Field application of an eDNA assay for the threatened white-clawed crayfish *Austropotamobius pallipes*

Siobhán Atkinson[1,2*], Jeanette E.L. Carlsson[2], Bernard Ball[2], Mary Kelly-Quinn[1], Jens Carlsson[2]

1. School of Biology and Environmental Science/Earth Institute, University College Dublin, Dublin, Ireland
2. Area52 Research Group, School of Biology and Environmental Science/Earth Institute, University College Dublin, Dublin, Ireland

*Corresponding author: Siobhán Atkinson, siobhan.atkinson@ucdconnect.ie

Abstract

The white-clawed crayfish *Austropotamobius pallipes* has undergone extensive declines within its native range in the last century. Because of its threatened status, European legislation requires the species to be regularly monitored and that Special Areas of Conservation (SACs) be designated for it. Knowledge of the distribution of this species is vital for addressing these needs. This study presents an environmental (e)DNA assay to detect *A. pallipes* in water samples, based on the mitochondrial cytochrome oxidase I (COI) gene, utilizing species-specific primers, a minor groove binding (MGB) probe and quantitative PCR. The results of this study indicate that eDNA is an effective tool for detecting *A. pallipes* in a lotic system, and could provide a valuable, non-invasive method for determining the distribution of this species.

Keywords: eDNA, qPCR, conservation, non-invasive, detection, native species, river.

Introduction

The white-clawed crayfish *Austropotamobius pallipes* is a relatively large, long-lived (>10 years) crustacean that inhabits both rivers and lakes (Reynolds et al. 2010). It requires alkaline conditions for survival and is commonly found in waterbodies overlying carboniferous limestone bedrock (Lucey and McGarrigle 1987). *A. pallipe*s is one of the five indigenous crayfish species in Europe (Holdich et al. 2009). This once abundant species has, however, become greatly reduced or locally extinct across large parts of its native range during the last century (Grandjean et al. 1997). Pollution (Demers and Reynolds 2002, Lyons and Kelly-Quinn 2003), habitat loss and disease (Matthews and Reynolds 1992) have contribution to this decline. Of particular concern is the crayfish plague, caused by the fungus *Aphanomyces astaci* (Holdich et al. 2009). *A. pallipes* possesses no resistance to this

fungus, so eradication of entire populations of *A. pallipes* is possible following an outbreak (Reynolds et al. 2010). Despite this, Ireland is considered one of the few remaining strongholds for *A. pallipes* within Europe. One of the reasons for this is that the invasive signal crayfish *Pacifastacus leniusculus,* which can impose wide-ranging impacts on ecosystems and act as a vector for the crayfish plague (Vaeßen and Hollert 2015), has not been reported in Ireland to date. However, crayfish plague has reached Ireland's rivers, and large changes in the distribution of *A. pallipes* have been attributed, at least in part, to past plague outbreaks (Demers et al. 2005). Additionally, there is at present an outbreak of crayfish plague in Ireland (National Biodiversity Data Centre 2018), and hence there is a pressing need for a non-invasive, rapid and cost-effective sampling method that can be used to establish the distribution of the species.

Because of its vital role in freshwater ecosystem functioning and its threatened status (Matthews and Reynolds 1992), *A. pallipes* is afforded protection under the EU Habitats Directive and is listed under Annexes II and V. This means Ireland is required to regularly monitor *A. pallipes*, and to designate Special Areas of Conservation (SACs) for the species under Natura 2000 (Reynolds et al. 2010). Knowledge of the geographic distribution of *A. pallipes* is essential for implementing these conservation measures. Traditional survey methods include night viewing with a strong torch, modified quadrat samplers (DiStefano et al. 2003), kick-sampling, baited traps and enclosures (Byrne and Lynch 1999), snorkeling surveys (Reynolds et al. 2010) and SCUBA diving (Matthews and Reynolds 1992). While many of these methods are effective, they are time-consuming, potentially costly, and may not be effective when *A. pallipes* occur in low abundance. Furthermore, a license is required for most traditional *A. pallipes* surveying methods. This is vital for ensuring the protection of *A. pallipes*, however, a non-invasive methodology that does not require a license for assessing

57 its distribution would be beneficial. A promising new tool that has been shown to be both
58 effective at detecting species and to save significant time in the field (Sigsgaard et al. 2015) is
59 environmental DNA (eDNA) analysis. Environmental DNA is the collective term for DNA
60 present freely in the environment that has been shed by organisms for example in the form
61 of mucus, excrement, gametes, or blood (Taberlet et al. 2012, Thomsen and Willerslev
62 2015). The analysis has been successful in detecting species that occur in low abundance
63 such as rare or endangered species (Mächler et al. 2014, Laramie et al. 2015, Sigsgaard et al.
64 2015, Boothroyd et al. 2016, Carlsson et al. 2017), or invasive alien species (Jerde et al. 2011,
65 Goldberg et al. 2013, Fujiwara et al. 2016). Numerous studies have also shown eDNA to be
66 more sensitive than traditional methods at detecting species at low abundance (Dejean et al.
67 2012, Smart et al. 2015, Dougherty et al. 2016). Environmental DNA assays have been
68 successfully deployed for other crayfish species including *Astacus astacus*, *Astacus*
69 *leptodactylus*, *P. leniusculus* (Agersnap et al. 2017, Larson et al. 2017), *Orconectes rusticus*
70 (Dougherty et al. 2016, Larson et al. 2017), *Procambarus clarkii* (Cai et al. 2017) and *Faxonius*
71 *eupunctus* (Rice et al. 2018).

