Targeted and passive environmental DNA approaches outperform established methods for detection of quagga mussels, *Dreissena rostriformis bugensis* in flowing water

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Abstract

1. The early detection of invasive non-native species (INNS) is important for informing management actions. Established monitoring methods require the collection or observation of specimens, which is unlikely at the beginning of an invasion when densities are likely to be low. Environmental DNA (eDNA) analysis is a highly promising technique for the detection of INNS – particularly during the early stages of an invasion.
2. Here, we compared the use of traditional kick-net sampling with two eDNA approaches (targeted detection using both conventional and quantitative PCR, and passive detection via metabarcoding with conserved primers) for detection of quagga mussel, *Dreissena rostriformis bugensis;* a high priority INNS, along a density gradient on the River Wraysbury, UK.
3. All three molecular tools outperformed traditional sampling in terms of detection. Conventional PCR and qPCR both had 100% detection rate in all samples, and outperformed metabarcoding when the target species was at low densities. Additionally, quagga mussel DNA copy number (qPCR) and relative read count (metabarcoding) were significantly influenced by both mussel density and distance from source population, with distance being the most significant predictor.
4. *Synthesis and application.* All three molecular approaches were more sensitive than traditional kick-net sampling for the detection of the quagga mussel in flowing water, and both qPCR and metabarcoding enabled estimates of relative abundance. Targeted approaches were more sensitive than metabarcoding, but metabarcoding has the advantage of providing information on the wider community, and consequently impacts of INNS.

44 # Introduction

45

46 Invasive non-native species (INNS) have wide ranging effects on ecosystems, including biodiversity loss
47 (Doherty et al., 2016; Gallardo et al., 2016) and economic impacts including damage to infrastructure
48 (Pimentel et al., 2005; Connelly et al., 2007). Eradication or containment methods are most effective
49 during the early stages of an invasion, both in terms of success and financial cost (Hulme, 2006; Mehta
50 et al., 2007). Early detection is particularly challenging for species that are small, elusive or cryptic and
51 these species often go unnoticed until they become established (Simmons et al., 2015; Blackman et al.,
52 2017). Monitoring organisms such as freshwater invertebrates generally relies on specimen collection
53 and morphological identification, and newly invading species can be easily overlooked. Alternative
54 methods that offer rapid and cost-effective detection of new INNS are crucial at a time when rapid
55 increase in trade and transport has caused a surge in the number of successful invasions to new
56 environments (Hulme, 2006).

57

58 The use of molecular methods to identify DNA taken from environmental samples (i.e. environmental
59 DNA or "eDNA") has been a major research focus in biodiversity monitoring over the last 10 years,
60 including the detection of INNS (Ficetola et al., 2008, Thomsen et al., 2012, Blackman et al., 2018). Two
61 broad approaches can be applied to detect a species of interest. The "targeted" approach utilises
62 species-specific primers with conventional PCR (cPCR), quantitative PCR (qPCR) or droplet digital PCR
63 (ddPCR) to detect a single species. By contrast, metabarcoding can be considered a "passive approach"
64 as it does not focus on single species (Lawson Handley, 2015; Creer et al., 2016; Deiner et al., 2017).
65 Metabarcoding utilises conserved primers to amplify DNA from a group of taxa e.g. fish and other
66 vertebrates (e.g. Kelly et al., 2014; Miya et al., 2015; Simmons et al., 2015), zooplankton (e.g. Brown et
67 al., 2016), or insects (e.g. Yu et al., 2012., Elbrecht et al., 2016). Amplicons are then sequenced using
68 High Throughput Sequencing (HTS) platforms and bioinformatically assigned to taxa. Metabarcoding

69 provides an opportunity to detect multiple species simultaneously, including those, which have not been
70 listed as priority INNS (Simmons et al., 2015; Blackman et al., 2017). Comparisons between traditional
71 methods for biodiversity monitoring and targeted (Dejean et al., 2012; Tréguier et al., 2014) or passive
72 (Smart et al., 2015; Hänfling et al., 2016; Wilcox et al., 2016) molecular approaches have demonstrated
73 an increased probability of detection using eDNA methods.
74
75 The targeted approach was first used by Ficetola et al. (2008) to detect invasive American bullfrog
76 *Lithobates catesbeianus*, in lentic waterbodies and has since been applied to over 100 target INNS
77 (Blackman et al., 2018). Conventional PCR is a well-established and widely practiced method for
78 detecting presence/absence of target DNA by visualisation of PCR products on agarose gels. However,
79 quantitative PCR (qPCR) has on many occasions been shown to be more sensitive than PCR (e.g. Nathan
80 et al., 2014; Simmons et al., 2015; De Ventura et al., 2016) and therefore more suitable for detecting
81 INNS at low density, e.g. the early stages of invasion. Quantitative PCR has the added advantage of
82 providing the number of DNA copies in a sample, which has been successfully linked to biomass or
83 abundance of some taxa (e.g. Amphibians, Thomsen et al., 2012; Fish, Takahara et al., 2012; Gastropods,
84 Goldberg et al., 2013). However, qPCR requires the construction of calibration curves from known
85 standard concentrations and is substantially more expensive and time-consuming than cPCR (Nathan et
86 al., 2014) and maybe less accessible to end-users than cPCR (Blackman et al., 2020).
87
88 The potential of eDNA metabarcoding for INNS monitoring has already been shown as an effective tool
89 (Simmons et al., 2015; Brown et al., 2016; Blackman et al., 2017; Borrell et al., 2017; Holman et al.,
90 2019). These studies include the detection of new INNS, which had been previously overlooked by
91 traditional methods. For example, Blackman et al. (2017) used a metabarcoding approach to investigate
92 macroinvertebrate diversity and discovered a non-native Gammaridae species, *Gammarus fossarum*,
93 which traditional methods had failed to identify for over 50 years in the UK. Similarly, Simmons et al.

94 (2015) detected the Northern Snakeshead, *Channa argus*, in the Muskingum River Watershed, Ohio,
95 which had not been included on INNS priority lists. Metabarcoding also has the potential to act as a
96 surveillance tool and has been applied to monitor high-risk pathways, such as ballast water (Ardura et
97 al., 2015; Zaiko et al., 2015), the live bait trade (Mahon et al., 2014) and ports (Brown et al., 2016;
98 Borrell et al., 2017). However, comparisons of targeted and passive approaches for INNS or other focal
99 species have been exemplified in the bighead carp, *Hypophthalmichthys nobilis* (Simmons et al., 2015)
100 and great crested newts, *Triturus cristatus* (Harper et al., 2018). These studies both showed that a
101 targeted approach had a higher sensitivity than the passive approach. Possible causes of this include
102 primer choice and PCR bias, i.e. species successfully amplifying and masking others in the PCR reaction
103 during metabarcoding (Harper et al., 2018). Further comparisons of these methods covering different
104 target taxa are required to better inform management strategies.

