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

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## Abstract

1. 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 distribution. However, this next step of eDNA-based applications requires a thorough method development.

2. We developed an eDNA assay for the white-clawed crayfish (*Austropotamobius pallipes*), a flagship species of conservation in the UK. 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.

3. The assay performed well in terms of specificity (no detection of non-target DNA) and sensitivity, which was higher than more established traditional species survey methods. Quantification of species biomass was, however, less reliable.

4. Comparison of eDNA sampling methods (precipitation vs. various filtration approaches) revealed that optimal sampling method differed across environments and might depend on inhibitor concentrations.

5. Finally, we applied our methodology together with established assays for crayfish plague and the invasive signal crayfish and demonstrated their significant interactions in a UK river system.

6. 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 practices.

37 **Introduction**

38 Since its initial conception as a method for aquatic ecological surveys (Ficetola et al., 2008), the use of
39 environmental DNA (eDNA) based methods are rapidly increasing in popularity (Biggs et al., 2015; Jerde
40 et al., 2013; . Advantages such as higher cost effectiveness compared to established traditional species
41 survey methods and the non-invasive sampling approach have been emphasised (Goldberg et al., 2016;
42 Huver et al., 2015). Nevertheless, the true potential of eDNA-based methods is just starting to be realized.
43 Currently, eDNA-based tools are mostly used for simple presence/absence surveys, while they could also
44 be used to study ecological interactions that determine species distribution and the conservation status
45 of target species. However, such advances in application require careful method evaluations and the
46 improvement of sampling approaches to increase reliability of detection and prevent false conclusions.

47 In the case of species-specific eDNA assays, the design and validation of the assay represents a critical first
48 step (Geerts et al., 2018). During assay design, it is fundamental to ensure a high target specificity
49 (Bylemans et al., 2018) by selecting suitable amplicon lengths, *in-silico* simulations and testing against
50 amplification of non-target DNA. *In-vitro* laboratory validation should then ascertain that the assay
51 complies with established guidelines (Bustin et al., 2009) and that limits of detection (LOD) and
52 quantification (LOQ) are established. Further, field comparisons with established traditional survey
53 methods are recommended to complement reliability assessments (Smart et al., 2015). However, both
54 traditional survey approaches and eDNA-based methods are affected by various error sources potentially
55 creating inconsistencies that require careful interpretation (Hinlo et al., 2017a).

56 Further, the reliability of eDNA-based tools is strongly influenced by sampling methodology (Hinlo et al.,
57 2017b). Currently, precipitation and various filtration methods are applied to concentrate eDNA during
58 field sampling. Filtration approaches have the advantage of collating eDNA from larger volumes of water

59 compared to precipitation-based methods (Mächler et al., 2016). However, they can also incorporate the
60 risk of missing particles below the filter pore size (Minamoto et al., 2016) and may lead to higher
61 concentrations of inhibitors preventing targeted eDNA amplification (Mauvisseau et al., 2019a). Previous
62 method comparisons have come to contrasting recommendations for difference species (Deiner *et al.*,
63 2015; Dickie *et al.*, 2018). Additionally, even for the same species the 'optimal' method for collecting eDNA
64 may vary between lentic (i.e. ponds or lakes) and lotic (i.e. rivers and canals) systems (Geerts et al., 2018)
65 and therefore careful method comparisons are recommended (Deiner et al., 2015).

66 In this study, we target the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), an
67 endangered and important umbrella species in the UK and Western Europe (Füreder et al., 2010). Range
68 reduction of white-clawed crayfish began in the 1860s, with declines rapidly accelerating in the UK after
69 the introduction of invasive non-native signal crayfish (*Pacifastacus leniusculus*, Dana, 1852) from North
70 America in the 1970s (Holdich et al., 2009). Moreover, the spread of crayfish plague *Aphanomyces astaci*
71 (Schikora 1906), an oomycete pathogen carried by signal crayfish, has greatly exacerbated the negative
72 impact of invasive competitors, pollution and habitat degradation (Holdich et al., 2009). Despite its
73 legislative protection (EU Habitats Directive), white-clawed crayfish has continued to decline by as much
74 as 50-80% between 2000 and 2010 (Füreder et al., 2010). Due (at least in part) to the now rarity of the
75 native species, traditional survey methods are having unsatisfactory success in monitoring populations
76 (Gladman et al., 2010; Holdich and Reeve, 1991), highlighting the urgent need to develop new survey
77 tools.

78 Consequently, the aim of this study was to develop a highly reliable eDNA assay for the detection of white-
79 clawed crayfish, that allows the assessment of interactions with competing species and pathogens which
80 threaten their survival. We designed a primer and probe set for the amplification of white-clawed crayfish
81 DNA and critically evaluated the sensitivity and specificity of the assay through extensive *in-silico, in-vitro*

82 and *in-situ* tests. Moreover, we evaluated the impact of different sampling methodologies on the
83 reliability of the assay in mesocosm experiments and field tests implemented in different habitat types.
84 Finally, this allowed us to assess in a UK river system the relationship between white-clawed crayfish,
85 signal crayfish and crayfish plague, demonstrating the applicability of eDNA-based approaches for in-
86 depth ecological investigations and ecosystem management.

87 **Materials and Methods**

88 ***Primer design and in-silico tests***

89 Primer/probe design and validation followed guidelines established by MacDonald and Sarre (2017) aimed
90 for assay development of species-specific eDNA methods. The primers and probe, targeting the
91 Cytochrome C Oxidase Subunit 1 (COI) mitochondrial gene of white-clawed crayfish, were designed *in-*
92 *silico* using Geneious Pro R10 (Kearse et al., 2012). The forward primer WC2302F 5' -
93 GCTGGGATAGTAGGGACTTCTTT - 3', reverse primer WC2302R 5' – CATGGGCGGTAACCACTAC - 3' and
94 probe WC2302P 5' - 6-FAM-CTGCCCGGCTGCCCTAATTC-BHQ-1 -3' amplified a 109bp fragment. To ensure
95 specificity, *in-silico* tests were run against published sequences of closely related and/or co-occurring
96 crayfish species.