72 The aim of this study was to develop an MGB based qPCR assay to detect the presence of
73 the white-clawed crayfish, *A. pallipes* in water samples, and to test the reliability of the assay
74 by comparing the results with field observation data.

75 **Methods**

76 *Study sites and crayfish distribution data*

77 Eight sampling locations within seven different rivers were selected for field validation of the
78 assay (Table 1). The research presented here was carried out as part of a larger study
79 assessing the impact of river obstacles on freshwater fauna (e.g. Atlantic salmon, *Salmo*

80 *salar*, Atkinson et al. 2018). As part of this study, eDNA samples were collected above and
81 below a number of river obstacles (weir, ford crossing or bridge apron) in order to determine
82 whether they imposed migratory barriers on *S. salar*. The same samples collected for this *S.*
83 *salar* study were used for the present study. Out of the eight sites sampled, four were in
84 rivers with SAC status in which *A. pallipes* featured as a species of interest. Study rivers
85 within the River Barrow and River Nore SAC included the Delour and Dinin rivers and study
86 rivers within the Lower River Suir SAC included the Multeen and Duag rivers. Although the
87 Burren River is a tributary of the River Barrow, it does not have SAC status.

88 The current distribution of *A. pallipes* in these rivers was extrapolated using data provided
89 by the Irish Environmental Protection Agency (EPA). These data are point data based on
90 casual field observations made of *A. pallipes* by EPA staff whilst undertaking routine
91 biological monitoring between the years 2007 and 2016 (W. Trodd, Irish Environmental
92 Protection Agency, personal communication). In addition, it was possible at some sites to
93 confirm the presence of crayfish by field observations made while electrofishing for *S. salar*.
94 For the purposes of this study, *A. pallipes* observations were extrapolated to the wider
95 subcatchment level. This meant that even if only one field observation of *A. pallipes*
96 occurred in a subcatchment, it was assumed that there were likely to be *A. pallipes* in the
97 rest of that subcatchment. This was considered the least ambigious way to present the data,
98 because the EPA were not specifically looking for *A. pallipes* when their observations were
99 made, so there is no certainty that *A. pallipes* did not occur in other parts of the
100 subcatchment.

101 *eDNA qPCR assay development*

102 Species-specific primers (forward primer: 5'- GGG TTA GTG GAG AGA GGG GT -3', and
103 reverse primer 5'- AAT CCC CAG ATC CAC AGA CG -3') and a 5'-6-FAM labelled TaqMan®
104 minor groove binding probe (5'- TCA GCT ATT GCC CAC GCA -3') for *A. pallipes*, which
105 targeted a locus within the mitochondrial cytochrome oxidase I (COI) region, were designed
106 in Primer Express 3.0 (Applied Biosystems-Roche, Branchburg, NJ) using the available Irish *A.*
107 *pallipes* COI sequences in the National Centre for Biotechnology Information (NCBI -
108 http://www.ncbi.nlm.nih.gov/). Including primers, the total amplicon size was 96 base pairs.
109 To verify the species specificity for the *A. pallipes* assay in silico, probe and primer sequences
110 were combined and queried against the National Centre for Biotechnology Information
111 (NCBI - http://www.ncbi.nlm.nih.gov/) nucleotide database with BLASTn (Basic Local
112 Alignment Search Tool). The BLASTn analyses showed 90% sequence identity for 100% of the
113 assay query only for the target species. The reason for not achieving 100% sequence identity
114 was due to gaps between the forward and reverse primers and the probe. Non-target
115 species showed a maximum of 37% query cover with 100% sequence identity indicating that
116 only the target species *A. pallipes* would bind to all three components of the assay (forward,
117 reverse primers and probe). To verify the species specificity of the assay in vitro, the assay
118 was tested with DNA extracted from the tissue of some freshwater species co-occuring with
119 *A. pallipes*, including brown trout *S. trutta*, sea lamprey *Petromyzon marinus*, Atlantic
120 salmon *S. salar*, allis shad *Alsoa alosa* and twaite shad *Alosa fallax,* by using conventional
121 PCR followed by visualization of amplicons on agarose. The qPCR assay was optimized using
122 tissue extracted from *A. pallipes*.

