish qPCR calibration curve. Limit of detection (LOD) and limit of quantification (LOQ) are illustrated by vertical lines (dashed-yellow and red respectively). (B) Change in detection probability with increasing DNA concentrations in calibration curve data (blue line). Red triangles represent field data (*in-situ* validation data) that is compared with the log-regression based on *in-vitro* calibration curve data. Three out of five field data points were outside the established confidence interval, indicating discrepancies between field and laboratory-based data sets. (C) Relationship between Ct values and white-clawed crayfish population monitored using traditional method. (D) Relationship between detection probability of eDNA and traditionally evaluated crayfish population sizes. The blue line and the light-blue shaded area reflect the results of a logit regression and its 95% confidence interval, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Comparison of eDNA sampling methods

In mesocosm experiments, sampling methodology had a significant impact on detection probability (ANOVA, $F_{(3,44)} = 74.48$, $p < 0.001$). Pairwise comparisons revealed that detection probabilities of all three filtration-based methods (2 μM, 0.22 μM and 0.45 μM) were comparable ($p > 0.05$). Notably, the *p*-value for the comparison between 0.45 μM and 2 μM was marginally non-significant ($p = 0.051$). Further, all were significantly different (resulting in a higher detection probability) than that of the precipitation method ($p < 0.001$, Fig. 3A). Similarly, methodologies also differed significantly in Ct (ANOVA $F_{(3,178)} = 90.1$, $p < 0.001$). However, in contrast to detection probability, pairwise tests revealed a difference between the 2 μM filtration method and all the other approaches ($p < 0.001$; Fig. 3B; only samples with positive detection were included).

In-situ comparisons of sampling methods in a lentic system were highly comparable to the mesocosm experiment (Fig. 4A–B). The precipitation method showed a significantly lower detection probability (*t*-test, $t = 3.55$, $df = 75.37$, $p < 0.001$) and a significantly higher Ct ($t = -2.46$, $df = 15.72$, $p < 0.05$) when compared to the filtration-based method (0.22 μM). However, contrasting results were attained in lotic systems. In this system, precipitation resulted in a higher detection probability (nested ANOVA $F_{(1,69)} = 13.77$, $p < 0.001$, Fig. 4C) and accordingly, lower Ct values (nested ANOVA; $F_{(1,34)} = 5.24$, $p = 0.028$; Fig. 4D). In this instance, we also assessed for the presence of crayfish plague. For this assay, the detection probability mirrored findings from other systems showing significantly higher detection probabilities for the 2 μM filtration method (nested ANOVA; $F_{(1,69)} = 4.92$, $p < 0.05$; Fig. 4E). Ct values were not significantly different, but also indicated a better performance of the filtration-based method (Fig. 4F).

3.4. Field tests of species co-occurrence

Finally, our joint assessment of white-clawed crayfish, signal crayfish and crayfish plague (Fig. 5) demonstrated that white-clawed crayfish occurrence was strongly related to both other species (Fig. 5B, C). Whilst univariate regressions were marginally non-significant (dependency of white-clawed crayfish on signal crayfish: $p = 0.063$; dependency of white-clawed crayfish on crayfish plague: $p = 0.051$), a multiple regression analysis revealed significant relationships ($y = -23.8x_1 + 13.1x_2 - 3.8$, $r^2 = 0.73$, $p = 0.016$; y, x_1 and x_2 represent detection probabilities of white-clawed crayfish, signal crayfish and crayfish plague, respectively). White-clawed crayfish were negatively impacted by the presence of signal crayfish (Fig. 5B), yet contrary to expectation they were positively related with crayfish plague (Fig. 5C). Signal crayfish and plague occurrence were shown to not be correlated (Fig. 5D).