97 ***In-vitro validation***

98 The specificity of the assay was further tested *in-vitro* against extracted DNA of either taxonomically
99 similar, or co-occuring crayfish species. These included; *Faxonius limosus* (Rafinesque, 1817), *P.*
100 *leniusculus*, *Astacus astacus* (Linnaeus, 1758), *Astacus leptodactylus* (Eschscholtz, 1823), *Procambarus*
101 *clarkii* (Girard, 1852), and *Procambarus virginalis* (Lyko, 2017). DNA was extracted from crayfish tissues
102 using the Qiagen DNeasy® Blood & Tissue kit, following manufacturers' instructions. PCRs were performed
103 using the primers and methods from Folmer et al. (1994) and sequenced by Eurofins Genomics (Germany)

104 to confirm species identity of all specimens. Specificity of the newly designed assay was then assessed
105 using qPCR.

106 The reactions for both tissue and all eDNA samples contained; 12.5µL TaqMan® Environmental Master
107 Mix 2.0 (Life Technologies®), 6.5µL $DH_20$, 1µL (10µm) of each primer, 1µL (2.5µm) of probe with the
108 addition of 3µL template DNA. qPCR's were performed with 6 technical replicates (i.e. qPCR replicates) of
109 each sample on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). qPCR conditions were as
110 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
111 1 min. Six no template controls (NTC's) were prepared using RT-PCR Grade Water (Ambion™) alongside a
112 duplicated serial dilution of control white-clawed crayfish DNA ($10^{-1}$-$10^{-3}$ ng $uL^{-1}$) for each qPCR plate that
113 was run. In each subsequent experiment, negative PCR controls were included in this fashion.

114 ***Limits of detection (LOD) and quantification (LOQ)***

115 The reliability of our assay was also assessed, following the Minimum Information for Publication of
116 Quantitative Real-Time PCR Experiments (MIQE) Guidelines, which recommend the establishment of a
117 calibration curve to determine LOD and LOQ (Bustin et al., 2009). We prepared a serial dilution of DNA
118 extracted from white-clawed crayfish starting from 0.79ng $\mu L^{-1}$ to $7.9x10^{-8}$ ng $\mu L^{-1}$ with 10 qPCR replicates
119 per dilution analysed. The LOD was defined as the last standard dilution that resulted in a detection of
120 target DNA with at least one qPCR replicate at a threshold cycle (Ct) of <45. The LOQ was defined as the
121 last standard dilution in which targeted DNA was detected and quantified in a minimum of 90% of qPCR
122 replicates of the calibration curve under a Ct of 45 (Mauvisseau et al., 2019b).

123 ***In-situ validation***

124 The reliability of the assay was further field tested by comparing eDNA-based and traditional capture-
125 mark-recapture sampling techniques at six sites of confirmed white-clawed crayfish presence (2017) in

126 the Centre-Val de Loire region, France. Each site was visited at least twice in subsequent nights between
127 22nd June and 1st of August 2018 (see supplementary information, Table S1 Individual white-clawed
128 crayfish were surveyed using a torching approach, counted and marked using a white waterproof marker
129 stain. In the second night the survey was repeated and marked, and non-marked crayfish were
130 differentiated. Population size was estimated using the Lincoln-Petersen Method $N = \left(\frac{Kn}{k}\right)$, where N is
131 the estimated population size, n is the number of crayfish marked on the 1st visit, K is the total number
132 captured on the 2nd visit and k is the number of those captured individual which were already marked on
133 the 2nd visit. (Petersen 1896, Lincoln 1930). Additionally, eDNA samples (two environmental replicates)
134 were collected at each site using the 0.22µm Sterivex filters (see below for detailed description). eDNA
135 samples were collected between the 22nd and 29th June 2018. The water volume filtered varied due to
136 cases of high turbidity (consistent minimum volume of 150mL, see Table S2 for list of all sample volumes).
137 Furthermore, additionally to the method below, eDNA filters were fixed with 2mL of ethanol to
138 accommodate for the longer storage and transport time between the field and the laboratory. All sampled
139 locations are part of an extensive monitoring programme for white-clawed crayfish population studies
140 and due to conservation reasons, locations of sites are not reported. The water temperature,
141 environmental variables and the volume of sample filtered for eDNA samples (varied due to variable
142 turbidity) were recorded at each site.

143 ***Comparison of eDNA sampling methodologies in mesocosms***

144 Further, our aim was to assess the impact of eDNA sampling methodology on both the probability of eDNA
145 detection and the signal strength (i.e. Ct) of its detection. We tested differences between the most
146 common eDNA sampling methods utilised to date, including (i) ethanol precipitation (Biggs et al., 2015),
147 (ii) 2µm pump-based filtration (Strand et al., 2014), (iii) 0.45µm pressure filtration and (iv) 0.22µm

148 pressure filtration (Spens et al., 2017). All methods were assessed in two mesocosms, housed at Bristol
149 Zoological Gardens, Bristol, UK during autumn 2018. Both mesocosms were designed to the same
150 specifications but contained different water volumes and crayfish numbers. Mesocosm 1 had a volume of
151 3000L and contained 249 individual adult white-clawed crayfish and sub-adults (between 17 months and
152 four years old) with a total biomass of 1.3kg. Water parameters of mesocosm 1 were; pH 8, temperature
153 11°C, and under natural light conditions. Mesocosm 2 contained a volume of 1000L of water with the
154 same pH (8) but with higher water temperatures (16°C) and under artificial light conditions. This
155 mesocosm held a larger number of crayfish (379) but all were juvenile (five months old). The sensitivity of
156 juveniles to handling did not allow us to obtain the exact biomass of this mesocosm, but biomass was
157 estimated as 250g. Both mesocosms were set up as recirculating 'flow through filtration systems',
158 ensuring high water quality at all times. Six samples for each method and mesocosm combination were
159 collected from both mesocosms.

