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

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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 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.

Key words: environmental DNA, sampling methodology, species interactions, white-clawed crayfish.

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 testing 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 (Rheyda. Hinlo et al., 2017).

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

63 during field sampling. Filtration approaches have the advantage of collating eDNA from larger volumes of
64 water compared to precipitation-based methods (Mächler et al., 2016). However, they can incorporate
65 the risk of losing particles below the filter pore size (Minamoto et al., 2016). They may also lead to higher
66 concentrations of inhibitors occurring, which would prevent the amplification of targeted eDNA
67 (Mauvisseau et al., 2019a). Previous method comparisons have come to contrasting recommendations, a
68 result which largely appears to be driven by the target species in question (Deiner *et al.*, 2015; Dickie *et*
69 *al.*, 2018). Additionally, even for the same species the 'optimal' method for collecting eDNA may vary
70 between lentic (i.e. ponds or lakes) and lotic (i.e. rivers and canals) systems (Geerts et al., 2018; Harper et
71 al., 2019). Careful method comparisons are therefore urgently needed and recommended (Deiner et al.,
72 2015).

73 In this study, we target the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), an
74 endangered and important umbrella species in the United Kingdom and Western Europe (Füreder et al.,
75 2010). Range reduction of white-clawed crayfish began in the 1860s, with declines rapidly accelerating in
76 the UK after the introduction of invasive non-native signal crayfish *Pacifastacus leniusculus* (Dana 1852),
77 which originated from North America in the 1970s (Holdich et al., 2009). Moreover, the spread of crayfish
78 plague *Aphanomyces astaci* (Schikora 1906), an oomycete pathogen carried by the signal crayfish, has
79 greatly exacerbated the negative impact of invasive competitors, pollution and habitat degradation
80 (Holdich et al., 2009). Despite its legislative protection (EU Habitats Directive), white-clawed crayfish
81 populations have continued to decline by as much as 50-80% between 2000 and 2010 (Füreder et al.,
82 2010). Due (at least in part) to the rarity of the native species, traditional survey methods are having
83 unsatisfactory success in monitoring populations (Gladman et al., 2010; Holdich and Reeve, 1991). This
84 highlights the urgent need for the development of novel, more sensitive survey tools/methods to assess
85 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-claw crayfish, earlier studies either lack a thorough field evaluation or are based on high resolution melt-curve (HMR) approaches (Robinson et al., 2018). Whist 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' -GCTGGGATAGTAGGGACTTCTTT - 3', reverse primer WC2302R 5' – CATGGGCGGTAACCACTAC - 3' and probe WC2302P 5' - 6-FAM-CTGCCCGGCTGCCCTAATTC-BHQ-1 -3' amplified a 109bp 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 samples (see below) contained a standard mix of; 12.5µL TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5µL DH_20, 1µL (10µM) of each primer, 1µL (2.5µM) of probe and 3µL of template DNA. qPCR's were performed with six replicates per sample technical replicates (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^{o}C for 5 min, denaturation at 95^{o}C for 8 min, followed by 50 cycles of 95^{o}C for 30 s and 55^{o}C for 1 min. Six no template controls (NTC's) were prepared using RT-PCR Grade Water (Ambion™) and run 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 uL^{-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 0.79ng μL^{-1} to $7.9x10^{-8}$ 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 0.22µM 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 did vary between sites, a result due to cases of high turbidity. That said, we ensured a consistent minimum volume of 150mL, 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 2mL of ethanol. It should be noted that all sampled locations for this part of

153 the study are part of an extensive monitoring programme for white-clawed crayfish populations,
154 therefore site locations are not reported for conservation reasons.

155 **2.5. Comparison of eDNA sampling methodologies in mesocosms**

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

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

eDNA samples taken with a 2µM pump-based filtration consisted of 2L of water (40 x 50mL, homogenized) and were filtered through a Millipore Glass fibre filter AP25, 47mm (2µM pore size) using a peristaltic pump (Masterflex E/S Portable Sampler, Cole-Parmer, USA). The filter was housed in an In-Line Filter Holder 47mm (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 -20ºC before extraction. Equipment was soaked and cleaned with 10% bleach between samples. Filters were extracted following procedures outlined in Spens et al. (2017). eDNA sample collections for 0.22µM and 0.45µM pressure filtration were undertaken in the same manner. 20 sub-samples were collected and a 50mL syringe (BD Plastipak™, Ireland) was then used to pressure filter 250mL of water through a sterile enclosed filter (Sterivex™, Merck®, Germany) with either a pore size of 0.22µM (Polyethersulfone membrane) or 0.45µM (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 1000m^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 436g; prior to release absence of signal and white-claw crayfish were confirmed by torching and eDNA-based surveys). Here, precipitation (sample volume: 90mL) was compared against 0.22µM pressure

197 filtration (sample volume: 250mL). Prior to sampling, the pond was confirmed absent of crayfish through
198 trapping and eDNA-based surveys. Sampling started on the 20th April 2018 and was repeated two hours,
199 seven days, 14 days and 35 days after crayfish release. At each sampling time, three field replicates eDNA
200 samples were taken from four different sites around the perimeter of the pond. This was undertaken for
201 both methods. Additionally, 20x 50mL sub-samples (taken from the entire pond perimeter) were pooled,
202 homogenised and sampled, again in triplicate and again for each method (this gave us n=60 environmental
203 samples of eDNA per method for across comparison analysis).