105

106 In this study we focus on the quagga mussel, *Dreissena rostriformis bugensis*, a highly invasive bivalve
107 from the Ponto-Caspian region, which has a significant impact on all trophic levels within the
108 environments it invades (Karatayev et al., 2007; Roy et al., 2014). In a horizon scanning exercise from
109 2014, the quagga mussel scored the highest out of 591 species evaluated for its potential to invade,
110 establish and have negative impact in the UK (Roy et al., 2014). Consistent with predictions, quagga
111 mussels were subsequently detected later the same year (Aldridge et al., 2014). Due to its wider
112 tolerance to environmental conditions, it has the potential to spread rapidly, and occupy a greater range
113 of habitat than the closely related zebra mussel, *Dreissena polymorpha,* which has already spread
114 throughout much of Europe and North America (Nalepa et al., 2010; Gallardo and Aldridge 2013; Quinn
115 et al., 2014). Motivated by a need for cost effective tools for quagga mussel detection, we previously
116 designed a cPCR assay, which provided 100% detection rate in field trials with quagga mussel
117 populations (Blackman et al., 2020). Here we further developed and validated this assay for abundance
118 estimation using a dye-based qPCR approach in mesocosm experiments and field trials. Secondly, we

directly compared the three molecular methods (cPCR, qPCR and metabarcoding) to traditional kick-net sampling for detection of quagga mussels in the field. Finally, we investigated whether qPCR and metabarcoding are suitable for estimating relative abundance of quagga mussels by sampling a density gradient away from the main population. We hypothesized that: (1) the probability of detecting quagga mussels will be higher for eDNA methods than traditional methods, (2) targeted methods will have a higher probability of detecting quagga mussel than the passive method, (3) qPCR will be more sensitive than cPCR for quagga mussel detection and (4) both read count and DNA copy number will decline with distance downstream of the main population.

Methods

Field sample collection, DNA capture and extraction

Quagga mussels were first detected in the UK in Wraysbury Reservoir, Berkshire, in October 2014. This site is considered the founding population of quagga mussels in the UK. The mussels spread through the outfall and along the adjacent River Wraysbury, which is a tributary of the River Colne. The species currently has a restricted distribution in the South East of England but has also been recorded in reservoirs adjacent to the River Lee, north of London, and in the River Thames. Samples were collected from Wraysbury Reservoir and River in May 2016, working in an upstream direction to ensure DNA availability and inhibitor levels were not altered by sampling activity. In order to compare established and molecular methods directly, we collected eDNA and kick-net samples at six sites across the river at each of three main sampling locations downstream of Wraysbury Reservoir: Wraysbury Weir (WW, 0.61 km downstream of Wraysbury Reservoir), Wraysbury Bridge (WB, 1.10 km), and Wraysbury Gardens (WG, 2.7 km) (orange diamonds, Fig. 1 and Supplementary Information I Table S1). Water samples from these three locations consisted of three 500 ml replicates, collected at each of six sites across the width of the river (n = 54). eDNA samples were collected from the river surface, using sterile gloves and Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd, UK) prior to kick-net sampling. Three-minute

144 kick-net samples were collected from the same six sites at each of these three locations, in accordance
145 with established methods (Murray-Bligh, 1999). eDNA sampling of three 500 ml replicates was also
146 carried out at four additional locations along Wraysbury River (Reservoir Outfall, RO, 0.2 km; Moor Lane,
147 ML, 1.7 km; Hale Street, HS, 2.72 km; and Upper Thames Confluence, UT, 2.75 km, blue diamonds, Fig. 1,
148 Supplementary Information I Table S1). These additional eDNA samples were collected to provide
149 additional fine-scale resolution in the eDNA analysis, and were analysed with PCR and qPCR only,
150 whereas samples from the three main locations were analysed with PCR, qPCR and metabarcoding.
151 Sampling locations within the river were chosen based primarily on accessibility, in accordance with
152 standard macroinvertebrate monitoring procedures carried out by UK agencies. We did not target
153 specific locations with previous records of quagga mussels, or their preferred habitat. At the time of
154 sampling, quagga mussels had not been reported upstream of the reservoir. We therefore collected an
155 eDNA sample upstream of the reservoir with the expectation that this would be negative for quagga
156 mussel (Waterside Car Park, n = 3). Sample bottles filled with ddH_2O were also taken into the field as
157 filtering blanks (n = 3). Hence the total number of eDNA samples collected in the field was n = 72. All
158 eDNA samples and blanks were stored in a cool box with ice packs and transported to the laboratory for
159 filtration within 24 hours. Water samples were filtered through 0.45 µm cellulose nitrate membrane
160 filters attached to a vacuum pump and DNA extractions performed following Brolaski et al. (2008) with
161 minor modifications (see Supplementary Information I and II for full details). Kick-net samples were
162 placed in individual 42 fluid oz. sterile Whirl-pak® bags (Cole-Palmer, Hanwell, London) and stored in a
163 cool box before being frozen at -20°C in the laboratory. Each sample was thawed at room temperature
164 and rinsed with ddH_2O separately prior to analysis. Samples were then sorted for the presence of *D. r.*
165 *bugensis* only and all specimens were counted and subsequently stored at -20°C.

166

167 *Performance of targeted molecular detection*

168 The performance of targeted DNA assays for the detection of invasive quagga mussel DNA was first
169 examined through laboratory-based mesocosm experiments. The objectives of these experiments were
170 a) to validate species-specific DNA assays, using both conventional and quantitative PCR, to target eDNA
171 detection of quagga mussels; b) to assess the duration and persistence of eDNA signal over a 42-day
172 period; and c) to examine a correlation between eDNA concentration and known densities of quagga
173 mussel. The mesocosm experiment was set up to monitor DNA production of mussels at three densities
174 (1, 5 and 20 individuals, with total and individual biomass weights recorded) over 21 days, with three
175 replicates of each density and a control tank with no mussels present. Fifteen litre plastic tanks with
176 individual aeration stones were set up in a climate controlled facility where temperature averaged 16 °C
177 (range 14–18 °C), with light:dark cycles of 16 h:8 h. Sampling events took place over 42 days at: 0hrs
178 (prior to specimens being added), 4 hrs, 8 hrs, 24 hrs, 48 hrs, 72 hrs, 7 days, 15 days and 21 days. On day
179 21, the specimens were removed, and sampling continued at: 22 days, 28 days, 35 days, 42 days (n =
180 130). Two hundred millilitre water samples were collected at each time point and each sample was
181 filtered through a single filter membrane following the same protocol as the field samples (Blackman et
182 al., 2020).