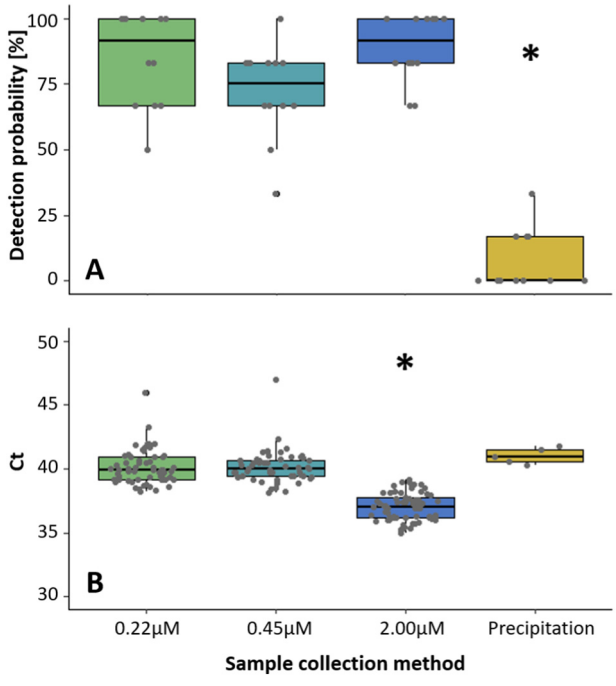

Fig. 3. Comparison of the detection probability (A) and Ct values (B) of white-clawed crayfish using different eDNA sampling methods (0.22 μM filtration (green), 0.45 μM filtration (light blue), 2 μM filtration (royal blue) and precipitation (yellow)) in a controlled mesocosm experiment (* indicates statistical significance). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Native crayfish species across Europe are threatened by invasive competitors and contraction of the crayfish plague. This has resulted in a downward trajectory of native species' abundance and distribution across much of their former range (Holdich et al., 2009). In this study, we present a novel assay for the detection of white-clawed crayfish, a flagship conservation species in Western Europe. In rigorous *in-vitro* and *in-situ* tests, we evaluated the reliability of our assay under various environmental conditions. Further, we applied our assay together with established eDNA-based methods to assess the drivers of white-clawed crayfish occurrence. Overall, we were able to demonstrate that our approach can not only be used for simple presence/absence surveys but also has the potential to reveal species co-occurrence and interactions. However, our results highlight that such applications are only meaningful after thorough method testing and validation.

Field comparisons indicated a higher sensitivity of the eDNA assay compared to conventional surveys, which incidentally only resulted in positive detection in five out of six sites surveyed. Often, traditional survey methods vary in their detection success (Gladman et al., 2010), in some cases leading to inaccurate presence/absence recording, complicating comparisons with eDNA-based approaches. Also, the two UK river systems, which we used in this study to compare different sampling approaches were since conventionally surveyed for the presence of crayfish (pers obvs, data not shown). In both systems, no white-clawed crayfish were found, despite historical presence records and our eDNA assay indicating a positive signal. This indicates that either our assay is more sensitive than traditional methods, supporting other eDNA based studies (Dejean et al., 2012; Jerde et al., 2011; Smart et al., 2015), or the occurrence of false positives in our sampling (Furlan et al., 2016). Such errors can occur *via* downstream transport of eDNA within river networks for example (Pont et al., 2018). Moreover, false positives may result from historic eDNA, which is still present after the extinction or emigration of the target species (Turner et al., 2015). In our case, this represents a valid hypothesis as all field sites were populated by white-clawed crayfish a year before our field surveys (C. Mauvisseau, personal observation). Although we took great care to avoid sediment disruption during sample collection (*e.g.* sampling of at least 20 cm above the riverbed), non-sampling related disturbance could have resulted in resuspension of ancient DNA. Therefore, it remains inconclusive whether the developed eDNA approach has a higher sensitivity (*i.e.* false negative of torching method) or, in fact, white-clawed crayfish were not present at the field sites in question. Further investigations into the impact of downstream flow and historic eDNA presence are required to obtain a more comprehensive picture in this regard. That said, a recent study has since shown that even whole crayfish carcasses resting on the bottom of a river (crayfish species *P. clarkii*) fail to produce detectable eDNA in a stream enclosure experiment (Curtis and Larson, 2020). Therefore, this result suggested that positive detections in field studies may be more confidently attributed to the presence of live organisms.

