Received: 12 February 2019 | Revised: 1 April 2019 | Accepted: 2 April 2019

DOI: 10.1002/edn3.12

ORIGINAL ARTICLE

WILEY Environmental DNA Open Access
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Monitoring the eradication of the highly invasive topmouth gudgeon (*Pseudorasbora parva*) using a novel eDNA assay

Chloe Victoria Robinson | Carlos Garcia de Leaniz | Matteo Rolla | Sofia Consuegra

Swansea University, Swansea, Wales, UK

Correspondence
Sofia Consuegra, Swansea University, Singleton Park, Swansea, Wales, UK.
Email: s.consuegra@swansea.ac.uk

Present Address
Chloe Victoria Robinson, Centre for Biodiversity Genomics, University of Guelph, 50 Stone Road E, Guelph, Ontario N1G 2W1, Canada

Funding information
H2020 Environment, Grant/Award Number: 689682 (AMBER); Higher Education Funding Council for Wales, Grant/Award Number: NRN-LCEE cluster AQUAWALES

Abstract

Aquatic invasive species (AIS) represent an important threat for Biodiversity and are one of the factors determining the ecological integrity of water bodies under the Water Framework Directive. Eradication is one of the most effective tools for the management of invasive species but has important economic and ecological trade-offs and its success needs to be carefully monitored. We assessed the eradication success of the topmouth gudgeon (*Pseudorasbora parva*), an invasive fish that poses significant risks to endemic aquatic fauna, in four ponds previously treated with the piscicide Rotenone using a novel qPCR-based environmental DNA (eDNA) assay. We validated the assay through successfully detecting DNA from topmouth gudgeon in two reservoirs with physically confirmed topmouth gudgeon populations. Topmouth gudgeon were detected in all four treated ponds using 750 ml water samples and in three of the ponds using 15 ml samples, despite the eradication treatment and lack of successful detection using conventional trapping methods. Our results highlight the difficulties of eradicating invasive fish and the need to incorporate reliable monitoring methods as part of a risk management strategy under the water framework directive (WFD).

KEYWORDS
aquatic invasive species, environmental DNA, invasion biology, water framework directive

1 | INTRODUCTION

Invasive species pose one of the main threats to global biodiversity (Sala et al., 2000) through their ability to modify the biological integrity and ecological functioning of native aquatic systems. The presence of aquatic invasive species (AIS) is one of the determinants of the ecological status of European water bodies under the water framework directive (WFD) (Cardoso, 2008). Eradication is considered the second most effective tool for the management of invasive species after prevention (Genovesi & Carnevali, 2011). However, eradications are typically costly and can have negative impacts on native ecosystems, so the decision to eradicate needs to be taken based on effectiveness, practicality, cost, impact, and likelihood of re-invasion (Booy et al., 2017). Critical to the application of risk management tools is the ability to detect the target species, even when they are at very low densities (Hulme, 2006). Early detection, followed by a rapid response, are critical for the success of eradication programmes (Simberloff, 2014), but these must be monitored to establish their success (Copp et al. 2010). Environmental DNA (eDNA) is increasingly being used for the early detection of invasive and endangered aquatic species (Rees, Maddison, Middleditch, Patmore, & Gough, 2014) and is beginning to be used as a monitoring tool under the WFD (Hering et al., 2018).

Methodology for monitoring AIS using eDNA can vary in terms of laboratory analyses. A number of species-specific assays using the qPCR primer-probe approach and/or end-point PCR have been implemented for detecting nonnative species such as Asian carp (Simmons, Tucker, Chadderton, Jerde, & Mahon, 2015), Ponto-Caspian gobies (Adrian-Kalchhauser & Burkhardt-Holm, 2016) and Burmese python (Hunter et al., 2015). More recently, quantitative PCR-High Resolution Melt (qPCR-HRM) analysis is starting to be used to detect closely related species (invasive and native), including crayfishes (Robinson, Uren Webster, Cable, James, & Consuegra, 2018) and cyprinid fish (Behrens-Chapuis et al., 2018), using a single qPCR reaction. High resolution melting (HRM) analysis is a qPCR-based method which facilitates identification of small variations in nucleic acid sequences by differences in the melting temperature of double stranded DNA depending on fragment length and sequence composition (Ririe, Pasmussen, & Wittwer, 1997). Analysis of HRM curves has been widely used for SNP genotyping as a fast method to discriminate species (Yang et al., 2009), including natives and invasives (Ramón-Laca et al., 2014). Alternatively, metabarcoding of eDNA samples is also used to determine presence/absence of invasive taxa within particular communities (Balasingham, Walter, & Heath, 2017; Borrell, Miralles, Do Huu, Mohammed-Geba, & Garcia-Vazquez, 2017; Klymus, Marshall, & Stepien, 2017). Yet, for questions related to target species, species-specific assays are cheaper and easier to design and analyse compared to a community-level approach (Valentini et al., 2015) and have been used previously for monitoring eradication, such as that of the topmouth gudgeon (*Pseudorasbora parva*; Davison, Copp, Créach, Vilizzi, & Britton, 2017).

