eDNA-based monitoring: Advancement in management and conservation of critically endangered killifish species

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Abstract
Ecosystems are currently changing at unprecedented rates due to anthropogenic influences. Application of appropriate management regimes and mitigation measures requires knowledge of ecological community composition and monitoring of any changes that occur. Environmental DNA-based monitoring is becoming increasingly common and offers substantial potential as a noninvasive method associated with highly repeatable and reliable results. In this study, we monitored river systems in Western Greece that have been strongly impacted by anthropogenic activities and the spread of an alien invasive fish species, the Eastern mosquitofish (Gambusia holbrooki). This invasive species has been credited as the major cause for the drastic decline of two endemic killifish species (Valencia letourneuxi and Valencia robertae). Here, we investigated the efficacy of an environmental DNA (eDNA-based) method of detection for all three species, as an alternative to conventional monitoring methods. Initially, a mesocosm experiment provided material for the design and validation of the sampling protocol. This was followed by two sampling periods in the field conducted in autumn 2017 and 2018, comparing the novel eDNA assays with the conventional surveying methods in six and 20 systems, respectively. eDNA detection consistently outperformed the traditional monitoring methods for both V. letourneuxi and V. robertae and was comparable for the invasive G. holbrooki. This supports the now increasing body of literature, highlighting the benefits of species-specific, targeted eDNA assays for the assessment of threatened and/or invasive species, one which can be utilized by conservation organizations and government bodies alike. However, we note that care should always be taken when designing such tools and strict validation steps should be adhered to, particularly with respect to minimizing the probability of false positives and negatives.

KEYWORDS
conservation, eDNA detection, endangered species, freshwater systems
1 | INTRODUCTION

Declining biodiversity, driven primarily by increasing anthropogenic activities such as habitat degradation and/or overexploitation, coupled with the effects of climate change, is significantly altering nearly every ecosystem on our planet (Cardinale et al., 2012; Harley, 2011; Hooper et al., 2012). The extent of the loss in species is now so high, that many describe this as a sixth mass extinction event (Ceballos et al., 2015). These losses are leading to negative impacts on ecosystem functioning, threatening the services they provide (Brooks et al., 2006; Ceballos et al., 2015). Therefore, there is an urgent need to reduce the rate of species loss through effective conservation management and regulating controls on land and water usage/management (Butchart et al., 2010; Hooper et al., 2012).

Wetlands and freshwater habitats, in particular, are now among the most endangered habitats in the world (Davidson, 2014; Reid et al., 2019). As well as impacts of climate change, these habitats are exposed to a number of other substantive threats, including anthropogenic development, pollution, impact caused by species introduction/biological invasions, and more general causes of habitat degradation (Reid et al., 2019). That said, there are a number of well-documented examples where effective habitat restoration has been undertaken in such ecosystems which involve relatively little effort and cost (Dolédec, Forcellini, Olivier, & Roset, 2015; Ng et al., 2011; Lyon et al., 2019; Morandí, Piégay, Lamouroux, & Vaudor, 2014; Palmer et al., 2005; Wohl et al., 2005). This gives hope to the protection and restoration of such important habitats and the species which reside within them. However, effective habitat restoration or habitat management requires knowledge of the current state of an ecosystem, as well as monitoring over time, in which the documentation of species presence is particularly important (Rosenberg, Bigford, & Leathery, 2000). Further, the early detection of invasive species would be beneficial in such instances in order to implement management plans quickly and thereby minimize any effect of such species (Vander Zanden, Hansen, Higgins, & Kornis, 2010; Xia et al., 2017). That said, it is often problematic to assess rare and invasive species, as in many instances they are in low abundances, which make the majority of traditional methods of surveying freshwater habitats (such as netting and electrofishing) ineffective as they require substantial amounts of time, effort, and expertise to yield useful results (Eiller, Löfgren, Hjerne, Nordén, & Saetre, 2018). These requirements lead to constraints on the number and extent of surveys that can be undertaken.

Environmental DNA (eDNA) detection is now widely utilized as an alternative tool for monitoring a number of species (Sepulveda et al., 2019; Thomas et al., 2019; Vilaça et al., 2020; Wacker et al., 2019). As eDNA is noninvasive in nature (relying on the detection of DNA traces left by living or dead organisms in their environment), species which are present even in low abundances can be efficiently detected with no impact on the sampled habitat or co-occurring species (Thomsen & Williams, 2015). Indeed, eDNA-based methods are already, for example, commercially available for the detection of endangered species in the United Kingdom (Harper et al., 2018). Utilization of eDNA-based detection methods can also allow for larger-scale surveys to be undertaken, with comparatively lower effort employed, compared to traditional methods (Yatsuyanagi et al., 2019).

