Title:

Environmental DNA consistently outperforms 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 to allow for informed 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) 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 read count (metabarcoding) had a significant negative relationship with distance from source population.
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.

Keywords: early detection, invasive non-native species, targeted assay, metabarcoding, PCR, qPCR

Introduction

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

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

monitoring and targeted (Dejean et al., 2012; Tréguier et al., 2014) or passive (Smart et al., 2015; Hänfling et al., 2016; Wilcox et al., 2016) molecular approaches have demonstrated an increased probability of detection using eDNA methods.

The targeted approach was first used by Ficetola et al. (2008) to detect invasive *Lithobates catesbeianus*, in lentic waterbodies and has since been applied to over 100 target INNS (Blackman et al., 2018). Conventional PCR is a well-established and widely practiced method for detecting presence/absence of target DNA by visualisation of PCR products on agarose gels. However, qPCR has on many occasions been shown to be more sensitive than PCR, (e.g. Nathan et al., 2014; Simmons et al., 2015; De Ventura et al., 2016) and therefore more suitable for detecting INNS at low density, e.g. the early stages of invasion. Quantitative PCR has the added advantage of providing the number of DNA copies in a sample, which in previous studies has been linked to biomass or abundance of some taxa (e.g. Amphibians, Thomsen et al., 2012; Fish, Takahara et al., 2012; Gastropods, Goldberg et al., 2013). With the addition of a probe-based qPCR assay there is added specificity not found with conventional or dye-based qPCR. However, qPCR requires the construction of calibration curves from known standard concentrations and is substantially more expensive and time-consuming than conventional PCR (Nathan et al., 2014) and maybe less accessible to end-users than conventional PCR.

The potential of eDNA metabarcoding for INNS monitoring has already been shown as an effective tool (Simmons et al., 2015; Brown et al., 2016; Blackman et al., 2017; Borrell et al., 2017; Holman et al., 2019). These studies include the detection of new INNS, which had been previously overlooked by traditional methods or targeted molecular tools. For example, Blackman et al. (2017) used a metabarcoding approach to investigate macroinvertebrate diversity and discovered a non-native Gammaridae species, *Gammarus fossarum*, which traditional methods had failed to identify for over 50 years in the UK. Similarly, Simmons et al. (2015) detected the Northern Snakeshead, *Channa argus*, in the Muskingum River Watershed, Ohio, which had not been included on INNS priority lists. Metabarcoding also has the potential to act as a surveillance tool and has been applied to monitor high-risk pathways, such as ballast water (Ardura et al.,

95 2015; Zaiko et al., 2015), the live bait trade (Mahon et al., 2014) and ports (Brown et al., 2016; Borrell et
96 al., 2017). However, comparisons of targeted and passive approaches for INNS or other focal species are
97 limited to a few exceptions including bighead carp, *Hypophthalmichthys nobilis* (Simmons et al., 2015) and
98 great crested newts, *Triturus cristatus* (Harper et al., 2018). These studies both showed that a targeted
99 approach had a higher sensitivity than the passive approach. Possible causes of this include primer choice
100 and PCR bias, i.e. species successfully amplifying and masking others in the PCR reaction during
101 metabarcoding (Harper et al., 2018), but further comparisons of these methods that include a more diverse
102 set of target taxa are required to better inform management strategies.