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

217 **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

241 population sizes. Differences in sample volumes between locations (due to turbidity) were accounted for
242 by including sample volume as a predictor in regression models, and log-log and untransformed models
243 were compared using AIC. Further, Ct and detection probability of different sampling methods were
244 compared using ANOVA analyses followed by Tukey's HSD post-hoc tests, and t-tests or nested ANOVA's
245 (lotic and lentic systems, where only two methods were compared). Although we acknowledge sample
246 volume differed between sampling methods, it was kept constant within samples of each method within
247 the methodology comparison and so we have not incorporated volume as a random effect in this
248 instance). Prior to ANOVAs, heteroscedasticity was evaluated, and data transformed if necessary. Finally,
249 the co-existence of white-clawed crayfish with signal crayfish and crayfish plague was tested in regression
250 models using detection probability of all three species. Parsimonious models were evaluated using Akaike
251 Information Criterion (AIC). All described statistical analyses were performed using R version 3.4.1 (R Core
252 Team (2017).

253 ## 3. Results

254 ### 3.1. Assay development and in-silico and in-vitro validation

255 The primers and probe were highly species-specific as *in-silico* and *in-vitro* tests did not reveal any matches
256 with non-target species (Table S4). Analysis of the standard curve (Fig. 2A) revealed a strong dependency
257 of Ct values on DNA concentrations (y=-1.73x+20.8, $p<0.001$, $r^2= 0.996$). Likewise, the detection
258 probability was also positively related to DNA concentration in the sample (y=-0.18x+1.39, $p=0.0016$,
259 $r^2=0.804$; Fig. 2B). This highlights the possibility of a quantifiable assay being developed. Method
260 sensitivity analyses revealed a LOD of 7.9 x 10^{-5}ng and a LOQ of 7.9 x 10^{-4}ng crayfish DNA extract per μL^{-1}.

261 ### 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.

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 (2µM, 0.22µM and 0.45µM) were comparable ($p>0.05$). Notably, the *p*-value for the comparison between 0.45µM and 2µM 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 2µM

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. 4 A-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 (0.22µM). 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 2µM 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. 5, B,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 was 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).

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

329 historic eDNA, which is still present after the extinction or emigration of the target species (Turner et al.,
330 2015). In our case, this represents a valid hypothesis as all field sites were populated by white-clawed
331 crayfish a year before our field surveys (C. Mauvisseau, personal observation). Although we took great
332 care to avoid sediment disruption during sample collection (e.g. sampling of at least 20cm above the
333 riverbed), non-sampling related disturbance could have resulted in resuspension of ancient DNA.
334 Therefore, it remains inconclusive whether the developed eDNA approach has a higher sensitivity (i.e.
335 false negative of torching method) or, in fact, white-clawed crayfish were not present at the field sites in
336 question. Further investigations into the impact of downstream flow and historic eDNA presence are
337 required to obtain a more comprehensive picture in this regard. That said, a recent study has since shown
338 that even whole crayfish carcasses resting on the bottom of a river (crayfish species *P. clarkii*) fail to
339 produce detectable eDNA in a stream enclosure experiment (Curtis and Larson, 2020). Therefore, this
340 result suggested that positive detections in field studies may be more confidently attributed to the
341 presence of live organisms.

342 Further, an important component of our method validation was the comparison of different field sampling
343 approaches. Precipitation and filtration protocols, aimed at concentrating eDNA from the environment
344 have been compared in a number of studies (Deiner et al., 2015; Spens et al., 2017) and the majority of
345 investigators endorse filtration approaches (Rheyda Hinlo et al., 2017; Spens et al., 2017; Vörös et al.,
346 2017). However, optimal filter pore size appears to differ among target species (Spens et al., 2017).
347 Moreover, method choice can also be environment-dependant. For example, Eichmiller, et al. (2016)
348 indicated filtration as the optimal eDNA-based method for surveying the common carp, *Cyprinus carpio*.
349 In contrast, Minamoto et al. (2016) highlighted precipitation performed better – a result likely induced by
350 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; (Garland et al. 2009; Goldberg et al. 2016; Mauvisseau et al. 2019b) 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 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.

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421

References

Agersnap, S., Larsen, W.B., Knudsen, S.W., Strand, D., Thomsen, P.F., Hesselsøe, M., Mortensen, P.B., Vrålstad, T., Møller, P.R., 2017. Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. PLoS One 12, 1–22. https://doi.org/10.1371/journal.pone.0179261

Atkinson, S., Carlsson, J.E.., Ball, B., Kelly-Quinn, M., Carlsson, J., 2019. Field application of an eDNA assay for the threatened white-clawed crayfish Austropotamobius pallipes. Freshw. Sci. 38, 000–000. https://doi.org/10.1086/704712

Baldigo, B.P., Sporn, L.A., George, S.D., Ball, J.A., 2017. Efficacy of Environmental DNA to Detect and Quantify Brook Trout Populations in Headwater Streams of the Adirondack Mountains, New York. Trans. Am. Fish. Soc. 146, 99–111. https://doi.org/10.1080/00028487.2016.1243578

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A., Foster, J., Wilkinson, J.W., Arnell, A., Brotherton, P., Williams, P., Dunn, F., 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). Biol. Conserv. 183, 19–28. https://doi.org/10.1016/J.BIOCON.2014.11.029

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Griffiths, R., Foster, J., Wilkinson, J., Arnett, A., Williams, P., Dunn, F., 2014. Analytical and methodological development for improved surveillance of the Great Crested Newt Appendix 5 . Technical advice note for field and laboratory sampling of great crested newt (Triturus cristatus) environmental DNA. Freshw. Habitats Trust. Oxford.