In this study, we assess the use of environmental DNA detection as a rapid and effective tool for monitoring the occurrence of two threatened freshwater killifish species: Valencia letourneuxi (Sauvage, 1880), Valencia robertae (Freyhof, Kärst, & Geiger, 2014), and of the alien invasive Gambusia holbrooki (Girard, 1859) in Greek aquatic systems. Once widely distributed in Western Greece (Barbieri, Daoulas, Psarras, Stoumboudi, & Economou, 2000), the distribution ranges of the two native species have now been drastically reduced over the last 40 years. This decline has been linked to anthropogenic habitat modification and competition from the non-native Eastern mosquitofish G. holbrooki (Kalogianni et al., 2010, 2019). G. holbrooki is originated from the United States and Mexico and is now widespread throughout much of Southern Europe where it was introduced in the early 1920s (Piria et al., 2018; Ribeiro & Leunda, 2012). G. holbrooki was initially introduced to control mosquito populations through predation of the larvae and is now the most widespread alien freshwater fish species in Greece (Economou et al., 2007; García-Berthou et al., 2005). Although successful to a degree in its aim, it spread rapidly and was uncontrollable due to its early maturation, viviparity, and high reproductive rates. The species was much more adaptable than originally thought and exhibited high behavioral plasticity further ensuring its success even in degraded habitats (Vargas & de Sostoa, 1996). Due to the spread of this invasive species and the other anthropogenic stressors mentioned above, V. letourneuxi has been listed among the “world’s 100 most threatened species” (Baillie & Butler, 2012; Freyhof et al., 2014). The range of both Valencia species is thought to now be restricted to only 12 systems, and these populations are thought to be vulnerable. Indeed, they have already gone extinct from two sites where they were historically found (Kalogianni et al., 2010; unpublished data). Due to the threatened status of these species, traditional methods for assessing their presence and absence (electrofishing and netting for example) are far from ideal due to their often destructive nature (Kalogianni et al., 2010; Vogiatzis et al., 2014). As both species are strictly protected by the Bern Convention (Appendix II) and by Presidential Decree (No. 67/1981 of the Greek state—Barbieri, Stoumboudi, & Economou, 2002; Freyhof et al., 2014; Kalogianni et al., 2010), finding a less invasive survey method is urgently needed to ensure effective management and mitigation of these endangered species.

Our study therefore aimed to investigate the use of eDNA detection as a reliable alternative tool for monitoring the two Valencia species and G. holbrooki across Western Greece. Species-specific assays were developed and validated, and a controlled mesocosm experiment allowed us to optimize our sampling protocol. Finally, two independent field surveys (combining both eDNA detection and conventional fish surveying methods) were conducted to assess the reliability of these methods illustrating a proof of concept for this method to be utilized in future conservation programs.
2 | METHODS

2.1 | Assay development

Species-specific primers and probes targeting the cytochrome C oxidase subunit 1 gene (COI) of Valencia letourneuxi, Valencia robertae, and Gambusia holbrooki were designed using our own sequences generated from specimens collected by the authors and existing sequences already deposited on GenBank. The primers and probe were developed using the Geneious Pro R10 Software (https://www.geneious.com; Appendix S1; Kearse et al., 2012). We sequenced our own samples in order to be sure of the existing NCBI data (See Appendix S1B for more details). Incidentally, our sequences were identical (100% match) to those already in the NCBI database (Appendix S1.A). For more exact details on the assay development, we refer readers to Appendix S1.B. Once designed, the specificity of the assays was tested in silico against DNA sequences retrieved from the NCBI database (National Centre for Biotechnology Information; https://www.ncbi.nlm.nih.gov/) from 23 fish species known to be and/or likely to be present in the same ecosystems with the targeted organisms (Appendix S1.A). The size of the fragments amplified was 113 bp in length for *V. letourneuxi*, 137 bp for *V. robertae*, and 167 bp for *G. holbrooki* (see Table 1). After in silico validation, the specificity of each assay was tested in vitro with PCR and qPCR using DNA extracted from the following co-occurring species: Anguilla anguilla (Linnaeus, 1758), Economidithys pygmaeus (Holly, 1929), G. holbrooki, Gasterosteus aculeatus (Linnaeus, 1758), Pelasgus thesproticus (Stephanidis, 1939), Pelasgus stymphalicus (Valenciennes, 1844), Squalius peloponensis (Valenciennes, 1844), V. letourneuxi, and V. robertae. DNA was extracted from tissue samples of these species using the Qiagen DNeasy® Blood and Tissue Kit following the manufacturer’s instructions.

2.2 | EDNA extraction

eDNA was extracted from the filters with the Qiagen DNeasy® Blood and Tissue Kit, following the extraction workflow for Sterivex filters outlined in Spens et al. (2017). Extraction of eDNA samples was performed in a separate clean PCR-free room (different than that used for extraction of the tissue samples identified above). This is in order to reduce potential cross-contamination between the samples. Two different types of “negative controls” were also utilized throughout this study. The 1st consisted of a field control whereby two independent 1 L samples of tap water were filtrated through a Sterivex filter in the field at the time of eDNA sampling. The 2nd consisted in two extraction controls, where water was substituted instead of the samples. All laboratory equipment was regularly disinfected and decontaminated under UV radiation throughout the whole analysis process. All other laboratory surfaces were disinfected using 10% bleach and ethanol prior to analysis.