160 In brief; eDNA samples classified hereafter as 'precipitation' samples were collected following the protocol
161 outlined in Biggs et al. (2014). 1L of water (20 x 50mL subsamples) was collected from ~20cm below the
162 surface and after homogenization, a subsample of 90 mL (6x 15mL) was aliquoted into sterile tubes
163 containing a pre-mixed buffer solution (Biggs et al., 2014). Samples were stored at -20$^{o}$C prior to extraction
164 and extracted following Tréguier et al. (2014). For batch of samples analysed, negative extraction controls
165 were included and processed analogously to other samples.

166 eDNA samples taken with a 2µm pump-based filtration consisted of 2L of water collected by the same
167 sub-sample method outlined above but were then filtered through a Millipore Glass fibre filter AP25,
168 47mm (2µm pore size) using a peristaltic pump (Masterflex E/S Portable Sampler, Cole-Parmer, USA). The
169 filter was housed in an In-Line Filter Holder 47mm (Merck) connected by silicone tubing. The combined
170 use of a peristaltic pump and a larger filter pore size allowed us to substantially increase the amount of

171 water filtered. The filter was then removed from the pump system and stored at -20°C before extraction.
172 Equipment was soaked and cleaned with 10% bleach between samples. Filters were extracted following
173 Spens et al. (2017). eDNA sample collections for 0.22µm and 0.45µm pressure filtration were undertaken
174 in the same manner. 20 sub-samples were collected and a 50mL syringe (BD Plastipak™, Ireland) was then
175 used to pressure filter 250mL of water through a sterile enclosed filter (Sterivex™, Merck®, Germany) with
176 either a pore size of 0.22µm (Polyethersulfone membrane) or 0.45µm (Polyvinylidene fluoride
177 membrane). All filters were stored at -20°C, and extracted following Spens et al. (2017).

178 ***In-situ comparison of eDNA sampling methodologies***

179 Complementary to the tests in the mesocosm experiment, we also evaluated sampling methodologies
180 under natural conditions. However, we performed only pairwise method comparisons in order to contain
181 sampling effort in the field. As a test in a lentic system, eDNA samples were collected from a 1000m$^2$ pond
182 in the South West of England after the release of 40 white-clawed crayfish individuals (equal juvenile-
183 adult and male-female ratios, total biomass of 436g). Here, precipitation (sample volume: 90mL) was
184 compared against 0.22µm pressure filtration (sample volume: 250mL). Sampling started on the 20$^{th}$ April
185 2018 and was repeated two hours, seven days, 14 days and 35 days after crayfish release. At each
186 sampling time, three field replicates were taken from four sites around the pond for each method.
187 Additionally, 20 50mL sub-samples taken from the entire pond perimeter were pooled, homogenised and
188 sampled with three field replicates per method.

189 Our second field test was conducted in a lotic system. We sampled 10 sites (situated approx. 1km apart)
190 along a chalk stream river in Dorset (UK), during September 2017, and 4 sites along a river in Derbyshire
191 (UK). Here, ethanol precipitation was used in comparison to pump-based filtration (2µm, sample volume:
192 2L), using three field replicates at each site per method ($n$ = 42). Samples collected in the river system (20

193 pooled sub-samples as described above) were taken in an interval of 1-2m along a diagonal downstream-
194 to-upstream transect across the river. In this field test, we also assessed the ability to screen for crayfish
195 plague using both sampling methods. qPCRs in this instance were run using the primers and probe
196 developed by Strand et al. (2014). For all of the field method comparisons, bottled distilled water was
197 sampled on site and processed as field negative controls.

198 **Field test of white-clawed crayfish, signal crayfish and crayfish plague co-existence**

199 Finally, we assessed the distribution of white-clawed crayfish, signal crayfish and crayfish plague in a river-
200 system in Derbyshire (UK). Two field replicates were taken at each of eight sites along the river in
201 November 2017. Six of these sites were located in proximity to the inflow of tributaries and two field
202 replicates were taken upstream and downstream of their confluence to capture the influence of
203 populations potentially present within tributaries (supplementary information, Fig. S1). The other two
204 sites were sampled with two field replicates. Sampling was conducted using the precipitation method
205 outlined above and water samples were tested for the occurrence of all three species. Protocols of
206 Mauvisseau *et al.*, (2018) for signal crayfish and of Strand *et al.*, (2014) for crayfish plague were applied.

207 ***Statistical Analysis***

208 Samples measured for the establishment of a standard curve were analysed using a linear regression to
209 evaluate the relationship between DNA dilution and Ct. A log-log data transformation decreased the
210 models Akaike Information Criterion (AIC; reduction by 23 units compared to second best model) and was
211 therefore used for downstream analyses. Residuals were tested for autocorrelation, normal distribution
212 and any remaining patterns (same procedure applied in all regression analyses). A logistic regression
213 analysis was also applied to test the relationship between DNA concentration and binomial detection data
214 assessing the change of detection probability with DNA concentrations. For the mesocosm and field

215 samples, the relationship between (i) the population density established by traditional sampling methods
216 and (ii) the Ct values and detection probability (calculated as the fraction of technical replicates that
217 resulted in positive detection) of eDNA measurements were examined in a linear regression model.
218 Differences in sample volumes between locations (due to turbidity) were accounted for by including
219 sample volume as a predictor in regression models, and log-log and untransformed models were
220 compared using AIC. Further, Ct and detection probability of different sampling methods were compared
221 using ANOVA analyses followed by Tukey's HSD post-hoc tests, and t-tests or nested ANOVA's (lotic and
222 lentic systems, where only two methods were compared). Prior to ANOVAs, heteroscedasticity was
223 evaluated, and data transformed if necessary. Finally, the co-existence of white-clawed crayfish with
224 signal crayfish and crayfish plague was tested in regression models using detection probability of all three
225 species. All described statistical analyses were performed using R version 3.4.1 (R Core Team (2017).