123 *eDNA collection, filtering and extraction*

Water samples for eDNA analysis were collected in PET bottles from two locations at each study site, above and below the river obstacle. The PET bottles were sterilized before use by soaking them for 30 minutes in 50% sodium hypochlorite solution. Prior to collecting the water samples, the bottles were rinsed at least five times to remove any remaining sodium hypochlorite. Water samples were filtered through disposable filter funnels on site using a peristaltic pump. The total volume filtered was 2 liters. To avoid contamination, water samples were taken in an upstream direction, prior to any individuals entering the river. At each sampling location, three replicate water samples were collected, one from each of the left margin, right margin and the center of the river channel. In addition, one negative field control consisting of ddH_20 was also filtered. This resulted in a total number of six water samples for eDNA analysis and two field controls collected per study site. All water samples were filtered through 47 mm glass microfiber filters (1.5 µm). Filters were placed in 2.0 mL Eppendorf tubes and subsequently stored in a cooler box on ice. The filters were frozen at -20˚C in the laboratory approximately 3-6 hours following collection. River flow conditions were low at each study site at the time of sampling. To reduce contamination risk, all eDNA work was performed in a dedicated Low Copy DNA laboratory. A modified CTAB (cetyltrimethylammonium bromide) protocol (Möller et al. 1992) was employed for eDNA extractions. Briefly, one-half of each filter was transferred to a 2.0 mL Eppendorf tube (the other half of each filter was archived to allow for future validation or queries). A total volume of 750 µL CTAB buffer (100 mM Tris-HCL, 20 mM EDTA, 1.4 M NaCl, 2% CTAB), and 7 µL of Proteinase K (20 mg mL^{-1}) were added to the tube. Samples were then vortexed for 10 seconds followed by incubation at 56˚C for 2 hours, after which 750 µL of phenol/chloroform/isoamyl alcohol (25:25:1 v/v) was added. The contents of the tube were manually mixed for 15 seconds and subsequently centrifuged (11,000 x g, 20 min). A new

148 tube contained 750 µL of chloroform/isoamyl alcohol (24:1 v/v) was prepared, and the
149 aqueous phase was transferred to it. The manual mixing followed by centrifugation steps
150 were repeated, and the aqueous phase was again transferred to a new tube. The DNA was
151 precipitated using one volume of isopropanol alcohol that was added to the aqueous phase
152 and incubated at -20˚C for 1 hour followed by centrifugation (11,000 x g, 20 min). The
153 resulting pellets were washed with 750 µL of 70% ethanol and centrifuged (11,000 x g, 5
154 min). The ethanol was removed, and care was taken to ensure that the pellet remained in
155 the tube. The tubes containing pellets were dried in a heat block (50˚C, 5 min) followed by
156 resuspension in 40 µL of molecular-grade ddH_2O. Environmental DNA concentrations were
157 determined with NanoDrop®-1000, Thermo Scientific, Wilmington, DE.

158 *eDNA assay deployment*

159 An Applied Biosystems ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City,
160 California, U.S.A.) quantitative thermocycler was used for eDNA amplification and
161 quantification. Briefly, the PCR profile consisted of 50°C for 2 min and 95°C for 10 min,
162 followed by 40 cycles between 95°C for 15 s and 60°C for 1 min. Standard curves were
163 generated from DNA originating from *A. pallipes* tissue extracts (quantified with NanoDrop®-
164 1000, Thermo Scientific, Wilmington, DE). This served as both a positive control and as a
165 means of calculating the concentration of *A. pallipes* eDNA in each sample. Seven 10:1 serial
166 dilutions ranging from 2.9 ng μL^{-1} to 2.9 x 10^{-6} ng μL^{-1} were used for the standard curve. Each
167 qPCR reaction was carried out in a total volume of 30 µL, consisting of 15 µL of TaqMan®
168 Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems, Foster City, CA), 3 µL
169 of each primer (final concentration of 2 µM), 3 µL probe (final concentration of 2 µM), 3 µL
170 eDNA/tissue extracted DNA template and 3 µL ddH_2O. Individual standard curves were

171 generated for each qPCR plate (y = -3.37x + 20.589, efficiency = 98.04 %, R^2 = 0.996 (1), y = -
172 3.442x + 20.392, efficiency = 95.22 %, R^2 = 0.999 (2) and y = -3.441x + 20.723, efficiency =
173 95.26 %, R^2 = 0.999 (3)). Each plate incorporated 2 no-template controls (NTCs) to identify
174 any contamination that may have occurred during preparation. Standard curve, field and
175 control samples were quantified in triplicate (3 technical replicates). A positive detection
176 was defined as being when at least 2 out of the 3 technical replicates contained amplifiable
177 DNA with quantitation cycle (Cq) differences not exceeding 0.5 (to allow for pipetting
178 errors), and when the samples were within the detection range of the standard curve. If 1
179 out of 3 technical replicates showed Cq differences exceeding 0.5, the replicate was
180 excluded from the study. In cases when the Cq value of 2 out of 3 technical replicates
181 differed by more than 0.5 Cq, that specific dilution series or field replicate was removed
182 from further study. As *S. trutta* was present in all rivers, both upstream and downstream of
183 the obstacles, this species was used as a positive field control to test for the presence of
184 amplifiable DNA in all samples. The *S. trutta* assay from previously published work
185 (Gustavson et al. 2015) was used for this analysis. Three replicates per location with 1
186 technical replicate were used.