Bylemans, J., Furlan, E.M., Gleeson, D.M., Hardy, C.M., Duncan, R.P., 2018. Does Size Matter? An Experimental Evaluation of the Relative Abundance and Decay Rates of Aquatic Environmental

443 DNA. Environ. Sci. Technol. 52, 6408–6416. https://doi.org/10.1021/acs.est.8b01071

444 Curtis, A.N., Larson, E.R., 2020. No evidence that crayfish carcasses produce detectable environmental
445 DNA (eDNA) in a stream enclosure experiment. PeerJ 9. https://doi.org/10.7717/peerj.9333

446 Deiner, K., Walser, J.-C., Mächler, E., Altermatt, F., 2015. Choice of capture and extraction methods
447 affect detection of freshwater biodiversity from environmental DNA. Biol. Conserv. 183, 53–63.
448 https://doi.org/10.1016/J.BIOCON.2014.11.018

449 Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., Miaud, C., 2012. Improved detection of
450 an alien invasive species through environmental DNA barcoding: the example of the American
451 bullfrog *Lithobates catesbeianus*. J. Appl. Ecol. 49, 953–959. https://doi.org/10.1111/j.1365-
452 2664.2012.02171.x

453 Dickie, I.A., Boyer, S., Buckley, H.L., Duncan, R.P., Gardner, P.P., Hogg, I.D., Holdaway, R.J., Lear, G.,
454 Makiola, A., Morales, S.E., Powell, J.R., Weaver, L., 2018. Towards robust and repeatable sampling
455 methods in eDNA-based studies. Mol. Ecol. Resour. 18, 940–952. https://doi.org/10.1111/1755-
456 0998.12907

457 Egan, D., Carlsson, J.J.E.L.L., Igoe, F., Collins, P.C., Farrell, E.D., Egan, D., Igoe, F., Carlsson, J., 2017. A
458 qPCR MGB probe based eDNA assay for European freshwater pearl mussel (Margaritifera
459 margaritifera L.). Aquat. Conserv. Mar. Freshw. Ecosyst. 27, 1341–1344.
460 https://doi.org/10.1002/aqc.2788

461 Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA
462 from water samples. Biol. Lett. 4, 423–5. https://doi.org/10.1098/rsbl.2008.0118

463 Füreder, L., Gherardi, F., Holdich, D., Reynolds, J., Sibley, P., Souty-Grosset, C., 2010. Austropotamobius

464 pallipes. The IUCN Red List of Threatened Species 2010: e.T2430A9438817 [WWW Document]. URL
465 http://www.iucnredlist.org/details/2430/0 (accessed 2.9.18).

466 Furlan, E.M., Gleeson, D., Hardy, C.M., Duncan, R.P., 2016. A framework for estimating the sensitivity of
467 eDNA surveys. Mol. Ecol. Resour. 16. https://doi.org/10.1111/1755-0998.12483

468 Geerts, A.N., Boets, P., Van den Heede, S., Goethals, P., Van der heyden, C., 2018. A search for
469 standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): A lab
470 and field evaluation. Ecol. Indic. 84, 564–572. https://doi.org/10.1016/j.ecolind.2017.08.068

471 Gladman, Z.F., Yeomans, W.E., Adams, C.E., Bean, C.W., McColl, D., Olszewska, J.P., McGillivray, C.W.,
472 McCluskey, R., 2010. Detecting North American signal crayfish (Pacifastacus leniusculus) in riffles.
473 Aquat. Conserv. Mar. Freshw. Ecosyst. 20, 588–594. https://doi.org/10.1002/aqc.1130

474 Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee,
475 A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S.,
476 Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., 2016. Critical
477 considerations for the application of environmental DNA methods to detect aquatic species.
478 Methods Ecol. Evol. 7, 1299–1307. https://doi.org/10.1111/2041-210X.12595

479 Harper, L.R., Buxton, A.S., Rees, H.C., Bruce, K., Brys, R., Halfmaerten, D., Read, D.S., Watson, H. V.,
480 Sayer, C.D., Jones, E.P., Priestley, V., Mächler, E., Múrria, C., Garcés-Pastor, S., Medupin, C.,
481 Burgess, K., Benson, G., Boonham, N., Griffiths, R.A., Lawson Handley, L., Hänfling, B., 2019.
482 Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds.
483 Hydrobiologia 826, 25–41. https://doi.org/10.1007/s10750-018-3750-5

484 Hinlo, Rheyda., Furlan, E., Suitor, L., Gleeson, D., 2017. Environmental DNA monitoring and management

485 of invasive fish: comparison of eDNA and fyke netting. Manag. Biol. Invasions 8, 89–100.
486 https://doi.org/10.3391/mbi.2017.8.1.09

487 Hinlo, Rheyda, Gleeson, D., Lintermans, M., Furlan, E., 2017. Methods to maximise recovery of
488 environmental DNA from water samples. PLoS One 12, e0179251.
489 https://doi.org/10.1371/journal.pone.0179251

490 Holdich, D.M., Reeve, I.A.N.D., 1991. Distribution of freshwater crayfish in the British Isles, with
491 particular reference to crayfish plague, alien introductions and water quality. Aquat. Conserv. Mar.
492 Freshw. Ecosyst. 1, 139–158. https://doi.org/10.1002/aqc.3270010204

493 Holdich, D.M.M., Reynolds, J.D.D., Souty-Grosset, C., Sibley, P.J.J., 2009. A review of the ever increasing
494 threat to European crayfish from non-indigenous crayfish species. Knowl. Manag. Aquat. Ecosyst.
495 11. https://doi.org/10.1051/kmae/2009025

496 Huver, J.R., Koprivnikar, J., Johnson, P.T.J., Whyard, S., 2015. Development and application of an eDNA
497 method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol. Appl. 25, 991–
498 1002. https://doi.org/10.2307/24432104

499 Jerde, C.L., Chadderton, W.L., Mahon, A.R., Renshaw, M.A., Corush, J., Budny, M.L., Mysorekar, S.,
500 Lodge, D.M., 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance
501 program. Can. J. Fish. Aquat. Sci. 70, 522–526. https://doi.org/10.1139/cjfas-2012-0478

502 Jerde, C.L., Mahon, A.R., Chadderton, W.L., Lodge, D.M., 2011. “Sight-unseen” detection of rare aquatic
503 species using environmental DNA. Conserv. Lett. 4, 150–157. https://doi.org/10.1111/j.1755-
504 263X.2010.00158.x

