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Targeting the invaders – targeted detection of four priority freshwater invasive non-native species using environmental DNA

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Early detection is paramount for attempts to remove invasive non-native species (INNS). Traditional methods rely on physical sampling and morphological identification, which can be problematic when species are in low densities and/or are cryptic. The use of environmental DNA (eDNA) as a monitoring tool in freshwater systems is becoming increasingly acceptable and widely used for the detection of single species. Here we demonstrate the development and application of standard PCR primers for the detection of four freshwater invasive species which are high priority for monitoring in the UK and elsewhere: Dreissenid mussels; *Dreissena rostriformis bugensis* (Andrusov, 1987) and *D. polymorpha* (Pallas, 1771), and Gammarid shrimps; *Dikerogammarus villosus* (Sowinsky, 1984) and *D. haemobaphes* (Eichwald, 1843). We carried out a rigorous validation process for testing the new primers, including DNA detection and degradation rate experiments in mesocosm, and a field comparison with traditional monitoring protocols. We successfully detected all four target species in mesocosms, but success was higher for mussels than shrimps. eDNA from single individuals of both mussel species could be detected within four hours of the start of the experiment. By contrast, shrimp were only consistently detected at higher densities (20 individuals). In field trials, the two mussel species and *D. haemobaphes* were detected at all sites where the species are known to be present, and eDNA consistently outperformed traditional kick sampling for species detection. However, *D. villosus* eDNA was only detected in one of five sites where the species was confirmed by kick sampling. These results demonstrate the applicability of standard PCR for eDNA detection of freshwater invasive species, but also highlight the importance of differences between taxa in terms of the detection ability.

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16 **Abstract**

17

18 Early detection is paramount for attempts to remove invasive non-native species (INNS).
19 Traditional methods rely on physical sampling and morphological identification, which can be
20 problematic when species are in low densities and/or are cryptic. The use of environmental DNA
21 (eDNA) as a monitoring tool in freshwater systems is becoming increasingly acceptable and
22 widely used for the detection of single species. Here we demonstrate the development and
23 application of standard PCR primers for the detection of four freshwater invasive species which
24 are high priority for monitoring in the UK and elsewhere: Dreissenid mussels; *Dreissena*
25 *rostriformis bugensis* (Andrusov, 1987) and *D. polymorpha* (Pallas, 1771), and Gammarid
26 shrimps; *Dikerogammarus villosus* (Sowinsky, 1984) and *D. haemobaphes* (Eichwald, 1843).
27 We carried out a rigorous validation process for testing the new primers, including DNA
28 detection and degradation experiments in mesocosm, and a field comparison with traditional
29 monitoring protocols. We successfully detected all four target species in mesocosms, but success
30 was higher for mussels than shrimps. eDNA from single individuals of both mussel species could
31 be detected within four hours of the start of the experiment. By contrast, shrimp were only
32 consistently detected at higher densities (20 individuals). In field trials, the two mussel species
33 and *D. haemobaphes* were detected at all sites where the species are known to be present, and
34 eDNA consistently outperformed traditional kick sampling for species detection. However, *D.*
35 *villosus* eDNA was only detected in one of five sites where the species was confirmed by kick
36 sampling. These results demonstrate the applicability of standard PCR for eDNA detection of
37 freshwater invasive species, but also highlight the importance of differences between taxa in
38 terms of the detection ability.

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39 **Introduction**

40

41 The rate of biological invasions has increased rapidly over the last 25 years due, at least in part,
42 to increasing globalisation (Sutherland et al., 2008; Hulme, 2009; Gallardo and Aldridge, 2013a).
43 Concern over the increasing number of invasive non-native species (INNS) has led to a number
44 of horizon scanning studies aimed at identifying and prioritizing the threat of potential INNS
45 (Gallardo and Aldridge, 2011; Gallardo and Aldridge, 2013b Roy et al., 2014). Roy et al., (2014)
46 for example, concluded the potential impact, risk of arrival and risk of establishment of quagga
47 mussels, *Dreissena rostriformis bugensis,* in the UK to be the highest out of 93 species
48 examined. As predicted, the quagga mussel was detected in the UK later the same year (Mills et
49 al., 2017). Quagga mussels are one of a number of Ponto-Caspian INNS that are currently
50 spreading throughout Europe, including the UK. Concerns have been raised about the risk of
51 'invasional meltdown' by Ponto-Caspian species, whereby INNS that have co-evolved in the
52 same region facilitate one another's spread (*sensu* Simberloff and Von Holl, 1999; Gallardo and
53 Aldridge, 2014a).

54

55 With growing pressure from legislators and limited funding to regulators to prevent further
56 introductions of new INNS, efficient and effective monitoring tools are in high demand. Recent
57 and rapid developments in molecular tools have meant a huge surge and investment in the use of
58 DNA methods for biodiversity monitoring, in particular the use of environmental DNA (eDNA)
59 (Lawson Handley, 2015). Environmental DNA refers to the DNA shed by an organism into its
60 environment, such as urine, faeces or sloughed cells (Taberlet et al., 2014; Rees et al., 2014;
61 Bohmann et al., 2014; Lawson Handley, 2015; Valentini et al., 2016). The first study to apply
62 this method to contemporary detection of an invasive species, analysed pond samples using
63 species-specific primers and standard PCR for the detection of American bull frog, *Lithobates*
64 *catesbeiana*. The method outperformed traditional monitoring approaches, producing reliable
65 positive detections even when bullfrogs were present at low densities (Ficetola et al., 2008;
66 Dejean et al., 2012). This case study was revolutionary, and there soon followed a succession of
67 studies utilising eDNA for single species detection of a range of taxa in lentic (e.g. red swamp
68 crayfish, *Procamabrus clarkia*, Tréguier et al., 2014), lotic (e.g. New Zealand mudsnail,

69 *Potamopyrgus antipordarum*, Goldberg et al., 2013) and marine systems (e.g North American
70 wedge clam, *Rangia cuneate*, Ardura et al., 2015).
71
72 Unlike traditional monitoring methods, the successful detection of a species using eDNA does
73 not rely on the collection of specimens. Instead, presence is determined by the detection of target
74 DNA which is shed by the organism. The amount of DNA present in the environment is
75 influenced by a combination of the species' DNA production rate, the degradation rate of the
76 shed DNA, and the transport of DNA within the environment (Barnes et al., 2014; Barnes et al.,
77 2015; Goldberg et al., 2015). The availability of eDNA is therefore highly dependent on the
78 species being studied (Jerde et al., 2011; Thomsen et al., 2012; Pilliod et al., 2013; Treguier et
79 al., 2014; Roussel et al., 2015; Klymus et al., 2015; Jane et al., 2015), and the environment in
80 which they are present (Jane et al., 2015; Jerde et al., 2016; Shogren et al., 2017), and these
81 variables therefore need to be considered during the development of species-specific primers.
82
83 The great majority of targeted eDNA studies have used either standard PCR or probe-based real-
84 time quantitative PCR (qPCR) for single species detection, although droplet digital PCR
85 (ddPCR) is also showing great promise (Nathan et al., 2014; Doi et al., 2015). qPCR is often
86 considered a more desirable approach than PCR due to its increased sensitivity for species
87 detection (Thomsen et al., 2012; Nathan et al., 2014) and, when using a probe-based assay, its
88 added specificity. However, for many applications, the sensitivity of standard PCR may be quite
89 adequate, and PCR may be preferable as it is cheaper and less technically challenging. Moreover,
90 studies that have directly compared the two approaches have indicated that PCR can be more
91 robust to PCR inhibitors than qPCR, which is important for avoiding false negatives (De Ventura
92 et al., 2017).
93
94 In this study, we explored the potential application of standard PCR (combined with validation
95 by Sanger sequencing) for the detection of key invasive species in UK freshwaters. Four high
96 priority species were targeted: quagga mussel (*Dreissena rostriformis bugensis*, Andrusov,
97 1897)*;* zebra mussel (*Dreissena polymorpha*, Pallas 1871)*;* killer shrimp *(Dikerogammarus*
98 *villosus*, Sowinsky, 1894) and demon shrimp (*Dikerogammarus haemobaphes*, Eichwald 1841).
99 These species all originate from the Ponto-Caspian area and have spread rapidly throughout their