2.3 | PCR and QPCR

Primers specificity was assessed using PCR before conducting qPCR. PCR amplifications were performed, each with two technical replicates on a Gen Amp PCR System 9,700 (Applied Biosystem) with each set of the three species-specific primers developed in this study (Table 1). PCR protocols and conditions were the same for the three targeted species. In brief; PCR reactions were conducted in a 25 µl reaction with 12.5 µl of PCRBIOSYSTEM Ultra Mix Red (PCRBIOSYSTEMS), 1 µl of each primer (10 µM), 9.5 µl of ddH₂O, and 1 µl of DNA template. Optimal PCR conditions were as follows: thermal cycling at 50°C for two min and 95°C for 10 min, followed by 35 cycles at 95°C for 15s and 62°C for 1 min. At least one positive (DNA extracted from tissue samples, *V. letourneuxi* (21 ng/µl), *V. robertae* (9.2 ng/µl), and *G. holbrooki* (9.7 ng/µl)) and one negative (no template) control were included for each PCR. PCR amplification was confirmed by electrophoresis on 2% agarose gel stained with 3 µl of GelRed™ Nucleic Acid Gel Stain, Biotium. Product sizes from amplified DNA were checked with visual comparison with PCRBio Ladder IV (PCRBIOSYSTEMS).

qPCR reactions were performed on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems) with the assays designed in this study (Table 1). The specificity of each assay was further confirmed by qPCR using two replicates of DNA extracted from the species mentioned above. qPCR protocols and conditions were the same across all three targeted species. These consisted of a 25 µl final volume, using 12.5 µl of PrecisionPlus qPCR Master Mix with ROX

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**TABLE 1** Primers and probes designed in this study for the detection of eDNA traces from *V. letourneuxi, V. robertae,* and *G. holbrooki* in freshwater systems

<table>
<thead>
<tr>
<th>Species</th>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Valencia letourneuxi</em></td>
<td><strong>Forward</strong></td>
<td>TGGGGGTTGTGGGCAACTGAC</td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>GAGAGGAAGAAAGACCGAGGGGGG</td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>CATAGCTCTCCCGGGATGAAAC</td>
<td>COI</td>
</tr>
<tr>
<td><em>Valencia robertae</em></td>
<td><strong>Forward</strong></td>
<td>ATGGGCATTCCTCCCGGAA</td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>GCTAAAGTTTTCCGCCAGGGG</td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>CTCCCTCGGGGTGTTGAGG</td>
<td>COI</td>
</tr>
<tr>
<td><em>Gambusia holbrooki</em></td>
<td><strong>Forward</strong></td>
<td>GTGCCCCAGACATAGCCCTT</td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>TACAGAAGGCTCCGGCATGTG</td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><strong>Probe</strong></td>
<td>AAGATGCGAGGAGGGAGG</td>
<td>COI</td>
</tr>
</tbody>
</table>
(Primer Design), 1 μl of each primer (10 μM), 1 μl of probe (2.5 μM), 6.5 μl of ddH2O, and 3 μl of extracted DNA. Optimal qPCR conditions were as follow: thermal cycling at 50°C for 2 min and 95°C for 10 min followed by 50 cycles of 95°C for 15s and 62°C for 1 min.

2.4 | QPCR analysis

Standard curves were established by analyzing a 1:10 dilution series of DNA extracted from tissue samples of *V. letourneuxi* (2.1 ng/μl, NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific), *V. robertae* (9.2 ng/μl), and *G. holbrooki* (9.7 ng/μl) following the MIQE Guidelines (Bustin et al., 2009; Mauvisseau, Burian, et al., 2019) (Appendices S2–S4). For the three species, the dilution series ranged from $10^{-1}$ to $10^{-8}$ using 10 “technical replicates” (i.e., qPCR replicates) for each dilution step, allowing for the assessment of the limit of detection (LOD) and limit of quantification (LOQ) (Figure 1) (Bustin et al., 2009; Mauvisseau, Burian, et al., 2019; Tréguier et al., 2014). We identified the LOD as the last dilution of the standard curve in which the targeted DNA is amplified with a cycle threshold (Ct) below 45 (Mauvisseau, Burian, et al., 2019; Mauvisseau, Davy-Bowker, et al., 2019). The LOQ was identified as the last dilution of the standard curve where the targeted DNA is amplified and quantified in at least 90% of the qPCR replicates with a cycle threshold below 45 (Mauvisseau, Burian, et al., 2019; Mauvisseau, Davy-Bowker, et al., 2019). When validating the specificity of each assay (with qPCR), at least two positive (tissue samples of the respective species) and two negative controls (filtered sterile water) were included. All eDNA samples were analyzed using six technical replicates. Each run analyzing eDNA samples also contained two replicates of each six dilution points ranging from $10^{-1}$ to $10^{-5}$ (the same as used for establishing the standard curves) as positive control and six negative controls (no template).

2.5 | Ex situ testing of the *V. robertae* EDNA assay

At the Zoological Society of London (ZSL), UK (https://www.zsl.org/), 128 *V. robertae* specimens were housed over three different mesocosms (“A,” “B,” and “C”) as a part of a conservation and breeding program. We used this opportunity for testing our sampling protocols and assessed the reliability of the developed assay (Appendix S5). Mesocosm “A” housed 40 juveniles in a 500 L aquarium (equating to a fish biomass of 10 g), “B” housed a mix of 12 adults and 10 juveniles in 626 L (biomass of 19.8 g), and “C” housed 66 adults in 723 L (biomass of 101.5 g) (Appendix S5). As part of the breeding program, eggs were collected on a daily basis and stored in mesocosm “B” for development and hatching. We recognize that

