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Research Article

Simple, sensitive and species-specific assays for detecting quagga and zebra mussels (*Dreissena rostriformis bugensis* and *D. polymorpha*) using environmental DNA

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Abstract

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 two freshwater invasive species which are high priority for monitoring in the UK and elsewhere: the Dreissenid mussels; *Dreissena rostriformis bugensis* (Andrusov, 1987) and *D. polymorpha* (Pallas, 1771). We carried out a rigorous validation process for testing the new primers, including DNA detection and degradation experiments in mesocosms, and a field comparison with traditional monitoring protocols. eDNA from single individuals of both mussel species could be detected within four hours of the start of the mesocosm experiment. In field trials, the two mussel species were detected at all sites where the species are known to be present, and eDNA consistently outperformed traditional kick-net sampling for species detection. These results demonstrate the applicability of standard PCR for eDNA detection of freshwater invasive species.

Key words: Dreissenidae, detection, invasive, mesocosm, molecular, PCR

Introduction

The rate of biological invasions has increased rapidly over the last 25 years due, at least in part, to increasing globalisation (Sutherland et al. 2013; Hulme 2009; Gallardo and Aldridge 2013a). Concern over the increasing number of invasive non-native species (INNS) has led to a number of horizon scanning studies aimed at identifying and prioritizing the threat of potential INNS (Gallardo and Aldridge 2013a, b; Roy et al. 2014). Roy et al. (2014) for example, concluded the potential impact, risk of arrival and risk of establishment of quagga mussels, *Dreissena rostriformis bugensis*

(Andrusov, 1987), in the UK to be the highest out of 93 species examined. As predicted, the quagga mussel was detected in the UK later the same year (Mills et al. 2017). Quagga mussels, together with closely related zebra mussels, *D. polymorpha* (Pallas, 1771), are examples of a number of Ponto-Caspian INNS that are currently spreading throughout Europe and North America. Concerns have been raised about the risk of "invasional meltdown" by Ponto-Caspian species, whereby INNS that have co-evolved in the same region facilitate one another's spread (*sensu* Simberloff and Von Holle 1999; Gallardo and Aldridge 2014).

Growing pressure from legislators, and limited funding to regulators to prevent further introductions and spread of new INNS, mean that cost-effective monitoring tools are in high demand. Recent developments in molecular biology are providing new tools and opportunities for biodiversity monitoring, in particular through the use of environmental DNA (eDNA) (Blackman et al. 2018). eDNA is the DNA shed by an organism into its environment, for example via gametes, urine, faeces or sloughed cells (Taberlet et al. 2012; Rees et al. 2014; Bohmann et al. 2014; Lawson Handley 2015; Valentini et al. 2016). The first study to apply eDNA for detection of an invasive species targeted the American bull frog, *Lithobates catesbeiana*, in pond samples using species-specific primers and standard PCR (Ficetola et al. 2008). The method outperformed traditional monitoring approaches, producing reliable positive detections even when bullfrogs were present at low densities (Ficetola et al. 2008; Dejean et al. 2012). This case study was revolutionary. There soon followed a succession of studies utilising eDNA for single species detection of a range of taxa in lentic (e.g. red swamp crayfish, *Procamabrus clarkia*, Tréguier et al. 2014), lotic (e.g. New Zealand mudsnail, *Potamopyrgus antipordarum*, Goldberg et al. 2013) and marine systems (e.g North American wedge clam, *Rangia cuneate*, Ardura et al. 2015).

An important consideration in eDNA studies is that the amount of DNA present in the environment is influenced by a combination of the species' DNA production rate, the degradation rate of the shed DNA, and the transport of DNA within the environment (Barnes et al. 2014; Barnes and Turner 2016; Goldberg et al. 2015). The availability of eDNA is therefore highly dependent on the species being studied (Jerde et al. 2011; Thomsen et al. 2012a; Pilliod et al. 2013; Tréguier et al. 2014; Roussel et al. 2015; Klymus et al. 2015; Jane et al. 2015), and the environment in which they are present (Jane et al. 2015; Jerde et al. 2016; Shogren et al. 2017), and these variables therefore need to be considered during the development and application of eDNA assays.

The great majority of targeted eDNA studies have used either standard PCR or probe-based real-time quantitative PCR (qPCR) for single species detection, although droplet digital PCR (ddPCR) is also showing great promise (Nathan et al. 2014; Doi et al. 2015). qPCR is often considered a

more desirable approach than PCR due to its increased sensitivity for species detection (Thomsen et al. 2012a; Nathan et al. 2014) and, when using a probe-based assay, its added specificity. However, for many applications, the sensitivity of standard PCR may be quite adequate, and PCR may be preferable as it is cheaper and less technically challenging. Moreover, studies that have directly compared the two approaches have indicated that PCR can be more robust to PCR inhibitors than qPCR, which is important for avoiding false negatives (De Ventura et al. 2017).

In this study, we focussed on the detection of quagga and zebra mussels, which are high priority species for monitoring in a number of countries, including the UK; the focus of our sampling. These species originate from the Ponto-Caspian area and have spread rapidly throughout their invasive ranges via boat transportation, canals and river basin connections (Timar and Phaneuf 2009; Bij de Vaate et al. 2002). The rapid spread has been aided by both human interaction and their unique ecology (Timar and Phaneuf 2009). Like other mussels, Dreissenids have a free-floating planktonic veliger life stage, during which young can be dispersed over a large area downstream of parental populations (Ricciardi et al. 1995; Karatayev et al. 2002, 2015). Compared to many other mussel species, Dreissenids exhibit unique abilities to colonise new environments by using protein-based byssal strands formed inside their shell to secure to hard surfaces, which can be a significant aid to transportation and establishment (Ricciardi et al. 1998; Karatayev et al. 2002; Aldridge et al. 2004; Timar and Phaneuf 2009; Peyer et al. 2009). Colonization of new areas and establishment has been facilitated by the ability of Dreissenids to survive out of water for up to 15 days (Ricciardi et al. 1995) and survive a wide range of environmental extremes (Gallardo and Aldridge 2013b). Both mussels are described as "ecological engineers" (Karatayev et al. 2002, 2007; Roy et al. 2014) having influences on all trophic levels. In some instances, mussels provide increase in shelter and habitat for benthic macroinvertebrates (Karatayev et al. 2002), however they also compete for food and decrease diversity, and have been directly linked to declines in native Unionid mussels (Ricciardi et al. 1996). Dreissenid feeding behaviour also has negative effects on phytoplankton and has been linked to greater numbers of cyanobacteria blooms (Karatayev et al. 2002). Economic impacts are also widely documented for both species. For example, between US$161–US$467 million was spent by water treatment and electric power facilities in North America on the control and removal of *D. polymorpha* between 1989–2004 (Connelly et al. 2007).

The traditional monitoring of *D. r. bugensis* and *D. polymorpha* poses a challenge due their morphological similarity (Peyer et al. 2011). eDNA is therefore a promising complementary tool for Dreissenid monitoring. Previous studies have designed and tested primers for detection of Dreissenid eDNA using either standard PCR (Bronnenhuber and Wilson

2013; De Ventura et al. 2017) or qPCR (Peñarrubia et al. 2016; De Ventura et al. 2017; Gingera et al. 2017; Amberg et al. 2019). However, the performance of these assays were not assessed in controlled mesocosm experiments or compared directly to traditional sampling methods in the field, and (in the case of Peñarrubia et al. 2016) do not distinguish the two species. eDNA assays based on microfluidic chip (Mahon et al. 2011), light transmission spectroscopy (LTM) technology (Egan et al. 2013) are also available for quagga mussels, and on loop-mediated isothermal amplification (LOOP) for both *Dreissena* species (Williams et al. 2017) although these tools, at present, are arguably less widely applicable. Our objective was to design and test species-specific standard PCR assays for detection of eDNA from quagga and zebra mussels, that can be deployed for routine monitoring in a simple and cost-effective way by both regulators and researchers. Our framework for development consisted of: 1. *in silico* and *in vitro* primer testing of *de novo* primer pairs; 2. mesocosm experiments to evaluate eDNA detection and degradation over time at three different densities (one, five and twenty individuals of each species); and 3. testing the efficiency of the eDNA assays compared to traditional kick-net sampling in the field.