The topmouth gudgeon (*P. parva*) is a highly invasive fish originating from Asia that can cause considerable damage to native communities (Britton, Davies, Brazier, & Pinder, 2007; Pinder, Gozlan, & Britton, 2005). The species is highly plastic, tolerates a wide range of environmental conditions, and is more fecund than many native fishes in Europe, traits that facilitate its invasion success (Beyer, Copp, & Gozlan, 2007; Britton, Davies, & Brazier, 2008; Pinder et al., 2005).

In Great Britain, *P. parva* has been reported in 23 locations across England and Wales, the majority being lentic systems, 10 of which are connected to major catchments (Britton, Davies, & Brazier, 2010; Britton et al., 2007). The species has been classified as highly impactive by the UK Technical Advisory Group on the WFD (Panov et al., 2009) due to its rapid reproductive rate, and competition for resources with native fish (Britton et al., 2007). In addition to direct competition, *P. parva* is also a vector for several fish pathogens, including *Sphaerothecum destruens*, the eel nematode parasite *Anguillicola crassus*, the trematode *Clinostomum complanatum* and the pike fry rhabdovirus (PFR). However, the actual impact of these pathogens on native fish populations is largely unknown (Britton et al., 2007).

Current methods for managing the spread of *P. parva* have focussed on eradication using the chemical piscicide Rotenone (Allen, Kirby, Copp, & Brazier, 2006; Britton et al., 2008). This has been reported to be effective at removing *P. parva* at small spatial scales (Ling, 2002). A common problem with the use of piscicides however, is nonspecificity, and the risk of impacting on nontarget native fish and invertebrates (Lemmens, Mergeay, Vanhove, Meester, & Declerck, 2015; Ling, 2002). The trade-off between eradication of invasive species and extirpation of native biodiversity needs to be considered, and this can render chemical eradication a nonviable option in some cases (Britton, Gozlan, & Copp, 2011; Ling, 2002).

Invasive species are often difficult to detect using traditional methods both during the early stages of invasion and posteradication due to their low abundance (Dejean et al., 2012; Jerde, Mahon, Chadderton, & Lodge, 2011; Takahara, Minamoto, & Doi, 2013). *Pseudorasbora parva* undergo boom and bust cycles in novel environments, most likely caused by a combination of biotic and abiotic factors, including temperature fluctuations and predation pressure (Britton et al., 2008; Britton et al., 2007). Trapping during low abundance or bust cycles may fail to yield any fish and result in false negatives (Davies & Britton, 2015). eDNA can be a more effective method for assessing presence/absence and could inform the success of eradication measures (Davison et al., 2017). eDNA shedding rates (i.e., mucous, faeces, sloughed cells) are likely higher among fish species (Barnes & Turner, 2016) than among other invasive organisms such as invertebrates or amphibians (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Tréguier et al., 2014), which facilitates detection and may allow the quantification of fish abundance by qPCR, at least in ponds and other closed systems (Klymus, Richter, Chapman, & Paukert, 2015; Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2016).

Rotenone has been used to eradicate *P. parva* from 15 of the 23 *P. parva* confirmed sites in the UK (Brazier, 2015), mainly in fishing and recreational lakes, small mountain lakes and village ponds across England (Allen et al., 2006; Britton et al., 2008,2010). In many lakes and ponds with direct connection to a stream, screens were installed to prevent dispersal of *P. parva* via rivers before eradication took place (Britton et al., 2008). Subsequent sampling using micromesh seine nets, capable of catching fish of >12 mm in length, failed to detect *P. parva* in all cases since eradication (Britton et al., 2010). In 2014, one pond within the Millennium Coastal Park (Llanelli, South Wales) was reported to have *P. parva*, despite two previous eradication attempts in 2012 and 2013 (Brazier, 2015). The use of eDNA was recommended as a more sensitive and robust alternative to netting and trapping to test for the presence/absence of *P. parva* before any further eradication attempts (Davies & Britton, 2015).

Previous methods for *P. parva* detection based on eDNA have used end-point (i.e., conventional) PCR (Davison et al., 2017). Here we developed and tested a highly sensitive and specific qPCR high resolution melt curve (qPCR-HRM) assay to test the efficiency of an eradication programme in four ponds and compared the results to traditional end-point PCR. We also assessed the effect of water volume on detection success by using two different protocols involving different water volumes.

2 | MATERIALS AND METHODS

2.1 | Sample sites

Four ponds (Morolwg (P1), Ashpits (P2), Turbine (P3), and Dyfatty (P4)) were sampled for eDNA at the Millennium Coastal Park (Llanelli,

Wales) in August 2017. eDNA sampling was targeted for this season due to increased activity of *P. parva* with warmer temperatures (Beyer, 2004; Beyer et al., 2007), which is more likely to result in successful DNA detection. The presence of *P. parva* at Ashpits pond had been confirmed by trapping and subsequent Rotenone treatment in 2011. Morolwg, Turbine and Dyfatty ponds were also treated with Rotenone in 2012, after confirmation of *P. parva* presence by trapping and electrofishing. Visual confirmation of *P. parva* in Ashpits pond in 2014 lead to concerns that *P. parva* could have been reintroduced into Morolwg pond due to existing stream between these two ponds. There had been no records of *P. parva* in Dyfatty or Turbine ponds since 2012 (Table 1; Figure 1).