226 **Results**

227 ***Assay development and* in-silico *and* in-vitro *validation***

228 Primers and probe were highly species-specific as *in-silico* and *in-vitro* tests did not reveal any matches
229 with non-target species (Table S3). Analysis of the standard curve (Fig. 1A) revealed a strong dependency
230 of Ct values on DNA concentrations (y=-1.73x+20.8, $p<0.001$, $r^2= 0.996$). Likewise, the detection
231 probability was also positively related to DNA concentration in the sample (y=-0.18x+1.39, $p=0.0016$,
232 $r^2=0.804$; Fig. 1B), highlighting the possibility of a quantifiable assay being developed. Method sensitivity
233 analyses revealed a LOD of $7.9 \times 10^{-5}$ng and a LOQ of $7.9 \times 10^{-4}$ng crayfish DNA extract per $\mu L^{-1}$.

234 ***In-situ validation***

235 Populations of white-clawed crayfish were found in five out of the six surveyed sites using traditional
236 survey methods. eDNA-based detection indicates the presence of white-clawed crayfish in all six sites,

237 though the site with no visual white-clawed crayfish sightings was characterised by a very low detection
238 probability. The Ct values from the six river sites were converted into DNA concentrations using the
239 calibration curve, which allowed us to compare the relationship between detection probability and DNA
240 concentration in laboratory and field samples (Fig. 1B). Four out of the six field sites lay outside of the 95%
241 confidence interval of the standard curve, indicating systematic differences between *in-vitro* validation
242 and field samples. As a direct result of limited recapture data, no recapture was observed in three out of
243 the six survey locations despite indication of the presence of a significantly sized population. Estimates of
244 population sites were therefore unable to be reliably made using the Lincoln-Petersen approach. The
245 relationship between the mean number of crayfish detected across all sampling sates was therefore
246 applied instead. The relationship of the mean number of crayfish detecting using conventional species
247 survey methods (torching) and detection probability of eDNA measurements (Fig. 1D) was significant, but
248 only when water temperature was included ($y=0.0118x_1-0.117x_2+1.77$; $x_1$=mean survey count,
249 $x_2$=temperature, $p=0.035$, $r^2=0.82$, best model resulted also in reduction of AIC by 10 units), with two out
250 of the six datapoints lying outside of the confidence interval. The relationship between Ct and the mean
251 number of crayfish detected using torching was marginally non-significant but showed a reasonable
252 model fit (Fig. 1C; $y=-0.00067\log(x)+3.76$, $p=0.079$, $r^2=0.47$, best model resulted in reduction of AIC by 5
253 units). Differences in filtered sample volume did not significantly influence results.

254 ***Comparison of eDNA sampling methods***

255 In mesocosm experiments, sampling methodology had a significant impact on detection probability
256 (ANOVA $F_{(3,44)}=74.48$, $p<0.001$). Pairwise comparisons revealed that detection probabilities of all three
257 filtration-based methods (2µm, 0.22µm and 0.45µm) were comparable ($p>0.05$) but differed significantly
258 from the precipitation method ($p<0.001$, Fig. 2A). However, the *p*-value for the comparison between
259 0.45µm and 2µm was marginally non-significant ($p=0.051$). Similarly, methodologies also differed

260 significantly in Ct (ANOVA $F_{(3,178)}$=90.1, $p$<0.001). However, in contrast to detection probability, pairwise
261 tests indicated a difference between the 2µm filtration method and all the other approaches (p<0.001;
262 Fig. 2B; only samples with positive detection were included in the analysis).

263 *In-situ* comparisons of sampling methods in a lentic system were highly comparable to the mesocosm
264 experiment (Fig. 3 A-B). The precipitation method showed a significantly lower detection probability (T-
265 test, t=3.55, df=75.37, $p$<0.001) and a significantly higher Ct (t=-2.46, df=15.72, $p$<0.05) than the filtration-
266 based method (0.22µm). However, contrasting results were attained in lotic systems. Here, we assessed
267 the method for both, white-clawed crayfish and the crayfish plague (not present in mesocosms or ponds).
268 The detection probability of crayfish plague mirrored findings from other systems showing significantly
269 higher detection probabilities for the 2µm filtration method (nested ANOVA; $F_{(1,69)}$=4.92, p<0.05; Fig. 3E).
270 Ct values were not significantly different, but also indicated a better performance of the filtration-based
271 method (Fig. 3F). However, the results for white-clawed crayfish contrasted all other results. In lentic
272 systems, precipitation resulted in a higher detection probability (nested ANOVA $F_{(1,69)}$=13.77, $p$<0.001, Fig.
273 3C) and accordingly, lower Ct values (nested ANOVA; $F_{(1,34)}$=5.24, $p$=0.028; Fig. 3D). Consequently,
274 filtration-based methods performed consistently better except in lentic systems where eDNA from white-
275 clawed crayfish was more reliably assessed with the precipitation method.