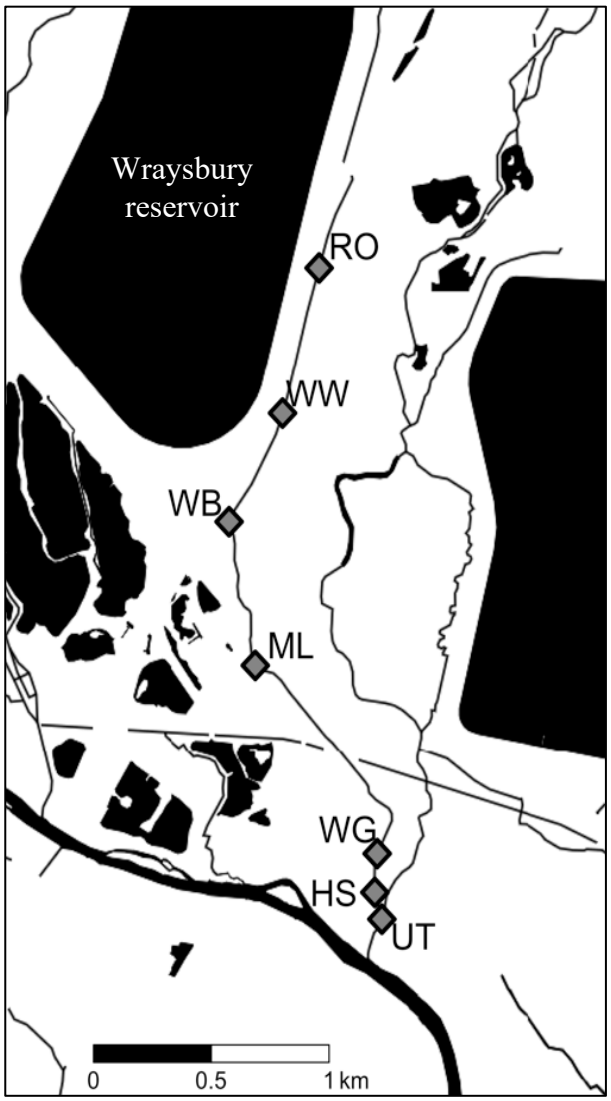
103

104 In this study we focus on the quagga mussel, *Dreissena rostriformis bugensis*, a highly invasive bivalve
105 from the Ponto-Caspian region, which has a significant impact on all trophic levels within the
106 environments it invades (Karatayev et al., 2007; Roy et al., 2014). The quagga mussel scored the highest
107 for its potential invasions to the UK in terms of impact, risk of invading and establishing in a study from
108 2014 (Roy et al., 2014), and was later detected that same year (Aldridge et al., 2014). Due to its wider
109 tolerance to environmental conditions, it has the potential to spread rapidly, and occupy a greater range of
110 habitat than the closely related zebra mussel, *Dreissena polymorpha,* which has already spread throughout
111 much of Europe and North America (Nalepa et al., 2010; Gallardo and Aldridge 2013; Quinn et al.,
112 2014). Motivated by a need for cost effective tools for quagga mussel detection, we previously designed a
113 conventional PCR assay, which provided 100% detection rate in field trials with quagga mussel
114 populations (Blackman et al., *in press*). Firstly, we further developed and validated this assay for
115 abundance estimation using a dye-based qPCR approach for quagga mussels in mesocosm experiments
116 and field trials. Secondly, we directly compared the three molecular methods (conventional PCR, qPCR
117 and metabarcoding) to traditional kick-net sampling for detection of quagga mussels in the field. Finally,
118 we investigated whether qPCR and metabarcoding are suitable for estimating relative abundance of
119 quagga mussels; by sampling a density gradient away from the main population. We hypothesized that:
120 (1) the probability of detecting quagga mussels will be higher for eDNA methods than traditional
121 methods, (2) targeted methods will have a higher probability of detecting quagga mussel than the passive

122 method, (3) qPCR will be more sensitive than conventional PCR for quagga mussel detection and (4) both
123 read count and DNA copy number will decline with distance downstream of the source population in the
124 Wraysbury Reservoir.
125
126 **Methods**
127
128 *Field sample collection, DNA capture and extraction*
129
130 Wraysbury Reservoir, Berkshire, is considered the founding population of quagga mussels in the UK. The
131 mussels spread through the outfall and along the adjacent River Wraysbury. Samples were collected by
132 working in an upstream direction to ensure DNA availability and inhibitor levels were not altered by
133 sampling activity. Environmental DNA samples comprised of 3 x 500 ml water samples collected from
134 the surface at points across the river width and combined into sterile sampling bottles (i.e. 1.5 L in total
135 per sample). In order to compare established and molecular methods directly, we collected six of each
136 sample type (6 x 1.5 L water samples and 6 x kick-net samples) at three main sampling sites along the
137 river. These sites were Wraysbury Weir (WW, 0.61 km downstream of Wraysbury Reservoir), Wraysbury
138 Bridge (WB, 1.10 km), and Wraysbury Gardens (WG, 2.75 km) (See Fig. 1 and Supplementary
139 Information I Table S1) (n = 54). In addition, we collected four eDNA samples for conventional and
140 qPCR analysis only. These sites were: Reservoir Outfall (RO, 0.2 km downstream from Wraysbury
141 Reservoir), Moor Lane (ML, 1.7 km), Hale Street (HS, 2.72 km), and Upstream Thames confluence (UT,
142 2.75 km) (See Fig. 1 and Supplementary Information I Table S1 for further sampling site details) (n = 12,
143 n = 66 total). An upstream sample from above the reservoir outfall acted as a negative control. Sample
144 bottles filled with ddH_2O were taken into the field to be used as sample blanks (n = 6). (See
145 Supplementary Information II and III for water filtration and DNA extraction methods details)
146

Kick-net sampling was performed using a standard three minute kick-net sampling method followed by a one minute hand search (Murray-Bligh, 1999). Kick-net samples were placed in labelled 42 fluid oz. sterile Whirl-pak® bag (Cole-Palmer, Hanwell, London) and stored in a cool box before being frozen at the laboratory. Each sample was defrosted and rinsed with ddH_2O separately prior to analysis. Samples were then sorted for the presence of *D. r. bugensis* only and all specimens were counted and subsequently stored at -20°C.