Further, an important component of our method validation was the comparison of different field sampling approaches. Precipitation and

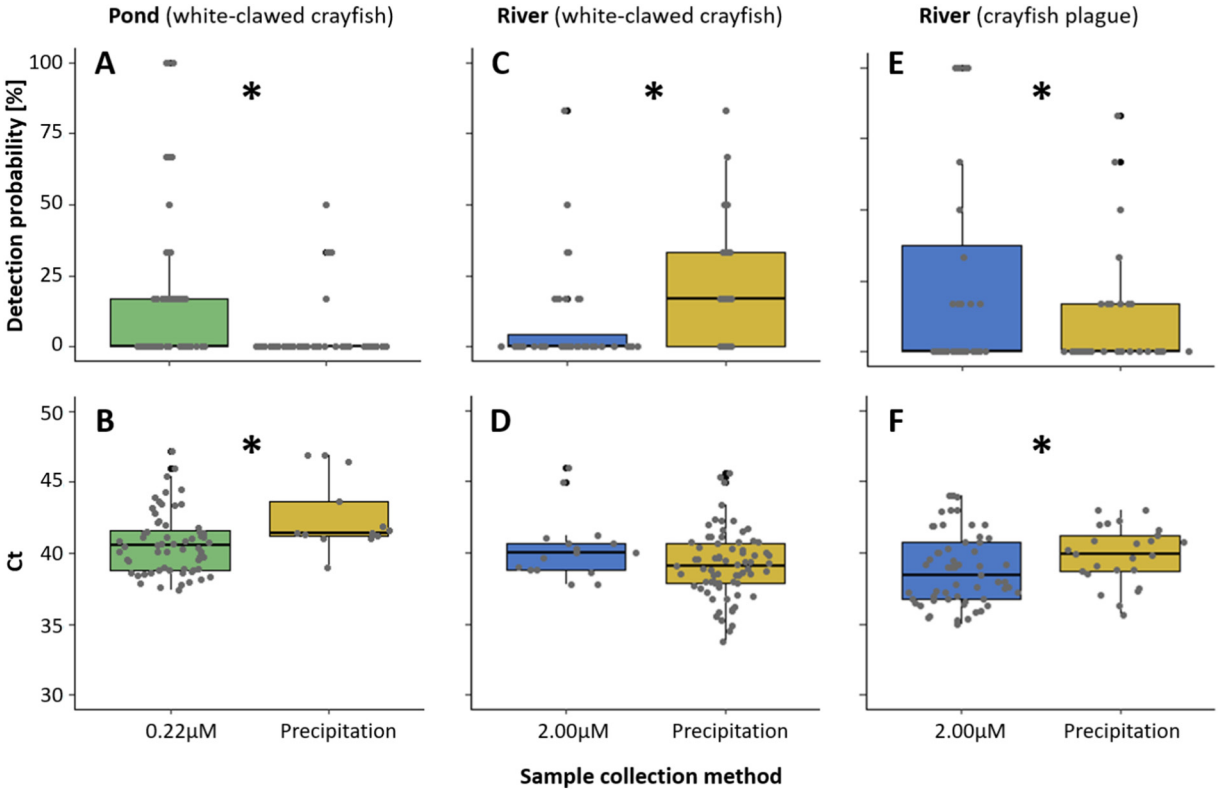

Fig. 4. Comparison of the detection probability (A, C, E) and Ct values (B, D, F) of different eDNA sampling methods (filtration and precipitation) for white-clawed crayfish in a lentic system (Pond, A–B) (filter pore size 0.22 µM) and for both white-clawed crayfish (River, C–D) and crayfish plague (River, E–F) in the same lotic system (filter pore size 2 µM) (* in panels signifies significant differences between pairwise method).