187 **Results and Discussion**

188 The present study was successful in detecting *A. pallipes* in silico, in vitro and in situ. The
189 assay was highly sensitive and could detect *A. pallipes* eDNA concentrations as low as 0.002
190 ng L^{-1} at Cq 38.2 (average over 3 technical replicates, standard deviation 0.000059 ng L^{-1}). No
191 cross-species amplification occurred. Furthermore, zero amplification occurred in the NTCs
192 and field controls. All field samples analysed yielded detectable DNA with the positive field
193 control (*S. trutta* assay), however, this was not the case for the *A. pallipes* assay. *A. pallipes*

194 was only detected in 5 out of 8 sites (Table 2, Fig. 1). Within the Burren, Duag and Multeen
195 rivers, positive detections were observed in all field replicates above and below the river
196 obstacles. At the Dinin (Crettyard) site, positive detection was observed in 2 out of 3 field
197 replicates above the obstacle, and 3 out of 3 field replicates below the obstacle. At the Dinin
198 (Castlecomer) site, positive detection of *A. pallipes* occurred in only 1 field replicate, which
199 was located above the obstacle (Table 2). The detection of *A. pallipes* eDNA above the
200 obstacle but not below it was a surprising result. Intuitively, one would expect that *A.*
201 *pallipes* would have been detected below the obstacle also, as eDNA travels downstream in
202 a river system. It is worth noting that it was at this site that the lowest concentration of *A.*
203 *pallipes* eDNA was detected (0.002 ng L^{-1}), and amplification of eDNA occurred in only 1 out
204 of 6 site replicates. It is possible that eDNA concentrations in the other field samples were
205 too low for a positive detection, or that false negative results were obtained. The density or
206 biomass of *A. pallipes*, environmental conditions such as flow, temperature, pH and
207 sediment, or natural inhibitors such as algae or humic substances, may have contributed to
208 this lack of amplification (Strickler et al. 2015, Goldberg et al. 2016, Stoeckle et al. 2017).
209 However, as all eDNA samples yielded detectable DNA with the positive field control (e.g. *S.*
210 *trutta*), it is unlikely that in this instance the lack of amplification of *A. pallipes* DNA was a
211 result of inhibition of the eDNA samples.

212 When compared with the EPA's *A. pallipes* observation data, the eDNA results reflected
213 what was observed in the field. In all subcatchments where *A. pallipes* was observed by EPA
214 staff, the eDNA results revealed a positive detection, and in all subcatchments where *A.*
215 *pallipes* was not observed by EPA staff, the eDNA results revealed a negative detection (Fig.
216 1). Furthermore, it is worth noting that the eDNA results also reflected the expected
217 distribution of *A. pallipes* based on environmental conditions. In all sites where *A. pallipes*

218 was not detected by either field observation or eDNA (Delour, Brown's Beck Brook and the
219 Dalligan), the dominant bedrock was siliceous. *A. pallipes* are known to occur in waterbodies
220 with underlying calcareous bedrock rather than siliceous bedrock (Lucey and McGarrigle
221 1987).

222 It was not possible in this study to discern the impact of the river obstacles on *A. pallipes*
223 using eDNA. This was because in most sites, *A. pallipes* was either detected both above and
224 below the obstacle, or as observed at the Dinin (Castlecomer) site, above the obstacle only.
225 However, studies where crayfish have been physically marked or tagged have shown that
226 river obstacles can hinder their upstream migration (Kerby et al. 2005, Bubb et al. 2008,
227 Rosewarne et al. 2013). Environmental DNA assays for diadromous species such as sea
228 lamprey *P. marinus* (Gustavson et al. 2015), Atlantic salmon *S. salar* (Atkinson et al. 2018) or
229 Chinook salmon *Oncorhynchus tshawytscha* (Laramie et al. 2015) are likely to be better
230 indicators for assessing the migratory impact of river obstacles. Alternatively, eDNA could be
231 used for monitoring whether invasive species have moved above the barriers designed to
232 block them (Cowart et al. 2018). Nonetheless, the present research shows that eDNA is a
233 reliable tool for detecting *A. pallipes* in river systems. Furthermore, while the focus of this
234 study was in lotic systems, the assay could be readily deployed in lentic systems such as
235 lakes or reservoirs where field sampling may be more demanding. For example, as noted by
236 Reynolds et al. (2010), different survey methods for monitoring *A. pallipes* can vary in their
237 efficiency depending on the habitat type being surveyed. Environmental DNA monitoring
238 may therefore lend itself to surveying more challenging habitats such as heavily vegetated
239 lake margins with soft substrates, or locations where trapping or night searching is not
240 possible or particularly difficult due to health and safety considerations. Environmental DNA
241 sampling requires one site visit, which involves simply collecting water samples, and filtering