Materials and methods

Specimen sampling and tissue DNA extraction

Dreissena polymorpha is widespread and common in the UK, having arrived in the 1820s potentially via the timber trade (Bij de Vaate et al. 2002; Quinn et al. 2014). *Dreissena rostriformis bugensis* is a much more recent invader, with the first UK record from 2014 in the River Wraysbury (Mills et al. 2017). This reservoir is subject to water transfers within the region and the mussel was subsequently found in neighbouring reservoirs. Specimens of both *Dreissena* species were collected at sites with known populations, two weeks prior to the beginning of each mesocosm experiment. These sites were as follows: *D. r. bugensis:* Wraysbury River, UK Grid. Ref. TQ 02680 73204 and D. *polymorpha:* Rutland Water, SK 92956 05963.

Specimens were kept in tanks with continuous aeration and fed dried *Cyclotella ad libitum*. Samples from the commonly co-occurring native mussel species (*Sphaerium corneum* and *Anadonta anatina*) were also collected for tissue DNA extraction and primer testing. Tissue samples from both individuals of each invasive species and native species were extracted using the DNeasy Blood and Tissue Kit® (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Species-specific primer development

Species-specific primers were designed and tested *in silico* with Primer BLAST (Ye et al. 2012) using all available *COI* reference sequences from GenBank (*D. r. bugensis* – 7 sequences, *D. polymorpha* – 31 sequences, see

Supplementary material Appendix 1 Table S1 for details of GenBank accession numbers). Suitable primers for *in vitro* testing were selected based on target species amplification, similarity of forward and reverse primer melting temperature, > 50% GC content and primers with an amplicon size of < 200 bp were preferentially selected to be suitable for amplification of degraded eDNA (Deagle et al. 2006; Jerde et al. 2011; Bronnenhuber and Wilson 2013; Mächler et al. 2014; Ardura et al. 2015). In total four primer pairs were tested *in vitro* for each species (see Appendix 1 Table S2 for all primer pairs tested). *In vitro* testing was carried out on tissue samples of the target INNS and three non-target taxa i.e. the congeneric INNS and two native taxa which are likely to co-occur in the same habitat. Serial dilutions of neat tissue-extracted target-species DNA (at approximate concentrations of 5 ng/µl, 0.5 ng/µl, 0.05 ng/µl, 0.005 ng/µl) were carried out to establish the Limits of Detection (LoD) for each primer pair (see Appendix 1 Table S3). PCRs were carried out in 25 µl volumes with 12.5 µl MyTaq Red Mix (Bioline, UK), 0.4 µM of each primer and 2 µl of DNA template. Temperature gradient PCRs were carried out on an Applied Biosystems Veriti Thermal Cycler in order to determine the correct PCR conditions. The following profile was selected after testing: 94 °C for 3 min, followed by 37 cycles of 94 °C for 30 s, 65 °C for 1 min and 72 °C for 1 min 30 s, with a final extension time of 10 min at 72 °C. PCR products from tissue samples were visualised by gel electrophoresis and stained with GelRed (Cambridge Bioscience Ltd, UK).

Based on PCR optimisation and the performance of the primer pairs, one primer pair from each species was selected. Four products using the selected primer was then. Sanger sequenced by Macrogen Europe in the forward direction. Sequences were compared with the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) using BLAST to confirm species identification (see Appendix 1 Figures S1 and S2 for sequence alignments).

Mesocosm experiments

Mesocosm experiments were carried out from January to April 2016 to test the sensitivity of the selected primer pairs under controlled densities and to investigate the rate of DNA accumulation and degradation. Each experiment was conducted in 15 L plastic tanks with fitted lids. Tanks were located in a climate controlled facility where temperature averaged 16 °C (range 14–18 °C) with light:dark cycles of 16 h:8 h. All tanks, aeration equipment and sampling equipment was sterilized in 10% commercial bleach solution for 10 minutes, then rinsed with 10% MicroSol detergent (Anachem, UK) and purified water prior to the experiment. Sampling and filtering equipment were also cleaned using the above method between each sampling event. Each tank was filled with water collected from Hotham Beck (SE 89133 32489) which has no prior record of Dreissenid mussels at the site or at any

location within the waterbody. Field-collected water was used in order to provide natural feeding opportunities for the mussels and limit stress caused. Tanks were supplied with constant air via sterile tubing and aeration stones for 48 hours prior to the start of the experiment and covered for the duration of the experiment with a fitted lid.

For each species, the experiment consisted of 10 tanks representing three replicates of three treatment densities (one, five and twenty individuals) and a control tank with no individuals. Specimens of similar total biomass were used in the density replicates in order to minimise any influence of different biomass (see Appendix 1 Tables S4 and S6 for biomass information gathered pre- and post-mesocosm experiments). Room temperature, control tank and water temperature were recorded prior to each sampling event (see Appendix 1 Tables S5 and S7 for temperature measurements taken during the mesocosm experiments). Tank water temperature was kept below 10 °C to minimise any potential spawning events. Before the specimens were added to the tanks, a water sample was collected and filtered to ensure no contamination from the target taxa; this sample was recorded as 0 hours. Tanks were sampled over 42 days at 4 hrs, 8 hrs, 24 hrs, 7 days, 15 days and 21 days with the species present. On day 21, the specimens were removed from the tanks and sampling continued at 22 days, 28 days, and 42 days.

A total of N = 100 water samples were collected per species. For each sampling event, the tank water was homogenised by stirring with a sterile spatula before collecting 200 ml water from each tank. Samples were vacuum filtered through sterile 47 mm diameter 0.45 µm cellulose nitrate membrane filters with pads (Whatman, GE Healthcare, UK) immediately after collection, using Nalgene filtration units (Thermo Fisher Scientific) in combination with a vacuum pump (15~20 in. Hg, Pall Corporation) in a dedicated eDNA laboratory at the University of Hull, UK. Filter papers were then placed in sterile petri dishes, sealed with parafilm and stored at −20 °C until extraction. The filtered water was then returned to the tank to maintain the water volume. This process was completed within one hour. The filtration units were cleaned with 10% commercial bleach solution and 10% MicroSol (Anachem, UK), and then rinsed thoroughly with deionized water after each filtration to prevent cross-contamination. All DNA extractions were carried out using a protocol modified from Brolaski et al. (2008) (for the full extraction protocol, see Appendix 2). Mesocosm samples were PCR amplified using the species-specific primers and visualised with conditions previously described. Three PCR products from each species/mesocosm experiment were Sanger sequenced to confirm primer specificity.