183 *Conventional PCR (cPCR) assay*: We used a cPCR assay to detect the quagga mussel, *D. r.*
184 *bugensis*, using a species-specific primer pair targeting a 188 bp fragment of the cytochrome oxidase I
185 (COI) gene (DRB1_F – 5'-GGAAACTGGTTGGTCCCGAT3'- and DRB1_R 5'-GGCCCTGAATGCCCCATAAT-3',
186 see Supplementary Material I for full PCR protocol). The DRB1 PCR assay has shown to be highly
187 sensitive (positive detection with 0.001 ng of target tissue DNA per reaction) and does not cross amplify
188 closely related Dreissenid or native mussel species (Blackman et al., 2020). All mesocosm (n=130), field
189 samples (n = 66) and control samples (field (3), filter (3) and PCR (6) blanks) were tested with the DRB1
190 assay. A tissue DNA sample from closely related zebra mussel was included as an additional negative
191 control.

192 *Quantitative PCR (qPCR) assay:* The DRB1 assay was further developed using fluorescent dye-
193 based qPCR (PowerUp SYBR green, Thermo Fisher, UK, see Supplementary Material I for full details).
194 Briefly, we performed serial dilutions of DNA from tissue samples (from 1 to 1 x 10^{-7} ng/µl) to estimate
195 the LoD, and performed the DRB1 qPCR assay on samples collected from the mesocosm experiment,
196 field an control samples. Zebra mussel tissue DNA was used as a negative control in the qPCR assays.
197 Amplification curves, Cq values and melt curves were analysed using StepOne-PlusTM software 2.0.
198 Quantification calibration curves using a synthesized gBlock of the target fragment was used to estimate
199 the DNA copy number for each sample (See supplementary Information I and IV for full details).
200

201 *Passive detection: eDNA metabarcoding*

202 Degenerate metazoan primers that target the conserved COI region were used for metabarcoding
203 (Geller et al., 2013 and Leray et al., 2013): jgHCO2198: TAIACYTCIGGRTGICC RAARAAYCA and mICOIintF:
204 GGWACWGGWTGAACWGTWTAYCCYCC. These primers were chosen as they i) target the same
205 mitochondrial gene as our targeted assays, ii) are the most commonly used COI metabarcoding primers
206 and are good candidates for studying the entire river community, and iii) we confirmed in previous work
207 that they amplify Dreissenid mussels. We used universal primers rather than mollusc specific
208 metabarcoding primers (which are also available, Klymus et al., 2017), since universal primers are more
209 likely to be useful in routine community monitoring of INNS. We confirmed amplification of the target
210 taxa from tissue extractions prior to library preparation. Full details of the library preparation are
211 provided in Supplementary Information I. In brief, the 54 field samples from WW, WB and WG (Fig. 1)
212 were sequenced, with the addition of 3 field blanks, 3 filter blanks, 6 PCR blanks and 3 positive non-
213 target tissue samples (2 each of *Triops cancriformis and Osmia bicornis total n* = 69). A two-step PCR
214 protocol was used for library preparation: first the target region was amplified for each sample and a
215 second amplification was performed to add the Illumina adaptor sequences onto the initial amplicons.
216 Samples were normalized following the second PCR using SequalPrep Normalization plates (Invitrogen,

217 UK) and samples were then pooled in equimolar concentrations. The final pooled library was
218 concentrated, cleaned with a QIAquick Gel Extraction Kit (Qiagen, UK), quantified and sequenced at 13
219 pM concentration with 20% PhiX, on an Illumina MiSeq platform using 2 x 250bp v2 chemistry at the
220 University of Hull.
221
222 *Bioinformatics and data analysis*
223 Full details of the bioinformatics pipeline are provided in Supplementary Material I. In brief, processing
224 of Illumina data and taxonomic assignment were performed using a custom pipeline, MetaBEAT
225 (metaBarcoding and eDNA Analysis Tool) v 0.8 (https://github.com/HullUnibioinformatics/metaBEAT)
226 but with minor modifications including filtering for the presence of the target taxa, *D. r. bugensis,* by
227 comparing against curated reference sequences from GenBank, listed in Supplementary Material I.
228
229 We normalised the metabarcoding data to account for uneven sequencing depth between samples, by
230 dividing the number of reads for each OTU within a sample by the total number of reads in that sample
231 and multiplying by 100 (hereafter referred to as relative read count).We used Generalized Linear Models
232 (GLMs) to investigate 1) the influence of mussel density, biomass and time on the DNA copy number
233 (qPCR) since the start of the mesocosm experiment (before the mussels were removed from the tanks
234 only), and 2) the influence of mussel density and distance from source population on the DNA copy
235 number (qPCR) from the field samples and relative read count (metabarcoding). Initial model tests
236 showed all data sets were overdispersed, therefore a negative binomial GLM with a log link function was
237 used to resolve this issue. To check for model suitability, we checked the residual deviance of each
238 model fitted a chi square distribution (see Supplementary Information III Tables S2, S3 and S4 for GLM
239 results). Finally, we assessed the relationship between DNA copy number and relative read count with
240 the distance along the river using all seven sites included in the qPCR experiment, using a Spearman's
241 Rank Correlation since the data were not normally distributed. All calculations and visualizations of the

242 data were carried out in R (R-Core-Team 2017), with GLMs performed using the MASS package
243 (Venables et al., 2002).
244

245 Results

246 We successfully adapted the DRB1 primer pair for detection of quagga mussel with the addition of SYBR
247 green. Across both traditional and eDNA field experiments we were able to detect quagga mussel with
248 kick-net sampling, cPCR, qPCR and metabarcoding. All negative controls (field blanks, filtration blanks,
249 PCR blanks and non-target tissue blanks were negative for cPCR and qPCR. Minor contamination was
250 detected in 3 field blanks with metabarcoding, with low reads of *Homo sapiens (5 and 14 reads), Triops*
251 *cancriformis* (the positive tissue extract) (10 reads) and unassigned reads (42-189 reads). We therefore
252 applied a 0.2% read threshold to remove possible contamination from the metabarcoding dataset (i.e.
253 sequences that had a frequency of 0.2% or less in each sample were removed).
254