242 them onsite. This sampling approach is considerably less labor intensive when compared
243 with traditional methods. Night searches, for example, typically require two site visits (for
244 health and safety purposes) and are dependent on water clarity and algal growth (Reynolds
245 et al. 2010). Trapping is time-consuming and requires two site visits, and snorkeling and
246 hand searches are habitat dependent. Snorkeling is also time-consuming as it requires time
247 to allow for disinfection and drying out of snorkeling gear between entering different
248 waterbodies (Reynolds et al. 2010). Relative to traditional sampling methods, eDNA sampling
249 requires substantially less field equipment. Herder et al. (2014) suggested that the eDNA
250 method may help reduce the risk of unintentional transmission of pathogens into new areas
251 due to the numerous contamination prevention precautions that must be taken when
252 sampling. Considering this, and the continued threat of crayfish plague in Ireland and
253 elsewhere, eDNA sampling may be an appropriate sampling technique to employ to
254 minimize the risk of further spread of crayfish plague by reducing the amount of field
255 equipment required, and therefore potential vectors of crayfish plague. This is an important
256 consideration, especially if surveys are being conducted in waterbodies harboring crayfish
257 plague.

258 To conclude, this assay provides an alternative, rapid method for locating *A. pallipes* in both
259 lentic and lotic ecosystems, allowing for more targeted, efficient surveying strategies.
260 Considering the current crayfish plague outbreak in Ireland (National Biodiversity Data
261 Centre 2018), and indeed other countries where it is present, there is an urgent need for a
262 non-invasive sampling methodology for the species. Large scale deployment of this assay
263 would enable managers to rapidly establish, with confidence, the distribution of *A. pallipes*,
264 which would support the designation of new conservation areas.

265 **Acknowledgements**

266 This research was funded by the Irish Environmental Protection Agency (Reconnect project -
267 2015-W-LS-8). The authors would also like to thank the Irish Environmental Protection
268 Agency for providing the data on *A. pallipes* distributions.

269 **Literature Cited**

270 Agersnap, S., W. B. Larsen, S. W. Knudsen, D. Strand, P. F. Thomsen, M. Hesselsøe, P. B.
271 Mortensen, T. Vrålstad, and P. R. Møller. 2017. Monitoring of noble, signal and narrow-
272 clawed crayfish using environmental DNA from freshwater samples. PLoS ONE 12:1–22.

273 Atkinson, S., J.E.L. Carlsson, B. Ball, D. Egan, M. Kelly-Quinn, K. Whelan, and J. Carlsson. 2018.
274 A quantitative PCR-based environmental DNA assay for detecting Atlantic salmon (*Salmo*
275 *salar* L.). Aquatic Conservation: Marine and Freshwater Ecosystems doi: 10.1002/aqc.2931.

276 Boothroyd, M., N. E. Mandrak, M. Fox, and C. C. Wilson. 2016. Environmental DNA (eDNA)
277 detection and habitat occupancy of threatened spotted gar (*Lepisosteus oculatus*). Aquatic
278 Conservation: Marine and Freshwater Ecosystems 26:1107–1119.

279 Bubb, D. H., T. J. Thom, and M. C. Lucas. 2008. Spatial ecology of the white-clawed crayfish in
280 an upland stream and implications for the conservation of this endangered species. Aquatic
281 Conservation: Marine and Freshwater Ecosystems 18:647–657.

282 Byrne, C., and J. Lynch. 1999. A sampling strategy for stream populations of white-clawed
283 crayfish, *Austropotamobius pallipes* (Lereboullet)(Crustacea, Astacidae). Biology and
284 Environment: Proceedings of the Royal Irish Academy 99b:89–94.

285 Cai, W., Z. Ma, C. Yang, L. Wang, W. Wang, G. Zhao, Y. Geng, and D. W. Yu. 2017. Using eDNA
286 to detect the distribution and density of invasive crayfish in the HongheHani rice terrace

World Heritage site. PLoS ONE 12:1–13.

Carlsson, J. E. L., D. Egan, P. C. Collins, E. D. Farrell, F. Igoe, and J. Carlsson. 2017. A qPCR MGB probe based eDNA assay for European freshwater pearl mussel (*Margaritifera margaritifera* L.). Aquatic Conservation: Marine and Freshwater Ecosystems 27:1341-1344.

Cowart, D. A., K. G. H. Breedveld, M. J. Ellis, J. M. Hull, and E. R. Larson. 2018. Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (Decapoda: Astacidea) through monitoring of invasive species barriers and relocated populations. Journal of Crustacean Biology doi:10.1093/jcbiol/ruy007.

Dejean, T., A. Valentini, C. Miquel, P. Taberlet, E. Bellemain and C. Miaud. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. Journal of Applied Ecology 49:953-959.