276 **Field tests of species co-occurrence**

277 Finally, our joint assessment of white-clawed crayfish, signal crayfish and crayfish plague (Fig. 4)
278 demonstrated that white-clawed crayfish occurrence was related to both other species (Fig. 4, B,C). Whilst
279 univariate regressions were marginally non-significant (dependency of white-clawed crayfish on signal
280 crayfish: $p$=0.063; dependency of white-clawed crayfish on crayfish plague: $p$= 0.051), a multiple
281 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

282 $x_2$ represent detection probabilities of white-clawed crayfish, signal crayfish and crayfish plague,
283 respectively). Unsurprisingly, white-clawed crayfish was negatively impacted by the presence of signal
284 crayfish (Fig. 4B), yet contrary to expectation they were shown to be positively related with increase in
285 detection of crayfish plague (Fig. 4C). There was no apparent correlation between signal crayfish and
286 plague (Fig. 4D).

287 **Discussion**

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

297 Field comparisons indicated a higher sensitivity of the eDNA assay compared to conventional surveys,
298 which only resulted in positive detection in five out of six sites. Such traditional surveys are reported to
299 vary in detection success (Gladman et al., 2010), therefore making comparisons between eDNA and these
300 methods challenging. Additionally, the two UK lotic river systems used to compare different sampling
301 approaches were since conventionally surveyed for the presence of crayfish, with no white-clawed
302 crayfish found, despite previous recorded presence, suggesting a higher sensitivity of the eDNA assay.
303 Whilst higher sensitivity is frequently reported for eDNA assays (Dejean et al., 2012; Smart et al., 2015),

304 and therefore likely represent a more reliable species detection approach, such results should be
305 interpreted with caution as eDNA-based approaches are associated with a risk of providing false positive
306 results (Furlan et al., 2016), for example via downstream transport of eDNA within river networks (Pont
307 et al., 2018). Moreover, false positives may result from historic eDNA, which is still present after the
308 extinction or emigration of the target species (Turner et al., 2015). In our case, this represents a valid
309 hypothesis as all field sites were populated by white-clawed crayfish a year before our field surveys (C.
310 Mauvisseau, personal communication). Consequently, it remains inconclusive whether the developed
311 eDNA approach truly has a higher sensitivity (i.e. false negative of torching method) or, in fact, white-
312 clawed crayfish was not present at the field site in question.

313 Further, an important component of our method validation was the comparison of different field sampling
314 approaches. Precipitation and filtration protocols to concentrate eDNA from the environment have
315 already been compared in a number of studies (Deiner et al., 2015; Spens et al., 2017). Most investigators
316 endorse filtration approaches (Hinlo et al., 2017b; Spens et al., 2017; Vörös et al., 2017), but optimal pore
317 size may differ between species (Spens et al., 2017). Moreover, method choice can also be environment-
318 dependant. For example, Eichmiller, et al. (2016) indicated filtration as the optimal eDNA-based method
319 for surveying the common carp, *Cyprinus carpio*. In contrast, Minamoto et al. (2016) highlighted
320 precipitation performed better – a result likely brought about by variations in the environment across
321 both studies. In our controlled mesocosm comparison, we found that a 2 µm filtration approach
322 outperformed precipitation and the other filtration methods tested. However, field comparisons revealed
323 contrasting results, again likely brought about by the different environments surveyed (Fig. 3). In this
324 scenario precipitation outperformed filtration (2 µm) in the lotic system.

325 One possible explanation for our divergent findings across different habitats is that target eDNA particles
326 differ in these environments. eDNA is exposed to continuous degradation through biotic (e.g. bacteria)

327 and abiotic (e.g. UV) factors (Strickler et al., 2015) and these degradation processes can affect eDNA
328 particle size distributions. Filtration has the advantage to collate eDNA from larger water volumes but is
329 linked to the risk of losing particles which are below filter pore sizes. Hence, the habitat-specific
330 differences in our method comparisons may be explained by the specific degradation processes within
331 the investigated river systems. A decrease of average eDNA particle size below the filter pore size would
332 substantially decrease detection probability of filtration approaches and explain our findings.

333 An alternative explanation for our results is linked to inhibition of eDNA amplification. Inhibitor
334 compounds (that interfere with qPCR processes), have been shown to affect target DNA amplification in
335 a non-linear way (Goldberg et al., 2016). If inhibitor concentration is low, amplification will not be strongly
336 impacted. However, if concentrations surpass a certain threshold, inhibitors may suppress the
337 amplification of even high concentrations of target eDNA (Mauvisseau et al., 2019a). Sampling methods
338 that differ in their water collection volumes and in the amount of concentrated target eDNA, will also
339 concentrate inhibitors to different degrees (Fig. 5). Consequently, sampling methods that reach higher
340 target eDNA concentrations may show a lower overall performance due to the non-linear relationship
341 between inhibitor concentrations and DNA amplification. This scenario will occur when inhibitors are
342 present in high concentrations and efficiently concentrated. Therefore, different ratios between target
343 eDNA and inhibitors in different environments can cause a shift in the relative performance of sampling
344 methods across habitats (Fig. 5). In our case, we did not include tests for inhibition, which include the
345 addition of synthetic DNA to qPCR reactions (i.e. failure to detect synthetic DNA indicates inhibition;
346 (Goldberg et al., 2016; Mauvisseau et al., 2019a). However, we recommend that such inhibition tests
347 should be included in future field method comparisons.

348 Both inhibition and different target-eDNA size distributions might also explain differences in method
349 comparisons between species in the same environment as observed for white-clawed crayfish and

350 crayfish plague in lotic habitats (Fig. 3). A fundamental distinction between the two species is that crayfish
351 plague depends for its proliferation on the frequent and abundant release of encapsulated spores (~8 µm
352 in diameter). It seems likely that these spores, which are designed for transport along large distances, will
353 show lower sensitivity to degradation than white-clawed crayfish DNA, which potentially could explain
354 our species-specific results.