100 invasive ranges via boat transportation, canals and river basin connections (Dick et al., 2002;
101 Timar and Phaneuf, 2009; Bij de Vaate et al., 2002). Both economic and ecological impacts are
102 widely documented for all four species (Karatayev et al., 2002; Dick et al., 2002; Karatayev et
103 al., 2007; Connelly et al., 2007; MacNeil et al., 2010; Roy et al., 2014). The UK invasion history
104 of these four species and the reasons for prioritising them for eDNA assay development are
105 discussed below.
106
107 *Dreissenidae mussels:*
108
109 *D. polymorpha* is widespread and common in the UK, having arrived in the 1820s potentially via
110 the timber trade (Bij de Vaate et al., 2002; Quinn et al., 2014). *D. r. bugensis* is a much more
111 recent invader, with the first UK record from 2014 in the River Wraysbury (Mills et al., 2017).
112 Subsequent surveys showed the mussel was extensively distributed in the neighbouring
113 reservoir; a facility used to supply drinking water and for leisure activities. This reservoir is
114 subject to water transfers within the region and the mussel was subsequently found in
115 neighbouring reservoirs. The monitoring of these two species within the UK poses a new
116 challenge to regulators due their morphological similarity (Peyer et al., 2011). The Dreissenid
117 mussels both have huge impacts on ecosystem structure and function (Karatayev et al., 2007) and
118 on the economy. For example, between US$161 - US$467 million was spent by water treatment
119 and electric power facilities in North America on the control and removal of *D. polymorpha*
120 between 1989- 2004 (Connelly et al., 2007).
121
122 The rapid spread and colonization of new waterbodies by Dreissenid mussels throughout the
123 world has been aided by both human interaction and their unique ecology (Timar and Phaneuf,
124 2009). Like other mussels, Dreissenids have a free floating planktonic veliger life stage, during
125 which young can be dispersed over a large area downstream of parental populations (Ricciardi et
126 al., 1995; Karatayev et al., 2002; Karatayev et al., 2015). Compared to many other mussel
127 species, Dreissenids exhibit unique abilities to colonise new environments by using protein-
128 based byssal strands formed inside their shell to secure to hard surfaces, which can be a
129 significant aid to transportation and establishment (Ricciardi et al., 1998; Karatayev et al., 2002;
130 Aldridge et al., 2004; Timar and Phaneuf, 2009; Peyer et al., 2009). Colonization of new areas

131 and establishment has been facilitated by the ability of Dreissenids to survive out of water for up
132 to 15 days (Ricciardi et al., 1995) and survive a wide range of environmental extremes (Gallardo
133 and Aldridge, 2013b).
134
135 Monitoring and preventing the spread of *D. r. bugensis* is a priority within the UK because of its
136 recent arrival and potential to spread. The quagga mussel is likely to be able to invade a wider
137 range of habitats than the zebra mussel, including areas with higher temperatures, lower rainfall,
138 greater water depth, and lower dissolved oxygen (Nalepa et al., 2010; Quinn et al., 2014).
139 Quagga mussels are also able to spawn at lower temperatures than zebra mussels (Roe and
140 MacIsaac, 2011), which suggests they will potentially thrive in the cool UK climate. Both
141 mussels are described as “ecological engineers” (Karatayev et al., 2002; Karatayev et al., 2007;
142 Roy et al., 2014) having influences on all trophic levels. In some instances, mussels provide
143 increase in shelter and habitat for benthic macroinvertebrates (Karatayev et al., 2002), however
144 they also compete for food and decrease diversity, and have been directly linked to declines in
145 native Unionid mussels (Ricciardi et al., 1996). Dreissenid feeding behaviour also has negative
146 effects on phytoplankton and has been linked to greater numbers of cyanobacteria blooms
147 (Karatayev et al., 2002).
148
149 Although previous studies have designed and tested primers for detection of Dreissenids, some
150 assays not all are suitable for discriminating between the two species (Peñarrubia et al., 2016).
151 Others discriminate the species using a two-step PCR protocol, which was designed for tissue
152 samples and may be less appropriate for eDNA due to its large amplicon size (Hoy et al., 2009).
153 Studies by Mahon et al., (2011) and Egan et al., (2015), have both focused on detection of
154 quagga mussel veligers in ballast water, using microfluidic chip and light transmission
155 spectroscopy (LTM) technology, respectively. Recently, De Ventura et al. (2017) demonstrated
156 the successful detection of eDNA from both species in the field with PCR and qPCR, using
157 mitochondrial *COI* primers developed by Bronnenhuber and Wilson (2013). However to our
158 knowledge, no previous study has designed and tested species-specific standard PCR primer
159 pairs in controlled experiments to evaluate the rate of DNA production and detection, nor
160 evaluated their performance for detecting eDNA in the field against traditional methods for
161 sampling.

162

163 *Dikerogammarus species:*

164

165 *Dikerogammarus villosus* and *Dikerogammarus haemobaphes* have spread in a similar way to
166 the Dreissenid mussels. Arriving in Germany by the late 1990s, *D. villosus* was first recorded in
167 the UK in September 2010 (MacNeil et al., 2010), and to date its spread has been limited to only
168 five further locations in the UK due to strict biosecurity measures (Check, Clean, Dry
169 www.nonnativespecies.org). *Dikerogammarus haemobaphes* on the other hand, has rapidly
170 colonized British waterways since its discovery in May 2012, spreading successfully through the
171 river and canal networks (Environment Agency, 2012). Both species are well documented as
172 having significant negative effects on the macroinvertebrate community, particularly out
173 competing native Gammaridae species (Dick et al., 2002; MacNeil et al., 2010).
174 *Dikerogammarus villosus* is particularly noted for its exceptional predatory capabilities (Dick et
175 al., 2002; MacNeil et al., 2010) and its high reproductive output (MacNeil et al., 2010). It has
176 also been known to survive for up to six days out of water, allowing for extensive transportation
177 on recreational kit, such as fishing equipment, as well as in ballast water (Martens and Grabow,
178 2008). Both *Dikerogammarus* species have been prioritised for monitoring by the UK
179 Environment Agency because of the potential for rapid spread and high impacts on native fauna.
180 To our knowledge, no species-specific primer pairs have been developed for *D. villosus* or *D.*
181 *haemobaphes*.

182

183 The overall objective of this study was to develop and test targeted PCR eDNA assays for the
184 four INNS named above. Our framework for developing and testing the assays, consisted of: 1.
185 *in silico* and *in vitro* primer testing; 2. single species mesocosm experiments to evaluate eDNA
186 detection over time at three different densities (one, five and twenty individuals) and eDNA
187 degradation; and 3. testing the efficiency of the targeted PCR eDNA assays compared to
188 traditional kick-net sampling in the field.