**FIGURE 1** All standard curves were established by analysing a 1:10 dilution series of DNA extracted from *V. letourneuxi* (2.12 ng/μL) (a), *V. robertae* (9.2 ng/μl) (b), and *G. holbrooki* (9.7 ng/μl) (c). All standard curves ranged from $10^{-1}$ to $10^{-9}$ with 10 technical replicates used for each dilution steps in order to assess the limit of detection (LOD) and limit of quantification (LOQ). The cycle threshold (i.e., Ct) represents the number of qPCR amplification cycles required for a positive amplification of each targeted DNA fragment.
We did not sample for fish at two of the sites ("2A" and "5A"), since these were the “recipient sites” of a then ongoing enhancement/translocation program (undertaken annually during 2015–2017), and we did not want to disturb populations there. Within the frame of this conservation program, V. letourneuxi individuals from "4A" and V. robertae individuals from "6A" were collected during this enhancement/translocation program with a variety of methods (seine net, D net and small nets) and transferred, bottled with water and oxygen, to the "recipient sites", that is, V. letourneuxi individuals to "2A," in order to establish a new population in this spring-fed stream, and V. robertae individuals to "5A" with the intention of enhancing the local population there (Table 2 and Appendix S6). All field equipment was disinfected with a chlorine solution between locations.

### 2.6 | In situ trial 1 (A) for V. robertae and V. letourneuxi

This component tested the newly designed assays for detecting eDNA from both V. letourneuxi and V. robertae in situ. Eight samples from six sites spanning six distinct aquatic systems (stream, wetland, or canal) were sampled over two days (from September 26, 2017, to September 27, 2017, Table 2, see also Appendix S6). These six aquatic systems are distributed in the two distinct geographical areas where V. letourneuxi and V. robertae are present (Appendix S6, Figure S1). More specifically, based on previous biodiversity assessment (Table 2), the presence of V. letourneuxi was expected at locations “1A” to “4A” and the presence of V. robertae was expected at locations “5A” and “6A.” However, all locations were analyzed with both Valencia’s assays, as a complementary step assessing their reliability. At sites “1A”-“4A,” a single sample, up to 1 L of water (depending on the turbidity), was taken from the water surface in the same manner as that detailed above. Two samples, spaced at least 20 m apart, were collected from sites “5A” and “6A” due to site characteristics. The samples were then mixed and filtered through a sterile 0.45 µm Sterivex™ HV filter (see results from ex situ methods). The filters were then immediately frozen at −20°C. To avoid contamination, disposable nitrile gloves were used during each step of the sampling.

After eDNA sampling, conventional methods of fish sampling were also applied at “1A,” “3A,” “4A,” and “6A” in order to assess for the presence or absence of V. letourneuxi and V. robertae. At three sites (“1A,” “3A,” and “4A”), sampling was conducted with a D-shaped frame net with a 1.5-m wooden handle (in conjunction with smaller dip nets). At “6A” (which was the only site that was wadable), we used a seine net (5 trials) in conjunction with smaller dip nets. Due to the variability of habitats and fishing method used, we present relative abundance (% contribution) data for purposes of comparison (See Appendix S6).

The second component of our study encompassed all three target species and was mapped against a more invasive conventional survey method (sampling using electrofishing or netting). Here, our first trial run (in situ trial 1 A) was expanded to a proof of concept for conservation practices, whereby twenty aquatic systems were surveyed using both our newly designed eDNA assays and the more traditional fishing methods (i.e., electrofishing or netting). This was conducted over a two-week period (from October 16, 2018, to October 28, 2018). It is important to note that the identifier codes used in the two different sampling events do not correspond to the same sampling site (i.e., sites “1A” and “1B” are two different locations).

All sites were sampled at one location, with eDNA sampling being performed before any fishing action. In trial 2 B, we collected two independent water samples up to 1 L. Variation in the volume of water filtered was dependant on the turbidity. Hereafter, we refer to these samples as natural replicates. They were collected from the surface and filtered through a sterile 0.45-µm Sterivex™ HV filter. The eDNA samples were then fixed and stored as described above.

The majority of the sites were sampled using electrofishing, with the exception of four sites, that were sampled using a D-shaped frame net (Table 3, Figure 3, see also Appendix S7, Table S2). This was due to high water salinity at these sites.

When electrofishing was undertaken at depths < 1.5 m (wadable sites), a single 100 m electrofishing pass was conducted from downstream to upstream. At sites that were > 1.5 m in depth (nonwadable sites), electrofishing was undertaken from the bank. Electrofishing was performed using a Honda GX160 3KW generator (150 m cable, 1.5 m anode pole, 5-10A DC output, voltage range 300–600 V). At the four brackish sites (“7B,” “8B,” “11B,” and “13B,” Appendix S7, Table S2), sampling was conducted with a D-shaped net (minimum 8 sweeps and maximum 22 sweeps). Due to the variability of habitats and fishing method used, we present relative abundance (% contribution) data for purposes of comparison, as well as information on historical presence of the target species (Appendix S7, Table S3).