255 *qPCR assay development and validation in mesocosms*

256 The limit of detection (LoD) determined from the serial dilutions of tissue DNA was 1 x 10^{-4} ng/µl per
257 reaction with both cPCR and qPCR, which equates to approximately 5 copies per reaction (See
258 Supplementary Information III Fig. S1 and Fig. S2 for amplification plots). Rapid DNA accumulation in the
259 first two days of the mesocosm experiments was followed by a depletion in DNA concentration until
260 removal of mussels on day 21 of the experiment (Fig. 2). This pattern of DNA accumulation and
261 depletion is highly consistent across all three density treatments. Time since the beginning of the
262 experiment (until removal of the mussels) had a significant effect on the DNA copy number in each
263 mesocosm experiment when all density treatments were analysed together (GLM, $Z = -7.224$, $P <$
264 0.0001, AIC = 912.95). There is still some overdispersion with this model, however the overdispersion
265 parameter indicates this is not substantial (overdispersion statistic = 1.3, Residual Deviance 91.142 on 70
266 df, χ^2 P = 0.0457). There is substantial overlap between the DNA copy number estimates for the three

267 density treatments (Fig. 2) and neither density nor total biomass significantly explained the number of
268 DNA copies amplified by qPCR (Density Z = -1.019, P = 0.308; Total biomass Z = -0.776, P = 0.438). DNA
269 was still detectable 24 hours after removal of the mussels, but not at 7 days after removal (Day 28) (Fig.
270 2). All blanks and mesocosm control tank samples were negative of target DNA throughout the
271 experiment.
272
273 *Detection of D. r. bugensis by traditional and molecular approaches in the field*
274
275 *Kick-net sampling*
276 The number of *D. r. bugensis* specimens varied from 14 at Wraysbury Weir, the location closest to the
277 reservoir outfall (source population), to a single specimen at Wraysbury Gardens, the location furthest
278 from the outfall (Table 1). Detection rate was also highest at Wraysbury Weir (mussels detected in 4/6
279 sampling events). Mussels were detected in 1/6 samples at each of the other two sampling locations
280 (Table 1).
281
282 *Targeted approaches*
283 Detection rate was 100% in all three field locations and all replicates tested for both targeted
284 approaches (cPCR and qPCR) (Table 1 and Fig. S3). Fig. 3A shows the DNA copy number ($\log_{10}$) of the
285 qPCR assay for *D. r. bugensis* found at each of the sampling locations. Both distance from source and
286 number of mussels found at the three locations, significantly influenced the DNA copy number, but
287 distance was the strongest predictor (Density Z = -3.367, p = 0.000761; Distance Z = -17.223, p < 0.0001;
288 AIC: 1392.1, Residual Deviance 57.621 on 51 df, χ^2 P = 0.244). A Spearman's Rank correlation between
289 the DNA copy number and distance from the source population at each of the seven locations showed a
290 weak correlation (ρ = -0.25, p = 0.5948). However, the low copy numbers found immediately

291 downstream of the reservoir outfall (Fig. 3A) affected this. If this site is treated as an outlier and
292 removed from the analysis, the correlation is significant (ρ = -1, p = 0.002778).
293
294 *Passive approach*
295 The total number of reads produced following quality control and chimera removal was 4,928,265, of
296 which 576,365 reads were assigned to the samples in this study. A total of 5.19% reads (29,936) were
297 assigned to *D. r. bugensis.* After applying a 0.2% threshold to remove minor contamination the average
298 read depth per sample was 2921 over all taxa, and 548 for *D. r. bugensis* (excluding controls). The
299 clustering procedure identified 81 OTUs revealing a range of macroinvertebrate and algae/diatom
300 species (Supplementary information III, Fig. S4). The proportion of reads assigned to *D. r. bugensis* from
301 each sample within each location varied greatly as seen in Fig. 3B (n = 54). The target species was
302 detected in all samples from both Wraysbury Weir and Wraysbury Bridge locations, while detection at
303 Wraysbury Gardens was much lower with only 3 of the 18 replicates present for *D. r. bugensis* (Table 1).
304 Detection rate was therefore 86%, and is lower than the targeted methods. The average raw read count
305 of *D. r. bugensis* was 843 and 801 at Wraysbury Bridge and Wraysbury Weir respectively, and only 1
306 read on average at Wraysbury Gardens. Distance was the strongest predictor of the relative read count
307 (Z = 13.47, p < 0.0001; AIC = 301.86, Residual Deviance 54.989 on 52 df, χ^2 P = 0.36) in agreement with
308 the qPCR results and when compared to the distance and density model (Distance- Z = -4.707, p <
309 0.0001; Density Z = -3.773, p = 0.0001; AIC = 258.64, Residual Deviance 49.696 on 51 df, χ^2 P = 0.820),
310 and density on its own (which was overdispersed).
311

312 # Discussion

313 This study investigates the detection, persistence and quantification of the invasive non-native quagga
314 mussel (*D. r. bugensis*) using kick-net sampling and environmental DNA (eDNA). Through experimental
315 mesocosms, we validated a species-specific DNA assay (cPCR) to detect quagga mussel and further
316 developed a dye-based quantitative PCR (qPCR) assay to evaluate the performance of both targeted
317 approaches. We carried out an eDNA survey of water samples and compared targeted (cPCR and qPCR)
318 and passive (DNA metabarcoding) approaches to traditional kick-net sampling for qualitative and semi-
319 quantitative detection in the River Wraysbury, UK. In line with our first hypothesis, detection rate was
320 higher for all three eDNA methods than with the traditional method. Secondly, targeted approaches
321 were more sensitive than the passive approach (100% detection rates versus 86%). Contrary to our third
322 hypothesis, we observed no increased sensitivity using a dye-based qPCR over cPCR. Lastly, both density
323 and distance from the source population were significant predictors of relative read count
324 (metabarcoding) and copy number (qPCR) indicating that both approaches could provide estimates of
325 quagga mussel abundance in the field.
326
327 *qPCR validation*
328 Mesocosm validation provides an opportunity to test the LoD of eDNA assays and to investigate the
329 relationship between density, time and DNA copy number (Dejean et al., 2011; Thomsen et al., 2012;
330 Blackman et al., 2020). DNA was detected with qPCR at all three densities (1, 5 and 20 individuals) within
331 4 hours of being present, in agreement with previous cPCR results (Blackman et al., 2020). The LoD from
332 serial dilutions of tissue DNA were also identical for cPCR and qPCR (1×10^{-4} ng/µl). Surprisingly, the
333 mesocosm experiments highlighted unexpected dynamics of DNA in closed systems. Firstly, time since
334 the start of the experiment was a significant predictor of DNA copy number, but rather than increasing
335 over time or reaching a plateau, DNA accumulated quickly in the first 48 hours then rapidly depleted,
336 and this trend was seen in all three density treatments. A possible explanation for this is stress induced