189

190 **Methods**

191

192 *Specimen sampling and tissue DNA extraction*

193

194 Specimens of all four target invasive species were collected at sites with known populations, two
195 weeks prior to the beginning of each mesocosm experiment. These sites were as follows: *D. r.*
196 *bugensis:* Wraysbury River, UK Grid. Ref. TQ 02680 73204; D. *polymorpha* and *D.*
197 *haemobaphes:* Rutland Water, SK 92956 05963; *D. villosus:* Grafham Water, TL 15081 67289.
198 Specimens were kept in tanks with continuous aeration and fed dried *Cyclotella* and leaf material
199 *ad libitum*. Samples from the most closely related native taxa were also collected for tissue DNA
200 extraction and primer testing (*Gammarus fossarum/pulex, Crangonyx pseudogracalis,*
201 *Sphaerium corneum* and *Anadonta anatina*). Tissue samples from four individuals of each
202 invasive species or native species were extracted using the DNeasy Blood and Tissue Kit®
203 (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

204

205 *Species-specific primer development*

206

207 For all four INNS, species-specific primers were designed and tested *in silico* with Primer
208 BLAST (Ye et al., 2012) using all available *COI* reference sequences from GenBank (*D. r.*
209 *bugensis* – 7 sequences, *D. polymorpha* – 31 sequences, *D. villosus* – 22 sequences and *D.*
210 *haemobaphes* – 7 sequences, see Supplementary Information I: Table S1 for details of GenBank
211 accession numbers). In total 38 primer pairs were tested *in silico* (See Supplementary
212 Information II for all primer pairs tested). Where possible suitable primers were preferentially
213 selected with an amplicon size < 200 bp, to be suitable for amplification of degraded eDNA
214 (Deagle et al., 2006; Jerde et al., 2011; Bronnenhuber and Wilson 2013; Mächler et al., 2014;
215 Ardura et al., 2015). Twenty-three primer pairs were tested *in vitro* on tissue samples of target
216 INNS and three non-target taxa i.e. the congeneric INNS (*D. r. bugensis – D. polymorpha* and *D.*
217 *villosus – D. haemobaphes*) and two native taxa which are likely to co-occur in the same habitat
218 (Supplementary Information I, Table S2). Serial dilutions of neat tissue-extracted DNA (3-5
219 ng/µl) to 1:1000 dilutions (0.003-0.005 ng/µl) were carried out to establish the Limits of
220 Detection (LoD) for each primer pair (Supplementary Information I, Table S2). PCRs were
221 carried out in 25 µl volumes with MyTaq Red Mix (Bioline, UK) following manufacturer's
222 instructions. The final concentration of forward and reverse primer was: 0.4 µM of each primer
223 and 2 µl of undiluted DNA template. PCRs were performed on an Applied Biosystems Veriti

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224 Thermal Cycler. In order to optimise the annealing temperature of all four pairs of primers,
225 temperature gradient PCRs were carried out in order to amplify the target fragments. The
226 following profile was used for both *Dreissena* species: initial denaturation at 94°C for 3 min,
227 followed by 37 cycles of denaturation at 94°C for 30s, annealing at 65°C for 1 min and extension
228 at 72°C for 1 min 30s, with a final extension time of 10 min at 72°C. For *Dikerogammarus*
229 species the extension time was reduced to 1 minute for *D. villosus* and 30 seconds for *D.*
230 *haemobaphes*. PCR products from tissue samples were visualised by gel electrophoresis and
231 stained with GelRed (Cambridge Bioscience Ltd, UK). Four PCR products per species were
232 Sanger sequenced by Macrogen Europe in the forward direction. All sequences were compared
233 with the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/)
234 using BLAST to confirm species identification (See Supplementary Information III for sequence
235 alignments).
236
237 *Mesocosm experiments*
238
239 Mesocosm experiments were carried out from January to August 2016 to test the sensitivity of
240 the selected primer pairs under controlled densities. Each experiment was conducted in 15 L
241 plastic tanks with fitted lids. Tanks were located in a climate controlled facility where
242 temperature averaged 16°C (range 14-18°C) with light:dark cycles of 16 h:8 h . All tanks,
243 aeration equipment and sampling equipment was sterilized in 10% commercial bleach solution
244 for 10 minutes, then rinsed with 10% MicroSol detergent (Anachem, UK) and purified water
245 prior to the experiment. Sampling and filtering equipment was also cleaned using the above
246 method between each sampling event. Each tank was filled with water collected from Hotham
247 Beck (SE 89133 32489) which has no previous records of the four target INNS. Tanks were
248 supplied with constant air via sterile tubing and aeration stones for 48 hours prior to the start of
249 the experiment and covered for the duration of the experiment with a fitted lid.
250
251 For each species, the experiment consisted of 10 tanks representing three replicates of three
252 treatment densities (one, five and twenty individuals) and a control tank with no individuals.
253 Specimens of similar total biomass were used in the density replicates in order to minimise any
254 influence of different biomass (see Supplementary Information I, Tables S3, S5, S7 and S9 for

255 biomass information gathered pre- and post-mesocosm experiments). Room temperature, control
256 tank and water temperature was recorded prior to each sampling event (see Supplementary
257 Information I, Tables S4, S6, S8 and S10 for temperature measurements taken during the
258 mesocosm experiments). Tank water temperature was kept below 10°C for both Dreissenidae
259 mesocosm experiments to minimise any potential spawning events. Before the experiments, all
260 specimens were examined to confirm species identification, and in the case of *Dikerogammarus,*
261 to eliminate females that may be carrying eggs or juveniles to avoid influencing the DNA
262 concentration. Before the specimens were added to the tanks, a water sample was collected and
263 filtered to ensure no contamination from the target taxa; this sample was recorded as 0 hours.
264 Tanks were sampled over 42 days at 4hrs, 8hrs, 24hrs, 7 days, 15 days and 21 days with the
265 species present to investigate eDNA detection over time and at different densities. On day 21, the
266 specimens were removed from the tanks and sampling continued at 22 days, 28 days, and 42
267 days to document the rate of DNA degradation. A total of N = 100 samples was collected per
268 species.
269
270 For each sampling event, the tank water was homogenised by stirring with a sterile spatula before
271 collecting 200 ml water from each tank. Samples were vacuum filtered through sterile 47 mm
272 diameter 0.45 µm cellulose nitrate membrane filters with pads (Whatman, GE Healthcare, UK)
273 immediately after collection, using Nalgene filtration units (Thermo Fisher Scientific) in
274 combination with a vacuum pump (15~20 in. Hg, Pall Corporation) in a dedicated eDNA
275 laboratory at the University of Hull, UK. Filter papers were then placed in sterile petri dishes,
276 sealed with parafilm and stored at -20 °C until extraction. The filtered water was then returned to
277 the tank to maintain the water volume. This process was completed within one hour. The filtration
278 units were cleaned with 10% commercial bleach solution and 10% MicroSol, and then rinsed
279 thoroughly with deionized water after each filtration to prevent cross-contamination. All DNA
280 extractions were carried out using a protocol modified from Bolaski et al. (2008) (for the full
281 extraction protocol, see Supplementary Information IV). Mesocosm samples were PCR amplified
282 using the species-specific primers and conditions previously described. PCR products were then
283 visualised on a 1.5% agarose gel stained with GelRed (Cambridge Bioscience Ltd, UK). Three
284 PCR products from each species/mesocosm experiment were sequenced to confirm primer
285 specificity (Macrogen Europe).