We targeted a number of sites due to previous knowledge of the species’ historical home ranges. Specifically, V. letourneuxi presence...
TABLE 2  Table depicting the sites sampled in 2017, the fish species targeted at each location, their suspected presence (based on past observation and more recent fish surveys), the fish sampling results (if the targeted fish were captured or not during this survey), eDNA detection results including the number of positive qPCR replicates, pH, water temperature, the number of locations sampled in each aquatic system, the volume filtered at each location, and the sampling date.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Species targeted</th>
<th>Suspected presence</th>
<th>Fish sampled</th>
<th>eDNA detection</th>
<th>pH</th>
<th>TC°</th>
<th>Locations sampled</th>
<th>Volume (mL)</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>V. letourneuxi</td>
<td>Yes(^a)</td>
<td>No</td>
<td>0/6</td>
<td>6.6</td>
<td>16.0</td>
<td>1</td>
<td>1,000</td>
<td>27/09/2017</td>
</tr>
<tr>
<td>2A</td>
<td>V. letourneuxi</td>
<td>Yes(^b)</td>
<td>NA</td>
<td>0/6</td>
<td>6.9</td>
<td>15.0</td>
<td>1</td>
<td>1,000</td>
<td>27/09/2017</td>
</tr>
<tr>
<td>3A</td>
<td>V. letourneuxi</td>
<td>Yes(^c)</td>
<td>No</td>
<td>0/6</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1,000</td>
<td>27/09/2017</td>
</tr>
<tr>
<td>4A</td>
<td>V. letourneuxi</td>
<td>Yes(^d)</td>
<td>Yes</td>
<td>1/6</td>
<td>6.8</td>
<td>17.9</td>
<td>1</td>
<td>300</td>
<td>27/09/2017</td>
</tr>
<tr>
<td>5A</td>
<td>V. robertae</td>
<td>Yes(^e)</td>
<td>NA</td>
<td>12/12</td>
<td>6.6</td>
<td>17.7</td>
<td>2</td>
<td>1,000</td>
<td>26/09/2017</td>
</tr>
<tr>
<td>6A</td>
<td>V. robertae</td>
<td>Yes(^f)</td>
<td>Yes</td>
<td>9/12</td>
<td>6.6</td>
<td>18.8</td>
<td>2</td>
<td>1,000</td>
<td>26/09/2017</td>
</tr>
</tbody>
</table>

Note: Fish sampling was not performed in sites “2A” and “5A,” as these systems were part of an ongoing enhancement/reintroduction conservation program (see NA: not performed). Only 300 ml of water was filtered at site “4A” due to high water turbidity.

\(^a\)Last confirmed record 2013.
\(^b\)Translocation action undertaken in 2015 and 2016.
\(^c\)Last confirmed record 2007.
\(^d\)Last confirmed record 2016.
\(^e\)Last confirmed record in 2015.
\(^f\)Last confirmed record in 2016 (based on unpublished HCMR data).

TABLE 3  Table depicting the sampled locations in 2018, the fishing results (i.e., whether the targeted fish were found during fish sampling conducted in 2018, see also Appendix S7), eDNA detection results including the number of positive qPCR replicates for each targeted species, pH and water temperature, the volume of water sampled for each natural replicate, and the sampling date.

<table>
<thead>
<tr>
<th>Sites</th>
<th>V. letourneuxi</th>
<th>V. robertae</th>
<th>G. holbrooki</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fishing</td>
<td>eDNA</td>
<td>Fishing</td>
</tr>
<tr>
<td>1B</td>
<td>Yes</td>
<td>0/12</td>
<td>/</td>
</tr>
<tr>
<td>2B</td>
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<td>0/12</td>
<td>/</td>
</tr>
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<td>0/12</td>
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<td>0/12</td>
<td>/</td>
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<td>3/12</td>
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<td>9B</td>
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<tr>
<td>18B</td>
<td>/</td>
<td>0/12</td>
<td>No</td>
</tr>
<tr>
<td>19B</td>
<td>/</td>
<td>0/12</td>
<td>Yes</td>
</tr>
<tr>
<td>20B</td>
<td>/</td>
<td>0/12</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Note: Due to high turbidity and pollution at the sampling location “4B,” only 750 ml was filtered for both natural replicates and no fishing was performed (see “NP”: not performed; see “/”: The site was outside of the known geographical area of the targeted fish). Sites “1B” to “12B” are within the geographical range or V. letourneuxi, while sites “14B” to “20B” are in the geographical range of V. robertae, with the exception of “17B.” Site “13B” is located at the junction of the ranges of the two Valencia species. Due to the detection of both Valencia species at site “13B,” the neighboring locations (i.e., “11B” and “14B”) were also investigated for both Valencia species using eDNA detection.
was expected and therefore assessed against 12 sites (“1B” to “12B”) and V. robertae presence was expected and assessed at seven sites (“13B” to “16B” and “18B” to “20B”). “17B” fell outside the known range of both species and was therefore used as a negative control (Table 3, Figure 3, Appendix S7). Sites, “11B,” “13B,” and “14B,” were at the border of the ranges of the two Valencia species, and therefore, we assessed for the presence of both V. letourneuxi and V. robertae. Further, G. holbrooki presence was assessed across all 20 sites (“1B” to “20B,” Table 3, Figure 3, Appendix S7). It should be noted that we expected G. holbrooki to be absent at five of the sampled locations (see Appendix S7 for more detail into why). We were unsure of the presence or absence of G. holbrooki at a further site, due to the absence of historical data (“2B”; Appendix S7).

Finally, at each sampling location, physicochemical water quality parameters, that is, water temperature (°C), salinity (ppt), and pH, were measured using a Portable multiparameter Aquaprobe AP-200 with a GPS Aquameter (Aquaread AP 2000) (Table 3, Appendix S7, Table S2). All field equipment was disinfected with a chlorine solution between locations.