2.2 | Physical surveys for detection of P. parva

Prior to and during eDNA sampling period, trapping of *P. parva* was attempted at four time points under permit EP/CW061-E-546/11141/02 and EP/CW061-E-546/12754/01, using a range of trapping methods. Trapping was designed to be conducted after the May spawning season and before the colder winter months (where possible), when *P. parva* is known to be more active (due to warmer temperatures and prey abundance), to increase the likelihood of trapping any existing fish (Beyer, 2004; Beyer et al., 2007; Britton, Pegg, & Gozlan, 2011). In July 2017, five standard minnow traps (20 cm × 20 cm × 60 cm) were placed by Natural Resources Wales at Ashpits pond at 1 m depth for 7 days, concentrating on the eastern side of the pond where the largest concentrations of *P. parva* had been previously seen. Traps were baited with fish pellets and checked daily. In February 2018 and June 2018, 10 standard minnow traps baited with fish pellets and algal-based bait were placed evenly around Ashpits (seine nets and traps) and Dyfatty Ponds (only traps) by Swansea University research staff at a depth of 1 m and checked after 3 hr (February) and 24 hr (June). Seine netting was also carried out in Ashpits, Morolwg, and Dyfatty ponds in both February and June 2018. In addition, eight larvae (<12 mm) were collected by hand-netting from Ashpits (n = 5) and Morolwg (n = 3) and transported back to the university before being euthanized following Schedule 1 protocol of overdose of 2-Phenoxyethanol. DNA was extracted from the eight larvae using Qiagen® DNeasy Blood and Tissue Kit (Qiagen, UK). Larvae DNA was eluted in 200 µl and amplified using both end-point PCR and the new qPCR 16S protocol developed for this study (see below). All amplified end-point PCR products were checked for the correct amplicon size using a 2% agarose gel electrophoresis and were sequenced on an ABI Prism 377 sequencer to confirm species identity.

2.3 | eDNA collection

Two different volumes of water were collected for this study; 250 ml (in triplicate) and a single sample of 15 ml. Water samples of 250 ml were collected in triplicate from 10 evenly distributed sampling points per pond. Samples were taken from the upper 30 cm of the water column using a sterile 1 m collection ladle before being pooled

TABLE 1 Site information for the four ponds sampled for presence of *Pseudorasbora parva* eDNA in South Wales during August 2017 and October 2018

Site	Pond Name	Latitude	Longitude	Pond Volume (m^3)	Average Depth (m)	Eradication Attempt	*Current Pseudorasbora parva* Status	Temp. at time of sampling (°C)	Number of eDNA Samples Collected[a]
1	Morolwg	51.687583	–4.2193952	15,640	1.7	Winter 2012	Unknown	19.3	11
2	Ashpits	51.688091	–4.2205920	89,538	1.5	2011	Present	19.1	11
3	Turbine	51.680326	–4.2430390	17,820	1.8	Winter 2012	Unknown	18.6	11
4	Dyfatty	51.683181	–4.2314910	56,579	1.9	Winter 2012	Unknown	19.6	11
5	Upper Lliedi	51.717176	–4.1538425	Unknown	Unknown	N/A	Present	13.2	2
6	Lower Lliedi	51.716366	–4.1509375	Unknown	Unknown	N/A	Present	13.6	2

Note: Pond volumes and average depth values were provided by Natural Resources Wales (NRW).
[a]Including negative controls

FIGURE 1 Location of *Pseudorasbora parva* eDNA and trapping sites. Four ponds were sampled for *Pseudorasbora parva* eDNA in the Millennium Coastal Park in Llanelli (P1 - Morolwg Pond, 2 - Ashpits Pond; P3 - Turbine Pond; P4 - Dyfatty Pond) at 10 sampling points per pond. Two additional sampling points were included from the Upper (R1) and Lower (R2) Lliedi reservoir. At each sampling point, 250 ml water samples were collected in triplicate (750 ml total per sampling point). For P1-P4, an additional 15 ml water sample was collected at each sampling point. Maps made using "Snazzy Maps" <http://snazzymaps.com/>, Created: March 2019

into a sterile 1 L Nalgene bottle (final volume 750 ml). This method of sampling was chosen to increase the coverage of the ponds (Eichmiller, Bajer, & Sorenson, 2014), whilst targeting locations where *P. parva* individuals were previously known to aggregate (i.e., east side of Ashpits). Filtration of 250 ml water volumes has been the recommended minimum volume of water to produce successful detection in a range of aquatic species (Dougherty et al., 2017; Goldberg et al., 2016; Hinlo, Gleeson, Lintermans, & Furlan, 2017; Olds et al., 2016). Sampling points were separated by between 20 and 100 m per pond and samples were collected from the bank to avoid releasing potential confounding eDNA from sediment material (Turner, Uy, & Everhart, 2015). The additional water sample of 15 ml was collected at each point per pond as in (Ficetola et al., 2008) to assess the sensitivity of this water volume to detect the target species in ponds. To each 15 ml sample, 33 ml of 99% ethanol and 1.5 ml 3 mol/L sodium acetate were added; tubes were kept upright on ice for transportation and subsequently stored at −20°C until DNA extraction. The ladle was used for sampling 2 ponds each day and was thoroughly decontaminated between ponds (Ashpits and Morolwg on day 1 and Turbine and Dyfatty on day 2) by thorough spraying with a 5% Virkon® solution followed by rinsing three times with ultrapure water to prevent potential DNA carryover, resulting in false positives (Szkuta, Oorschot, & Ballantyne, 2017). Virkon® was used as a nontoxic, nonbleaching alternative to decontaminating with sodium hypochlorite in the field (Stockton-Fiti & Moffitt, 2017; Szkuta et al., 2017). In between the two sampling days (i.e., between sampling Morolwg and Turbine), the ladle was autoclaved at 121°C. Virkon® has previously been used to remove DNA (Szkuta et al., 2017) and to ensure no carryover of DNA between ponds, we collected negative controls directly after each decontamination (see below).