Demers, A., J. Lucey, M. L. McGarrigle, and J. D. Reynolds. 2005. The distribution of the white-clawed crayfish, *Austropotamobius pallipes*, in Ireland. Biology & Environment: Proceedings of the Royal Irish Academy 105b:65–69.

Demers, A., and J. D. Reynolds. 2002. A survey of the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet), and of water quality in two catchments of eastern Ireland. Bulletin Francais de Pêche et de Pisciculture 367:729–740.

DiStefano, R. J., C. M. Gale, B. A. Wagner, and R. D. Zweifel. 2003. A sampling method to assess lotic crayfish communities. Journal of Crustacean Biology 23:678–690.

Dougherty, M. M., E. R. Larson, M. A. Renshaw, C. A. Gantz, S. P. Egan, D. M. Erickson, and D. M. Lodge. 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. Journal of Applied Ecology 53:722–732.

309 Fujiwara, A., S. Matsuhashi, H. Doi, S. Yamamoto, and T. Minamoto. 2016. Use of
310 environmental DNA to survey the distribution of an invasive submerged plant in ponds.
311 Freshwater Science 35:748–754.

312 Goldberg, C. S., A. Sepulveda, A. Ray, J. Baumgardt, and L. P. Waits. 2013. Environmental
313 DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus*
314 *antipodarum*). Freshwater Science 32:792–800.

315 Goldberg, C. S., C. R. Turner, K. Deiner, K. E. Klymus, P. F. Thomsen, M. A. Murphy, S. F.
316 Spear, A. McKee, S. J. Oyler-McCance, R. S. Cornman, M. B. Laramie, A. R. Mahon, R. F.
317 Lance, D. S. Pilliod, K. M. Strickler, L. P. Waits, A. K. Fremier, T. Takahara, J. E. Herder and P.
318 Taberlet. 2016. Critical considerations for the application of environmental DNA methods to
319 detect aquatic species. Methods in Ecology and Evolution 7: 1299-1307.

320 Grandjean, F., C. Souty-Grosset, R. Raimond, and D. M. Holdich. 1997. Geographical variation
321 of mitochondrial DNA between populations of the white-clawed crayfish *Austropotamobius*
322 *pallipes*. Freshwater Biology 37:493–501.

323 Gustavson, M. S., P. C. Collins, J. A. Finarelli, D. Egan, R. Ó. Conchúir, G. D. Wightman, J. J.
324 King, D. T. Gauthier, K. Whelan, J. E. L. Carlsson, and J. Carlsson. 2015. An eDNA assay for
325 Irish *Petromyzon marinus* and *Salmo trutta* and field validation in running water. Journal of
326 Fish Biology 87:1254–1262.

327 Herder, J. E., A. Valentini, E. Bellemain, T. Dejean, J. J. C. W. van Delft, P. F. Thomsen, and P.
328 Taberlet. 2014. Environmental DNA—a review of the possible applications for the detection
329 of (invasive) species. Report no. 2013–104. Stichting RAVON, Nijmegen.

330 Holdich, D. M., J. D. Reynolds, C. Souty-Grosset, and P. J. Sibley. 2009. A review of the ever

331 increasing threat to European crayfish from non-indigenous crayfish species. Knowledge and
332 Management of Aquatic Ecosystems 394-395:11.

333 Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. "Sight-unseen" detection
334 of rare aquatic species using environmental DNA. Conservation Letters 4:150–157.

335 Kerby, J. L., S. P. D. Riley, L. B. Kats, and P. Wilson. 2005. Barriers and flow as limiting factors
336 in the spread of an invasive crayfish (*Procambarus clarkii*) in southern California streams.
337 Biological Conservation 126:402–409.

338 Laramie, M. B., D. S. Pilliod, and C. S. Goldberg. 2015. Characterizing the distribution of an
339 endangered salmonid using environmental DNA analysis. Biological Conservation 183:29–37.

340 Larson, E. R., M. A. Renshaw, C. A. Gantz, J. Umek, S. Chandra, D. M. Lodge, and S. P. Egan.
341 2017. Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and
342 *Pacifastacus leniusculus* in large lakes of North America. Hydrobiologia 800:173–185.

343 Lucey, J., and M. L. McGarrigle. 1987. The distribution of the crayfish *Austropotamobius*
344 *pallipes* (Lereboullet) in Ireland. Irish Fisheries Investigations A29:1–13.

345 Lyons, R., and M. Kelly-Quinn. 2003. An investigation into the disappearance of
346 *Austropotamobius pallipes* (Lereboullet) populations in the headwaters of the Nore River,
347 Ireland and the correlation to water quality. Bulletin Français de la Pêche et de la Pisciculture
348 370-371:139–150.

349 Mächler, E., K. Deiner, P. Steinmann, and F. Altermatt. 2014. Utility of environmental DNA
350 for monitoring rare and indicator macroinvertebrate species. Freshwater Science 33:1174–
351 1183.