Field trials

Water samples were collected at sites with previous records of the target INNS to test and verify the efficiency of each INNS assay in the field (Figure 1). We collected a total of 54 samples per INNS from their respective

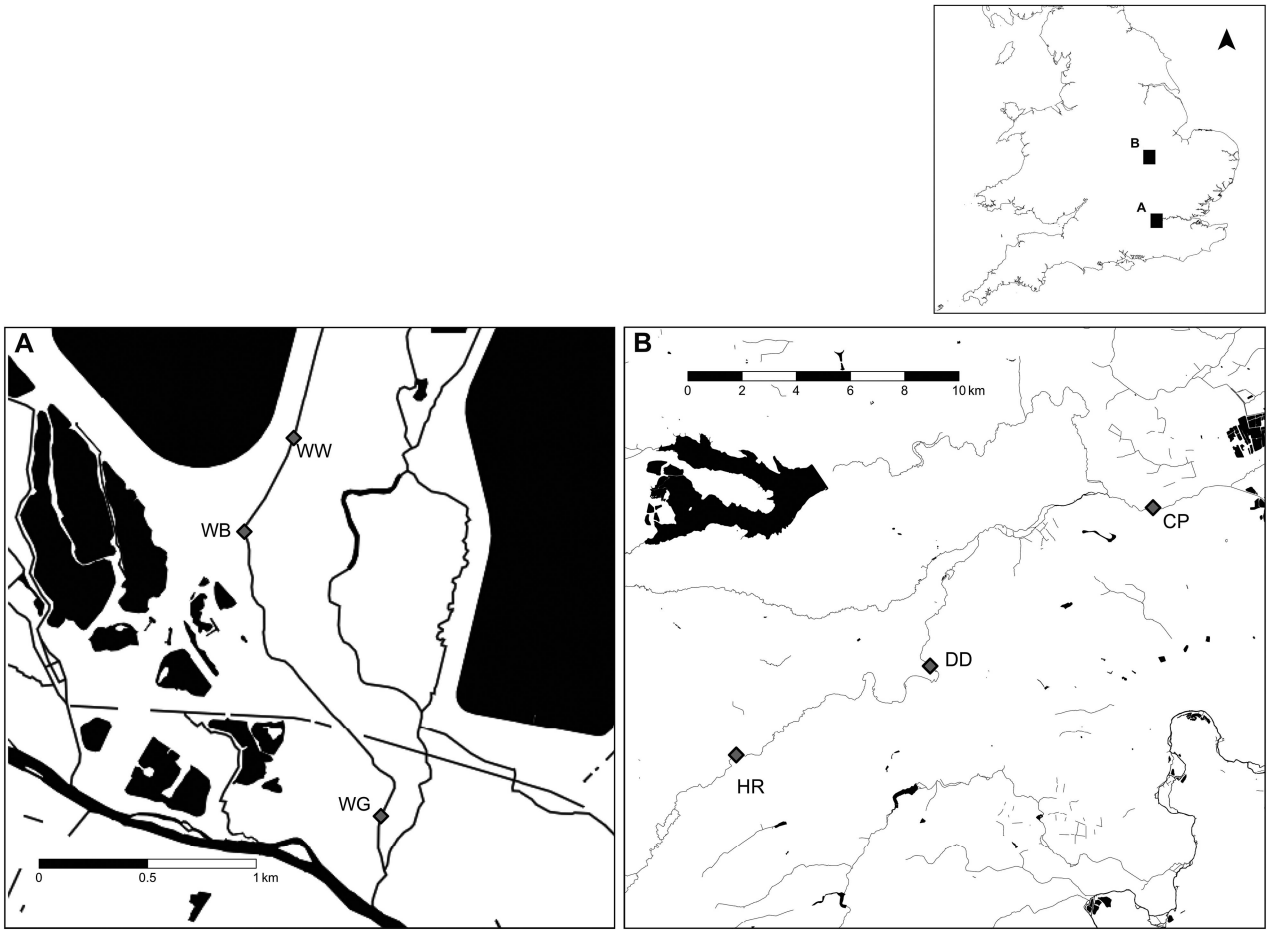

Figure 1. Site locations for field trials. Site locations are highlighted in blue boxes. All sample sites are referred to from upstream to downstream, waterbodies are in black and sample points are marked with a diamond. A – sampling sites from *D. r. bugensis* were on the River Wraysbury at Wraysbury 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).

river catchments (River Wraysbury for *D. r. bugensis* and River Welland for *D. polymorpha*). For each waterbody we collected 6 samples from three selected UK Environment Agency macroinvertebrate monitoring sites. Each sample consisted of 500 ml of water collected across the width of the river and was replicated 3 times (N samples = 18 per site). Each replicate was filtered and extracted independently. Sample bottles filled with ddH_2O were taken into the field as sample blanks. Samples were processed within 24 hours using the same method as the mesocosm samples. Each replicate was PCR amplified three times. PCR products from a total of 4 field samples for each species were Sanger sequenced to confirm primer specificity. Sites were surveyed after eDNA sample collection using standard 3-minute kick-net samples also (Murray-Bligh 1999). Using the same approach as eDNA sampling we collected 6 × 3-minute kick-net samples at each of the selected sites.

Data Analysis

Positive detection of the target INNS in both mesocosm experiment and field trial was determined by a band found on the electrophoresis gel at the

Table 1. Species specific primers. Primer pairs designed for this study and used for the detection of the two target INNS.

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	

correct size. The bands strength was not used to determine a positive detection, as we believe any positive detection (including a faint band strength) found using the primers described in this paper will result in implementation of management strategies to confirm detection visually and carry our control measures. Binomial Generalized Linear Models (GLMs) with a logit link function were used to investigate the influence of density or total biomass and time since the start of the experiment (until the taxa were removed from the mesocosm) on the detection in the mesocosms. Models were checked by testing whether the residual deviance fitted a chi squared distribution. The best supported model was identified by the lowest AIC value, and models with Δ AIC < 2 were also considered equivalent (Burnham and Anderson 2002). All data analyses were performed in R v.3.3.1. (R Core Team 2017), with GLMs performed using the MASS package (Venables et al. 2002) and plots created in ggplot2 (Wickham 2016). To ensure full reproducibility of this study the raw data and code can be accessed (https://github.com/RosettaBlackman/Blackman_et_al_Dreissenidae).

Results

Primer specificity

One primer pair for each species (*DRB1* for *D. r. bugensis* and *DP1* for *D. polymorpha*, Table 1) was selected based on the criteria of good target amplification with no cross-amplification of non-target species. *DRB1* amplified 29 published *D. rostriformis, D. bugensis* and *D. rostriformis bugensis* sequences *in silico* with no mismatches. *DP1,* amplified 45 published *D. polymorpha* and subspecies *(D. p. polymorpha, D. p. gallandi* and *D. p. anatoli*a) *in silico*. Of the published *D. polymorpha* sequences, one had a mismatch in the forward primer (Accession number AF510508) and a second sequence had two mismatches in the forward and one in the reverse primer (Accession number JQ435817) (see Appendix 1 Table S8). Note that the forward primer pair selected for *D. polymorpha* shares a 16 bp overlap with DpoCOI3F designed by Bronnenhuber and Wilson (2013) but our primer pair, *DP1*, amplifies a much shorter sequence (73 bp, as opposed to 164 bp).

Species-specific primer testing on target tissue samples yielded positive PCR amplification of a single band at the expected size for both selected assays (Figure 2) following the above prescribed PCR chemistry and PCR settings. It should be noted that using less stringent conditions (e.g., lower annealing temperature) may result in non-target amplification or and must