337 on the mussel caused by a change in environment, as reported in other eDNA studies (Li et al., 2019).
338 Dreissenid mussels, however, are known to “shut-down” in times of stress, such as chemical dosing, and
339 can remain with their shells closed for up for three weeks (Aldridge et al., 2006). A further explanation
340 for the rapid accumulation in DNA may be due to the production of protein based byssal threads which
341 allow the mussels to attach to the substrate (Ricciardi et al., 1998; Aldridge et al., 2004; Timar and
342 Phaneuf, 2009; Peyer et al., 2009). During the first 48 hours of the experiment, the mussels are likely to
343 secure themselves to the new substrate. All mussels were found to have done this when they were
344 removed at the end of the experiment. The mussels are also filter-feeders and were actively feeding (on
345 microorganisms found in the river water) throughout the experiment producing sloughed cells, faeces
346 and pseudofaeces (Sansom and Sassoubre, 2017). This feeding behaviour is likely to be a major
347 contributor to successful detection in the field, and in a closed system, effect the DNA copy number
348 detected with the qPCR assay.
349
350 The second surprising result from the mesocosm experiments was that we found no effect of density or
351 biomass on DNA copy number. This contradicts several previous studies that have demonstrated a
352 relationship between biomass or density and DNA copy number (Thomsen et al., 2012; Takahara., et al
353 2012; Doi et al., 2015). This could in part be due to the physiology and feeding behaviour of mussels,
354 since eDNA is being consumed as well as produced (Friebertshauser et al., 2019). The complex pattern
355 of eDNA accumulation and depletion together with the lack of discrimination between different density
356 treatments presents challenges for estimation of quagga mussel abundance. However, eDNA is unlikely
357 to accumulate in the same way in natural (particularly lotic) environments, and this case highlights that
358 mesocosm experiments may poorly reflect the true relationship between DNA copy number and mussel
359 density in the field. It would be worthwhile to examine whether this pattern is also observed with other
360 filter feeding species. In keeping with previous studies (e.g. Thomsen et al., 2012), rapid degradation of
361 eDNA was observed in mesocosm experiments, with a complete loss of signal by one week after mussel

362 removal. Unfortunately, our experiments did not allow us to fully investigate the degradation rate of
363 eDNA in mesocosms since our sampling was too limited. We recommend future studies carry out more
364 intensive sampling once specimens have been removed.
365
366 *Comparison of eDNA and kick-net sampling for quagga mussel detection*
367 The lower sensitivity of kick-net sampling is highlighted by the failure to collect a *D. r. bugensis* specimen
368 in any of the first kick-net sampling attempts at each site. As demonstrated in a previous study, species
369 in low abundance are likely to be missed, with only 62% of macroinvertebrate families detected in a
370 three-minute kick-net sample (Furse et al., 1981). This highlights the need for multiple replicates for
371 detection of low abundance species if using kick-net sampling. It should be noted that surveillance
372 methods specifically for *D. r. bugensis* would be better focussed on the preferred habitat of mussels i.e.
373 hard substrates such as man-made structures (Aldridge et al., 2014), which is more likely to detect
374 quagga mussels than the randomised method used here. However, randomised kick-net sampling along
375 a range of substrate types is the most widely used sampling method for freshwater macroinvertebrate
376 monitoring, and therefore our experiment reflects the most likely methods to detect new INNS through
377 routine monitoring schemes.
378
379 Of the three molecular approaches compared in this study, we show that both targeted approaches
380 were more sensitive for detecting *D. r. bugensis* than metabarcoding, but that metabarcoding still
381 performed encouragingly well, given that the assay targeted metazoan in general rather than the focal
382 species. Few studies have compared targeted approaches with metabarcoding; however, our findings
383 are similar to those of Simmons et al., (2015) and Harper et al., (2018), who showed that metabarcoding
384 using conserved primers was less sensitive than targeted approaches. In this study, there was no
385 increase in the detection of *D. r. bugensis* when the 0.2% contamination threshold was removed.
386 However, it would be pertinent to examine the raw output of metabarcoding runs before thresholds are

387 applied in order to identify any potential INNS with low read counts. As this form of INNS monitoring is
388 still in its infancy, quality control measures to ensure these detections are correct are yet to be put in
389 place (Darling et al., 2020). We would therefore recommend any new INNS detections with
390 metabarcoding are followed up with species-specific primers or collection of specimens prior to
391 management actions to verify the detection.
392
393 A significant advantage of metabarcoding with conserved primers is the added information from non-
394 target species detection: in our study a further 81 OTUs were identified, and although no additional
395 INNS were detected, previous studies have demonstrated the power of this approach for detecting new
396 and unexpected INNS (Blackman et al., 2017). Metabarcoding also provides the opportunity to monitor
397 changes in community composition as the result of INNS or other ecological stressors (Simmons et al.,
398 2015; Hänfling et al., 2016), which is extremely promising for better understanding INNS impacts for
399 more effective prioritisation and management. This information on the wider community is a
400 justification for the use of universal primers rather than more taxon-specific metabarcoding assays, but
401 if more targeted information is needed, more specific metabarcoding primers can be developed. Indeed,
402 specific mollusc metabarcoding primers have recently been developed, and are very promising for more
403 targeted investigation of mollusc communities (Klymus et al., 2017). We hypothesize that more focussed
404 metabarcoding assays will yield even higher detection rates than the one reported here, reducing the
405 difference in sensitivity between targeted and passive approaches. It could also be possible to improve
406 the power of metabarcoding by increasing spatial sampling. Future studies should investigate how many
407 samples are needed in order to maximise the probability of detecting rare species with metabarcoding
408 and also the required read depth needed per sample in order to maximise species detection at a given
409 sampling site.
410