2.8 Statistical analysis

In order to assess the effect of the filters pore size on the cycle threshold (Ct) for the detection of V. robertae in the mesocosm experiment, a one-way ANOVA analysis was performed. For each of our natural replicates, we ran six technical replicates. Therefore, where we only took one natural replicate, we have six technical replicates, and while where we have two natural replicates, we have twelve technical replicates (Table 2 and Table 3). Statistical analyses were performed with R, version 3.4.1 (R Core Team, 2018).

3 RESULTS

All assays designed in this study were species-specific to the intended targeted fish species using both PCR and qPCR. The standard curves gave a LOD for Valencia letourneuxi, at 0.02 pg/μl of 36.30 ± 0.82 Ct (8/10 qPCR replicates), and the LOQ was indicated at 0.2 pg/μl at 34.10 ± 0.88 Ct (10/10 qPCR replicates) (Slope = −3.392, Y-inter = 18.288, R² = .985, Eff = 97.137) (Figure 1a).

For Valencia robertae, the LOD was 0.92 pg/μl at 39.26 ± 0.20 Ct (2/10 qPCR replicates) and the LOQ was 9.2 pg/μl at 37.10 ± 0.67 Ct (10/10 qPCR replicates) (Slope = −3.243, Y-inter = 30.234, R² = .841, Eff = 103.416) (Figure 1b). For Gambusia holbrooki, the LOD was 0.97 pg/μl at 41.96 ± 2.65 Ct (5/10 qPCR replicates) and the LOQ was 9.7 pg/μl at 36.71 ± 0.74 Ct (10/10 qPCR replicates) (Slope = −3.959, Y-inter = 24.922, R² = .951, Eff = 78.889) (Figure 1c). All negative controls showed no amplification for any species throughout the experiment.

In the mesocosm experiment, all natural and technical replicates showed a positive amplification of V. robertae regardless of fish abundance and biomass. There was no significant difference between the Ct values acquired from either the 0.22-μm or 0.45-μm filters (ANOVA, df = 1, F-value = 0.138, p = .711) nor between the Ct values acquired with the different fish biomass (ANOVA, df = 1, F-value = 0.793, p = .374). However, it is worthy of note that system “B” (which housed the eggs) had the lowest mean Ct value (24.14 ± 0.54). This is compared to system “A” (housing low biomass and a medium number of fish [29.98 ± 0.57]) and system “C” (the highest biomass and highest number of fish [28.34 ± 0.32]).

In the first of our in situ trials (conducted in six aquatic systems in Autumn 2017), we were able to confirm the reliability of the designed assays for the two killifish species (Table 2, Appendix S6, Table S1). All eDNA samples collected during this trial were tested with both killifish assays as a complimentary step for assessing the specificity and reliability of our method. V. letourneuxi was detected using both eDNA and fish sampling in one site (“4A”). This matched historical survey data (see legend of Table 2, Appendix S6, Table S1). At two sites (“1A” and “3A”), we were unable to detect V. letourneuxi with either eDNA or fish sampling. This was contrary to historical presence but matched more recent data (Table 2, Appendix S6, Table S1). Finally, there was no eDNA signal at a site where a translocation action had been previously undertaken (“2A”). V. robertae was detected using both eDNA and fish sampling at one site (“6A”). We detected the presence of V. robertae at one further site (“5A”) only with eDNA. This site is a very small wetland where the fish had been detected in the past (Appendix S6, Table S1). There was no fish
sampling performed at site “5A” due to an enhancement program being undertaken a year before this sampling event. *V. robertae* was not detected at any of the sites where *V. letourneuxi* was found to be present.

In the second in situ trial (October 2018), we were able to detect *V. letourneuxi* at two sites using fish sampling and six with eDNA (only site 10B was positive using both methods, Table 3, Figure 3a, see also Appendix S7, Table S2 and Table S3). For
V. robertae, we detected its presence at four sites using fish sampling and six with eDNA. Here, three of the seven sites where the fish were detected were positive for both methods (Table 3, Figure 3b). The non-native and invasive species G. holbrooki was detected at fifteen sites; twelve using fish sampling; and twelve with eDNA, and nine of these were positive across both methodologies (Table 3, Figure 3a,b).

4 | DISCUSSION

In this study, we developed three species-specific assays which were laboratory validated and then tested in the field. We targeted two threatened killifish and one non-native invasive species, known to be spreading across Europe at a rapid rate (Freyhof et al., 2014; Grapputo, Bisazza, & Pilastro, 2006). After an in vitro validation step, following the protocol outlined in Appendix S7 (Tréguier et al., 2014), all assays were shown to be species-specific and gave high reliability in both in situ and ex situ trials. Interestingly, a few recent studies have indicated that the fragment size that eDNA-based assays target can influence detection rates (Byelemans, Furlan, Gleeson, Hardy, & Duncan 2018). Although the target fragments of our assays ranged between 113 and 167 bp, we saw no such variation in the reliability of the assays, all performing equally well and accurately. However, it is important to note that the sensitivity of the assays varied substantially. The standard curves of all three indicated a similar limit of detection, all be it relatively high compared to some other species-specific assays such as those reported by Klymus et al. (2019). There were slight differences with regard to the limit of quantification, which was lower for V. robertae for example. Further, the assays for V. letourneuxi and G. holbrooki had an $R^2 > .95$, while V. robertae $R^2 < .095$. It should also be noted that the efficiency of the assay targeting G. holbrooki fell outside the standard range ($80 < \text{Efficiency} < 120$). These LOD, $R^2$, and efficiency values therefore indicate that there is a level of risk of false negatives occurring with these assays. Such a result which is also common with physical monitoring methods can and often does mean management decisions designed on such data is not 100% perfect, notably, as failure to detect the organism in question is not proof of absence. However, that said, the noninvasive nature of eDNA sampling and the comparable results in the field between our assays and more traditional survey methods suggest eDNA is still a sufficient tool for the detection of these species. It should also be noted that although all assays were shown to be effective in both the laboratory and in the field, we were only able to complete optimization for V. letourneuxi and those targeting V. robertae and G. holbrooki would benefit from further testing.