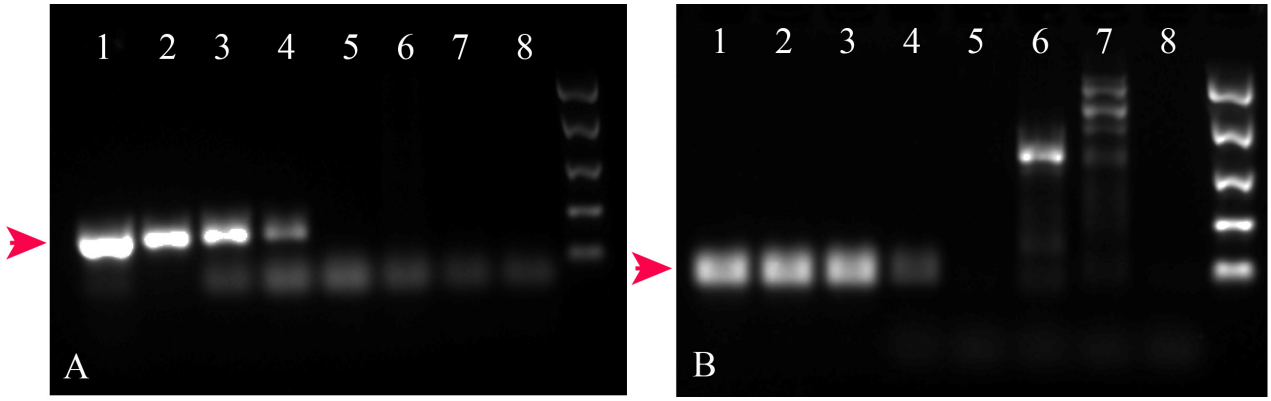

Figure 2. Results of in vitro primer testing. A – *Dreissena rostriformis bugensis* (primer pair DRB1) and B – *Dreissena polymorpha* (DP1). Lane 1 contains undiluted target INNS tissue DNA (5 ng/µl per reaction), lanes 2–4 contain a dilution series of the target tissue (lane 2 1:10 dilution, ~ 0.5 ng/µl per reaction; lane 3 1:100 dilution, ~ 0.05 ng/µl per reaction; lane 4 1:1000 dilution, ~ 0.005 ng/µl per reaction). Lanes 5 and 6 contain closely related native species found in the UK: *Anadonta anatina* and *Sphaerium corneum*, Lane 7 contains the paired INNS and lane 8 is a PCR negative (ddH_2O). The final lane in both gels EasyLadder I (Bioline, UK) with fragment sizes of 100 bp, 250 bp, 500 bp, 1000 bp and 2000 bp.

be avoided. The LoD for both *DRB1* and *DP1* was ~ 0.005 ng/µl DNA per reaction (1:1000 dilutions of neat tissue DNA, Figure 1A and B). No bands of the expected size were obtained in the cross amplification tests; however, much larger, non-specific bands, were seen in non-target species for *DP1* (Figure 1B). Due to the substantial size difference these non-specific bands are easily distinguishable from the target band size and will not lead to false positive detections. Sequences generated from PCR products from all tissue, mesocosm and field samples were verified as being from the correct target species for *D. rostriformis bugensis*. However due to the short amplicon length of the DP1 product, we were unable to determine a good quality sequence length greater than the reverse primer (see Appendix 1 Figures S1 and S2) and therefore all positive detection for *D. polymorpha* was determined on band size alone.

Mesocosm experiments

We had no contamination of tanks prior to target species being added (see Figure 3 and also Appendix 3 Figures S1 and S2). Both Dreissenid mussel primers showed positive detection of their target species at all three density treatments at the four hour sampling event (Figures 3 and 4). *Dreissena polymorpha* was detected at all three replicates for each density treatment after 4 hours, whereas *D. r. bugensis* was detected in 1/3, 2/3 and 3/3 replicates for the 1, 5, and 20 individual treatments respectively. At least one positive replicate was obtained for every sampling point over the first 21 days. However, the number of detections did not increase linearly over time or density. The 20 individual density treatment was quite consistent over time, with positive detections in 3/3 replicates from 4 h to 21 days for both species, with just one exception (*D. r. bugensis* after 7 days). For *D. r. bugensis,* time and total biomass significantly influenced the detection by standard PCR. Of these two measures, total biomass was the more significant predictor

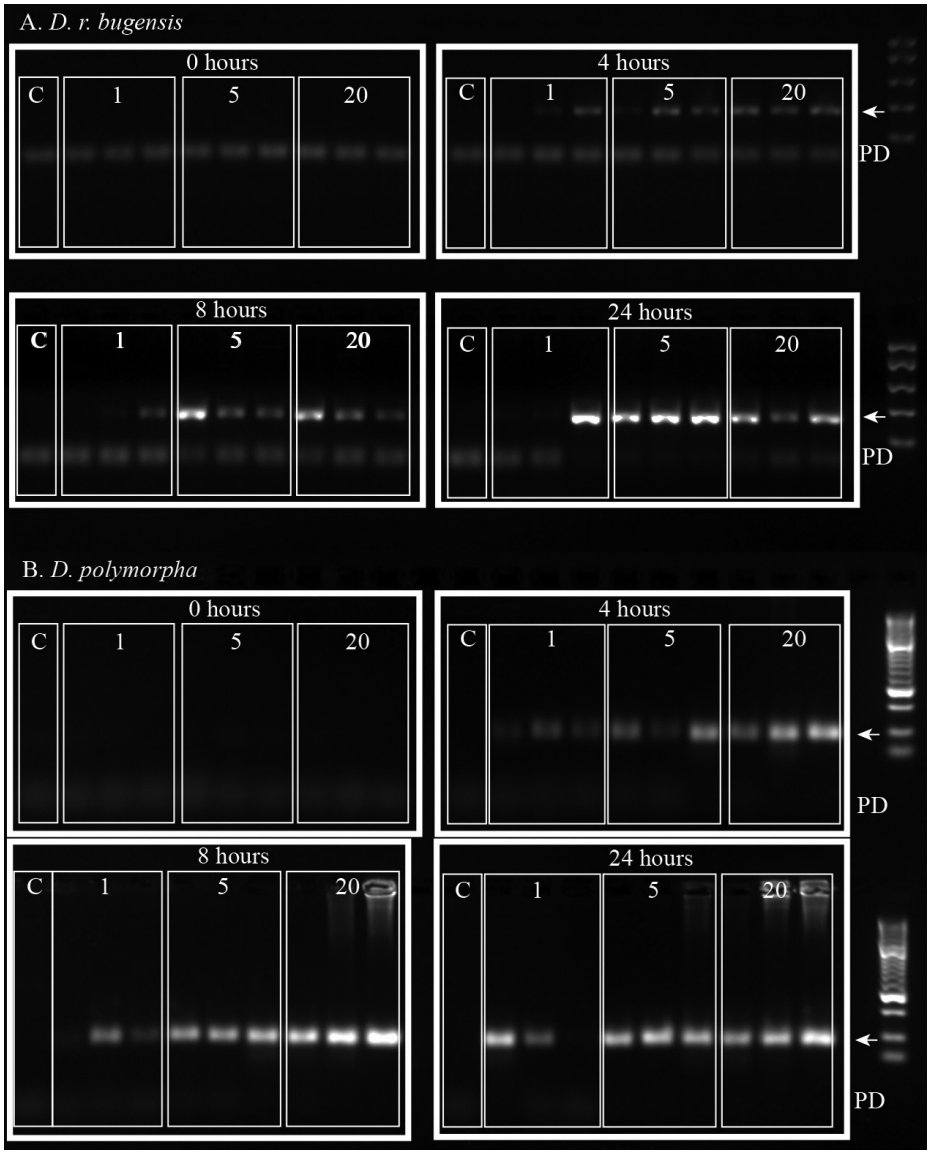

Figure 3. Species detection in mesocosm experiments. These images show the results from the sampling events at 0, 4, 8 and 24 hour during the experiment. The white arrow shows the target band for each species (A. *D. r. bugensis* 188 bp and B. *D. polymorpha* 73 bp), the labels are as follows: C – control tank which contained no target species, 1 – one specimen, 5 – five specimens, 20 – twenty specimens, PD – primer dimer and L – ladder. Ladder A - EasyLadder I (Bioline, UK) with fragment sizes of 100 bp, 250 bp, 500 bp, 1000 bp and 2000 bp. Ladder B – DNA Hyperladder 50 bp (Bioline, UK) with fragment sizes of 50 bp, 100 bp, 200 bp, 300 bp – 2000 bp.

in GLMs and generated the lowest AIC (GLM, z = 2.262, P = 0.023, AIC 55.368). After removal of *D. r. bugensis,* DNA was only detected in tanks with the highest mussel density (20 individuals) 24 hours after removal. DNA from these tanks was no longer detected at day 28 (7 days after removal). For *D. polymorpha,* both time and density were significant predictors of detection. Of the two measures, density was the strongest predictor with the lowest AIC (GLM, z = 1.969, P = 0.049, AIC 32.823). DNA from *D. polymorpha* persisted to day 42 (21 days after removal) in two of the three density treatments (see Appendix 1 Tables S9 and 10 for full GLM breakdown).