Negative controls, consisting of ultrapure water in place of pond water, were taken at each pond for both methods (750 ml and 15 ml) prior to sampling and after decontamination of each ladle before sampling a new pond (n = 4 for 750 ml and n = 4 for 15 ml). Environmental conditions, including water temperature, and sample

water depth (m) were recorded at each site (Table 1). Pond volumes were provided by Natural Resources Wales (NRW).

2.4 | Primer design and DNA extraction

Species-specific qPCR primers (PparvaF 5′-CGAGCCCAAATAAC AGAGGGT-3′ [Tm: 60°C, GC: 52.3%] and PparvaR 5′-CAGGC GAGGCTTATGTTTGC-3′ [Tm: 59°C, GC: 55%]) were designed for *P. parva* using NCBI Primer-BLAST and amplify a 147 bp fragment of the 16S mtDNA gene (product melt temperature 78.8°C). NCBI Primer-BLAST was also used to check for cross-amplification using NCBI Primer-BLAST (Ye, McGinnis, & Madden, 2006). Primers were aligned with 16S sequences from closely related fish species to *P. parva* (common bream *Abramis brama*; common roach *Rutilus rutilus*; silver rudd *Scardinius erythrophthalmus*; common carp *Cyprinus carpio*; common dace *Leuciscus leuciscus* and common minnow *Phoxinus phoxinus*), using BioEdit (v 7.0.5.3) to assess number and positions of mismatches (Figure S1). Primers were then tested in vitro for nonspecific amplification against four of these species (*C. carpio*, *S. erythrophthalmus*, *A. brama* and *R. rutilus*), which are known to inhabit similar water systems as *P. parva* (Davison et al., 2017) and were previously trapped in these ponds prior to eDNA sampling (no additional fish species found). DNA from these species was assessed for amplification in triplicate using qPCR, at 20 ng/µl, 5 ng/µl, and 0.1 ng/µl concentrations, with a positive control consisting of 0.1 ng of *P. parva* DNA (Davison et al., 2017). qPCR reactions were undertaken using SsoFast™ EvaGreen® Supermix (Bio-Rad, UK) and the 16S protocol. All nontarget species failed to amplify and no subsequent products were produced in qPCR-HRM analysis. Primers were then assessed in vitro for specific amplification using positive control tissue (caudal muscle) from 15 *P. parva* individuals caught locally in Wales during 2012/13. DNA was extracted using the Qiagen® DNeasy Blood and Tissue Kit (Qiagen), eluted in 200 µl, and amplified in end-point PCR using BioMix™ (Bioline, UK) in the following 16S protocol: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s with a final elongation step of 72°C for 10 min. All amplified PCR products were checked for the correct amplicon size using a 2% agarose gel electrophoresis.

2.5 | qPCR optimization

Specific in vitro testing of qPCR-HRM was performed for *P. parva* to confirm the amplification efficiency. The limit of detection (LOD) and limit of quantification (LOQ) were determined through running a 10-fold dilution series (Atkinson et al., 2018; Wilcox et al., 2013) ranging from 5 ng/µl to 5×10^{-7} ng/µl, using a *P. parva* DNA pool (consisting of DNA from 15 individual *P. parva*). The annealing temperature of 16S primers was optimized at 61°C and yielded an efficiency of 91.1%, $R^2 = 0.981$ (Figure S2; Table S1 and S2). For optimization, the qPCR-HRM was undertaken using SsoFast™ EvaGreen® Supermix (Bio-Rad, UK) and the 16S protocol. An additional HRM step was applied to the end of qPCR reactions, ranging from 55°C to 95°C in 0.1°C increments to assess the consistency of amplicon melt temperature (Tm). qPCR-HRM analysis was conducted on 15 individuals from three different populations to account for any degree of intraspecific variation in qPCR product melt temperature (Tm).

2.6 | Assay field validation

To validate the assay in the field, we collected 3 × 250 ml water samples from the Upper (high abundance of *P. parva*) and 3 × 250 ml samples from the Lower (low abundance of *P. parva*) Lliedi reservoirs in October 2018. *P. parva* presence in this system had been confirmed by trapping in June 2018 using standard minnow traps baited as above (permit: EP/CW061-E-546/12754/01; Table 1; Figure 1). Negative controls (ultrapure water) were collected as described previously for the Millennium Coastal Park and water samples were processed following the previously described filtration, extraction, and qPCR protocols. Subsequently, samples which displayed a melt peak consistent with *P. parva* in at least one of three replicates in qPCR were re-amplified in end-point PCR using 16S protocol and sequenced to confirm species identity.