As mentioned above, the assay validation (within the laboratory) was extensive one of our targeted species, V. robertae. This was due to a close collaboration with a current conservation program led jointly by the Zoological Society of London (ZSL) and the Hellenic Centre for Marine Research (HCMR). For this species, we utilized controlled ex situ mesocosms and assessed the effect eDNA sampling had on the reliability of the results. Surprisingly, there was no variation in the detection rate when sampling with either of the two filter sizes tested. Meaning that (at least in this instance), either filter could be utilized without compromising the assays effectiveness. However, we opted for the larger pore size of the two as, in the field, filters can get clogged by sediment, affecting the amount of water that can be filtered (Goldberg et al., 2016). It is well known that filtering larger volumes of water is optimum (for any type of eDNA sampling applied) and improves detection probability (Hunter, Ferrante, Meigs-Friend, & Ulmer, 2019; Mächler, Deiner, Spahn, & Altermatt, 2015; Sepulveda et al., 2019). In this same system, we were also able to test the effect of biomass on the eDNA assay. However, there was no indication of biomass or fish abundance affecting the detection rate for V. robertae. This contrasts to some studies which have indicated that quantification of eDNA (especially with regard to fish) may be used to estimate biomass (Evans et al., 2016; Mauvisseau, Burian, et al., 2019). This could be due to the experimental set up utilized in our controlled experiment (i.e., various fish sizes, stage of life). Interestingly, the mesocosm, which additionally held the eggs prior to hatching, did result in higher Ct values of eDNA amplification compared to the two other mesocosms. This suggests that the eggs may have increased the amount of eDNA in the system; alternatively, the result may also be explained by an increase in the sloughing rates of the fishes tissue and/or mucus directly (Klymus, Richter, Chapman, & Paukert, 2015). Regardless of the reason, this result implies that the best sampling time in the field for eDNA for this species is likely to be during the spawning period. Finally, the lack of detection of V. letourneuxi (with both eDNA and traditional sampling) at sites “1A” and “3A” during the first trial is not surprising, as both sites have been highly degraded in recent years and no records of the species have been noted since 2013 and 2007 respectively (HCMR unpublished data).

Regarding the conservation actions currently being undertaken, our results from the first field trial (in 2017) indicate a positive eDNA signal for the site “5A,” where an enhancement action was undertaken for V. robertae in 2015–2016. This maps with a last confirmed presence as late as 2015 at this pristine habitat. However, there was unfortunately no positive signal at site “2A,” where V. letourneuxi were translocated in 2015–2016. It was hoped that the utilization of a more sensitive technique (compared to the traditional more invasive fish sampling methods previously used) would have indicated the species presence, even if it was at very low abundances (Mächler, Deiner, Steinmann, & Altermatt, 2014; Sigsgaard, Carl, Møller, & Thomsen, 2015; Thomsen et al., 2011). However, as this was not the case, the translocation may not have been successful. That said, it should be noted that during this first field trial, we only took one natural replicate for our eDNA analysis and more natural replicates would have been preferable (Erickson et al., 2019) and where possible the greatest number of replicates which can be affordably analyzed should always be taken.
In our second in situ validation trial, two natural replicates were taken at each of the sites (conducted in 2018), effectively doubling the confidence in our results. During this survey, we again demonstrated that the eDNA approach outperformed conventional survey techniques (Table 3). Using our novel assays, we were able to highlight six sites where *V. letourneuxi* were found and six sites for *V. robertae*. These locations fell within the historical range of the targeted species but had steep banks, abundant vegetation cover, and/or deep waters, making traditional surveying difficult (Kalogianni et al., 2010, 2019). Therefore, eDNA-based methods offer a non-invasive and safer alternative for monitoring these species, especially at locations which present difficulties of applying more traditional sampling methodologies. Interestingly, there were two locations, one for *V. letourneuxi* and one for *V. robertae* (sites “1B” and “14B”), where we obtained a negative signal in the eDNA sample but found fish via traditional method sampling. These two confirmed “false negatives” were occurring for our eDNA assay, thereby indicating a level of limitation with the newly developed assays. This could be down to possible insufficient sensitivity associated with the assays (as stated previously) which has been linked to the LOD, the R² value, and the efficiency value—which all indicate that false positives were indeed likely. However, such discrepancies could also be explained by a number of other factors, such as (a) hydrology, (b) the low abundance of the target species, (c) inhibition, and/or (d) issues with the sampling of the eDNA sample, such as insufficient number of natural replicates or insufficient volume of water collected. More specifically, site “14B” is a large riverine habitat with high discharge, and therefore, eDNA is likely to be considerably diluted and may be rapidly removed from the site of origin. Site “1B,” in contrast, is a much smaller system but with abundant vegetation cover, and both inhibition and/or insufficient number of natural replicates, combined with an insufficient volume of water collected, could explain this false-negative result. Reducing the chance of false negatives should be a priority in an eDNA-based survey, and therefore, increasing the number of natural replicates, as well as increasing the filtering capacity should be considered, in combination with the utilization of an internal positive control aimed at assessing levels of inhibition (Goldberg et al., 2016; Mauvisseau, Burian, et al., 2019; Mauvisseau, Davy-Bowker, et al., 2019; Sepulveda et al., 2019).