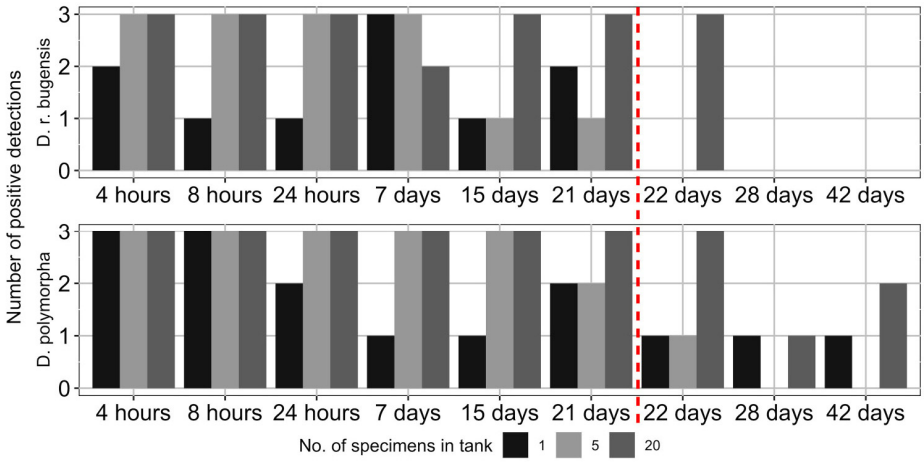

Figure 4. Species detection in mesocosm experiments. A – *Dreissenia rostriformis bugensis* and B – *Dreissena polymorpha*. Each graph indicates the number of positive detections from three replicates taken from each treatment (specimen density) during the 42 day experiment. Specimens were removed after 21 days (indicated by the dashed red line), the tanks were sampled for a further 21 days to monitor the degradation in detection rates.

Table 2. Species detection in field experiments. Summary of the number of positive detections from each field sample at each site (eDNA sample results are out of 3 PCR reactions and kick-net sample results are the number of specimens collected in a 3-minute sample). Green: high confidence of presence (eDNA – 3/3 positive detection and kick-net sample – specimen collection), orange medium confidence (eDNA 2/3 positive detection), red low confidence (eDNA 1/3 positive detection), grey no evidence of presence. Sample site codes: *D. r. bugensis* sites on the River Wraysbury at Wraysbury weir (WW), Wraysbury bridge (WB) and Wraysbury Gardens (WG). *Dreissena polymorpha* sites were on the River Welland at Harrington (HR), Duddington (DD) and Copthill (CP).

		Sample Number											
	Site	*1*		*2*		*3*		*4*		*5*		*6*	
		eDNA	*Kick-net*	*eDNA*	*Kick-net*	*eDNA*	*Kick-net*	*eDNA*	*Kick-net*	*eDNA*	*Kick-net*	*eDNA*	*Kick-net*
D. r. bugensis	*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
D. polymorpha	*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	0	0	0	0	0	0	1	0	0	0	1	0

Field trials

Dreissenid mussels: *D. r. bugensis* specimens were found by kick-net sampling at all 3 sites surveyed, but the number of individuals found decreased with distance along the River Wraysbury from the main source population at Wraysbury Reservoir (Table 2). Detection by kick-net sampling was 33% (6 samples out of 18). Positive eDNA detections were obtained for every sampling replicate at each of the three sites along the River Wraysbury, hence eDNA detection was 100% (Table 2 and Appendix 3 Figure S5). *Dreissena polymorpha* was found by kick-net sampling in only one of three sites (Duddington, Table 2) although the species is known to be present throughout the sampled catchment. The number of positive detections for kick-net sampling was 11% (2/18 samples). Positive eDNA detections for *D. polymorpha* were obtained in 77.7% (14/18) of samples

and 53.7% (29/54) of the PCR replicates, including in sites where specimens of *D. polymorpha* were not found (Table 2 and Appendix 3 Figure S6). Both primer sets showed positive detections of their respective target taxa in field, however for detection of *D. polymorpha* we do not see the clear bright bands observed in the mesocosm experiments, this could be due to inhibition in the samples.

Discussion

Rapid, cost-effective tools are needed for detection of newly invading, or spreading invasive non-native species. Here, we designed and tested PCR primer pairs for two highly invasive non-native species: *D. r. bugensis* and *D. polymorpha,* which are high priority for monitoring. Primers were tested *in silico* and *in vitro,* then in a series of mesocosm experiments and field trials. The two primer pairs amplify target tissue at a low concentration (0.005 ng/µl) which is broadly in line with other eDNA species-specific standard PCR primer assays, (e.g. detection limit of 0.00046 ng/µl (Davison et al. 2016) to 0.4 ng/µl (Ardura et al. 2015), with no cross-species amplification with each other, or with two native mussel species present in the UK. Both species were detected from eDNA collected from water samples in both laboratory and field trials.

eDNA could be detected in mesocosms within 4 hours of the start of the experiment and detection at this first time point was possible from just one individual. Dreissenid eDNA was detected at every sampling point at all three densities in the mesocosms, and outperformed kick-net sampling for detection in the field. Below we highlight the range of factors that likely interact to determine the success of eDNA detection in real-world applications.

Mesocosm trials

Mesocosm experiments have been advocated (De Ventura et al. 2017), and performed by previous studies (Dejean et al. 2011; Thomsen et al. 2012b; Sansom and Sassoubre 2017) to allow information on species-specific DNA production rates, persistence and degradation over time, which informs users whether the method is appropriate for the detection of target taxa. Here, both abundance variables (density and total biomass) were significant predictors of detection for both species. Hence, there is a positive relationship between abundance and detection, as found in previous studies (e.g. Thomsen et al. 2012b). Mesocosm experiments also demonstrated the depletion of DNA once the specimens were no longer present in the tank; 7 days after removal for *Dreissena rostriformus bugensis* in agreement with similar studies (Dejean et al. 2011; Thomsen et al. 2012b). However, DNA for *D. polymorpha* was still detecting at the end of the experiment, 21 days after removal of the species. This indicates there

are differences between species in terms of detectability and DNA did not accumulate or degrade in a linear fashion over time, as discussed below.

The mesocosm experiments performed in this study were useful for determining the assay sensitivity and for identifying differences in detectability between species. Our experiments revealed that the *Dreissena* primers are sensitive and robust – being able to detect single individuals within four hours and then consistently throughout the course of the experiment. The high rate of detection for Dreissenid mussels is likely due to the fact they were able to continuously filter feed on algae and phytoplankton present in the water column during our experiments, as they would in the wild, enabling them to maintain an active metabolism. However, it is clear from our study and others that DNA production and its availability in the water column is a complex topic and can vary substantially even between closely related species.

We might expect that as long as DNA production rate is greater than the degradation rate, (as seen in models produced by Thomsen et al. 2012a), eDNA availability should increase over the course of the experiment. Under this prediction, we expect the DNA concentration and the number of positive detections to increase over time, and for there to be an interaction with density. Alternatively, DNA concentrations may increase at first and then plateau, when an equilibrium is reached between DNA production and degradation (Klymus et al. 2015; Sansom and Sassoubre 2017; Nevers et al. 2018). As we are using standard PCR, rather than qPCR we are unable to determine DNA concentration, however we do see an increase in band strength in both Dreissenid mesocosm experiments between 4 and 24 hours. However, overall the number of positive detections fluctuates rather than showing an accumulation or a plateau over time. Time was also a significant predictor of detection probability for both species. The fluctuation in the number of detections over time may be due to a combination of the activity of the organisms, the balance between DNA production and degradation, and/or changes in the concentration of PCR inhibitors. In Dreissenid mussels, filter feeding may both release and uptake DNA, so the amount of DNA present in a controlled environment may reach an equilibrium. Further experiments with a quantitative method such as qPCR or ddPCR are needed to fully understand the dynamics of DNA concentration over the course of the experiment, and the influence of feeding and other behaviours on the rate of DNA production.