2.7 | Processing and analysis of eDNA field samples

Three different analysis approaches were undertaken in this study; (a) "PCR" – consisting of end-point PCR of eDNA from 750 ml samples using primers from Davison et al. (2017); (b) "qPCR1" – consisting of qPCR eDNA from 750 ml samples using 16S primers developed here and (c) "qPCR2" – consisting of qPCR of eDNA from 15 ml samples using 16S primers developed here.

Samples of 750 ml of pooled water (3 × 250 ml at each sampling site) from each of the study ponds were filtered the same day of collection using the same filtration protocol as described previously for the Virkon® decontamination validation. DNA filtration and extraction took place in a designated eDNA area in a laboratory where no previous *P. parva* DNA or tissue had been handled. Filters from water samples were stored in individual Eppendorfs at −20°C until subsequent DNA extraction. *P. parva* DNA from filter papers was extracted using Qiagen® DNeasy Blood and Tissue Kit (Qiagen), following the Qiagen Blood Spot extraction protocol with an adjustment to the elution volume (from 200 to 50 µl) to maximize DNA concentration.

The 15 ml water samples were centrifuged at 6°C for 45 min at 5,000 *g* (Ficetola et al., 2008) and the supernatant was poured off to allow DNA extraction from the resulting pellet. DNA pellets were extracted using DNeasy PowerLyzer PowerSoil kit (Qiagen®), which produces higher yields of DNA for this method of eDNA capture (Hinlo et al., 2017), following a standard protocol with a reduction in elution volume (from 60 to 50 µl, to increase DNA concentration). All samples (750 ml and 15 ml) were amplified in triplicate in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK), in 10 µl reactions consisting of 5 µl SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.25 µl each forward and reverse primer, 2.5 µl HPLC water and 2 µl of extracted DNA. Amplifications were carried out in triplicate using the

standard 16S qPCR protocol as described above and only samples which amplified in one of three replicates at the target DNA product Tm (78.8°C ± 0.3), with a melt rate (the rate at which dsDNA denatures and dye dissociates as a function of temperature) above 200 -d(RFU)/dT were considered to be a positive result. qPCR reactions were prepared in a separate room from the eDNA extractions room under a PCR hood with laminar flow. We added a *P. parva* positive control to each plate after all the eDNA samples had been loaded and sealed to prevent any false positives from cross-contamination. As negative amplification controls we used ultrapure water which, along with extraction negative controls, were added to the same well locations on each plate to test for eDNA contamination.

Field samples of 750 ml were also amplified in triplicate in end-point PCR with a second set of species-specific primers targeting the COI gene in *P. parva* (method: PCR; Davison et al., 2017), using both the PCR protocol described in Davison et al. (2017) and an adjusted version of the same protocol increasing the number of cycles to 40. Subsequent PCR products were checked for the correct amplicon size using a 2% agarose gel. Sensitivity of COI primers was conducted by amplifying a dilution series of pooled *P. parva* DNA in end-point PCR, ranging from 5 ng/µl to 5×10^{-7} ng/µl, and assessing production of visible bands in a 2% agarose gel. Any positive reactions for *P. parva* DNA with both primer sets were sequenced on an ABI Prism 377 sequencer (Applied Biosystems) to confirm species identity.

2.8 | Statistical analysis

We employed a generalized linear modelling approach in R v.3.4.3 (R Core Team, 2014) to model detection success (i.e., the proportion of sites that tested positive for *P. parva* at each pond) as a function of assay type (three assays: conventional PCR on 750 ml of water [PCR], qPCR on 750 ml of water [qPCR1], and qPCR on 15 ml of water [qPCR2]) and pond identity (n: 4 ponds). We considered that *P. parva* was present at a site if one of the three replicates tested positive for that site. We attempted model fitting using a binomial logit-link but this resulted in overdispersion (i.e., the residual deviance was greater than the residual degrees of freedom), probably caused by clustering of observations and a large number of zeroes. Following Crawley (2007), we refitted the model using a quasi-binomial logit-link to correct for overdispersion and produce more conservative and reliable confidence intervals and tests of significance.

3 | RESULTS

3.1 | Sensitivity and detection limits ex-situ

The limit of detection (LOD) for *P. parva* DNA was 0.005 ng/µl, determined through a 10-fold dilution series in qPCR. The detection threshold for *P. parva* DNA was 38 cycles (Table S1) and the product melt temperature (Tm) was consistent throughout the dilution series (Table S2). Tests for nonspecific amplification with nontarget fish species resulted in no amplifications for any of the species for all DNA concentrations. The newly designed 16S primers amplified *P. parva* DNA pools at 0.05 ng/µl and above, whereas the previously described COI primers (Davison et al., 2017) failed to produce any visible bands for *P. parva* DNA at 0.05 ng/µl, indicated the greater sensitivity of 16S primers (Figure S3).