Figure 1: Sampling sites on the Wraysbury River, downstream of the main source population of *D. r. bugensis* in the Wraysbury Reservoir. Sampling sites were the 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). Full grid references are provided in Supplementary Information I Table S1.



154 Conventional PCR

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156 We used the species-specific primer set published by Blackman et al. (*in press*) for PCR amplification,
157 which targets a 188 bp fragment of the cytochrome oxidase I (COI) gene (DRB1_F -
158 GGAAACTGGTTGGTCCCGAT and DRB1_R GGCCCTGAATGCCCCATAAT). We previously
159 demonstrated that *D. r. bugensis* can be detected in mesocosms using this assay after four hours and at
160 three densities (1, 5, and 20 individuals in 15 L water, Blackman et al., *in press*). This assay is highly
161 sensitive for PCR (Limit of Detection [LoD] 0.0005 ng/µl) and does not cross amplify closely related
162 Dreissenid or native mussel species (Blackman et al., *in press*). All samples (n = 66), sampling blanks (n
163 = 6), filter blanks (n = 6), and PCR blanks (n = 6) were tested with the DRB1 assay, including tissue
164 samples from the closely related zebra mussel, acting as an additional control and to check for cross
165 contamination (See Supplementary Information II for full details).

166

167 qPCR assay development

168

169 The DRB1 assay was further developed using fluorescent dye-based qPCR. We carried out a serial
170 dilution of DNA from tissue samples (from 1 to 1 x 10^{-7} ng/µl) to estimate the Limit of Detection (LoD),
171 and tested the assay in the same mesocosm experiment previously used for validating the conventional
172 PCR assay (Blackman et al., *in press*) to ensure the assay detected *D. r. bugensis* DNA only with no cross
173 amplification. In brief, the mesocosm experiment monitored DNA production from mussels in three
174 densities (1, 5 and 20 individuals) over 21 days, with 3 replicates of each density and a control tank with
175 no mussels present (See Blackman et al., *in press* for further details). Fifteen litre tanks were sampled
176 over 21 days at: 0hrs (prior to specimens being added), 4hrs, 8hrs, 24hrs, 48 hrs, 72 hrs, 7 days, 15 days
177 and 21 days. On day 21, the specimens were removed, and sampling continued at: 22 days, 28 days, 35
178 days, 42 days (n = 130). Two hundred millilitres of water was collected at each time point and each
179 sample was filtered through a single filter membrane following the same protocol as the field samples.

All samples (n = 66), sampling blanks (n = 6), filter blanks (n = 6), and PCR blanks (n = 6) were tested with DRB1 qPCR assay. Each qPCR plate had the addition of tissue DNA from a zebra mussel to act as an additional negative control (See supplementary Information II for full qPCR protocol). Amplification curves, Cq values and melt curves were analysed using StepOne-PlusTM software 2.0. DNA copy number for each sample was calculated using Cq value and standard curve results (See supplementary Information II and IV for full details).

Passive detection: eDNA metabarcoding

To match the region used for conventional and qPCR we chose the COI region for metabarcoding, which also has the broadest taxonomic coverage for macroinvertebrates in public sequence databases. The metabarcoding library was constructed using universal primers described by Geller et al., 2013 and Leray et al., 2013. We confirmed amplification of the target taxa with tissue extractions prior to library preparation. A two-step protocol was used for library preparation: briefly, this method amplifies the target region during the first PCR and sample identification tags are added during a reduced cycle second PCR, full details of the workflow are in Supplementary Information II. In total 54 samples were sequenced, with the addition of 6 field blanks, 6 PCR blanks and 4 positive non-target tissue samples (n= 70).

Bioinformatics pipeline

The bioinformatics approach as described in Blackman et al., (2017) was followed with minor modifications (See Supplementary Information II). In brief, sequences were filtered for the presence of the target taxa only, *D. r. bugensis,* using GenBank sequences: EF080862, JX945980, EF080861, U47651, AF495877, EU484436, KJ881409 (See Supplementary Information II for full details).