Field application

In the field tests, eDNA outperformed kick-net sampling for detection of both *D. r. bugensis* (100% eDNA *vs* 33% kick-net samples) and *D. polymorpha* (89% *vs* 11%). Below we discuss the reasons for the discrepancies between eDNA and kick-net samples, and the performance of both assays in the field.

There are numerous influences on the persistence of eDNA in waterbodies, that have been well documented such as: pH, microbial activity and transportation (Deiner and Altermatt 2014; Jane et al. 2015; Jerde et al. 2016; Shogren et al. 2017, see Barnes et al. 2014 for further discussion). In our study, we reported higher detection rates from eDNA compared to kick-net samples in both species. These samples were collected from lotic systems, and therefore detection is likely to come from both local populations and eDNA being transported from upstream sources (Deiner and Altermatt 2014; Jane et al. 2015). However, to what extent the DNA is being transported is still largely unknown. Previous work on river morphology states substrate type and the related flow regime, are huge influences on DNA transportation, substrate retention and subsequent resuspension (Shogren et al. 2017; Jerde et al. 2016).

Our results show no decrease in band strength for the detection of *D. r. bugensis* across the population density gradient along the River Wraysbury (2 km). In part this is likely to be down to transported DNA. However, it is also likely to be attributed to an increase in water mixing caused by rainfall before the samples were collected. As eDNA is not uniformly distributed through a river (Macher and Leese 2017) the rainfall is likely to increase the dispersion of eDNA in a waterbody (Shogren et al. 2017). We therefore see a greater number of positive detections. Similarly, variation in the eDNA detection throughout the river for *D. polymorpha* may be due to the relatively lower flows during these surveys which have caused a reduction in DNA distribution across the river. However, there is a fluctuation in the number of detections across the samples at each site for this species and we detect DNA at sites where they were not physically collected (as we also see with *D. r. bugensis*). This is further evidence of eDNA being transported down the catchment rather than a false positive result. This greater variability in detection due to the lower flow conditions is likely to demonstrate the true variation encountered when surveying lotic systems for target species.

In this study we were unable to determine successful detection of *D. polymorpha* by Sanger sequencing the PCR product generated from our primer pair, DP1. This was due to the short amplicon length (73 bp). Although several studies may demonstrate species detection with small amplicon length (< 200 bp, Deagle et al. 2006; Jerde et al. 2011; Bronnenhuber and Wilson 2013; Mächler et al. 2014; Ardura et al. 2015; Takeuchi et al. 2019) there may be an optimal size to use when considering species detection from eDNA samples. As we know eDNA from water samples is known to be a combination of cellular and extracellular DNA (Taberlet et al. 2012) we must therefore opt for species determination over detection and aim to design primers based on a larger size amplicon (> 100 bp) for successful species determination (Meusnier et al. 2008).

Conclusion

This study provides targeted eDNA assays for two priority invasive non-native species, and demonstrates a simple framework for assay development that can be used by regulatory bodies with responsibility for invasive species monitoring. It is important to highlight that by using eDNA it not only allows detection but also easily differentiate between cryptic species, such as the ones in this study. Standard PCR outperformed established kick-net sampling for both 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 even by closely related species.

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Author's Contribution

RB and LLH designed the experiments. RB designed the primers and carried out *in silico*, *in vitro* and mesocosm experiments. RB, MB and LH carried out the fieldwork. RB and LLH analysed the data and all authors contributed to the writing of the manuscript.

References

Aldridge DC, Elliott P, Moggridge GD (2004) The Recent and Rapid Spread of the Zebra Mussel (*Dreissena polymorpha*) in Great Britain. *Biological Conservation* 119: 253–261, https://doi.org/10.1016/j.biocon.2003.11.008

Amberg JJ, Merkes CM, Stott, Rees CB, Erikson RA (2019) Environmental DNA as a tool to help inform zebra mussel, *Dreissena polymorpha*, management in inland lakes. *Management of Biological Invasions* 10: 96–110, https://doi.org/10.3391/mbi.2019.10.1.06

Ardura A, Zaiko A, Martinez JL, Samulioviene A, Semenova A, Garcia-Vazquez E (2015) eDNA and Specific Primers for Early Detection of Invasive Species - A Case Study on the Bivalve *Rangia cuneata*, Currently Spreading in Europe. *Marine Environmental Research* 112: 48–55, https://doi.org/10.1016/j.marenvres.2015.09.013

Barnes MA, Turner CR (2016) The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics* 17: 1–17, https://doi.org/10.1007/s10592-015-0775-4

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM (2014) Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology* 48: 1819–1827, https://doi.org/10.1021/es404734p

Bij de Vaate A, Jazdzewski K, Ketelaars HAM, Gollasch S, Van der Velde G (2002) Geographical Patterns in Range Extension of Ponto-Caspian Macroinvertebrate Species in Europe. *Canadian Journal of Fisheries and Aquatic Sciences. Journal Canadien Des Sciences Halieutiques et Aquatiques* 59: 1159–1174, https://doi.org/10.1139/f02-098

Blackman RC, Hänfling B, Lawson Handley L (2018) The use of environmental DNA as an early warning tool in the detection of new freshwater invasive non-native species. *CAB Reviews* 13: 1–15, https://doi.org/10.1079/PAVSNNR201813010

Bohmann K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Yu DW de Bruyn M (2014) Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution* 29: 358–367, https://doi.org/10.1016/j.tree.2014.04.003

Brolaski MN, Venugopal RJ, Stolow D (2008) Kits and Processes for removing contaminants from nucleic acids in environmental and biological samples. US Patent

Bronnenhuber JE, Wilson CC (2013) Combining Species-Specific COI Primers with Environmental DNA Analysis for Targeted Detection of Rare Freshwater Species. *Conservation Genetics Resources* 5: 971–975, https://doi.org/10.1007/s12686-013-9946-0

Burnham AM, Anderson DR (2002) Model Selection and Multi-Model Inference: A Practical Information-Theoretic Approach, 2nd Edition. Springer, New York, pp 49–96

Connelly NA, O'Neill CR, Knuth B, Brown TL (2007) Economic Impacts of Zebra Mussels on Drinking Water Treatment and Electric Power Generation Facilities. *Environmental Management* 40: 105–112, https://doi.org/10.1007/s00267-006-0296-5

Davison PI, Créach V, Liang W-J, Andreou D, Britton JR, Copp GH (2016) Laboratory and Field Validation of a Simple Method for Detecting Four Species of Non-Native Freshwater Fish Using eDNA. *Journal of Fish Biology* 89: 1782–1793, https://doi.org/10.1111/jfb.13086

Deagle BE, Eveson JP, Jarman SN (2006) Quantification of Damage in DNA Recovered from Highly Degraded Samples–a Case Study on DNA in Faeces. *Frontiers in Zoology* 16: 3–11, https://doi.org/10.1186/1742-9994-3-11

Deiner K, Altermatt F (2014) Transport Distance of Invertebrate Environmental DNA in a Natural River. *PLoS ONE* 9: e88786, https://doi.org/10.1371/journal.pone.0088786

Dejean T, Valentini A, Duparc A, Pellier-Cuit S, Pompanon F, Taberlet P, Miaud C (2011) Persistence of Environmental DNA in Freshwater Ecosystems. *PLoS ONE* 6: e23398, https://doi.org/10.1371/journal.pone.0023398

Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C (2012) Improved Detection of an Alien Invasive Species through Environmental DNA Barcoding: The Example of the American Bullfrog *Lithobates catesbeianus*. *The Journal of Applied Ecology* 49: 953–959, https://doi.org/10.1111/j.1365-2664.2012.02171.x