3.2 | Assay validation in-situ

Results of qPCR confirmed a positive detection for *P. parva* in one out of three replicates in both the upper (Ct: 36.6) and lower (Ct: 36.5) Lliedi reservoir samples. All negative controls failed to amplify and *P. parva* DNA control amplified in all three replicates (av. Ct: 24.5). Results of Sanger Sequencing confirmed *P. parva* in both upper and lower reservoirs.

3.3 | Detection success

No adult *P. parva* were caught in any of the two trappings at Ashpits, Morolwg, or Dyfatty ponds for any of the sampling events. Despite this, larval DNA sequencing and qPCR profiles confirmed that two larvae hand-netted from Ashpits pond matched 100% with *P. parva* on BLAST, despite lack of adult *P. parva* being caught in traps and seine netting at trapping events. Remaining larvae failed to amplify and remained unidentified. Results of qPCR indicated the presence of *P. parva* DNA in all ponds (Figure S4; Table S3). Mean Ct values for positive 750 ml samples ranged from 28.8 to 38.8 in Morolwg, 27.5 to 36.7 in Ashpits, 32.1 to 36.5 in Turbine and 27.1 to 38.5 in Dyfatty (Table S6). Morolwg Pond (MP) yielded the highest proportion of sampling sites amplifying for *P. parva* (8 out of 10 sites), whereas Turbine Pond (TP) had the lowest proportion of positive sampling sites (six out of 10 sites; Table 2). The 15 ml water samples detected *P. parva* in three out of the four ponds, with no *P. parva* DNA being detected in Dyfatty Pond (Figure S5; Table S4). Mean Ct values for positive 15 ml samples ranged from 34.5 to 37.8 in Morolwg, 37.2 to 37.9 in Ashpits and 36.8 to 37.8 in Turbine (Table S6). In comparison, results of end-point PCR with species-specific COI primers (Davison et al., 2017) failed to produce any positive amplification for *P. parva* unless the PCR reaction was undertaken with 40 cycles. End-point PCR results also showed that *P. parva* DNA was present in all four ponds, however, most positive samples were only observed in one of the three triplicates and product bands were faint (Table 2; Figure S6). Sequencing of the 350 bp (Davison et al., 2017) and 147bp products produced a 100% species match on BLAST (Ye et al., 2006), with target species (for both primer sets), confirming species presence in all positive amplifications. All negative controls from the field and laboratory failed to amplify in qPCR and end-point PCR (both at 40 cycles), indicating that both the decontamination with Virkon® and laboratory protocols applied successfully prevented cross-contamination among pond samples.

Detection success varied significantly depending on eDNA assay (deviance = 31.32, $df = 2$, $p < 0.001$) but not on pond identity (deviance = 3.36, $df = 3$, $p = 0.582$l; Figure 2). Our novel qPCR

Pond	Method	Sampling point									
		1	2	3	4	5	6	7	8	9	10
P1	PCR	0	0	0	0	3	0	0	0	0	0
P1	qPCR1	2	3	3	2	3	3	2	1	0	3
P1	qPCR2	0	0	0	2	0	0	1	0	0	1
P2	PCR	0	1	0	1	0	0	1	1	1	1
P2	qPCR1	1	3	0	2	0	3	2	1	3	3
P2	qPCR2	1	0	0	2	0	1	0	0	0	0
P3	PCR	0	1	0	2	1	1	0	0	0	1
P3	qPCR1	0	2	0	3	3	3	0	0	2	3
P3	qPCR2	0	2	0	0	0	2	0	0	0	0
P4	PCR	0	0	0	1	0	0	0	0	2	0
P4	qPCR1	2	3	0	2	2	2	0	3	3	3
P4	qPCR2	0	0	0	0	0	0	0	0	0	0

Number = number of replicates (out of three) resulting in *Pseudorasbora parva* positive melt peak for 16S (78.8°C ± 0.3) or a positive band at 350 bp for COI.

TABLE 2 Amplifications for *Pseudorasbora parva* in 750 ml water samples amplified with previously designed COI primers (Davison et al., 2017; method: PCR), 750 ml water samples amplified in qPCR with our designed 16S primers (method: qPCR1) and 15 ml water samples amplified with designed 16S primers (method: qPCR2) from all four ponds (P1 - Morolwg Pond, 2 - Ashpits Pond; P3 - Turbine Pond; P4 - Dyfatty Pond) at each sampling point (1–10) in optimized SsoFast™ EvaGreen® qPCR assay

750 ml eDNA assay detected the presence of *P. parva* (31/40 or 77.5%) in a significantly higher proportion of sites (t = 2.962, p = 0.016) than conventional PCR with the same water volume (14 out of 40 samples or 35.0%) or qPCR with 15 ml of water (eight out of 40 samples or 20.0%). The novel assay was 4.2 times more likely to detect *P. parva* in an individual water sample than conventional end-point PCR, and 2.2 times more likely to detect its presence at a sampling site when multiple samples are collected (Table S5).