Data analysis

207

208 In order to normalise the sequencing depth of each sample from the metabarcoding data the number of
209 sequences assigned to *D. r. bugensis* was divided by the total number of sequences from that sample. We
210 used Generalized Linear Models (GLMs) to investigate 1) the influence of mussel density, biomass and
211 time on the DNA copy number (qPCR) since the start of the mesocosm experiment (before the mussels
212 were removed from the tanks only), and 2) the influence of mussel density and distance from source
213 population on the DNA copy number (qPCR) from the field samples and read count (metabarcoding).
214 Initial model tests showed all data sets were overdispersed, therefore a negative binomial GLM with a log
215 link function was used to resolve this issue. To check for model suitability, we checked the residual
216 deviance of each model fitted a chi square distribution (see Supplementary Information I for GLM
217 results). Finally, we assessed the relationship between DNA copy number and read count with the
218 distance along the river using all seven sites included in the qPCR experiment, using a Spearman's Rank
219 Correlation since the data were not normally distributed. All calculations and visualizations of the data
220 were carried out in R (R-Core-Team 2017), with GLMs performed using the MASS package (Venables et
221 al., 2002).

222

223 **Results**

224

225 *qPCR assay development and validation in mesocosms*

226

227 The LoD determined from the serial dilutions of tissue DNA was 1 x 10^{-4} ng/µl per reaction with both
228 conventional PCR and qPCR, which equates to approximately 5 copies per reaction (See Supplementary
229 Information I Fig. S1 and Fig. S3 for amplification plots). We previously demonstrated that *D. r. bugensis*
230 can be detected in mesocosms by conventional PCR after four hours and at all three densities using the
231 same mesocosm experiment as described in this study (Blackman et al., *in press*). This result was
232 confirmed here using qPCR (Fig. 2). Rapid DNA accumulation in the first two days of the mesocosm
233 experiments was followed by a depletion in DNA concentration until removal of mussels on day 21 of the

experiment. This pattern of DNA accumulation and depletion is highly consistent across all three density treatments (Fig. 2). Time since the beginning of the experiment (until removal of the mussels) had a significant effect on the DNA copy number in each mesocosm experiment when all density treatments were analysed together (GLM, Z = -7.224, P < 0.0001, AIC = 912.95). There is still some overdispersion with this model, however the overdispersion parameter indicates this is not substantial (overdispersion statistic = 1.3, Residual Deviance 91.142 on 70 df, χ^2 P = 0.0457). There is substantial overlap between the DNA copy number estimates for the three density treatments (Fig. 2) and neither density nor total biomass significantly explained the number of DNA copies amplified by qPCR (Density Z = -1.019, P = 0.308; Total biomass Z = -0.776, P = 0.438). DNA was still detectable 24 hours after removal of the mussels, but not at the at 7 days after removal (Day 28) (Fig. 2). All blanks and mesocosm control tank samples were clear of target DNA throughout the experiment.