505 Jo, T., Arimoto, M., Murakami, H., Masuda, R., Minamoto, T., 2019. Particle Size Distribution of

506 Environmental DNA from the Nuclei of Marine Fish. Environ. Sci. Technol. 53, 9947–9956.
507 https://doi.org/10.1021/acs.est.9b02833

508 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A.,
509 Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic:
510 an integrated and extendable desktop software platform for the organization and analysis of
511 sequence data. Bioinformatics 28, 1647–9. https://doi.org/10.1093/bioinformatics/bts199

512 Klymus, K.E., Merkes, C.M., Allison, M.J., Goldberg, C.S., Helbing, C.C., Hunter, M.E., Jackson, C.A., Lance,
513 R.F., Mangan, A.M., Monroe, E.M., Piaggio, A.J., Stokdyk, J.P., Wilson, C.C., Richter, C.A., 2019.
514 Reporting the limits of detection and quantification for environmental DNA assays. Environ. DNA
515 edn3.29. https://doi.org/10.1002/edn3.29

516 Larson, E.R., Renshaw, M.A., Gantz, C.A., Umek, J., Chandra, S., Lodge, D.M., Egan, S.P., 2017.
517 Environmental DNA (eDNA) detects the invasive crayfishes Orconectes rusticus and Pacifastacus
518 leniusculus in large lakes of North America. Hydrobiologia 800, 173–185.
519 https://doi.org/10.1007/s10750-017-3210-7

520 MacDonald, A.J., Sarre, S.D., 2017. A framework for developing and validating taxon-specific primers for
521 specimen identification from environmental DNA. Mol. Ecol. Resour. 17, 708–720.
522 https://doi.org/10.1111/1755-0998.12618

523 Mächler, E., Deiner, K., Spahn, F., Altermatt, F., 2016. Fishing in the Water: Effect of Sampled Water
524 Volume on Environmental DNA-Based Detection of Macroinvertebrates. Environ. Sci. Technol. 50,
525 305–312. https://doi.org/10.1021/acs.est.5b04188

526 Martín-Torrijos, L., Campos Llach, M., Pou-Rovira, Q., Diéguez-Uribeondo, J., 2017. Resistance to the

crayfish plague, Aphanomyces astaci (Oomycota) in the endangered freshwater crayfish species, Austropotamobius pallipes. PLoS One 12, e0181226. https://doi.org/10.1371/journal.pone.0181226

Mauvisseau, Q., Coignet, A., Delaunay, C., Pinet, F., Bouchon, D., Souty-Grosset, C., 2018. Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. Hydrobiologia 805, 163–175. https://doi.org/10.1007/s10750-017-3288-y

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., Sweet, M., 2019a. Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. Sci. Rep. 9, 1–9. https://doi.org/10.1038/s41598-019-50571-9

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., Sweet, M., 2019b. Improving detection capabilities of a critically endangered freshwater invertebrate with environmental DNA using digital droplet PCR. bioRxiv 661447. https://doi.org/10.1101/661447

Mauvisseau, Q., Kalogianni, E., Zimmerman, B., Bulling, M., Brys, R., Sweet, M., 2020. eDNA-based monitoring: Advancement in management and conservation of critically endangered killifish species. Environ. DNA edn3.92. https://doi.org/10.1002/edn3.92

Mauvisseau, Q., Tönges, S., Andriantsoa, R., Lyko, F., Sweet, M., 2019c. Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems, Management of Biological Invasions.

Minamoto, T., Naka, T., Moji, K., Maruyama, A., 2016. Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. Limnology 17, 23–32.

548 https://doi.org/10.1007/s10201-015-0457-4

549 Niemiller, M.L., Porter, M.L., Keany, J., Gilbert, H., Fong, D.W., Culver, D.C., Hobson, C.S., Kendall, K.D.,
550 Davis, M.A., Taylor, S.J., 2018. Evaluation of eDNA for groundwater invertebrate detection and
551 monitoring: a case study with endangered Stygobromus (Amphipoda: Crangonyctidae). Conserv.
552 Genet. Resour. 10, 247–257. https://doi.org/10.1007/s12686-017-0785-2

553 Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H.,
554 Dejean, T., 2018. Environmental DNA reveals quantitative patterns of fish biodiversity in large
555 rivers despite its downstream transportation. Sci. Rep. 8, 10361. https://doi.org/10.1038/s41598-
556 018-28424-8

557 R Core Team (2017). R: A language and environment for statistical computing. R Foundation for
558 Statistical Computing, Vienna, A.U. https://www. R. org/., n.d. No Title.

559 Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., Gough, K.C., 2014. REVIEW: The detection
560 of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology.
561 J. Appl. Ecol. 51, 1450–1459. https://doi.org/10.1111/1365-2664.12306

562 Robinson, C.V., Uren Webster, T.M., Cable, J., James, J., Consuegra, S., 2018. Simultaneous detection of
563 invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using
564 environmental DNA. Biol. Conserv. 222, 241–252. https://doi.org/10.1016/j.biocon.2018.04.009

565 Smart, A.S., Tingley, R., Weeks, A.R., Van Rooyen, A.R., McCarthy, M.A., 2015. Environmental DNA
566 sampling is more sensitive than a traditional survey technique for detecting an aquatic invader.
567 Ecol. Appl. 25, 1944–1952. https://doi.org/10.1890/14-1751.1

568 Spear, S.F., Groves, J.D., Williams, L.A., Waits, L.P., 2015. Using environmental DNA methods to improve

detectability in a hellbender (Cryptobranchus alleganiensis) monitoring program. Biol. Conserv. 183, 38–45. https://doi.org/10.1016/j.biocon.2014.11.016

Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E., Hellström, M., 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods Ecol. Evol. 8, 635–645. https://doi.org/10.1111/2041-210X.12683