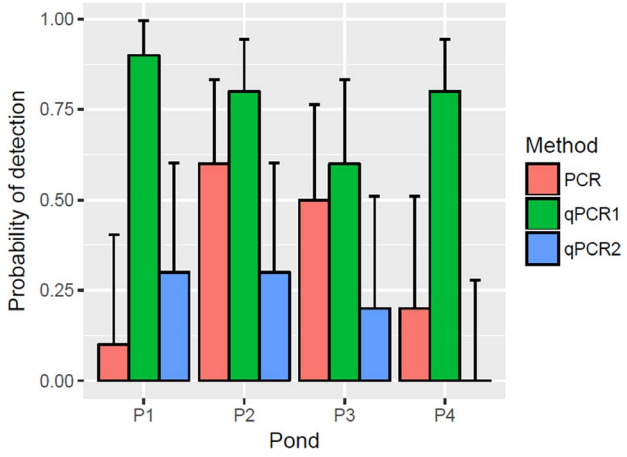

FIGURE 2 Probability of detection (± 95% Confidence Interval) of top mouth gudgeon (*Pseudorasbora parva*) at each pond (P1 - Morolwg Pond, 2 - Ashpits Pond; P3 - Turbine Pond; P4 - Dyfatty Pond) using different eDNA assays. Methods: PCR - end point-PCR on 750 ml of water using COI primers; qPCR1 - qPCR on 750 ml of water using 16S qPCR primers; qPCR2 - qPCR on 15 ml of water using 16S qPCR primers

4 | DISCUSSION

The application of our novel qPCR *P. parva* assay detected the presence of *P. parva* DNA at sites where the species was thought to have been eradicated or had not been detected by trapping. This serves to highlight the difficulties of inferring species absences from traditional survey methods (Ficetola et al., 2015; Jerde et al., 2011) and the superior sensitivity of qPCR-based eDNA methods over traditional approaches. The assay was validated in the field at a location where the species had been confirmed by trapping earlier this year, and all qPCR-HRM assays were validated through sequencing, including positive field samples. The qPCR assay described here successfully amplified the target species in very small volumes of sample water (15 ml), albeit with lower sensitivity, which should greatly facilitate the collection of multiple replicated field samples, particularly in remote/inaccessible areas. Our results show that high resolution melt curve analysis (HRM) based on species-specific melt curve profiles (Héritier, Verneau, Breuil, & Meistertzheim, 2017; Jaiswal, Tripathi, & Malhotra, 2017) offers greater sensitivity and quicker results (Martinou, Mancuso, & Rossi, 2010; Nathan, Simmons, Wegleitner, Jerde, & Mahon, 2014; Wood et al., 2013) than traditional end-point PCR for the detection of *P. parva* using eDNA. The advantages of using an qPCR-HRM approach over end-point PCR include increased sensitivity and the diagnostic nature of the melt peak analysis (Winchell, Wolff, Tiller, Bowen, & Hoffmaster, 2010). Thus, for species-specific assays such as this, the presence of the target species will produce an amplification peak that will melt at a temperature specific of the DNA fragment amplified (Héritier et al., 2017; Jaiswal et al., 2017; Robinson et al., 2018). HRM-qPCR assays can be then validated through sequencing, as we did here.

The use of eDNA to detect and monitor invasive species at low densities has numerous advantages over traditional techniques,

namely higher sensitivity and ease of collection (Evans, Shirey, Wieringa, Mahon, & Lamberti, 2017; Jones, 2013; Tucker et al., 2016). For example, eDNA from European weather loach (*Misgurnus fossilis*) and redfin perch (*Perca fluviatilis*) was detected at sites where fishing had previously failed to find these species (Bylemans, Furlan, Pearce, Daly, & Gleeson, 2016; Sigsgaard, Carl, Møller, & Thomsen, 2015). *P. parva* has a small size, and trapping can on occasions be unsuccessful, particularly during the early life stages (Jerde et al., 2011; Magnuson, Benson, & Mclain, 1994). The detection threshold for *P. parva* in closed systems using traditional methods (electrofishing and traps) is approximately 0.5 fish per m^2, which could suggest low *P. parva* densities in both Ashpits and Dyfatty ponds (Britton, Pegg, et al., 2011). The existence of false-negatives poses a particular problem for the management of AIS because they tend to occur at low population abundance, particularly during the early stages of invasion (Fitzpatrick, Preisser, Ellison, & Elkinton, 2009).

Absence of adult *P. parva* during the some of the trapping events could be explained by low temperatures (<10°C), short trapping time (<4 hr), shallow deployment and below-threshold densities, while size-selection biases can explain their absence during the 2016 surveys (Britton, Gozlan, et al., 2011). Posteradication survey methods at the Millennium Coastal Park, including micromesh seine netting and trapping (plastic bottle and big mesh "minnow" traps) is highly size-selective and it is possible that smaller colonizing individuals (<20 mm) could have evaded nets and traps, potentially resulting in false negatives (Davies & Britton, 2015). Even if eradication had been initially successful, it is possible that proximity to other invaded sites could have allowed fish to disperse through interconnecting streams (Britton et al., 2008; Copp, Vilizzi, & Gozlan, 2010; Pinder et al., 2005) and/or during flooding events, as reported for other AIS (Diez et al., 2012; Rahel & Olden, 2008; Scott, Arbeider, Gordon, & Moore, 2016). However, the closest site of known *P. parva* is the Lliedi reservoirs, which are over 5 km North-East of the ponds with no direct connection via a watercourse, rendering the Lliedi reservoirs as a *P. parva* DNA source impossible (Balasingham, Walter, Mandrak, & Heath, 2018). Inadvertent translocation of eggs and small larvae by local anglers across ponds is also a possible source of *P. parva* individuals as shown previously for this and other AIS (Britton et al., 2007; Johansson et al., 2018; Pinder et al., 2005).