411 Conventional PCR and dye-based qPCR had identical detection rates for *D. r. bugensis* in our study. This
412 is somewhat surprising as qPCR is generally considered to be more sensitive than cPCR (e.g. Thomsen et
413 al., 2012, Nathan et al., 2014). Conventional PCR has the advantage of being quick and cost-effective,
414 requiring limited lab equipment and up to half the time for sample processing than qPCR (Davison et al.,
415 2016). Drawbacks to cPCR are typically lower sensitivity, and gel-quality issues that make interpretation
416 of results more subjective than with qPCR. This is dependent on the primer specificity and optimisation
417 of the PCR. Our conventional *D. r. bugensis* assay is highly specific and fully optimised, yielding clear
418 bands on agarose gels even in samples with low concentration of target species DNA (as inferred from
419 qPCR). However, we acknowledge that our experience with this assay may be unusual. An obvious
420 advantage to qPCR is the quantitative information it provides, in addition to increased sensitivity. The
421 added information on DNA concentration from qPCR in this study yielded important insights into the
422 mussel density and distance from the source population (see below), which was not possible with cPCR.
423 An important consideration is whether the assay could be improved by using probe-based rather than
424 dye-based qPCR. Addition of a probe is generally considered beneficial to prevent the possibility of non-
425 target amplification. Given the high specificity of the DRB1 primer pair described here, we believe the
426 addition of a probe is not necessary in this case, but we stress that adding a probe should be considered
427 if an extra layer of specificity is needed and/or if non-target amplification is possible.
428
429 *Quagga mussel eDNA declines with distance from the source and decreasing mussel density*
430 Results from field trials for both dye-based qPCR and metabarcoding demonstrate a significant decrease
431 in DNA copy number and relative read count of quagga mussel, respectively, with increasing distance
432 from the main source population (Wraysbury reservoir) and with decreasing mussel density. Kick-net
433 sample data also indicates that mussel density decreases along the course of the river, with only one
434 mussel found at Wraysbury Gardens, 2.7 km downstream of the reservoir. The only site that did not fit
435 this trend was the first site, 20 m downstream of the outfall from Wraysbury reservoir, which had a very

436 low DNA copy number. The decrease in concentration of quagga mussel eDNA detected with distance
437 from Wraysbury Reservoir, confirmed with both qPCR and metabarcoding, reflects both the decreasing
438 density of mussels and a gradient in the transport of DNA downstream. The fact that eDNA
439 concentration is not homogeneously distributed in the river suggests that it may be possible, at least in
440 some cases, to infer the source population in cases where it is unknown. This is somewhat surprising
441 given that our sampling took place after a period of heavy rain and eDNA distribution in rivers is often
442 considered to be uniform and heavily confounded by flow rate and mixing (e.g Jane et al., 2015).
443 Characteristics including discharge (Jane et al. 2015), physical structure and substrate type (Wilcox et al.,
444 2016; Shogren et al., 2017) and water chemistry (Seymour et al., 2018) all influence the transport and
445 retention of eDNA in lotic systems. Further empirical studies in rivers of all sizes and characteristics,
446 ideally in combination with hydrological modelling (Carraro et al., 2018), are needed to fully understand
447 the influence of flow dynamics and substrate type on retention and transport of eDNA in rivers.
448
449 Estimating abundance with eDNA is challenging because of the combined physical, chemical and
450 biological factors that influence production, transport and retention of DNA in a water body (Barnes et
451 al., 2014, Barnes and Turner 2015). Abundance estimation is expected to be particularly challenging in
452 rivers due to their complex hydrodynamics, and the transport of eDNA from upstream (e.g. Jerde et al.,
453 2016). Abundance estimation with metabarcoding is also controversial as read counts are influenced by
454 bias during sampling, laboratory and bioinformatics steps (Valentini et al., 2016). Despite these
455 challenges, our data add to the growing body of evidence that both qPCR and metabarcoding can yield
456 insight into relative biomass, which has rarely been demonstrated in lotic environments (but see e.g. Doi
457 et al., 2016, Akamatsu et al., 2020). Temporal sampling and controlling for environmental variables,
458 combined with improved statistical approaches such as site occupancy modelling, which is becoming
459 increasingly popular in eDNA studies (e.g. Schmidt et al., 2013, Pilliod et al., 2013, Chambert et al., 2018,
460 Sales et al., 2020) or spatially explicit approaches that take hydrological conditions into account (e.g.

461 Shogren et al., 2017, Carraro et al., 2018) will provide better quantitative information, and allow
462 changes in biomass to be monitored.
463
464 *Conclusions and practical considerations for future INNS biomonitoring with eDNA*
465 We have demonstrated that eDNA from quagga mussels can be successfully detected using cPCR, qPCR
466 and metabarcoding in a UK river. Both qPCR and metabarcoding provide quantitative information, with
467 DNA copy number and relative read count significantly decreasing with a combination of distance from
468 the source population and mussel density. In our previous work we demonstrated that metabarcoding is
469 an effective tool for passive detection of a previously unrecorded cryptic non-native species, *Gammarus*
470 *fossarum* (Blackman et al. 2017). The present study provides further demonstration that metabarcoding
471 with conserved metazoan primers is suitable as an early warning tool for non-targeted monitoring or
472 routine surveillance even at a very low population density. The optimal approach for detection of INNS
473 using eDNA will depend on the objectives of the research or monitoring programme. For early detection
474 of previously unrecorded INNS, we recommend a comprehensive, randomised sampling design,
475 combined with metabarcoding using universal primers, which can be supplemented with targeted
476 approaches if additional verification is needed. Targeted qPCR assays provide optimal sensitivity and
477 specificity and are particularly suited to surveys of INNS that have been previously recorded. In terms of
478 future monitoring of quagga mussels, we believe that the sampling and laboratory methods adopted in
479 the present study are widely applicable, but a temporal study would be beneficial to determine the
480 optimum timing for sampling, and greater spatial coverage is needed to investigate the influence of
481 environmental variables on detection rates.
482
483

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Author contributions

The design and concept of this study was developed by RCB and LLH. RCB and LH carried out the field work. RCB, KKSL and PS performed the lab work. RCB analysed the data. RCB and LLH wrote the paper. All authors have approved the final manuscript.