Strand, D.A., Johnsen, S.I., Rusch, J.C., Agersnap, S., Larsen, W.B., Knudsen, S.W., Møller, P.R., Vrålstad, T., 2019. Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. J. Appl. Ecol. 56, 1365-2664.13404. https://doi.org/10.1111/1365-2664.13404

Strand, D.A., Jussila, J., Johnsen, S.I., Viljamaa-Dirks, S., Edsman, L., Wiik-Nielsen, J., Viljugrein, H., Engdahl, F., Vrålstad, T., 2014. Detection of crayfish plague spores in large freshwater systems. J. Appl. Ecol. 51, 544–553. https://doi.org/10.1111/1365-2664.12218

Strickler, K.M., Fremier, A.K., Goldberg, C.S., 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol. Conserv. 183, 85–92. https://doi.org/10.1016/J.BIOCON.2014.11.038

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., Kawabata, Z., 2012. Estimation of Fish Biomass Using Environmental DNA. PLoS One 7, e35868. https://doi.org/10.1371/journal.pone.0035868

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M.A., Roussel, J.-M., 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish Procambarus clarkii in freshwater ponds. J. Appl. Ecol. 51, 871–879.

https://doi.org/10.1111/1365-2664.12262

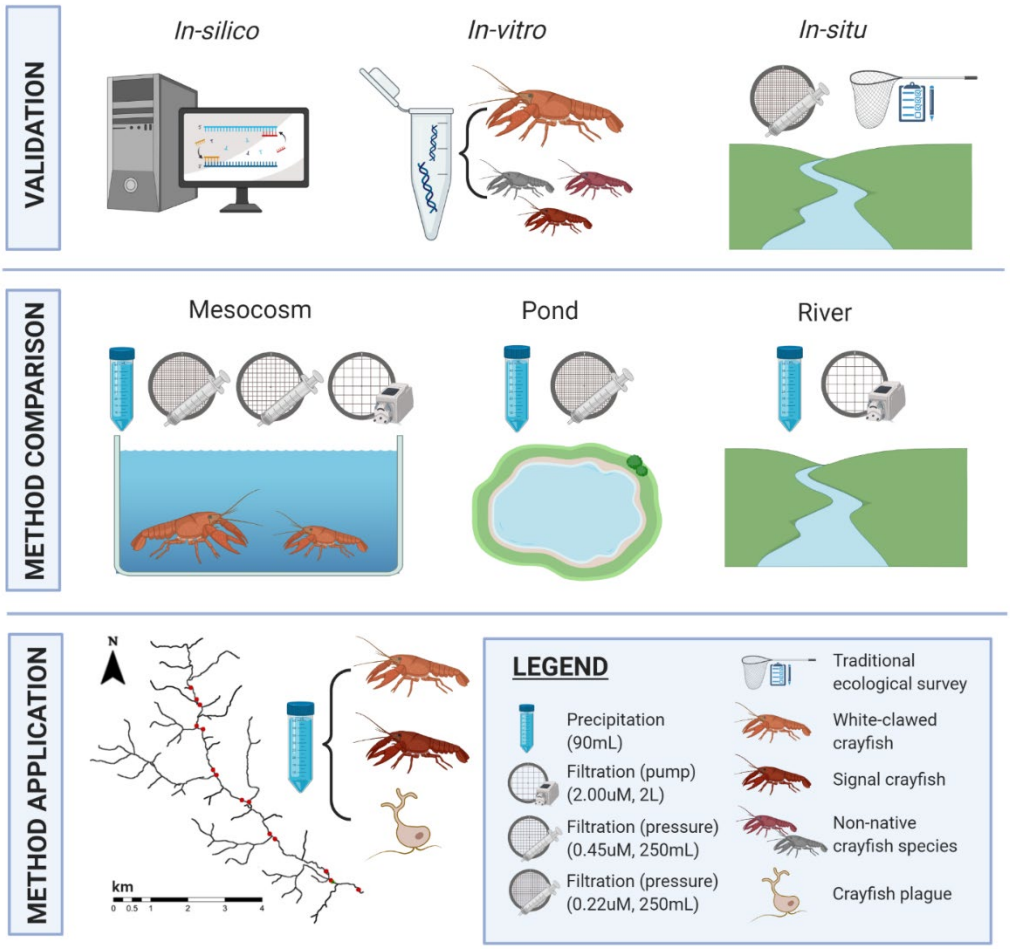
Turner, C.R., Uy, K.L., Everhart, R.C., 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biol. Conserv. 183, 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

Vörös, J., Márton, O., Schmidt, B.R., Gál, J.T., Jelić, D., 2017. Surveying Europe's Only Cave-Dwelling Chordate Species (Proteus anguinus) Using Environmental DNA. PLoS One 12, e0170945. https://doi.org/10.1371/journal.pone.0170945