352 Matthews, M., and J. D. Reynolds. 1992. Ecological impact of crayfish plague in Ireland.

Hydrobiologia 234:1–6.

Möller, E. M., G. Bahnweg, H. Sandermann, and H. H. Geiger. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Research 20:6115–6116.

National Biodiversity Data Centre. 2018. Crayfish plague. (Available from: http://www.biodiversityireland.ie/projects/invasive-species/crayfish-plague/)

Reynolds, J. D., W. O'Connor, C. O'Keeffe, and D. Lynn. 2010. A technical manual for monitoring white-clawed crayfish *Austropotamobius pallipes* in Irish lakes. (Available at: https://www.npws.ie/sites/default/files/publications/pdf/IWM45.pdf)

Rice, C. J., E. R. Larson, and C. A. Taylor. 2018. Environmental DNA detects a rare large river crayfish but with little relation to local abundance. Freshwater Biology 63:443–455.

Rosewarne, P. J., A. T. Piper, R. M. Wright, and A. M. Dunn. 2013. Do low-head riverine structures hinder the spread of invasive crayfish? Case study of signal crayfish (*Pacifastacus leniusculus*) movements at a flow gauging weir. Management of Biological Invasions 4:273–282.

Sigsgaard, E. E., H. Carl, P. R. Møller, and P. F. Thomsen. 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation 183:46–52.

Smart, A.S., R. Tingley, A. R. Weeks, A. R. van Rooyen, and M. A. McCarthy. 2015. Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. Ecological Applications 25:1944-1952.

Stoeckle, B. C., S. Beggel, A. F. Cerwenka, E. Motivans, R. Kuehn and J. Geist. 2017. A

375 systematic approach to evaluate the influence of environmental conditions on eDNA
376 detection success in aquatic ecosystems. PloS one 12: e0189119.

377 Strickler, K.M., A. K. Fremier and C.S. Goldberg. 2015. Quantifying effects of UV-B,
378 temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation
379 183: 85-92.

380 Taberlet, P., E. Coissac, M. Hajibabaei, and L. H. Rieseberg. 2012. Environmental DNA.
381 Molecular Ecology 21:1789–1793.

382 Thomsen, P. F., and E. Willerslev. 2015. Environmental DNA - An emerging tool in
383 conservation for monitoring past and present biodiversity. Biological Conservation 183:4–18.

384 Vaeßen, S., and H. Hollert. 2015. Impacts of the North American signal crayfish (*Pacifastacus*
385 *leniusculus*) on European ecosystems. Environmental Sciences Europe 27:1–6.

386 Table 1. Summary of sampling information.

River	Obstacle Type	Sampling Location	Distance from Obstacle (m)	Latitude	Longitude	Sampling Date
Burren	Weir	Upstream	1	52.83300	-6.92534	18/07/2017
		Downstream	215	52.83388	-6.92822	18/07/2017
Delour	Weir	Upstream	193	52.97709	-7.54606	19/07/2017
		Downstream	267	52.97957	-7.54100	19/07/2017
Nore (Castlecomer)	Weir	Upstream	11	52.80599	-7.20527	20/07/2017
		Downstream	57	52.80535	-7.20558	20/07/2017
Duag	Weir	Upstream	8	52.27196	-8.01319	21/07/2017
		Downstream	45	52.27174	-8.01248	21/07/2017
BBB	Ford	Upstream	1	53.01627	-6.62311	24/07/2017
		Downstream	60	53.01573	-6.62322	24/07/2017
Dalligan	Weir	Upstream	15	52.11876	-7.51938	25/07/2017

		Downstream	92	52.11862	-7.51803	25/07/2017
Muilteen	Bridge Apron	Upstream	101	52.61304	-8.00872	27/07/2017
		Downstream	34	52.61182	-8.00966	27/07/2017
Nore (Crettyard)	Bridge Apron	Upstream	8	52.84534	-7.12758	28/07/2017
		Downstream	68	52.84584	-7.12891	28/07/2017

387

388 Table 2. Environmental DNA concentrations (ng L^{-1}) from the *A. pallipes* assay at each study
389 river. Concentrations are based the average eDNA concentration in a minimum of 2 technical
390 replicates and between 1 and 3 field replicates. Confidence intervals (95%) are given, where
391 possible, for each location (upstream or downstream of the river obstacle).