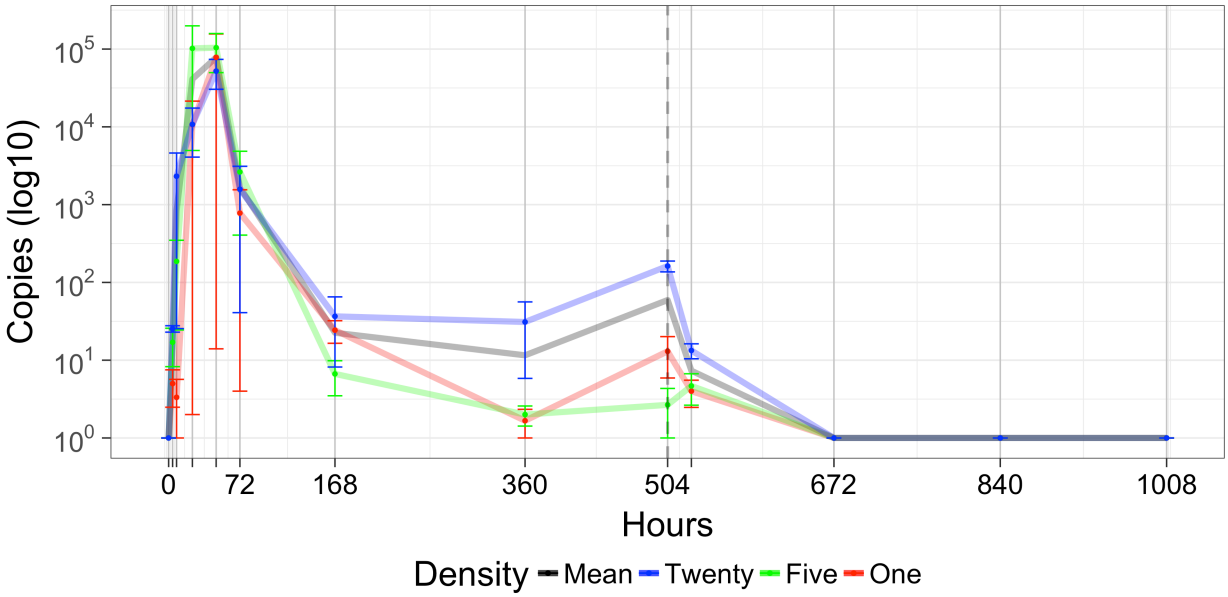

Figure 2: *Dreissena rostriformis bugensis* qPCR mesocosm assay validation. The graph shows the mean and standard deviation of DNA copy number recorded for each density treatment during the mesocosm experiment: red line – 1 specimen, green line – 5 specimens, blue line – 20 specimens and the black line is the mean copy number for all three densities. The vertical dashed line indicates when the mussels were removed from the mesocosm experiment.

Detection of D. r. bugensis by traditional and molecular approaches in the field

Kick-net sampling

The number of *D. r. bugensis* specimens varied from 14 at Wraysbury Weir, the site closest to the reservoir outfall (source population), to a single specimen at Wraysbury Gardens, the site furthest from the outfall (Table 1). Detection rate was also highest at Wraysbury Weir (mussels detected in 4/6 sampling events). Mussels were detected in 1/6 samples at each of the other two sampling sites (Table 1).

Targeted approaches

Detection rate was 100% in all three field sites and all replicates tested for both targeted approaches (conventional PCR and qPCR) (Table 1 and Fig. S2). For both conventional PCR and qPCR, all negative and non-target tissue samples were clear of amplification for *D. r. bugensis* DNA. Fig. 3A shows the DNA copy number ($\log_{10}$) for *D. r. bugensis* found at each of the sampling sites. Both distance from source and number of mussels found at the three sites, significantly influenced the DNA copy number, but distance was the strongest predictor (Density $Z = -3.367$, $P = 0.000761$; Distance $Z = -17.223$, $P < 2e-16$; AIC: 1392.1, Residual Deviance 57.621 on 51 df, χ^2 $P = 0.244$). A Spearman's Rank correlation between the DNA copy number at each of the seven sites screened by qPCR and distance from the source population showed a weak correlation ($\rho = -0.25$, $P = 0.5948$). However, the low copy numbers found immediately downstream of the reservoir outfall (Fig. 3A) likely affected this. If this site is treated as an outlier and removed from the analysis, the correlation is significant ($\rho = -1$, $P = 0.002778$).

Passive approach

Metabarcoding was less sensitive than the targeted methods at the lowest quagga mussel density (detection rate = 86%). The total assigned read count passing quality control after chimera removal was

4,928,265, of which 0.6% of reads (i.e. 29,570 reads) were assigned to *D. r. bugensis* after application of a 0.2% threshold to remove minor contamination. The clustering procedure identified 281 OTUs in total including a range of macroinvertebrate and algae/diatom species (Supplementary information I, Fig. S4). The proportion of reads assigned to *D. r. bugensis* from each sample within each site varied greatly as seen in Fig. 3B (n=54). The target species was detected in all samples from both Wraysbury Weir and Wraysbury Bridge sites, while detection at Wraysbury Gardens was much lower with only 3 of the 18 replicates having *D. r. bugensis* reads (Table 1). Furthermore, the raw number of *D. r. bugensis* reads was substantially lower with average read counts of 843 and 801 at Wraysbury Bridge and Wraysbury Weir, respectively and only 1 read on average at Wraysbury Gardens. The read count shows a strong relationship with both the number of mussels collected at the three sites and the distance from the source populations, but distance was the strongest best predictor (Distance $Z = -4.707$, $P < 0.0001$; Density $Z = -3.773$, $P = 0.0001$; AIC = 258.64, Residual Deviance 49.696 on 51 df, χ^2 $P = 0.820$), in agreement with the qPCR results.