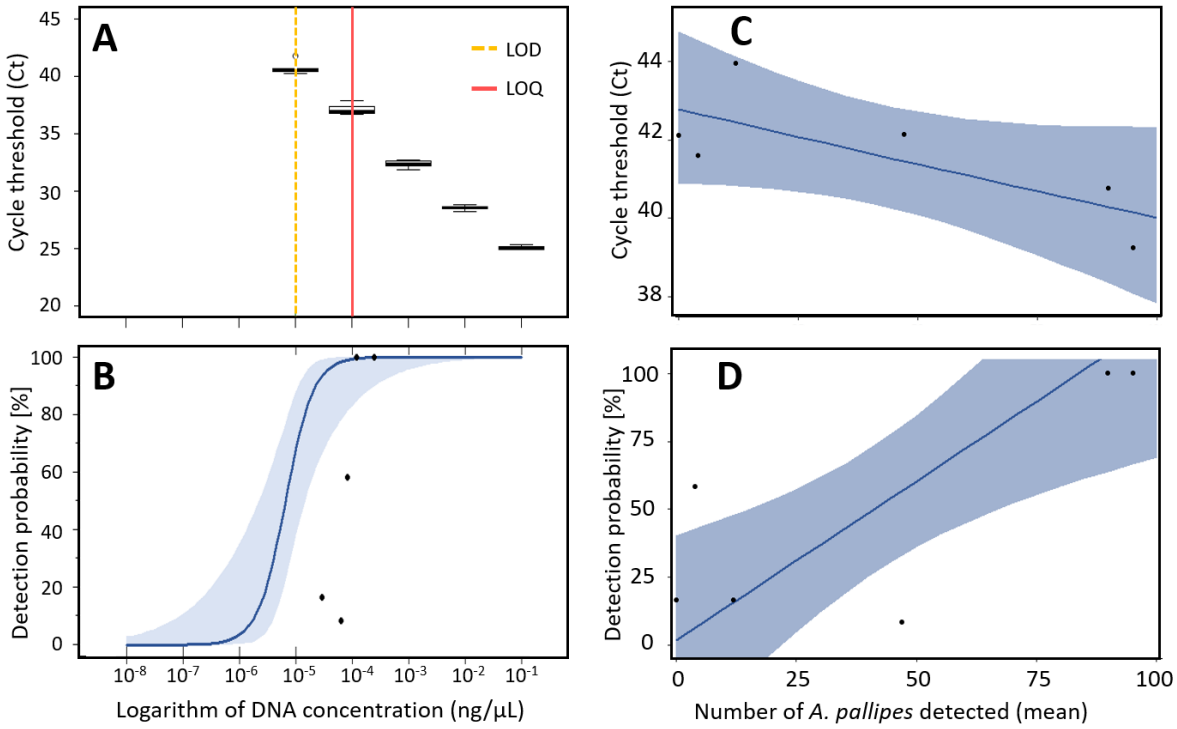
Yang, S., Ramachandran, P., Rothman, R., Hsieh, Y.H., Hardick, A., Won, H., Kecojevic, A., Jackman, J., Gaydos, C., 2009. Rapid identification of biothreat and other clinically relevant bacterial species by use of universal PCR coupled with high-resolution melting analysis. J. Clin. Microbiol. 47, 2252–2255. https://doi.org/10.1128/JCM.00033-09

Author Contributions

C.T., M.S. and J.N. designed the experiment and methodology, C.T., Q.M., J.N. and C.M. collected field samples, C.T., and Q.M. performed extraction and PCR, C.T., A.B. and M.B. analysed the data. The manuscript was written by C.T., M.S. and A.B. and reviewed by all authors.



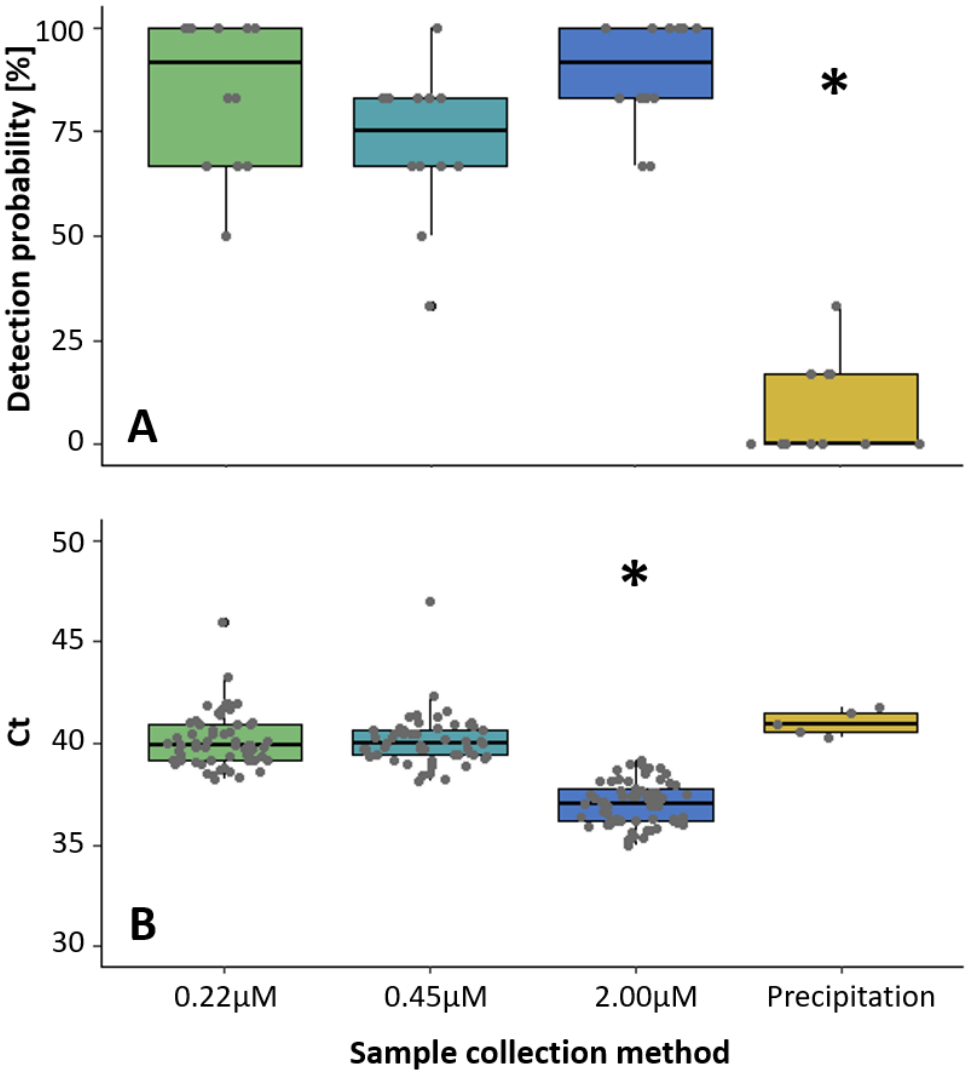
607
608 **Figure 1.** Different steps of method development and application performed in this study. Method
609 validation encompassed three key steps: *In-silico* (computer simulations of assay sensitivity), *in-vitro*
610 (performance tests using target and non-target DNA) and *in-situ* assessments (quantitative comparison
611 between eDNA and established field methods). Additionally, we examined possibilities to enhance assay
612 performance in an extensive method comparison of different eDNA collating methods in controlled
613 mesocosm experiments and field tests in lotic and lentic environment. The potential of the assay to
614 evaluate interactions between different crayfish and crayfish plaque was investigated in an extensive
615 monitoring campaign of a target catchment. Water collation methods inherently differed in the water
616 volumes collected, but for all method comparisons and applications, sampled water within each method
617 was kept constant, facilitating site comparisons.