Data availability statement

Data generated in this study is available from the GitHub repository:

https://github.com/RosettaBlackman/Blackman_et_al_quagga

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Figures and Table:

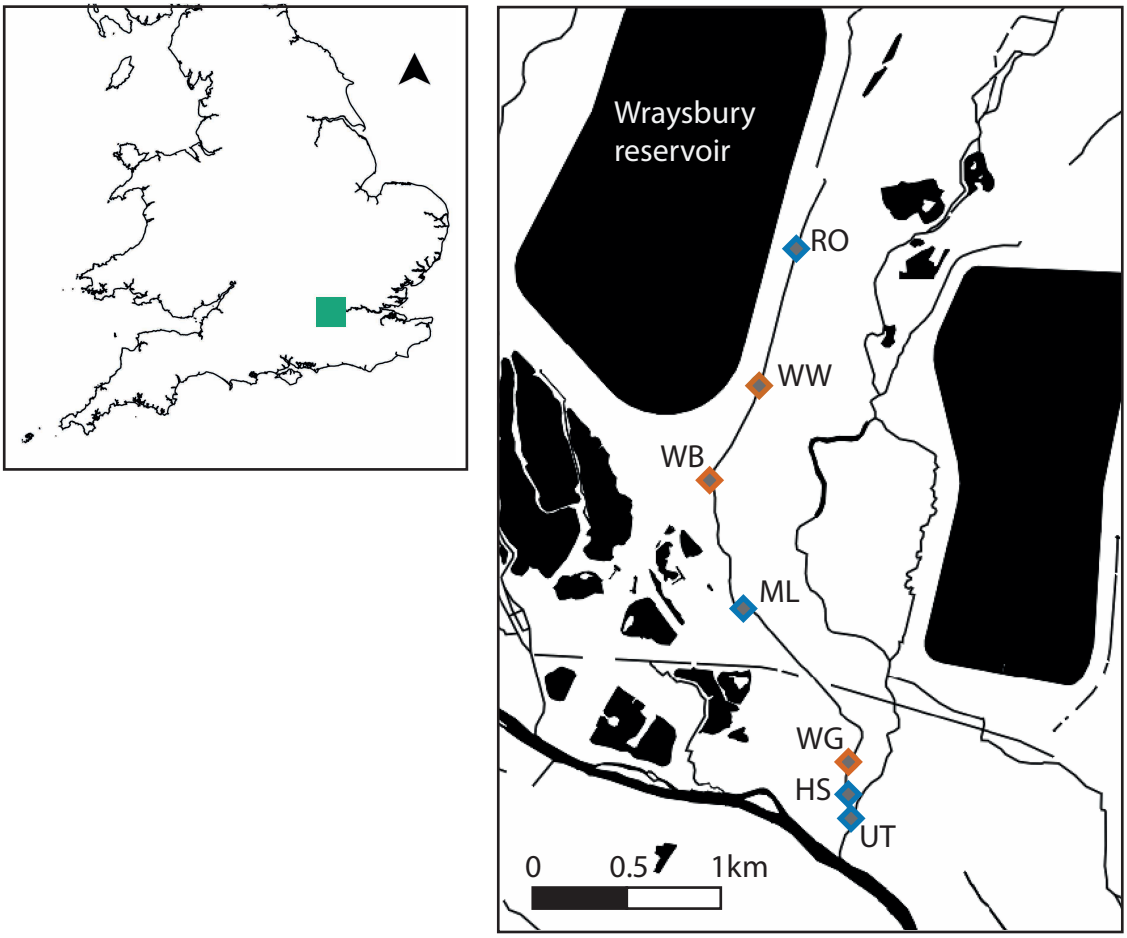
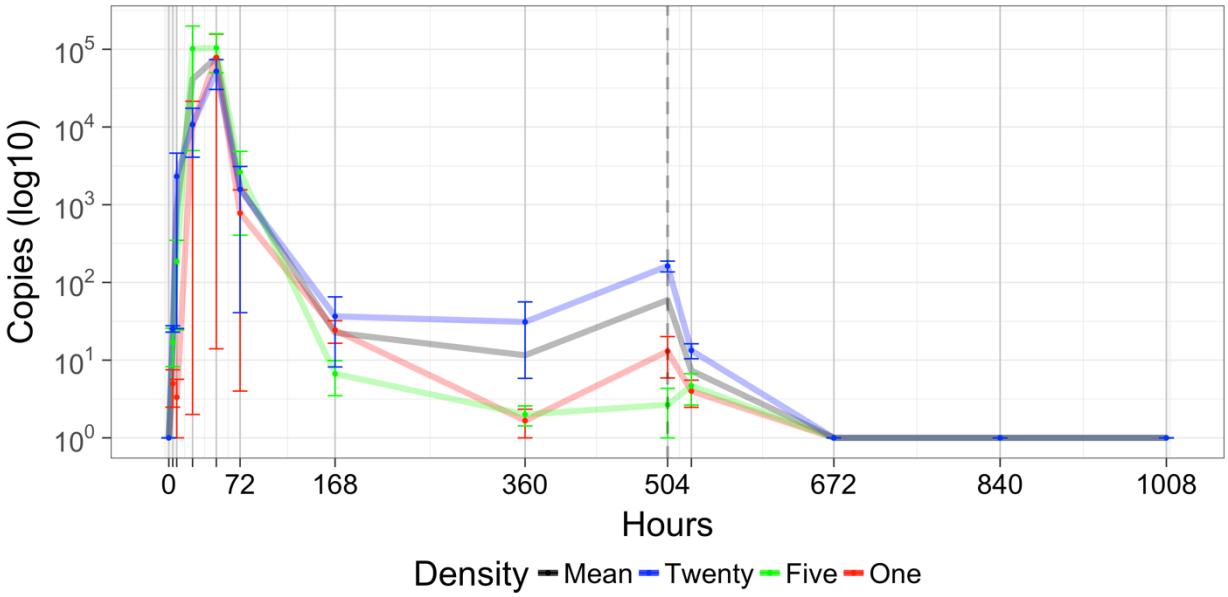


Figure 1: Sampling locations on the Wraysbury River. Diamond symbols represent the sampling locations: Reservoir Outfall (RO, 0.2 km downstream of the reservoir), Wraysbury Weir (WW, 0.61 km), Wraysbury Bridge (WB, 1.10 km), Moor Lane (ML, 1.7 km), Wraysbury Gardens (WG, 2.7 km), Hale Street (HS, 2.72 km), and Upstream Thames confluence (UT, 2.75 km). eDNA and kick-net samples were collected from sites with an orange outline and sites sampled for eDNA only have a blue outlined symbol (Full grid references are provided in Supplementary Information I Table S1).