filtration protocols, aimed at concentrating eDNA from the environment have been compared in a number of studies (Deiner et al., 2015; Spens et al., 2017) and the majority of investigators endorse filtration approaches (Hinlo et al., 2017a, 2017b; Spens et al., 2017; Vörös et al., 2017). However, optimal filter pore size appears to differ among target species (Spens et al., 2017). Moreover, method choice can also be environment-dependant. For example, Eichmiller et al. (2016) indicated filtration as the optimal eDNA-based method for surveying the common carp, *Cyprinus carpio*. In contrast, Minamoto et al. (2016) highlighted precipitation performed better – a result likely induced by variations in the environment across both studies. Such contrasting results were also found in our study. Filtration approaches outperformed precipitation in the controlled mesocosm and lentic environments, yet precipitation was the superior method for white-clawed detection in lotic systems (Fig. 4).

One possible explanation for our divergent findings across habitats is that the nature of target eDNA particles differ in these environments. eDNA is exposed to continuous degradation through biotic (*e.g.* bacteria) and abiotic (*e.g.* UV) factors (Strickler et al., 2015). Degradation processes such as these can severely affect eDNA particle size distributions (Jo et al., 2019). Filtration has the advantage to collate eDNA from larger water volumes but could be linked to the risk of losing particles which are below filter pore sizes. Hence, the habitat-specific differences in our method comparisons may be explained by the specific degradation processes within the investigated river systems. A decrease of average eDNA particle size (below the filter pore size) would substantially decrease detection probability of filtration approaches therefore, at least in part explaining our findings.

An alternative explanation is linked to inhibition of eDNA amplification in our samples. Inhibitor compounds that interfere with qPCR processes, have been shown to affect target DNA amplification in a non-linear way (Goldberg et al., 2016). If inhibitor concentration is low, amplification will not be strongly impacted. However, if concentrations surpass a certain threshold, inhibitors may suppress the amplification of even high concentrations of target eDNA (Mauvisseau et al., 2019a). Sampling methods that differ in their water collection volumes, and in the amount of concentrated target eDNA, will also concentrate inhibitors to different degrees (Fig. 6). Consequently, sampling methods that reach higher target eDNA concentrations may show a lower overall performance due to the non-linear relationship between inhibitor concentrations and DNA amplification. This scenario will occur when inhibitors are present in high concentrations and are efficiently concentrated. Therefore, different ratios (between target eDNA and inhibitors in different environments) can cause a shift in the relative performance of sampling methods across habitats (Fig. 6). In our case, we did not include tests for inhibition, which include the addition of synthetic DNA to qPCR reactions (*i.e.* failure to detect synthetic DNA indicates inhibition; (Goldberg et al., 2016; Mauvisseau et al., 2019a) and this should certainly be factored in for any future assay development studies. If synthetic markers are not desirable, there is also the option to use a downstream inhibitor removal kit (Niemiller et al., 2018) which should effectively clean up any eDNA sample for later processing.

Both inhibition and different target-eDNA size distributions might also explain differences in method comparisons between species in the same environment as observed for white-clawed crayfish and crayfish plague in lotic habitats (Fig. 4). A fundamental distinction between the two species is that crayfish plague depends for its proliferation on the frequent and abundant release of encapsulated spores (~8 µM in diameter). It seems likely that these spores, which are designed for transport along large distances, will show lower sensitivity to degradation than white-clawed crayfish DNA, which potentially could explain our species-specific results.

Finally, we demonstrated that our approach can also be used for wider scoping investigations into the ecological relationships determining the distribution of endangered species. Our simultaneous assessments of