618

619 **Figure 2. (A)** Relationship between cycle threshold (Ct) and DNA concentration from White-clawed
620 crayfish qPCR calibration curve. Limit of detection (LOD) and limit of quantification (LOQ) are illustrated
621 by vertical lines (dashed-yellow and red respectively). **(B)** Change in detection probability with increasing
622 DNA concentrations in calibration curve data (blue line). Red triangles represent field data (in-*situ*
623 validation data) that is compared with the log-regression based on in-*vitro* calibration curve data. Three
624 out of five field data points were outside the established confidence interval, indicating discrepancies
625 between field and laboratory-based data sets. **(C)** Relationship between Ct values and white-clawed
626 crayfish population monitored using traditional method. **(D)** Relationship between detection probability
627 of eDNA and traditionally evaluated crayfish population sizes. The blue line and the light-blue shaded area
628 reflect the results of a logit regression and its 95% confidence interval, respectively.

629



630
631 **Figure 3.** Comparison of the detection probability **(A)** and Ct values **(B)** of white-clawed crayfish using
632 different eDNA sampling methods (0.22µM filtration (green), 0.45µM filtration (light blue), 2µM filtration
633 (royal blue) and precipitation (yellow)) in a controlled mesocosm experiment (* indicates statistical
634 significance).

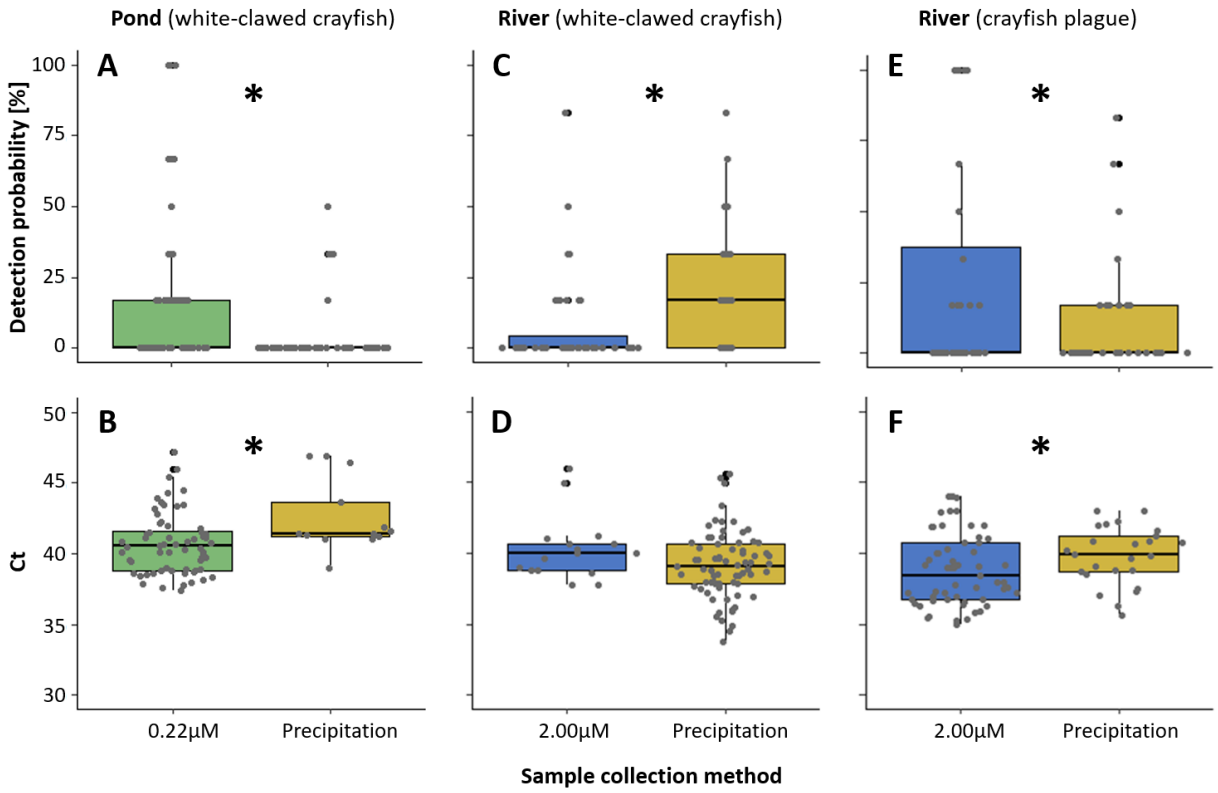


Figure 4. Comparison of the detection probability **(A, C, E)** and Ct values **(B, D, F)** of different eDNA sampling methods (filtration and precipitation) for white-clawed crayfish in a lentic system **(Pond, A-B)** (filter pore size 0.22µM) and for both white-clawed crayfish **(River, C-D)** and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