286

287 *Field trials*

288

289 Water samples were collected at sites with previous records of the target INNS to test and verify
290 the efficiency of each INNS assay in the field. For *D. r. bugensis, D. polymorpha* and
291 *Dikerogammarus haemobaphes,* three UK Environment Agency macroinvertebrate monitoring
292 sites were selected on three river catchments (Colne, Welland and Nene) (n = 9) (Fig. 1 A-C).
293 Six samples were collected at each of the three sites. Each sample consisted of 3 x 500 ml (n =
294 54 per INNS). Field samples for *D. villosus* were collected from the shoreline of the Grafham
295 Water reservoir using the same protocol as for the lotic samples (3 sites x 3 x 500 ml replicates,
296 hence n = 9, Fig. 1D). Each 500 ml sub-sample was filtered and extracted independently. For *D.*
297 *villosus,* additional single 2 L water samples from Wroxham Broad and Pitsford Water (Fig. 1E
298 and F respectively), collected during a different study, were also tested. Sites were surveyed after
299 eDNA sample collection using standard 3-minute kick samples (Murray-Bligh, 1999). Sample
300 bottles filled with ddH_2O were taken into the field as sample blanks. Samples were processed
301 within 24 hours using the same method as the mesocosm samples. Each technical replicate was
302 PCR amplified three times to avoid false negatives. To confirm primer specificity, PCR products
303 from a total of 8 samples (for each species) were Sanger sequences (1 tissue sample, 3 mesocosm
304 samples and 4 field samples)

305

306 *Data Analysis*
307

308 Binomial Generalized Linear Models (GLMs) with a logit link function were used to investigate
309 the influence of density or total biomass and time since the start of the experiment (until the taxa
310 were removed from the mesocosm) on the detection in the mesocosms. Models were checked by
311 testing whether the residual deviance fitted a chi squared distribution. The best supported model
312 was identified by the lowest AIC value, and models with Δ AIC <2 were also considered
313 equivalent (Burnham & Anderson, 2002). All data analyses were performed in R v.3.3.1. (R
314 Core Team 2017), with GLMs performed using the MASS package (Venables et al., 2002) To
315 ensure full reproducibility of this study the raw data and code can be accessed
316 (https://github.com/RosettaBlackman/targeting_the_invader).

317
318 **Results**
319
320 *Primer specificity*
321
322 Of the thirty-eight primer pairs tested *in silico*, twenty-three pairs were selected for *in vitro*
323 testing. Four primer pairs (one for each species: *DRB1, DP1, DV1* and *DH2*, Table 1) were then
324 selected based on our criteria of good target amplification with no cross-amplification of non-
325 target species. The *D. r. bugensis* primer pair, *DRB1,* amplified 29 published *D. rostriformis, D.*
326 *bugensis* and *D. rostriformis bugensis* sequences *in silico* with no mismatches. The *D.*
327 *polymorpha* primer pair, *DP1,* amplified 45 published *D. polymorpha* and subspecies *(D. p.*
328 *polymorpha, D. p. gallandi* and *D. p. anatoli*a) *in silico*. Of the published *D. polymorpha*
329 sequences, one had a mismatch in the forward primer (Accession number AF510508) and a
330 second sequence had two mismatches in the forward and one in the reverse primer (Accession
331 number JQ435817). Note that the forward primer pair selected for *D. polymorpha* shares a 16 bp
332 overlap with DpoCOI3F designed by Bronnenhuber and Wilson (2013) but our primer pair, *DP1*,
333 amplifies a much shorter sequence (73 bp, as opposed to 164 bp). The *D. villosus* primer pair,
334 *DV1,* amplified 23 *D. villosus* sequences *in silico.* Two published sequences of *D. villosus* from
335 the Ukraine had mismatches to our primer pair; 1 mismatch with the forward primer and two in
336 the reverse (Accession numbers KM208873 and EF570297). Finally, the *D. haemobaphes* primer
337 pair, *DH1,* amplified 7 published sequences *in silico.* Three of these sequences are non-target
338 species of marine gastropod: *Thuridilla albopustulosa* (Accession number KM086443),
339 *Hemicycla pouchadan* (Accession number GU598226), *Caucasotachea calligera* (Accession
340 number KT794407). Since these are marine species and currently not recorded in the UK, it is
341 unlikely they will generate false positives in our tests but this should be considered for wider
342 applications.
343
344 Species-specific primer testing on target tissue samples yielded positive PCR amplification of a
345 single band at the expected size for all four selected assays (Fig. 2). The LoD for *DRB1, DP1,*
346 and *DH1* primer pairs was ~0.005 ng/µl DNA per reaction (1:1000 dilutions of neat tissue DNA,
347 Fig. 2 A, B, D). For *DV1,* the LoD was a 1:100 dilution, which corresponds to approximately

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348 0.03 ng/µl of target DNA (Fig. 2 C). No bands of the expected size were obtained in the cross
349 amplification tests; however, much larger, non-specific bands, were seen in non-target species
350 for *DP1,* and *DV1* (Fig. 2 B and C). Due to the substantial size difference these non-specific
351 bands are easily distinguishable from the target band size and will not lead to false positive
352 detections. Sequences generated from PCR products from all tissue, mesocosm and field samples
353 were verified as being from the correct target species. Some sequences generated from D.
354 villosus and D. polymorpha were of poor quality due to the short amplicon length (See
355 Supplementary Information III), and highlights the difficulty of using primers with small
356 amplicon lengths.
357
358 *Mesocosm experiments*
359
360 We had minor contamination of a single tank prior to target species being add (*D. haemobaphes*,
361 tank 8, time = 0). Target DNA was also found in the control tank for *D. haemobaphes* at 8 and
362 24 hours, for a single replicate, however there was no target DNA detected prior to these sample
363 events or from the subsequent sampling events (see Supplementary Information V, Fig. S1, S2,
364 S3 and S4 for gel images of all mesocosm samples).
365
366 Both Dreissenid mussel primers showed positive detection of their target species in all three
367 replicates at the four hour sampling event (Fig. 3A and B). At least one positive replicate was
368 obtained for every sampling point over the first 21 days. For *D. r. bugensis,* time and total
369 biomass significantly influenced the detection by standard PCR. Of these two measures, total
370 biomass was the more significant predictor in GLMs and generated the lowest AIC (GLM, z =
371 2.262, *P* = 0.023, AIC 55.368). After removal of *D. r. bugensis,* DNA was only detected in tanks
372 with the highest mussel density (20) 24 hours after removal. DNA from these tanks was no
373 longer detected at day 28 (7 days after removal). For *D. polymorpha,* both time and density were
374 significant predictors of detection. Of the two measures, density was the strongest predictor with
375 the lowest AIC (GLM, z = 1.969, *P* = 0.049, AIC 32.823). DNA from *D. polymorpha* persisted
376 to day 42 (21 days after removal) in two of the three density treatments (see Supplementary
377 Information I Table S12 – 15 for full GLM data).
378