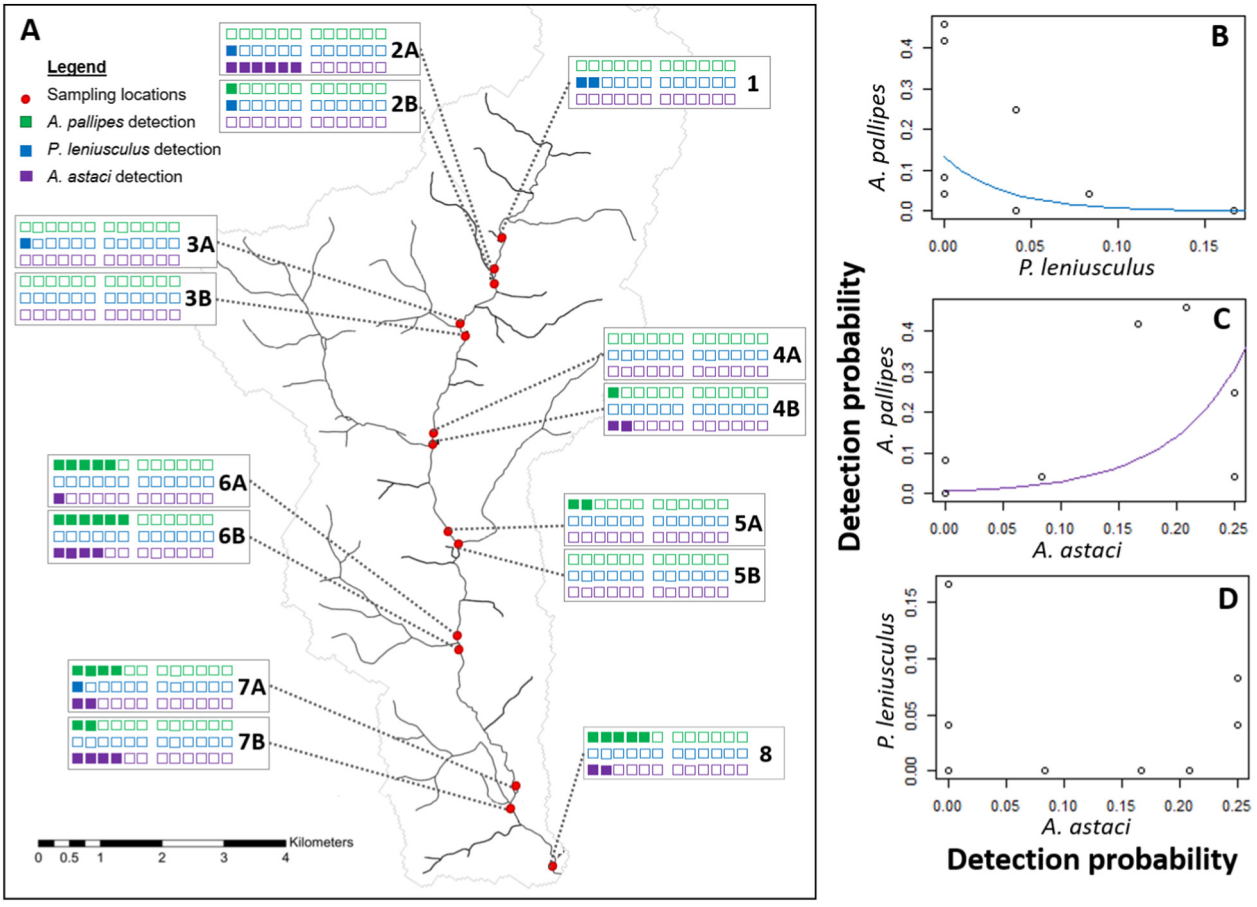

Fig. 5. (A) Detection of eDNA from white-clawed crayfish (green squares), signal crayfish (blue squares) and crayfish plague (purple squares) in a river catchment in Derbyshire. Eight locations were sampled and are represented by red dots. The empty squares represent the negative qPCR replicates. (B) Indicates the relationship between the detection probability of white-clawed crayfish and detection probability of signal crayfish. (C) The relationship between the detection probability of white-clawed crayfish and detection probability of crayfish plague. (D) The relationship between the detection probability of signal crayfish and detection probability of crayfish plague. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