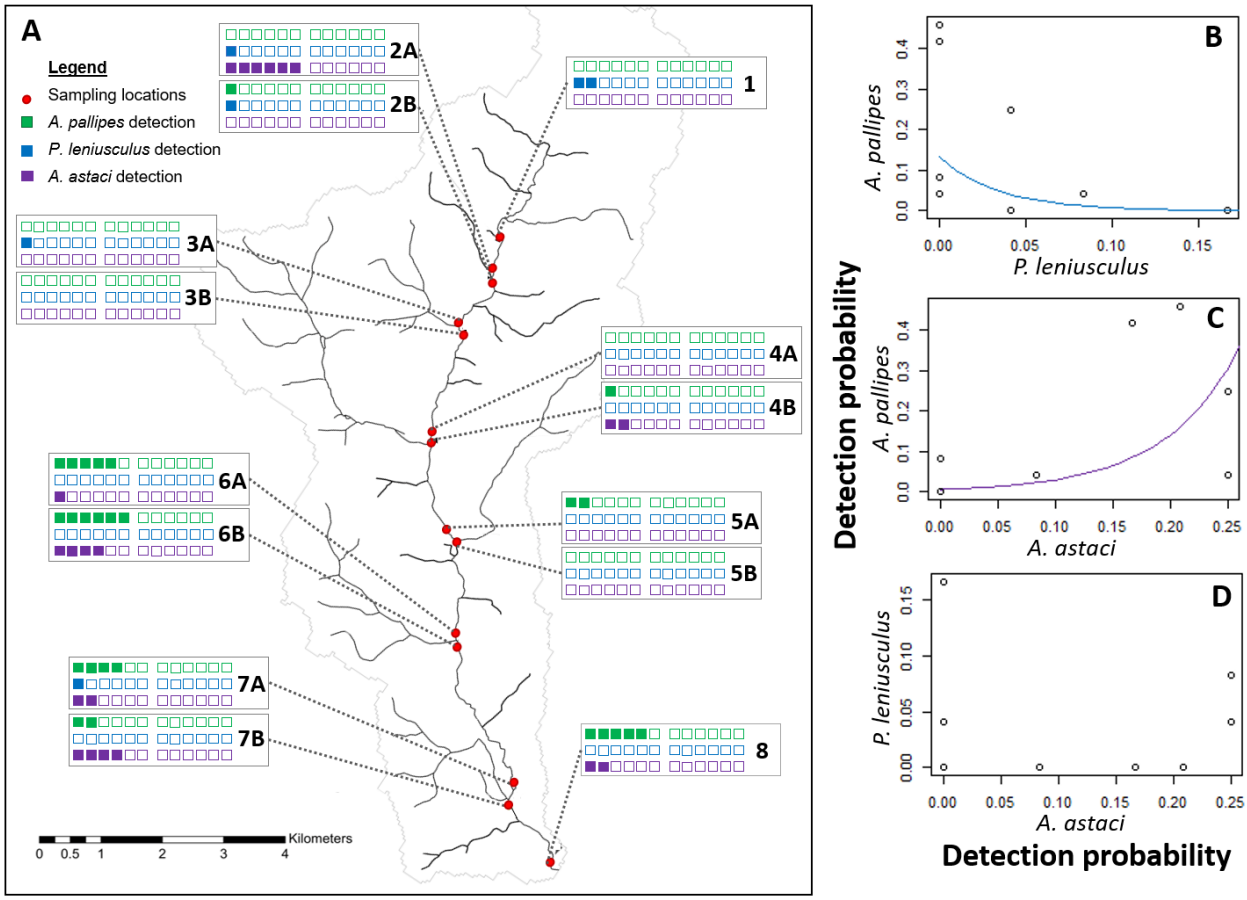


Figure 5. (A) Detection of eDNA from white-clawed crayfish (green squares), signal crayfish (blue squares) and crayfish plague (purple squares) in a river catchment in Derbyshire. Eight locations were sampled and are represented by red dots. The empty squares represent the negative qPCR replicates. **(B)** 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.

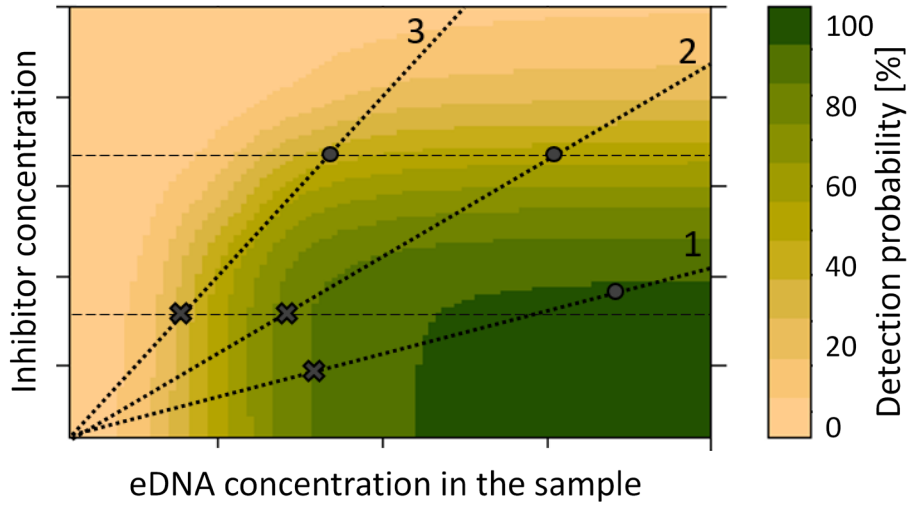


Figure 6. Schematic of the co-dependency of detection probability on target eDNA and inhibitors concentrations in water samples. Detection probability increases with eDNA concentration and decreases with inhibitor concentrations but is low when both variables are high. Each water body is characterised 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 other decrease (Line 2) detection probability. The same is true when different eDNA assays in the same water body are considered. While 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).