An alternative explanation could be that the eDNA we detected originated not from live fish but from carcasses remaining at the bottom of the lake after the eradication or from lake sediment, where it could have been trapped and potentially released into the water (Turner et al., 2015). However, the eradication took place more than 6 years ago and eDNA from fish carcasses in lake systems has been suggested not to be detectable in water form more than 70 days (Dunker et al., 2016). Yet, while vertical migration (leaching) of DNA from sediments to the surrounding aquatic environment has not previously been observed in pond or lake sediments (Parducci et al., 2017; Sjögren et al., 2017), it cannot be completely ruled out as a DNA source in this study, as the persistence of eDNA in sediment depends largely on the origin of the DNA, its concentration and the nature of the sediment (Pietramellara et al., 2009). Sediment can have a higher content of eDNA than water (Pietramellara et al., 2009), and consequently longer temporal resolution (annual to decadal-centennial; Bálint et al., 2018). Yet, reported rates of eDNA degradation in sediment compared to water range from lower to up to 100 times higher (Dell'Anno & Corinaldesi, 2004; Turner et al., 2015). Recent studies have also shown that higher detection rates of fish DNA in sediment can occur in the deeper sediment (>150 cm below surface) in comparison to more recently deposited sediment (<50 cm below surface; Olajos et al., 2018) and that surficial sediment (depending on local conditions) can be incapable at retaining DNA for target fish species, for example in running waters (Baldigo, Sporn, George, & Ball, 2017). As the duration of eDNA in sediments is highly variable and there could be potential for resuspension into the water, the problem of temporal false positives when analysing eDNA could be resolved by determining the most likely age of DNA through use of RNA-based markers (Pochon, Zaiko, Fletcher, Laroche, & Wood, 2017), which would assist in determining whether the DNA signal observed shows past or present occupancy. Still, the finding of *P. parva* larvae in one of the eDNA positive lakes confirms that, at least in that lake, the species remains physically present after eradication.

The volume of environmental samples collected can greatly influence the rate of detection of eDNA from a range of aquatic species (Pilliod, Goldberg, Arkle, & Waits, 2013; Rees et al., 2014). In our study, the probability of detection by qPCR was c. 4–6 times higher with a large water volume (750 ml) compared to a smaller volume (15 ml). This is not unexpected considering the predicted low abundance of target DNA, the 50x reduction in water volume and the large size of the ponds sampled (Table 1, Table S5). In addition, the 15 ml samples were stored and processed differently from the larger volume which could have been affected the results. Yet, using the same laboratory and field protocols as for the 750 ml samples, would have likely the resulted in even lower eDNA concentrations (Hinlo et al., 2017; Rees et al., 2014). Collecting smaller volumes of water with more replicates could offset the problem of sampling ponds and reservoirs without much additional effort (Goldberg, Strickler, & Pilliod, 2015; Rees et al., 2014).

In summary, routine monitoring with the assay developed here can provide valuable data regarding the expansion and dispersal of invasive *P. parva* populations and, with further optimization and calibration, might also be used to obtain relative estimates of species abundance (Lacoursière-Roussel et al., 2016; Sassoubre, Yamahara, Gardner, Block, & Boehm, 2016). Thus, the application of qPCR-based eDNA analyses can be used to monitor eradication programmes and help inform risk management strategies for *P. parva* and other AIS under the Water Framework Directive.

ACKNOWLEDGMENTS

We would like to thank Gereint Mortimer (Afan Valley Angling Club) and Chris Lloyd for providing fish tissue samples for qPCR optimization, Teja Muha for assistance in processing of DNA samples, Dr.

Peter Jones for time and effort trapping topmouth gudgeon, and Emma Keenan (Natural Resources Wales) for assisting with eDNA sample collection and providing information on sampling sites. This research was funded by the Welsh Government and Higher Education Funding Council for Wales (HEFCW) through the Sêr Cymru National Research Network for Low Carbon Energy and Environment (NRN-LCEE; AQUAWALES cluster) and the EU Horizon 2020 AMBER project (www.amber.international) under EC grant agreement No 689682.

CONFLICT OF INTEREST

Authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

SC & CVR designed the study; CVR performed the analyses with advice from SC; CGL performed statistical analyses; MR carried out the field work and contributed to the writing; CVR, SC and CGL wrote the paper.

ORCID

Chloe Victoria Robinson https://orcid.org/0000-0003-3776-9166

Sofia Consuegra https://orcid.org/0000-0003-4403-2509

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Robinson CV, Garcia de Leaniz C, Rolla M, Consuegra S. Monitoring the eradication of the highly invasive topmouth gudgeon (*Pseudorasbora parva*) using a novel eDNA assay. *Environmental DNA*. 2019;1:74–85. https://doi.org/10.1002/edn3.12