794

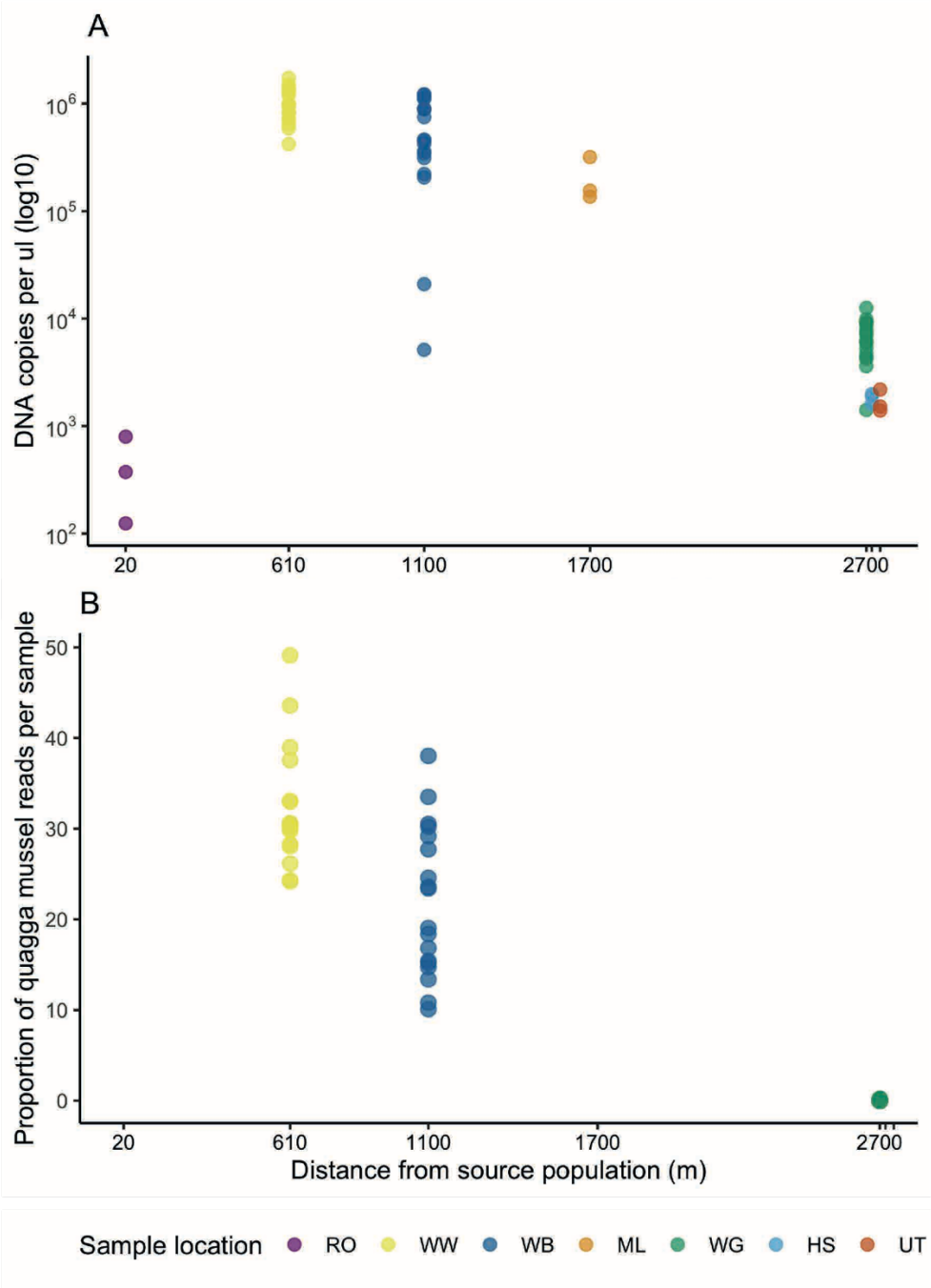
795 **Figure 2: *Dreissena rostriformis bugensis* qPCR mesocosm assay validation.** The graph shows the mean

796 and standard deviation of DNA copy number recorded for each density treatment during the mesocosm

797 experiment: red line – 1 specimen, green line – 5 specimens, blue line – 20 specimens and the black line

798 is the mean copy number for all three densities. The vertical dashed line indicates when the mussels

799 were removed from the mesocosm experiment.



800

801 **Figure 3: *Dreissena rostriformis bugensis* DNA signal recorded at field sites.** A - qPCR DNA copy number

802 per µl (log^{10}) at all 7 sample sites. B – Normalised relative read count of taxa assigned to quagga mussel

803 at the three locations sequenced.

804

805

806 **Table 1 A - C: Comparison of methods for the detection of *D. r. bugensis* at three locations on the**
807 **Wraysbury River.** "+" indicates a positive detection and "–" indicates no detection for each technical
808 replicate (i.e. three per sample for the eDNA methods and one for the kick sample). The numbers in
809 parentheses under Kick Sampling correspond to the number of individuals found in each sample.

810 A. Wraysbury Weir (WW)

	Kick sampling	**Environmental DNA**		
Sample No.	No. of individuals	PCR	qPCR	Metabarcoding
1	-	+ + +	+ + +	+ + +
2	+ (4)	+ + +	+ + +	+ + +
3	+ (4)	+ + +	+ + +	+ + +
4	-	+ + +	+ + +	+ + +
5	+ (3)	+ + +	+ + +	+ + +
6	+ (3)	+ + +	+ + +	+ + +

811

812 B. Wraysbury Bridge (WB)

	Kick sampling	**Environmental DNA**		
Sample No.	No. of individuals	PCR	qPCR	Metabarcoding
1	-	+ + +	+ + +	+ + +
2	-	+ + +	+ + +	+ + +
3	-	+ + +	+ + +	+ + +
4	-	+ + +	+ + +	+ + +
5	+ (4)	+ + +	+ + +	+ + +
6	-	+ + +	+ + +	+ + +

813

814 C. Wraysbury Gardens (WG)

	Kick sampling	Environmental DNA		
Sample No.	No. of individuals	PCR	qPCR	Metabarcoding
1	-	+ + +	+ + +	- + -
2	-	+ + +	+ + +	- - +
3	+ (1)	+ + +	+ + +	- - +
4	-	+ + +	+ + +	- - -
5	-	+ + +	+ + +	- - -
6	-	+ + +	+ + +	- - -

815