De Ventura L, Kopp K, Seppälä K, Jokela J (2017) Tracing the Quagga Mussel Invasion along the Rhine River System Using eDNA Markers: Early Detection and Surveillance of Invasive Zebra and Quagga Mussels. *Management of Biological Invasions* 8: 101–112, https://doi.org/10.3391/mbi.2017.8.1.10

Doi H, Takahara T, Minamoto T, Matsuhashi S, Uchii K, Yamanaka H (2015) Droplet Digital Polymerase Chain Reaction (PCR) Outperforms Real-Time PCR in the Detection of Environmental DNA from an Invasive Fish Species. *Environmental Science & Technology* 49: 5601–5608, https://doi.org/10.1021/acs.est.5b00253

Egan SP, Barnes MA, Hwang CT, Mahon AR, Feder JL, Ruggiero ST, Tanner CE, Lodge DM (2013) Rapid Invasive Species Detection by Combining Environmental DNA with Light Transmission Spectroscopy. *Conservation Letters* 6: 402–409, https://doi.org/10.1111/conl.12017

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species Detection Using Environmental DNA from Water Samples. *Biology Letters* 4: 423–425, https://doi.org/10.1098/rsbl.2008.0118

Gallardo B, Aldridge DC (2013a) Priority Setting for Invasive Species Management: Risk Assessment of Ponto-Caspian Invasive Species into Great Britain. *Ecological Applications: A Publication of the Ecological Society of America* 23: 352–364, https://doi.org/10.1890/12-1018.1

Gallardo B, Aldridge DC (2013b) The 'dirty Dozen': Socio-Economic Factors Amplify the Invasion Potential of 12 High-Risk Aquatic Invasive Species in Great Britain and Ireland. *The Journal of Applied Ecology* 50: 757–766, https://doi.org/10.1111/1365-2664.12079

Gallardo B, Aldridge DC (2014) Is Great Britain Heading for a Ponto-Caspian Invasional Meltdown? *The Journal of Applied Ecology* 52: 1, https://doi.org/10.1111/1365-2664.12348

Gingera T, Bajno R, Docker MF, Reist JD (2017) Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. *Management of Biological Invasions* 8: 287–300, https://doi.org/10.3391/mbi.2017.8.3.03

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP (2013) Environmental DNA as a New Method for Early Detection of New Zealand Mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science* 32: 792–800, https://doi.org/10.1899/13-046.1

Goldberg CS, Strickler KM, Pilliod DS (2015) Moving Environmental DNA Methods from Concept to Practice for Monitoring Aquatic Macroorganisms. *Biological Conservation* 183: 1–3, https://doi.org/10.1016/j.biocon.2014.11.040

Hulme PE (2009) Trade, Transport and Trouble: Managing Invasive Species Pathways in an Era of Globalization. *The Journal of Applied Ecology* 46: 10–18, https://doi.org/10.1111/j.1365-2664.2008.01600.x

Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR (2015) Distance, Flow and PCR Inhibition: eDNA Dynamics in Two Headwater Streams. *Molecular Ecology Resources* 15: 216–227, https://doi.org/10.1111/1755-0998.12285

Jerde CL, Mahon AR, Lindsay Chadderton W, Lodge DM (2011) 'Sight-Unseen' Detection of Rare Aquatic Species Using Environmental DNA. *Conservation Letters* 4: 150–157, https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jerde C L, Olds BP, Shogren AJ, Andruszkiewicz EA, Mahon AR, Bolster D, Tank JL (2016) Influence of Stream Bottom Substrate on Retention and Transport of Vertebrate Environmental DNA. *Environmental Science & Technology* 50: 8770–8779, https://doi.org/10.1021/acs.est.6b01761

Karatayev AY, Burlakova LE, Padilla DK (2002) Impacts of Zebra Mussels on Aquatic Communities and their Role as Ecosystem Engineers. In: Leppäkoski E, Gollasch S, Olenin S (eds), Invasive Aquatic Species of Europe. Distribution, Impacts and Management, Springer, Dordrecht, pp 433–446, https://doi.org/10.1007/978-94-015-9956-6_43

Karatayev AY, Padilla DK, Minchin D, Boltovskoy D, Burlakova LE (2007) Changes in global economies and trade: the potential spread of exotic freshwater bivalves. *Biological Invasions* 9: 161–180, https://doi.org/10.1007/s10530-006-9013-9

Karatayev AY, Burlakova LE, Padilla DK (2015) Zebra versus quagga mussels: a review of their spread, population dynamics, and ecosystem impacts. *Hydrobiologia* 746: 97–112, https://doi.org/10.1007/s10750-014-1901-x

Klymus KE, Richter CA, Chapman DC, Paukert C (2015) Quantification of eDNA Shedding Rates from Invasive Bighead Carp *Hypophthalmichthys nobilis* and Silver Carp *Hypophthalmichthys molitrix*. *Biological Conservation* 183: 77–84, https://doi.org/10.1016/j.biocon.2014.11.020

Lawson Handley L (2015) How will the "molecular revolution" contribute to biological recording? *Biological Journal of the Linnean Society* 115: 750–766, https://doi.org/10.1111/bij.12516

Macher J, Leese F (2017) Environmental DNA Metabarcoding of Rivers: Not All eDNA Is Everywhere, and Not All the Time. bioRxiv. https://doi.org/10.1101/164046

Mächler E, Deiner K, Steinmann P, Altermatt F (2014) Utility of Environmental DNA for Monitoring Rare and Indicator Macroinvertebrate Species. *Freshwater Science* 33: 1174–1183, https://doi.org/10.1086/678128

Mahon AR, Barnes MA, Senapati S, Feder JL, Darling JA, Chang H, Lodge DM (2011) Molecular Detection of Invasive Species in Heterogeneous Mixtures Using a Microfluidic Carbon Nanotube Platform. *PLoS ONE* 6: e17280, https://doi.org/10.1371/journal.pone.0017280

Meusnier I, Singer GAC, Landry J, Hickey DA, Hebert PDN, Hajibabaei M (2008) A Universal DNA Mini-Barcode for Biodiversity Analysis. *BMC Genomics* 9: 214, https://doi.org/10.1186/1471-2164-9-214

Mills DN, Chadwick MA, Francis RA (2017) Impact of invasive quagga mussel (*Dreissena rostriformis bugensis*, Bivalva: Dreissenidae) on the macroinvertebrate community structure of a UK river. *Aquatic Invasions* 4: 509–521, https://doi.org/10.3391/ai.2017.12.4.08

Nathan LM, Simmons M, Wegleitner BJ, Jerde CL, Mahon AR (2014) Quantifying Environmental DNA Signals for Aquatic Invasive Species across Multiple Detection Platforms. *Environmental Science & Technology* 48: 12800–12806, https://doi.org/10.1021/es5034052

Nevers MB, Byappanahalli MN, Morris CC, Shively D, Przybyla-Kelly K, Spoljaric AM, Dickey J, Roseman EF (2018) Environmental DNA (eDNA): A tool for quantifying the abundant but elusive round goby (*Neogobius melanostomus*). *PLoS ONE* 13: e0191720 https://doi.org/10.1371/journal.pone.0191720

Peñarrubia L, Alcaraz C, Bij de Vaate A, Sanz N, Pla C, Vidal O, Viñas J (2016) Validated Methodology for Quantifying Infestation Levels of Dreissenid Mussels in Environmental DNA (eDNA) Samples. *Scientific Reports* 6: 39067, https://doi.org/10.1038/srep39067

Peyer SM, Hermanson JC, Lee CE (2011) Effects of shell morphology on mechanics of zebra and quagga mussel locomotion. *The Journal of Experimental Biology* 214: 2226–2236, https://doi.org/10.1242/jeb.053348

Peyer SM, McCarthy AJ, Lee CE (2009) Zebra Mussels Anchor Byssal Threads Faster and Tighter than Quagga Mussels in Flow. *The Journal of Experimental Biology* 212: 2027–2036, https://doi.org/10.1242/jeb.028688