River	**Location**	**No. eDNA samples collected**	**No. of positive detections**	**eDNA concentration ng L^{-1} (95% CI)**	**Visual observation**
Burren	Upstream	3	3	0.027 (0.01-0.02)	+
	Downstream	3	3	0.02 (0.01-0.04)	
Duag	Upstream	3	3	0.027 (0.005-0.05)	+
	Downstream	3	3	0.157 (0.09-0.23)	
Multeen	Upstream	3	3	0.034 (0.02-0.04)	+
	Downstream	3	3	0.06 (0.04-0.08)	
Dinin (Crettyard)	Upstream	3	2	0.011 (0.004-0.02)	+
	Downstream	3	3	0.006 (0.002-0.01)	
Dinin (Castlecomer)	Upstream	3	1	0.002 [a]	+
	Downstream	3	0	0	
Delour	Upstream	3	0	0	-
	Downstream	3	0	0	
Dalligan	Upstream	3	0	0	-
	Downstream	3	0	0	
Brown's Beck Brook (BBB)	Upstream	3	0	0	-
	Downstream	3	0	0	

392 [a] It was not possible to calculate a 95% confidence interval for this site as a positive detection was

393 only detected in one out of three site replicates

Figure Heading

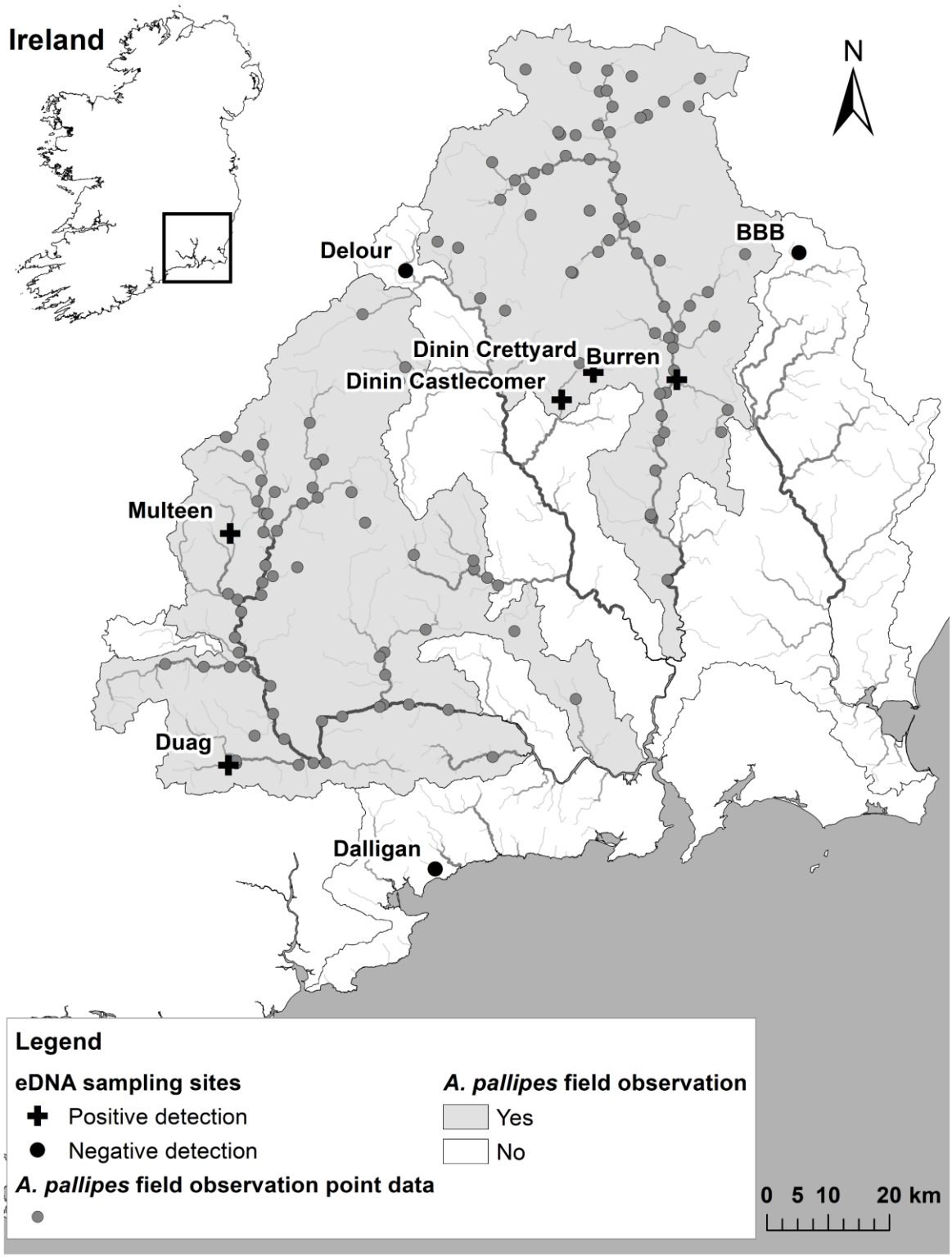

Figure 1. Map showing the sampling locations for the eDNA analysis. Crosses indicate sites where a positive detection of *A. pallipes* was made in at least one field replicate. Circles

398 indicate sites where no positive detection of *A. pallipes* was made in all field replicates. River
399 subcatchments in which field observations of *A. pallipes* were made between the years 2007
400 and 2016 are highlighted in grey. Grey circles indicate the actual locations where field
401 observations of *A. pallipes* were made.