379 *Dikerogammarus* species: DNA from both target species was consistently detected at each
380 sampling point between 4 hours and 21 days for the 20 individual density treatment (Fig. 3 C and
381 D). *Dikerogammarus haemobaphes* was detected at every sampling point between 4 hours and
382 21 days in the 5 and 20 individual density treatments (Fig. 3D), whereas *D. villosus* was only
383 consistently detected at the highest density treatment (Fig. 3C). *D. villosus* and *D. haemobaphes*
384 primers amplified non-specific DNA during the mesocosm experiment. However, these non-
385 specific bands were substantially different in size to the target band in both cases (*D. villosus*
386 non-target bands > 300 bp, *D. haemobaphes* non-target band size < 100 bp) (See Supplementary
387 Information V Fig. S3 and S4 for agarose gels from each *Dikerogammarus* mesocosm
388 experiment). Density or total biomass significantly influenced the detection for both shrimp
389 species. Total biomass was the most significant predictor for *D. villosus* and had the lowest AIC
390 (GLM, $z = 4.346$, $P < 0.001$, AIC: 40.372). Both models including time since the start of the
391 experiment (plus total biomass or density) influenced the number of positive detections by
392 standard PCR. Of these two models, total biomass was the more significant predictor (GLM total
393 biomass, $z = 2.652$, $P = 0.008$, AIC: 64.595), however the Δ AIC value score for the time and
394 density model differed by <2 and therefore could be considered the equivalent for predicting the
395 detection of *D. haemobaphes* (GLM density, $z = 2.577$, $P = 0.009$, AIC: 65.239). Positive
396 detection of *D. villosus* after removal at any density was lost within 24 hours of the specimens
397 being removed, however detection of *D. haemobaphes* remained in a single tank (with a
398 specimen density of 5) for 24 hours.
399
400 *Field trials*
401
402 Dreissenid mussels: *D. r. bugensis* specimens were found by kick-sampling at all sites surveyed,
403 but the number of individuals found decreased with distance along the River Wraysbury from the
404 main source population at Wraysbury Reservoir (Table 2A). Detection by kick-sampling was
405 33% (6 samples out of 18). Positive eDNA detections were obtained for every sampling replicate
406 at each of the three sites along the River Wraysbury, hence eDNA detection was 100% (Table
407 2A and Supplementary Information V Fig. S5). *D. polymorpha* was found by kick-sampling in
408 only one of three sites (Duddington, Table 2A) although the species is known to be present
409 throughout the sampled catchment. The number of positive detections for kick-sampling was

410 11% (2/18 samples). Positive eDNA detections for *D. polymorpha* were obtained in 88.9%
411 (16/18) of samples and 61.1% (33/54) of the PCR replicates, including in sites where specimens
412 of *D. polymorpha* were not found (Table 2A and Supplementary Information V Fig. S6).
413
414 *Dikerogammarus* species: *D. haemobaphes* was detected in all kick-samples at two of the three
415 sites sampled (detection in 66.7% of samples, Fig. 4 D). Positive eDNA detections for *D.*
416 *haemobaphes* were obtained in 77.7% (14/18) of samples and 74.1% (40/54) of PCR replicates
417 (Table 2A). Positive detections were obtained for three of the six samples at Flore Road Bridge,
418 where the species was not detected by kick-sampling (Fig. 3 D and Supplementary Information
419 V, Fig. S8). *D. villosus* was detected in all five kick samples obtained at varying density (Table
420 2B). However, the species was only detected in one of the 5 samples using eDNA (detection in
421 20% of samples, Table 2 and Supplementary Information V S7). We observed considerable
422 differences in the application of the *Dikerogammarus* and Dreissenid primers in the field, with
423 the *Dikerogammarus* primer pairs producing weaker target bands and more non-specific
424 amplification (Supplementary Information V, Figs S7-8).
425
426 **Discussion**
427
428 Rapid, cost-effective tools are needed for detection of newly invading, or spreading invasive
429 non-native species. Here, we designed and tested PCR primer pairs for four invasive non-native
430 species: *D. r. bugensis, D. polymorpha, D. villosus,* and *D. haemobaphes,* which are high priority
431 for monitoring in the UK and beyond. Primers were tested *in silico* and *in vitro,* then in a series
432 of mesocosm experiments and field trials. The four primer pairs amplify target tissue at a low
433 concentration (0.005-0.03 ng/μl) which is in line with other eDNA species-specific standard PCR
434 primer assays, (e.g. detection limit of 0.00046 ng/μl (Davison et al., 2006) to 0.4 ng/μl (Ardura et
435 al., 2015), with no cross-species amplification with closely related or native species present in
436 the UK. All four species were detected from eDNA collected from water samples in both
437 laboratory and field trials.
438
439 eDNA could be detected in mesocosms within 4 hours of the start of the experiment and, with
440 the exception of *D. villosus,* detection at this first time point was possible from just one

441 individual. Dreissenid eDNA was detected at every sampling point at all three densities in the
442 mesocosms, and outperformed kick-sampling for detection in the field. Detection of
443 *Dikerogammarus* eDNA was more challenging in both the mesocosm and field experiments but
444 both species were consistently detected in the mesocosms at high density (20 individuals) and *D.*
445 *haemobaphes* was also consistently detected at medium density (5 individuals). Field detection
446 was higher for eDNA than kick-sampling for *D. haemobaphes* but detection of *D. villosus* was
447 lower. Below we highlight the range of factors that likely interact to determine the success of
448 eDNA detection in real-world applications.
449
450 *Mesocosm trials*
451
452 Mesocosm experiments have been advocated (De Ventura et al., 2016), and performed by
453 previous studies (Dejean et al., 2011; Thomsen et al., 2012b; Sansom and Sassoubre, 2017) to
454 allow information on species-specific DNA production rates, persistence and degradation over
455 time. This information will inform users whether the method is appropriate for the detection of
456 target taxa. Here, both abundance variables (density and total biomass) were significant
457 predictors of detection for all four species. Hence, there is a positive relationship between
458 abundance and detection, as found in previous studies (e.g. Thomsen et al., 2012b). Mesocosm
459 experiments also demonstrated the rapid depletion of DNA once the specimens were no longer
460 present in the tank; 24 hours after removal for *Dikerogammaurs villosus*, 7 days after removal
461 for *Dreissena rostriformus bugensis* and *Dikerogammarus haemobaphes*, and 21 days of
462 removal for *Dreissena polymorpha,* also in agreement with similar studies (Dejean et al., 2011;
463 Thomsen et al., 2012b). However, there were differences between species in terms of
464 detectability and DNA did not accumulate in a linear fashion over time, as discussed below.
465
466 The mesocosm experiments performed in this study were useful for determining the assay
467 sensitivity and for identifying differences in detectability between species. Our experiments
468 revealed that the *Dreissena* primers are highly sensitive and robust – being able to detect single
469 individuals within four hours and then consistently throughout the course of the experiment. The
470 *Dikerogammarus* assays were less sensitive than those for the mussels, but *D. haemobaphes* was
471 still consistently detected at medium and high densities, and *D. villosus* consistently detected in

472 the highest density treatment. This likely reflects both differences between the assays in terms of
473 primer robustness and physiological differences between the two species pairs. The high success
474 for Dreissenid mussels is likely due, at least in part, to the fact they were able to continuously
475 filter feed on algae and phytoplankton present in the water column during our experiments, as
476 they would in the wild, enabling them to maintain an active metabolism.By contrast,
477 *Dikerogammarus* metabolism may have been limited by the availability of only phytoplankton
478 and algae as their diet in the wild is much more varied. Furthermore, no evidence of moulting
479 was found during the course of the *Dikerogammarus* experiment, which is likely to be a main
480 source of eDNA in the wild. Previous studies have suggested that organisms with exoskeletons
481 (such as Crustacea) can be hard to detect with eDNA, potentially due to low shedding rates
482 (Tréguier et al., 2014, Dunn et al., 2017). It is clear from our study and others that DNA
483 production and its availability in the water column is a complex topic and can vary substantially
484 between species.
485
486 Although differences in species physiology may explain the differences in detection of the
487 Dreissenid and *Dikerogammarus* species pairs, it does not explain differences within pairs,
488 where anatomy and physiology are very similar. This difference in eDNA detection was
489 observed for *D. villosus* and *D. haemobaphes.* The detection of *D. haemobaphes* but not *D.*
490 *villosus* in the single density treatment could at least partly be explained by a substantial
491 difference in biomass (means 0.97 g and 0.13 g respectively). However, this explanation is less
492 likely to account for differences in detection in the five individual density treatment since
493 biomass was more similar for the two species (total biomass *D. haemobaphes* 2.10 g and *D.*
494 *villosus* 1.82 g). Higher sensitivity and/or robustness of the *D. haemobaphes* primer pair, is likely
495 an important contributing factor.
496
497 We might expect that as long as DNA production rate is greater than the degradation rate, (as
498 seen in models produced by Thomsen et al., 2012), eDNA availability should increase over the
499 course of the experiment. Under this prediction, we expect the DNA concentration and the
500 number of positive detections to increase over time, and for there to be an interaction with
501 density. Alternatively, DNA concentrations may increase at first and then plateau, when an
502 equilibrium is reached between DNA production and degradation (Klymus et al., 2015; Sansom