355 Finally, we demonstrated that our approach can also be used for investigating ecological relationships
356 determining the distribution of endangered species. Our simultaneous assessments of white-clawed
357 crayfish, signal crayfish and crayfish plague revealed a negative impact of signal crayfish on white-clawed
358 crayfish (Fig. 4). Such negative impacts of invasive competitors on native crayfish species have been
359 frequently highlighted before (Holdich et al., 2009) and demonstrate the applicability of our approach.
360 Interestingly, however, we illustrated a positive relationship between white-clawed crayfish and crayfish
361 plague, which went against our expectation. Such co-occurrence might result from the one-time nature
362 of our sampling approach and reflect a disease outbreak within the crayfish population, which most
363 probably would result in local extinction (Strand et al., 2019). However, recent discoveries have also
364 indicated the potential of plague resistance in some white-clawed crayfish strains (Martín-Torrijos et al.,
365 2017). Such increased disease tolerance might facilitate a permanent co-existence of pathogen and host.
366 Consequently, further in-depth monitoring of species dynamics together with genetic profiling and
367 disease susceptibility tests should be a primary objective of future conservation planning.

368 **Conclusions**

369 Currently, many species-specific eDNA assays only cover *in-silico*, *in-vitro* and sometimes basic *in-situ*
370 validation steps (Dickie et al., 2018; Lacoursière-Roussel et al., 2016). Already published white-clawed
371 crayfish eDNA assays have shown some promising first results but yet need to go through the required

372 thorough level of *in-situ* evaluation (Atkinson et al., 2019; Robinson et al., 2018). Here we illustrate that
373 sampling methods can differ strongly in performance and recommend rigorous testing of eDNA assays to
374 optimise sampling strategies. However, our contrasting results of method comparisons across habitats
375 and species highlight that there might not be something like a universal 'optimal sampling method', but
376 that adjustments to account for local conditions are required. The resulting higher method reliability
377 increases the applicability of eDNA assays and paves the way for more detailed ecological studies to
378 improve species management and conservation.

379

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386

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## 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.

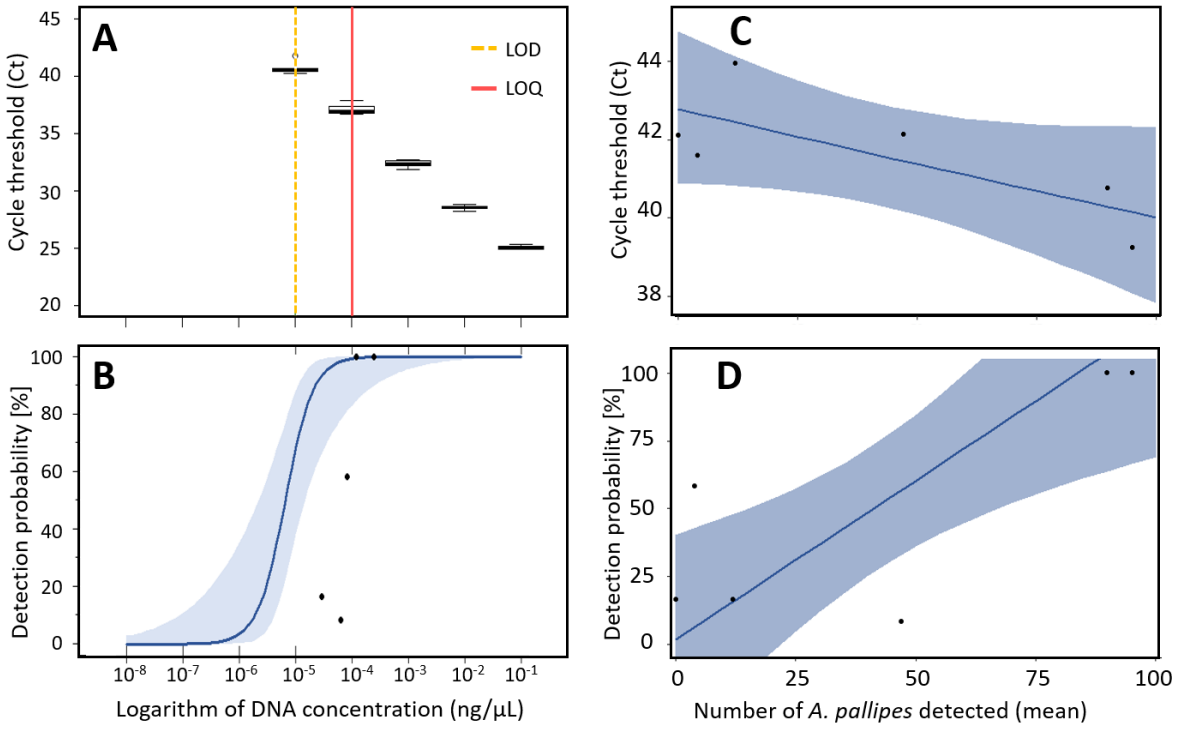


**Figure 1. (A)** Relationship between cycle threshold (Ct) and DNA concentration from White-clawed crayfish qPCR calibration curve. Limit of detection (LOD) and limit of quantification (LOQ) are illustrated by vertical lines (dashed-yellow and red respectively). **(B)** Change in detection probability 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.