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

A. Wraysbury Weir (WW)

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

B. Wraysbury Bridge (WB)

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

C. Wraysbury Gardens (WG)

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

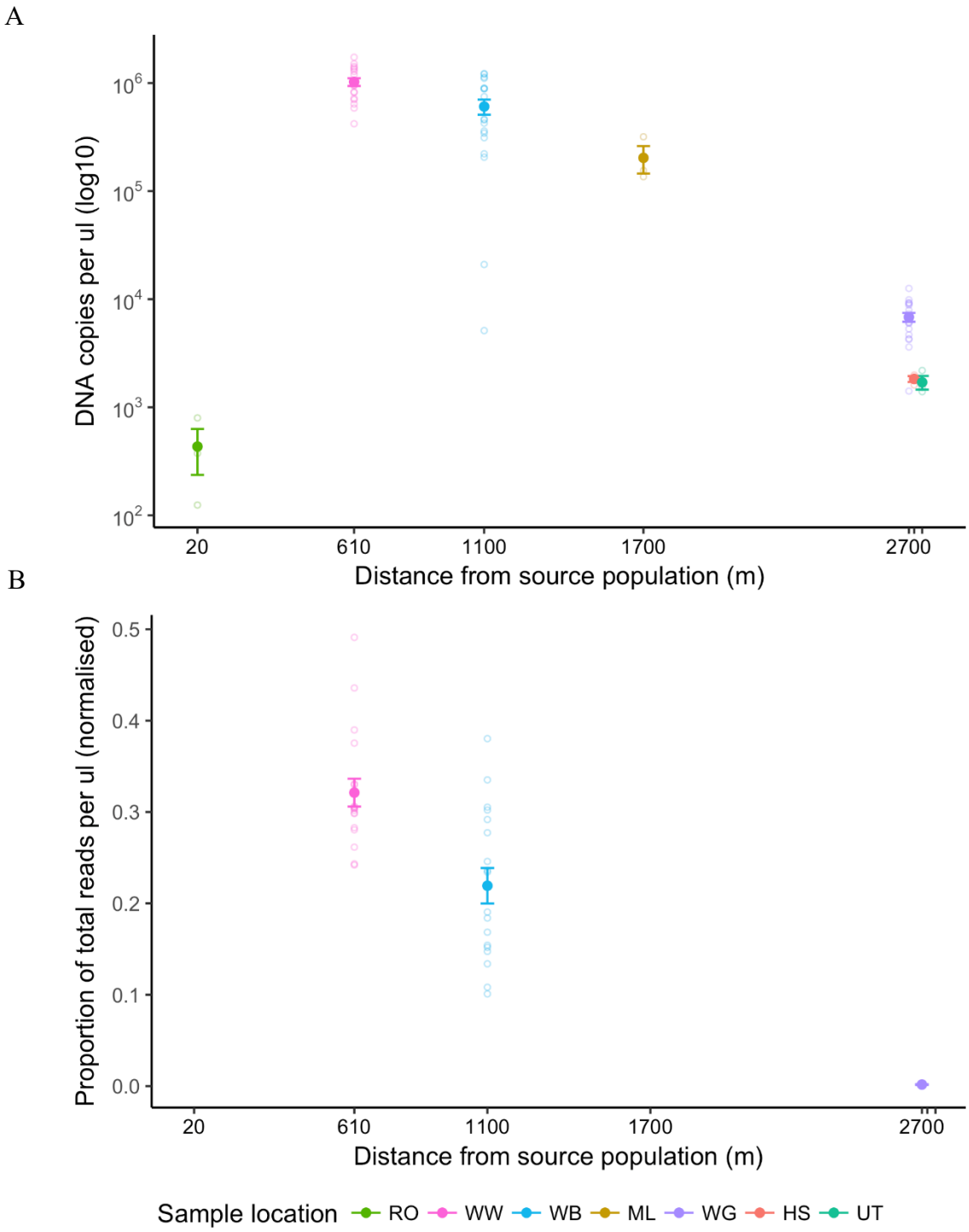

Figure 3: *Dreissena rostriformis bugensis* DNA signal recorded at field sites. A - qPCR DNA copy number per µl ($\log^{10}$) at all 7 sample sites. B - Metabarcoding read count per sample at each of the three sites sequenced. The graph shows the read number for each site normalised over the total number of reads obtained from each sample.