503 and Sassoubre 2017; Nevers et al., 2018). As we are using standard PCR, rather than qPCR we
504 are unable to determine DNA concentration, however we do see an increase in band strength in
505 both Dreissenid mesocosm experiments between 4 and 24 hours, and for the high density
506 *Dikerogammarus* mesocosm tanks, suggesting increasing DNA concentration in the early stages
507 of the experiment. However, overall the number of positive detections fluctuates rather than
508 showing an accumulation or a plateau over time. Of the 4 mesocosm experiments, times was a
509 significant predictor of detection probability for the two Dreissenidae mussels only. The
510 fluctuation in the number of detections over time may be due to a combination of the activity of
511 the organisms, the balance between DNA production and degradation, and/or changes in the
512 concentration of PCR inhibitors. In Dreissenid mussels, filter feeding may both release and
513 uptake DNA, so the amount of DNA present in a controlled environment may reach an
514 equilibrium. Activity of the shrimp may have fluctuated over the course of the experiment with
515 reduction of food resources in the water column. PCR inhibition has been reported in other
516 mesocosm tests (Sassoubre et al., 2016), however this seems unlikely in our experiment as we do
517 not see any consistency in PCR failure within individual tanks. Further experiments with a
518 quantitative method such as qPCR or ddPCR are needed to fully understand the dynamics of
519 DNA concentration over the course of the experiment, and the influence of feeding and other
520 behaviours on the rate of DNA production.

521

522 *Field application*

523

524 In the field tests, eDNA outperformed kick sampling for detection of three target INNS: *D. r.*
525 *bugensis* (100% eDNA vs 33% kick samples), *D. polymorpha* (89% vs 11%) and *D.*
526 *haemobaphes* (83% vs 67%) but not for *D. villosus* (20% vs 100%). Below we discuss the
527 reasons for the discrepancies between eDNA and kick-samples for all four species.

528

529 There are numerous influences on the persistence of eDNA in waterbodies, that have been well
530 documented such as: pH, microbial activity and transportation (Deiner and Altermatt, 2014; Jane
531 et al., 2015; Jerde et al., 2016; Shogren et al., 2017, see Barnes et al., 2014 for further
532 discussion). In our study, we reported higher detection rates from eDNA compared to kick-

533 samples in the three species sampled in lotic environments, but lower eDNA detection compared
534 to kick-samples in a lentic species. In the lotic samples, detection is likely to come from both
535 local populations and eDNA being transported from upstream sources (Deiner and Altermatt,
536 2014; Jane et al., 2015). However, to what extent the DNA is being transported is still largely
537 unknown. Previous work on river morphology states substrate type and the related flow regime,
538 are huge influences on DNA transportation, substrate retention and subsequent resuspension
539 (Shogren et al., 2017; Jerde et al., 2016).
540
541 In this study our results show no decrease in band strength for the detection of *D. r. bugensis*
542 across the population density gradient along the River Wraysbury (2km). In part this is likely to
543 be down to transported DNA. However, it is also likely to be attributed to an increase in water
544 mixing caused by rainfall before the samples were collected. As eDNA is not uniformly
545 distributed through a river (Macher and Leese, 2017) the rainfall is likely to increase the
546 dispersion of eDNA in a waterbody (Shogren et al., 2017). We therefore see a greater number of
547 positive detections. Similarly, variation in the eDNA detection throughout the rivers, for both *D.*
548 *polymopha* and *D. haemobaphes*, may be due to the relatively lower flows during these surveys
549 which have caused a reduction in DNA distribution across the river. However, there is a
550 fluctuation in the number of detections across the samples at each site for these species and we
551 detect DNA at sites where they were not physically collected. This is further evidence of eDNA
552 being transported down the catchment rather than a false positive result. This greater variability
553 in detection due to the lower flow conditions is likely to demonstrate the true variation
554 encountered when surveying lotic systems for target species.
555
556 Reasons for the greater difficulty detecting *D. villosus* in the wild maybe due to the lower DNA
557 shedding rates and poorer assay performance as discussed above. Combined with localisation of
558 eDNA, this suggests that a greater sampling resolution may be required to detect *D. villosus* and
559 other species with low shedding rates in lentic water bodies. Due to the high amount of non-
560 specific bands shown on the field samples for both *Dikerogammarus* species, we would suggest
561 carrying out Sanger sequencing of PCR product on samples where similar sized target bands are
562 observed.
563

Conclusion

This study provides targeted eDNA assays for four priority invasive non-native species, and demonstrates a cost-effective framework for assay development that can be used by regulatory bodies with responsibility for invasive species monitoring. Standard PCR outperformed established kick sampling for three out of four target species, and provides a simple and effective detection method without significant investment in qPCR, ddPCR or Next Generation Sequencing facilities. However, more quantitative methods are needed to provide deeper insights into the rate of DNA accumulation and degradation in both mesocosms and field experiments. This study also highlights some of the challenges for designing and implementing eDNA assays for different species, emphasizing the need to understand the dynamics of DNA production and degradation by different species.

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Figure 1

Site locations for field trials.

All sample sites are referred to from upstream to downstream, waterbodies are in black, sample points are marked with a diamond. A - Sampling sites from *D. r. bugensis* were on the River Wraysbury at Wraysbury weir (WW), Wraysbury bridge (WB) and Wraysbury Gardens (WG). B - Sample sites for *D. polymorpha* were on the R. Welland at Harrington (HR), Duddington (DD) and Copthill (CP). C - Samples sites for *D. haemobaphes* were on the R. Nene at Flore's Road Bridge (FR), Duston Mill (DM) and Cogenhoe (CG). D, E, F - Sample sites for *D. villosus* were carried out on three reservoirs: Grafham Water (GW1, GW2, GW3), Wroxhom Broad (WB) and Pitsford Water (PW).