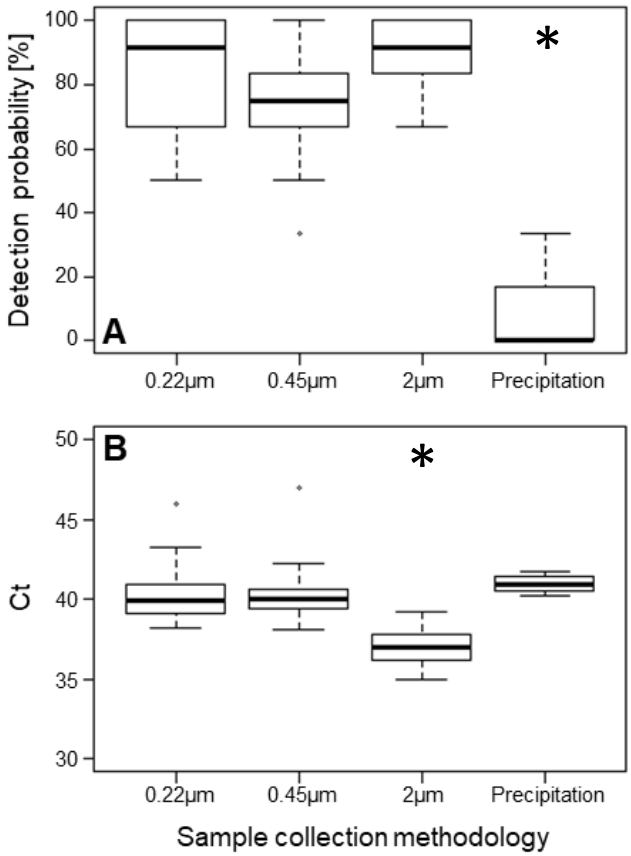


**Figure 2.** Comparison of the detection probability **(A)** and Ct values **(B)** of white-clawed crayfish using different eDNA sampling methods (0.22µm filtration, 0.45µm filtration, 2µm filtration and precipitation) in a controlled mesocosm experiment (* indicates statistical significance).

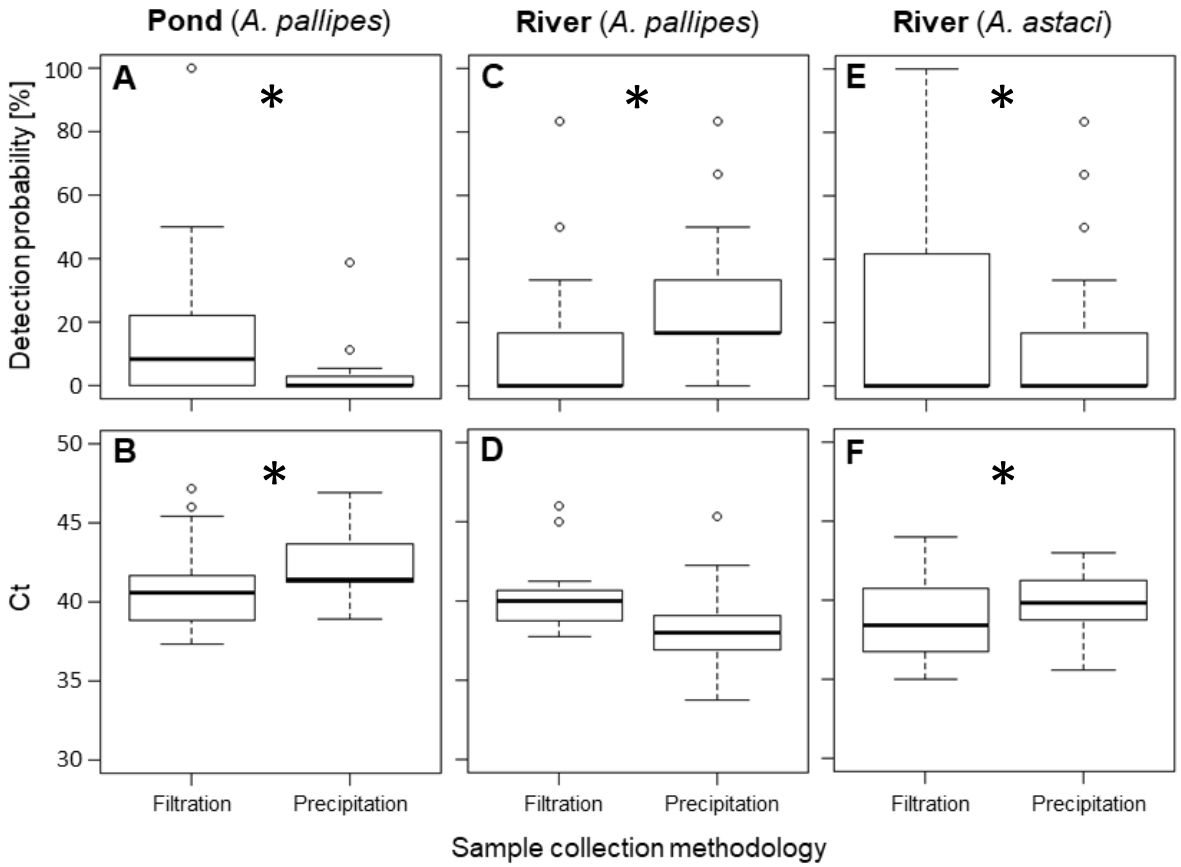


**Figure 3.** Comparison of the detection probability **(A, C, E)** and Ct values **(B, D, F)** of different eDNA sampling methods (filtration and precipitation) for white-clawed crayfish in a lentic system **(Pond, A-B)** (filter pore size 0.22 µm) and for both white-clawed crayfish **(River, C-D)** and crayfish plague **(River, E-F)** in the same lotic system (filter pore size 2 µm) (* in panels signifies significant differences between pairwise method).