308 **Discussion**

309

310 In this study, we developed and validated a dye-based qPCR assay for *D. r. bugensis* then compared
311 targeted (conventional PCR and qPCR) and passive (metabarcoding) approaches to traditional kick-net
312 sampling for qualitative and semi-quantitative detection in the field. In line with our first hypothesis,
313 detection rate was higher for all three eDNA methods than the traditional method. Secondly, targeted
314 approaches were more sensitive than the passive approach. Contrary to our third hypothesis, we saw no
315 increased sensitivity using a dye-based qPCR over conventional PCR. Lastly, both density and distance
316 from the source population were significant predictors of read count (metabarcoding) and copy number
317 (qPCR) indicating that both approaches could provide some indication of quagga mussel abundance in the
318 field.

319

320 *qPCR validation*

321

322 Mesocosm validation provides an opportunity to test the LoD of eDNA assays and to investigate the
323 relationship between density, time and DNA copy number (Dejean et al., 2011; Thomsen et al., 2012;
324 Blackman et al., *in press*). DNA was detected with qPCR at all three densities (1, 5 and 20 individuals)
325 within 4 hours of being present, in agreement with previous conventional PCR results (Blackman et al., *in*
326 *press*). The LoD from serial dilutions of tissue DNA were also identical for conventional and qPCR (1 x
327 10^{-4} ng/µl). Surprisingly, the mesocosm experiments highlighted unexpected dynamics of DNA in closed
328 systems. Firstly, time since the start of the experiment was a significant predictor of DNA copy number,
329 but rather than increasing over time or reaching a plateau, DNA accumulated quickly in the 48 hours then
330 rapidly depleted, and this trend was replicated in all three density treatments. A possible explanation for
331 this is stress induced on the mussel caused by a change in environment, as reported in other eDNA studies
332 (Li et al., 2019). Dreissenid mussels, however, are known to "shut-down" in times of stress, such as
333 chemical dosing, and can remain with their shells closed for up for three weeks (Aldridge et al., 2006). A

334 further explanation for the rapid accumulation in DNA may be due to the production of protein based
335 byssal threads which allow the mussels to attach to the substrate (Ricciardi et al., 1998; Aldridge et al.,
336 2004; Timar and Phaneuf, 2009; Peyer et al., 2009). During the first 48 hours of the experiment, the
337 mussels are likely to secure themselves to the new substrate, all mussels were found to have done this
338 when they were removed at the end of the experiment. The mussels are also filter-feeders and were
339 actively feeding (on microorganisms found in the river water) throughout the experiment producing
340 sloughed cells, faeces and pseudofaeces (Sansom and Sassoubre., 2017). This feeding behaviour is likely
341 to be a major contributor to successful detection in the field and in a closed system effect the amount
342 DNA copy number detected with the qPCR assay.
343
344 The second surprising result from the mesocosm experiments was that we found no effect of density or
345 biomass on DNA copy number. This contradicts several previous studies that have demonstrated a
346 relationship between biomass or density and DNA copy number (Thomsen et al., 2012; Takahara., et al
347 2012; Doi et al., 2015). This could in part be due to the physiology and feeding behaviour of mussels,
348 since eDNA is being consumed as well as produced. The complex pattern of eDNA accumulation and
349 depletion together with the lack of discrimination between different density treatments presents
350 challenges for estimation of quagga mussel abundance. However, eDNA is unlikely to accumulate in the
351 same way in natural (particularly lotic) environments, and this case highlights that mesocosm experiments
352 may poorly reflect the true relationship between DNA copy number and mussel density in the field. It
353 would be worthwhile to test whether this result is confirmed with other filter feeding species. More in
354 keeping with previous studies (e.g. Thomsen et al., 2012), rapid degradation of eDNA was observed in
355 mesocosm experiments, with a complete loss of signal by one week after mussel removal.
356
357 *Comparison of eDNA and kick-net sampling for quagga mussel detection*
358
359 The lower sensitivity for kick-net sampling is highlighted by the failure to collect a *D. r. bugensis*
360 specimen in any of the first kick-net sampling attempts at each site. As demonstrated in a previous study,

species in low abundance are likely to be missed with only 62% of macroinvertebrate families detected in a three minute kick-net sample (Furse et al., 1981), and therefore highlights the need for multiple replicates for detection of low abundance species if using kick-net sampling. It should be noted that surveillance methods for *D. r. bugensis* should be focussed on the preferred habitat of mussels i.e. hard substrates such as man-made structures (Aldridge et al., 2014), which is more likely to detect quagga mussels than the randomised method used here. However, randomised kick-net sampling along a range of substrate types is the most widely used sampling method for freshwater macroinvertebrate monitoring, and therefore the most likely to detect new INNS.