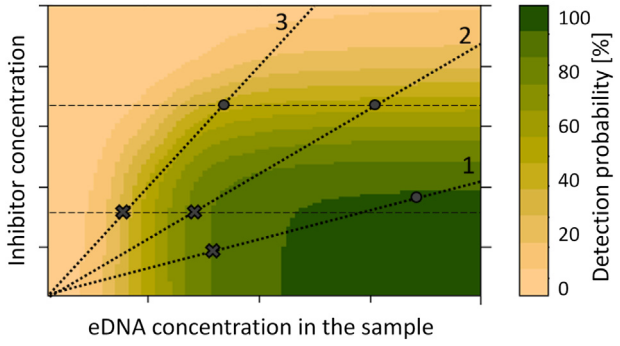

Fig. 6. Schematic of the co-dependency of detection probability on target eDNA and inhibitors concentrations in water samples. Detection probability increases with eDNA concentration and decreases with inhibitor concentrations but is low when both variables are high. Each water body is characterised by a certain ratio between inhibitor and target eDNA concentrations represented by black dotted lines (1–3). A change in sampling methods accompanied by a change in the sampled water volume will result in different concentrations of target eDNA and inhibitors in the sample and in shifts along dotted lines (grey crosses and dots). An increase in sampled water volume will therefore in some water bodies increase (Line 1) and in others decrease (Line 2) detection probability. The same is true when different eDNA assays in the same water body are considered. Whilst eDNA concentrations of two targets may differ, inhibitor concentrations will be the same. Consequently, samples with the same water volume will have the same inhibitor concentrations (horizontal dashed lines). Nevertheless, changes in sampling volume and method can result in increased detection probability for one target (Line 3) but not for the other (Line 2).

white-clawed crayfish, signal crayfish and crayfish plague revealed a negative impact of signal crayfish on white-clawed crayfish (Fig. 5). Such negative impacts of invasive competitors on native crayfish species have been frequently highlighted before (Holdich et al., 2009), demonstrating the applicability of our approach. Interestingly, however, we also illustrated a positive relationship between white-clawed crayfish and crayfish plague. This contrasts against our initial expectations. Such co-occurrence might result from the one-time nature of our sampling approach, possibly reflecting a disease outbreak within the crayfish population for example. One which (if occurring) would most probably result in local extinction (Strand et al., 2019). However, another recent study has highlighted the potential of plague resistance in some white-clawed crayfish populations (Martín-Torrijos et al., 2017). If this is indeed proven to occur, such increased disease tolerance might facilitate a permanent co-existence of pathogen and host offering a ray of hope for the survival of the white clawed crayfish in Europe. Consequently, further in-depth monitoring of species dynamics together with genetic profiling and disease susceptibility tests should be a primary objective of future conservation planning.

5. Conclusions

Currently, many species-specific eDNA assays only cover *in-silico*, *in-vitro* and sometimes basic *in-situ* validation steps (Baldigo et al., 2017; Dickie et al., 2018; Egan et al., 2017; Mauvisseau et al., 2020). Already published white-clawed crayfish eDNA assays have shown some

promising first results but yet need to go through the required thorough level of *in-situ* evaluation as undertaken in this study (Atkinson et al., 2019; Robinson et al., 2018). Here we illustrate that sampling methods can differ strongly in performance and recommend rigorous testing of eDNA assays to optimise sampling strategies. However, our contrasting results of method comparisons (across habitats and species), highlight that there might not be a universal 'optimal sampling method', but instead, adjustments will likely need to be made to account for local conditions as required. The resulting higher method reliability (when such validation tests are undertaken), increases the applicability of any novel eDNA assays designed and paves the way for more detailed ecological studies to be undertaken aimed primarily at improving species management and conservation.

CRediT authorship contribution statement

Christopher R. Troth: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft. **Alfred Burian:** Formal analysis, Supervision, Validation, Visualization, Writing - review & editing. **Quentin Mauvisseau:** Data curation, Formal analysis, Validation, Visualization, Writing - review & editing. **Mark Bulling:** Formal analysis, Validation, Visualization, Writing - review & editing. **Jen Nightingale:** Conceptualization, Data curation, Validation, Writing - review & editing. **Christophe Mauvisseau:** Data curation, Writing - review & editing. **Michael J. Sweet:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.141394.

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