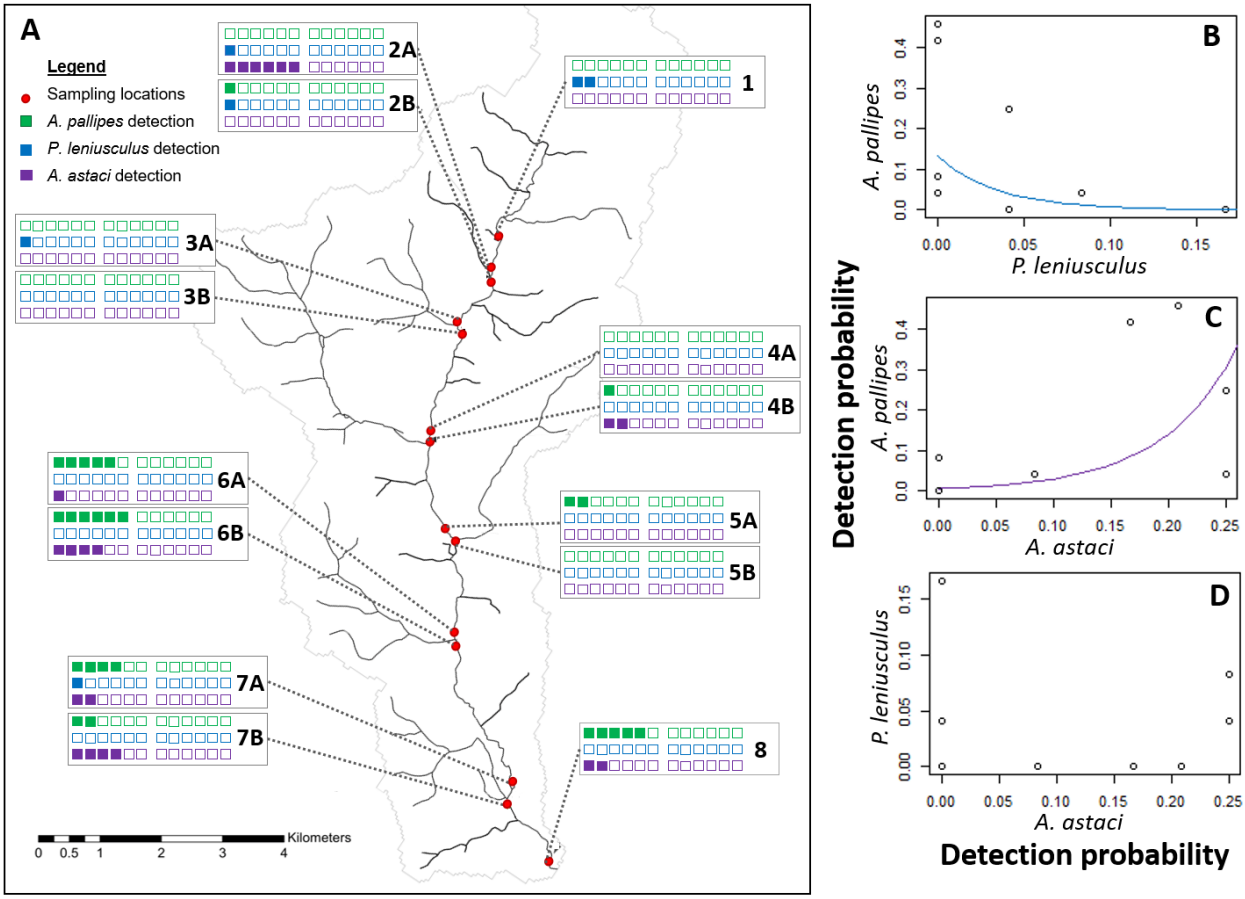


**Figure 4. (A)** Detection of eDNA from white-clawed crayfish (green squares), signal crayfish (blue squares) and crayfish plague. (purple squares) in a river catchment in Derbyshire. Eight locations were sampled and are represented by red dots. The empty squares represent the negative qPCR replicates. **(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.

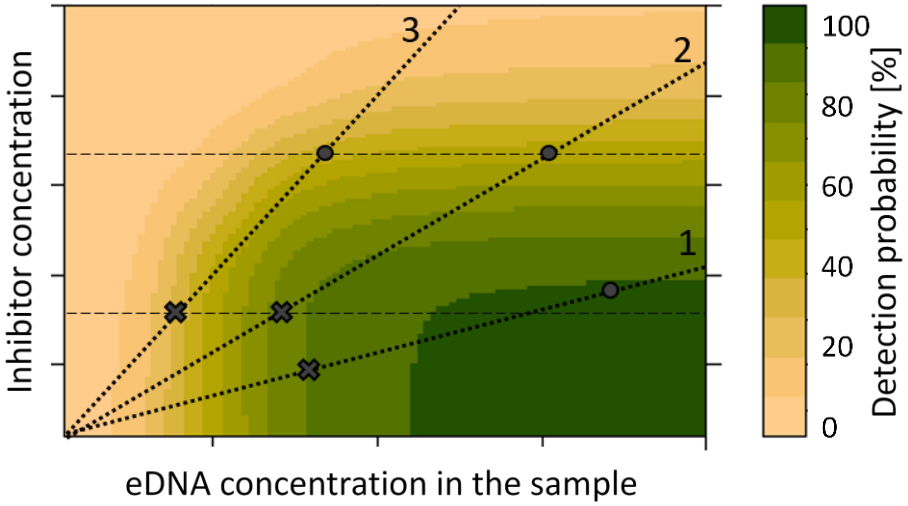


**Figure 5.** Schematic of the co-dependency of detection probability on target eDNA and inhibitors concentrations in water samples. Detection probability increases with eDNA concentration and decreases with inhibitor concentrations but is low when both variables are high. Each water body is characterised 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).