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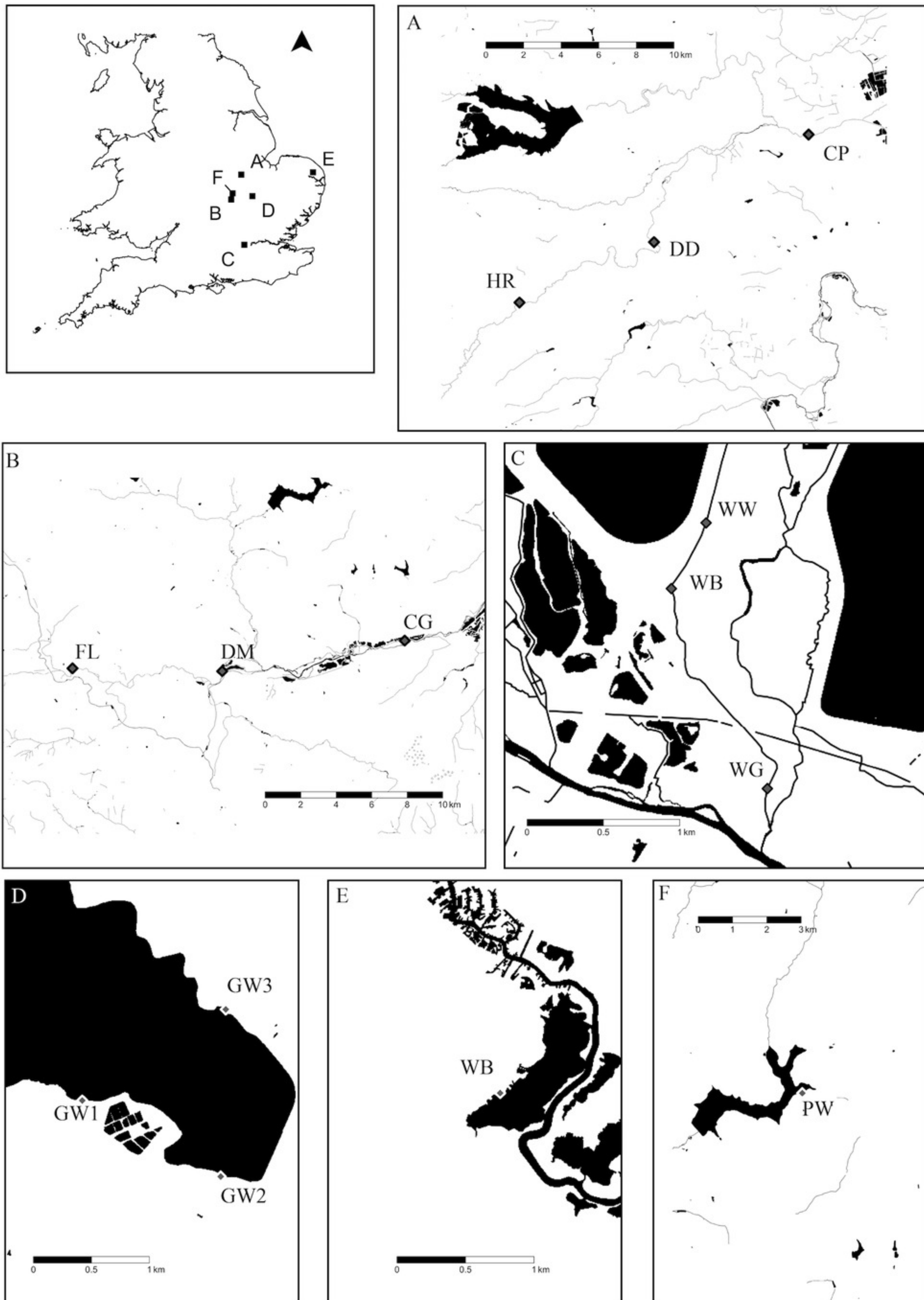
A
B
C
D
E
F
0 2 4 6 8 10 km
CP
DD
HR
FL
DM
CG
WW
WB
WG
0 0.5 1 km
GW3
GW1
GW2
0 1 2 3 km
PW

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Figure 2

Results of *in vitro* primer testing.

A – *Dreissena rostriformis bugensis* (primer pair *DRB1)*, B – *Dreissena polymorpha* (*DP1),* C – *Dikerogammarus villosus* (*DV1*) and D – *Dikerogammarus haemobaphes (DH2)*. Lane 1 contains undiluted target INNS tissue DNA (3-5 ng/µl per reaction), lanes 2-4 contain a dilution series of the target tissue (lane 2 1:10 dilution, ~0.3-0.5 ng/µl per reaction; lane 3 1:100 dilution, ~0.03-0.05 ng/µl per reaction; lane 4 1:1000 dilution, ~0.003-0.005 ng/µl per reaction). Lanes 5 and 6 contain closely related native species found in the UK: for the Dreissenid mussels (A and B): *Anadonta anatina* and *Sphaerium corneum,* and for the *Dikerogammarus* species (C and D): *Gammarus fossarum/pulex,* and *Crangonyx pseudogracalis*. Lane 7 contains the paired INNS and lane 8 is a PCR negative (ddH_2O). The final lane is DNA EasyLadder

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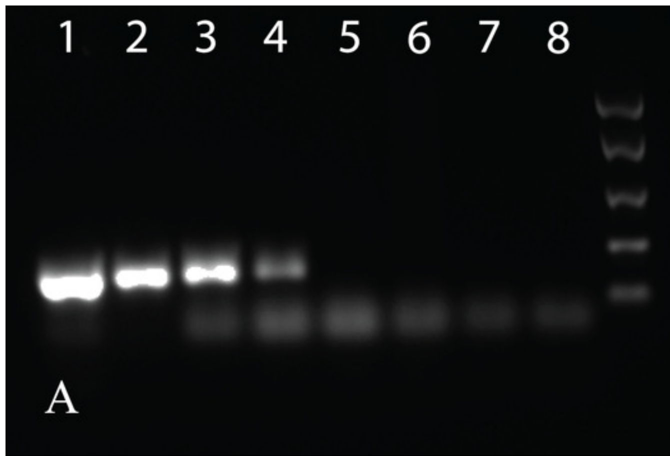
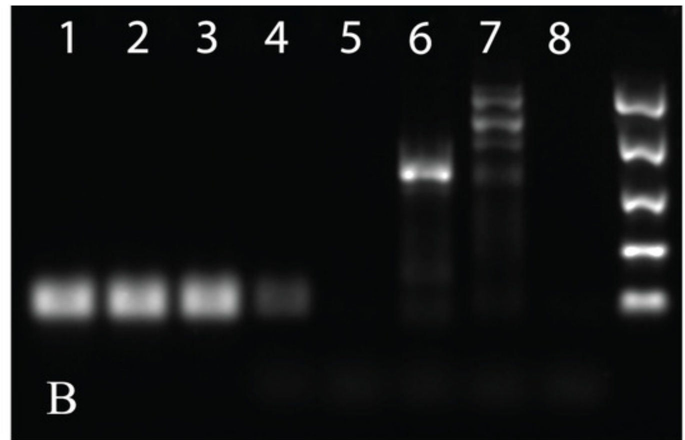

1 2 3 4 5 6 7 8
A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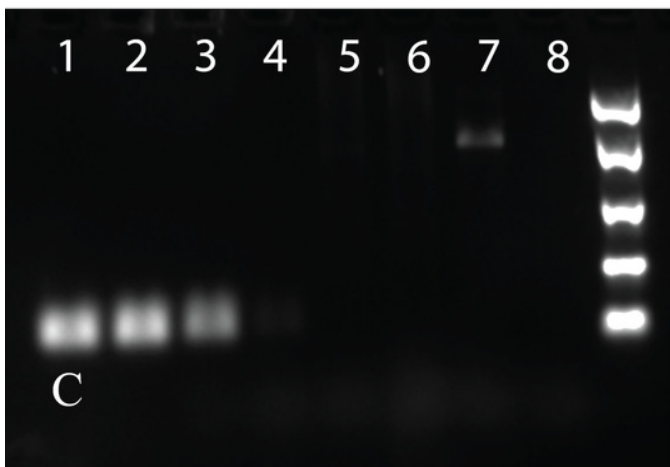
B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C