Of the three molecular approaches compared in this study, we show that both targeted approaches were more sensitive for detecting *D. r. bugensis* than metabarcoding. Few studies have compared targeted approaches with metabarcoding; however, our findings are similar to those of Simmons et al., (2015) and Harper et al., (2018), who showed that metabarcoding was less sensitive than targeted approaches. In this study, there was no increase in the detection of *D. r. bugensis* without a contamination threshold applied (0.2%). However, it would be pertinent to examine the raw output of metabarcoding runs before thresholds are applied in order to identify any potential INNS with low read counts, and to verify detection of priority INNS with a targeted assay. A significant advantage of metabarcoding is the added information it provides on non-target species. A further 281 OTUs were identified in this dataset, and although no additional INNS were detected, previous studies have demonstrated the power of this approach for detecting new and unexpected INNS (Blackman et al., 2017). Metabarcoding also provides the opportunity to monitor changes in community composition as the result of INNS or other ecological stressors (Simmons et al., 2015; Hänfling et al., 2016), which is extremely promising for better understanding INNS impacts for more effective prioritisation and management. It could also be possible to improve the power of metabarcoding by increasing spatial sampling. Future studies should investigate how many samples are needed in order to maximise the probability of detecting rare species with metabarcoding.

388 Conventional PCR and dye-based qPCR had identical performance for *D. r. bugensis* detection in our
389 study. To increase specificity eDNA qPCR studies often use the addition of a probe to prevent non-target
390 amplification. However, given the already high specificity of the DRB1 primer set described here, we
391 believe the addition of a probe is not necessary for INNS monitoring needs. The chosen approach will
392 depend on the specific requirements of the monitoring programme. If quantitative information is not
393 required, conventional PCR is easily accessible to end-users. It offers a quick and cost-effective approach
394 for the detection of new INNS, requiring limited lab equipment and up to half the time for sample
395 processing than qPCR (Davison et al., 2016). If quantitative information is required, for example when
396 estimating population sizes and monitoring areas where eradication attempts have been carried out, qPCR
397 will be more appropriate.
398
399 *Estimating quagga mussel abundance with eDNA*
400
401 Results from field trials for both dye-based qPCR and metabarcoding demonstrate a significant decrease
402 in DNA copy number and read count, respectively, with increasing distance from the main source
403 population (Wraysbury reservoir). Kick-net sample data also indicates that mussel density decreases
404 along the course of the river, with only one mussel found at Wraysbury Gardens, 2.7 km downstream of
405 the reservoir. Quantitative data from qPCR and metabarcoding is particularly informative in this case as
406 the concentration gradient suggests that the source population may be inferred in cases where it is
407 unknown. The only site that did not fit this trend was the first site, 20 m downstream of the outfall from
408 Wraysbury reservoir, which had a very low DNA read and number.
409
410 There are two main processes which are known to influence the transport of DNA: firstly, the effect of
411 stream discharge on transport (Jane et al., 2015), and secondly, the fundamental structure of the river
412 (Wilcox et al., 2016; Shogren et al., 2017). During this study, sampling took place at a period of increased
413 flow (following heavy rain). In contrast to the findings of Jane et al., (2015), the DNA signal was not
414 uniform along the stretch as expected by higher flows and increased mixing. It is therefore likely other

factors were influencing the DNA signal across the river length. Shogren et al., (2017) in particular, summarised the elements most influential on the movement of DNA as "Transport, Retention and Resuspension". Our data indicates distance has a significant correlation with DNA copy number along the 2.75 km stretch of river. It is highly likely that our data are reflect both a decrease in DNA being shed by populations of decreasing size as well as the effect of DNA being retained by the substrate in slower flow areas (slacks, glides and pools) and re-suspended in higher velocity flows (riffles and runs). To what extent these factors are influencing the DNA signal is currently unclear, and further studies during different flow rates are needed in order to explore these variables further.

Practical consideration for future INNS biomonitoring and conclusions

1. Successful early detection of INNS monitoring requires tools fit for purpose: current methods are unsuitable for the detection of species in low densities and often rely on expertise, which are unable to predict or identify new or unexpected INNS. Here we have demonstrated that eDNA is a viable alternative for both targeted and community surveys.
2. Here we have also shown that dependant on the information required (quantitative, presence/absence or community level surveillance) we are able to tailor molecular tools to answer different questions about INNS dynamics.
3. The optimal approach we believe is to combine both molecular approaches: carry out routine surveillance monitoring for those new or unexpected INNS using a metabarcoding approach followed by verification with targeted assays. Targeted assays should be applied for those INNS expected to arrive or those that have established already and are at risk of spreading.

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