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating Occupancy and Abundance of Stream Amphibians Using Environmental DNA from Filtered Water Samples. *Canadian Journal of Fisheries and Aquatic Sciences. Journal Canadien Des Sciences Halieutiques et Aquatiques* 70: 1123–1130, https://doi.org/10.1139/cjfas-2013-0047

Quinn A, Gallardo B, Aldridge DC (2014) Quantifying the Ecological Niche Overlap between Two Interacting Invasive Species: The Zebra Mussel (*Dreissena polymorpha*) and the Quagga Mussel (*Dreissena rostriformis bugensis*). *Aquatic Conservation: Marine and Freshwater Ecosystems* 24: 324–337, https://doi.org/10.1002/aqc.2414

Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) REVIEW: The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology* 51: 1450–1459, https://doi.org/10.1111/1365-2664.12306

Ricciardi A, Serrouya R, Whoriskey FG (1995) Aerial Exposure Tolerance off Zebra and Quagga Mussels (Bivalvia: Dreissenidae): Implications for Overland Dispersal. *Canadian Journal of Fisheries and Aquatic Sciences. Journal Canadien Des Sciences Halieutiques et Aquatiques* 52: 470–477, https://doi.org/10.1139/f95-048

Ricciardi A, Whoriskey FG, Rasmussen JB (1996) Impact of the (*Dreissena*) Invasion on Native Unionid Bivalves in the Upper St. Lawrence River. *Canadian Journal of Fisheries and Aquatic Sciences. Journal Canadien Des Sciences Halieutiques et Aquatiques* 53: 1434–1444, https://doi.org/10.1139/f96-068

Ricciardi A, Neves RJ, Rasmussen JB (1998) Impending Extinctions of North American Freshwater Mussels (Unionoida) Following the Zebra Mussel (*Dreissena polymorpha*) Invasion. *The Journal of Animal Ecology* 67: 613–619, https://doi.org/10.1046/j.1365-2656.1998.00220.x

Roussel J, Paillisson J, Tréguier A, Petit E (2015) The Downside of eDNA as a Survey Tool in Water Bodies. *The Journal of Applied Ecology* 52: 823–826, https://doi.org/10.1111/1365-2664.12428

Roy HE, Peyton J, Aldridge DC, Bantock T, Blackburn TM, Britton R, Clark P, Cook E, Dehnen-Schmutz K, Dines T, Dobson M, Edwards F, Harrower C, Harvey MC, Minchin D, Noble DG, Parrott D, Pocock MJO, Preston CD, Roy S, Salisbury A, Schonrogge K, Sewell J, Shaw RH, Stebbing P, Stewart AJA, Walker KJ (2014) Horizon Scanning for Invasive Alien Species with the Potential to Threaten Biodiversity in Great Britain. *Global Change Biology* 20: 3859–3871, https://doi.org/10.1111/gcb.12603

Sansom BJ, Sassoubre LM (2017) Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. *Environmental Science and Technology* 51: 14244–14256, https://doi.org/10.1021/acs.est.7b05199

Simberloff D, Von Holle B (1999) Positive interactions of nonindigenous species: invasional meltdown. *Biological Invasions* 1: 21–32, https://doi.org/10.1023/A:1010086329619

Shogren AJ, Tank JL, Andruszkiewicz E, Olds B, Mahon AR, Jerde CL, Bolster D (2017) Controls on eDNA Movement in Streams: Transport, Retention, and Resuspension. *Scientific Reports* 7: 5065, https://doi.org/10.1038/s41598-017-05223-1

Sutherland WJ, Bardsley S, Clout M, Depledge MH, Dicks LV, Fellman L, Fleishman E (2013) A Horizon Scan of Global Conservation Issues for 2013. *Trends in Ecology & Evolution* 28: 16–22, https://doi.org/10.1016/j.tree.2012.10.022

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012) Environmental DNA. *Molecular Ecology* 21: 1789–1793, https://doi.org/10.1111/j.1365-294X.2012.05542.x

Takeuchi A, Sado T, Gotoh RO, Watanabe S, Tsukamoto K, Miya M (2019) New PCR Primers for Metabarcoding Environmental DNA from Freshwater Eels, Genus *Anguilla*. *Scientific Reports* 9: 7977, https://doi.org/10.1038/s41598-019-44402-0

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012a) Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS ONE* 7: e41732, https://doi.org/10.1371/journal.pone.0041732

Thomsen PF, Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert TP, Orlando L, Willerslev E (2012b) Monitoring Endangered Freshwater Biodiversity Using Environmental DNA. *Molecular Ecology* 21: 2565–2573, https://doi.org/10.1111/j.1365-294X.2011.05418.x

Timar L, Phaneuf DJ (2009) Modeling the Human-Induced Spread of an Aquatic Invasive: The Case of the Zebra Mussel. *Ecological Economics: The Journal of the International Society for Ecological Economics* 68: 3060–3071, https://doi.org/10.1016/j.ecolecon.2009.07.011

Tréguier A, Paillisson J, Dejean T, Valentini A, Schlaepfer MA, Roussel J (2014) Environmental DNA Surveillance for Invertebrate Species: Advantages and Technical Limitations to Detect Invasive Crayfish *Procambarus clarkii* in Freshwater Ponds. *Journal of Applied Ecology* 51: 871–879, https://doi.org/10.1111/1365-2664.12262

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F, Gaboriaud C, Jean P, Poulet N, Roset N, Copp GH, Geniez P, Pont D, Argillier C, Baudoin JM, Peroux T, Crivelli AJ, Olivier A, Acqueberge M, Le Brun M, Møller PR, Willerslev E, Dejean T (2016) Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology* 25: 929–942, https://doi.org/10.1111/mec.13428

Venables WN, Ripley BD (2002) Modern Applied Statistics with S. 4th edition. Springer, New York, 497 pp, https://doi.org/10.1007/978-0-387-21706-2

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York

Williams MR, Stedtfeld RD, Engle C, Salach P, Fakher U, Stedtfeld T, Dreelin E, Stevenson RJ, Latimore J, Hashsham SY (2017) Isothermal amplification of environmental DNA (eDNA) for direct field-based monitoring and laboratory confirmation of *Dreissena* sp. *PLoS ONE* 12: e0186462, https://doi.org/10.1371/journal.pone.0186462

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012) Primer-BLAST: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction. *BMC Bioinformatics* 13: 134, https://doi.org/10.1186/1471-2105-13-134

Supplementary material

The following supplementary material is available for this article:

Appendix 1. Primer design and GLM testing

Table S1. GenBank accession numbers used for designing species specific primers for INNS target taxa.
Table S2. Species specific primer design.
Table S3. Tissue samples used in species specific primer testing.
Table S4. Specimen biomass pre and post mesocosm experiment, *D. r. bugenisis*.
Table S5. Temperature measurements during mesocosm experiment, *D. r. bugenisis*.
Table S6. Specimen biomass pre and post mesocosm experiment, *D. polymorpha*.
Table S7. Temperature measurements during mesocosm experiment, *D. polymorpha*.
Table S8. Species specific primer mismatches.
Table S9. *D. r. bugensis* mesocosm GLM.
Table S10. *D. polymorpha* mesocosm GLM.
Figure S1. AliView alignment for *D. polymorpha* sequencing.
Figure S2. AliView alignment for *D. r. bugensis* sequencing.

Appendix 2. DNA extraction protocol

Appendix 3. Gel Images

Figure S3. Agarose gel images from *Dreissena rostriformis bugensis* mesocosm experiment.
Figure S4. Agarose gel images from *Dreissena polymorpha* mesocosm experiment.
Figure S5. Agarose gel images from *Dreissena rostriformis bugensis* field samples.
Figure S6. Agarose gel images from *Dreissena polymorpha* field samples.

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