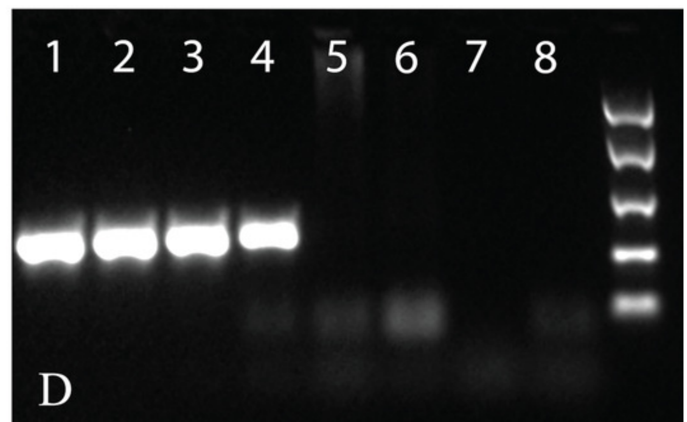
1 2 3 4 5 6 7 8
D

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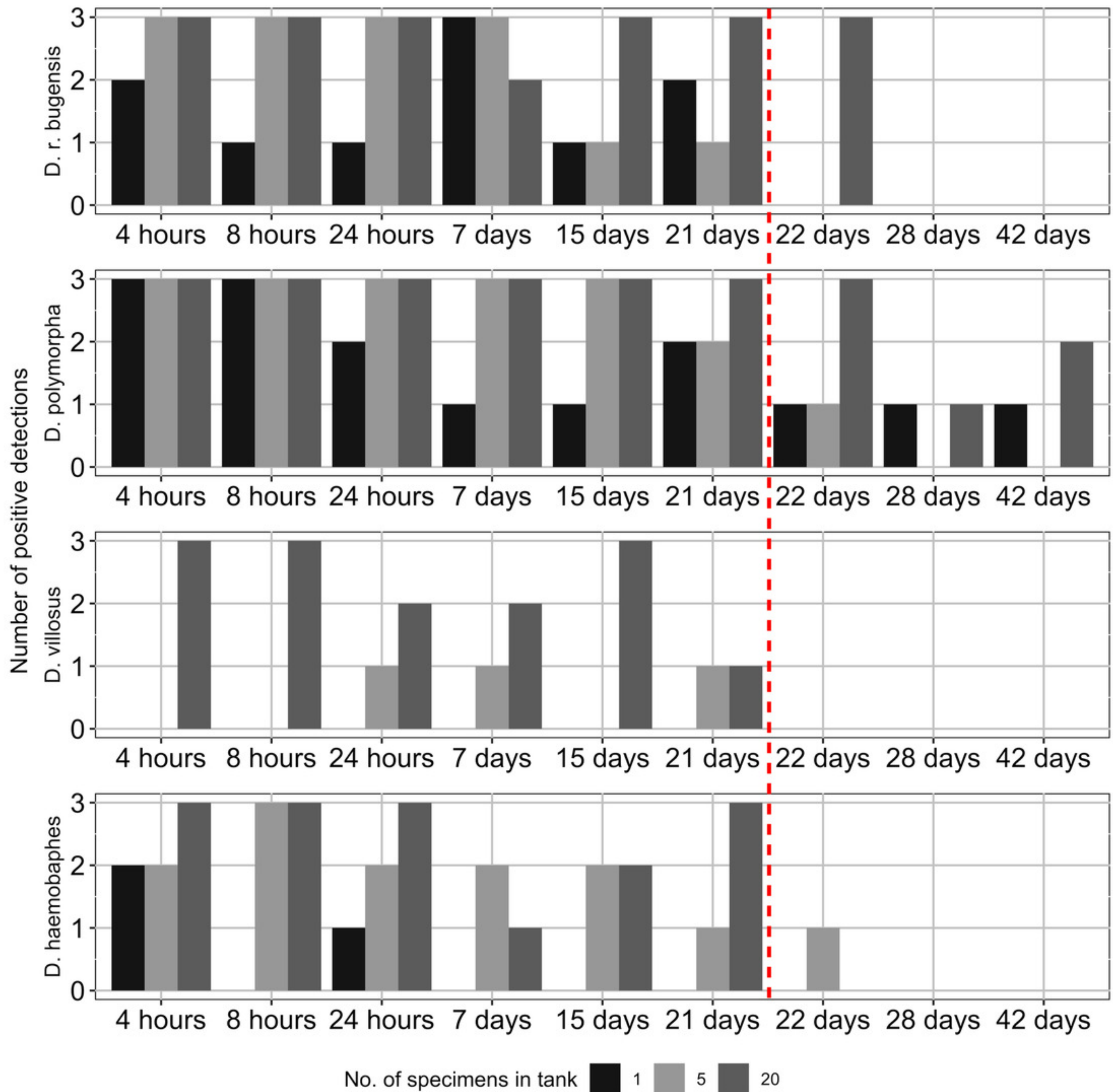
Figure 3

Species detection in mesocosm experiments

Each graph indicates the number of positive detections from three replicates taken from each treatment (specimen density) during the 42 day experiment. Specimens were removed after 21 days (indicated by the dashed red line). A – Dreissenia rostriformis bugensis, B - Dreissena polymorpha, C - Dikerogammarus villosus and D -Dikerogammarus haemobaphes.



Number of positive detections
D. r. bugensis
D. polymorpha
D. villosus
D. haemobaphes
0
1
2
3
4 hours
8 hours
24 hours
7 days
15 days
21 days
22 days
28 days
42 days
No. of specimens in tank
1
5
20

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Table 1 (on next page)

Primer pairs designed for this study and used for the detection of 4 target INNS.

1 Table 1. Primer pairs designed for this study and used for the detection of 4 target INNS.

2

Target species	Primer	Primer sequence	Amplicon length (bp)
Dreissena rostriformis bugensis	*DRB1_F*	GGAAACTGGTTGGTCCCGAT	188
	DRB1_R	GGCCCTGAATGCCCCATAAT	
Dreissena polymorpha	*DP1_F*	TAGAGCTAAGGGCACCTGGAA	73
	DP1_R	AGCCCATGAGTGGTGACAAT	
Dikerogammarus villosus	*DV1_F*	TCTTGGCAGGTGCCATTACG	87
	DV1-R	GAATAGGATCACCCCCGCCT	
Dikerogammarus haemobaphes	*DH2_F*	TAGGTCACAGGGGTGCTTCT	295
	DH2_R	AAGTGCTGGTAAAGAATAGGATCT	

3

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Table 2 (on next page)

Species detection in field experiments

Summary of the number of positive detections from each field sample at each site (eDNA sample out of 3 PCRs, kick sample the number of specimens collection in a 3 minute sample) DRB - *Dreissenia rostriformis bugensis*– Wraysbury River, DP - *Dreissena polymorpha*– River Welland and DH - *Dikerogammarus haemobaphes*– River Nene. B – Comparison of detection for DV- *Dikerogammarus villosus*at 5 locations: GW1 – 3 – Grafham Water, PW – Pitsford Water and WB – Wroxham Broad (eDNA sample out of 1 PCRs, kick sample the density of specimens found after a 3 minute sample).

1 A.

	Site	1		2		3		4		5		6	
		Sample Number											
		eDNA	Kick	eDNA	Kick	eDNA	Kick	eDNA	Kick	eDNA	Kick	eDNA	Kick
DRB	**WW**	3	0	3	4	3	4	3	0	3	3	3	3
	WB	3	0	3	0	3	0	3	0	3	4	3	0
	WG	3	0	3	0	3	1	3	0	3	0	3	0
DP	**HR**	2	0	2	0	3	0	3	0	3	0	3	0
	DD	1	0	3	0	2	0	3	0	1	1	2	2
	CP	1	0	1	0	0	0	1	0	0	0	2	0
DH	**FR**	2	0	1	0	0	0	2	0	1	0	1	0
	DM	2	34	0	14	2	8	2	12	3	13	1	14
	CG	0	4	0	16	2	4	2	6	1	7	1	10

2

3 B.

	Site	*Sample* 1	
		eDNA	Kick
DV	**PW**	0	Low
	WB	0	High
	GW1	0	Medium
	GW2	1	Low
	GW3	0	Low

4

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