Interestingly, our third assay (targeting the non-native and highly invasive Eastern mosquitofish - *G. holbrooki*) showed no variation between eDNA sampling and traditional fish sampling. *G. holbrooki* is known to be present in considerably higher densities than the two critically endangered Valencia species (Kalogianni et al., 2010). Such high densities may therefore be playing a yet undetermined role on the reliability or sensitivity of this assay. Alternatively, the result may have been driven more from variation with regard to the accessibility of the sites, inhibition, and/or the number of natural replicates taken at any given time. Future studies should therefore focus on these aspects in order to more fully understand the complex interaction between biotic and abiotic factors affecting the sensitivity of these targeted assays and their performance against the more traditional survey methods. That said, our mesocosm study (with *V. robertae*) showed no apparent effect of biomass on the reliability of detection. This contradiction in results may be explained by the very different characteristics of the two studied systems (i.e., small closed artificial system and larger open natural and dynamic systems with high levels of water flow).

There were also some instances of false negatives throughout the study. Regardless of the cause, such a result highlights an important issue which needs to be dealt with. That said, this is the same for both eDNA detection and traditional methods and is acknowledged as a norm in the vast majority of survey techniques. In an attempt to try and ascertain the impact of such false negatives on the end result (and therefore the management/conservation plan which would be implemented), occupancy modeling has shown some promise (Dorazio & Erickson, 2018; Mackenzie et al., 2002; Mackenzie & Royle, 2005). Further, such models can help to assess the influence that specific environmental factors have on the probability of detection or account for the imperfect detection of eDNA (Dorazio & Erickson, 2018; Hunter, Meigs-Friend, Ferrante, Smith, & Hart, 2019; Hunter et al., 2015; Lahoz-Monfort, Guillerma-Arroita, & Tingley, 2016; Schmelze & Kinziger, 2015; Schmidt, Kéry, Ursenbacher, Hyman, & Collins, 2013; Sutter & Kinziger, 2019).

However, in our case (due to the limited number of habitats hosting our target species and thus the relatively low number of locations surveyed), occupancy modeling may not have helped in this regard. The uniformity of environmental characteristics of the sampled locations, combined with a relatively low number of natural replicates, would have led to incorrect estimation from an occupancy model. Further, we intentionally targeted the killifishes preferred habitats in this study (spring-fed wetlands with clear waters and rich surface vegetation) and focused on sites which had known historical presence of the species (Kalogianni et al., 2010, 2019). This meant that the environment surveyed was largely uniform across the various samples. And as stated earlier, the number of habitats hosting the targeted species in this study was limited due to the endangered nature of the two Valencia species. Further, as of yet we do not understand the temporal effect of eDNA sampling on either the killifish or *G. holbrooki* and further studies should assess whether there is an optimal time for sampling or more importantly when results are less reliable (a time not to survey). Finally, the number of replicate samples taken will undoubtedly play a role in the reliability of the end result. Interestingly, the number of replicates needed for eDNA studies is currently in dispute with some studies showing as little as 2 or 3 is sufficient (Mauvisseau, Burian, et al., 2019), while others suggest much more would be appropriate (≤15 natural replicates for common species and up to 45–90 samples per site for rare species—Erickson et al., 2019). This is clearly a research question which warrants greater attention as we move more into the regular use of eDNA as a survey tool in ecological monitoring and conservation.

In conclusion, our field efforts confirmed the usefulness of eDNA monitoring for the detection of both threatened killifishes and a non-native and highly invasive species, regarded as responsible for the decline of many native, and often threatened freshwater species across Europe (Freyhof et al., 2014; Grapputo et al., 2006).
One major benefit of this new tool for surveying these species is the noninvasive nature of the technique. Electrofishing in particular should be reconsidered as a method as it can be potentially harmful to the fish (both target and nontarget alike) (Miranda & Kidwell, 2010; Snyder, 2003). That said, despite the very promising initial results of all three eDNA assays we recommend more intensive field tests be undertaken in the future. Such field trials should, where possible, include a more balanced distribution of sites with known presence or absence of the target species (Farrington et al., 2015; Guan et al., 2019). That said, when dealing with critically endangered species, this is obviously not always possible, and so, such a balance was only possible for the invasive species in this context. Regardless, the advent of novel eDNA approaches aimed at targeting specific species of conservation priority and/or non-native invasive species that have deleterious effects on native wildlife (as demonstrated here) is always going to be a logical step forward in environmental monitoring, due to the noninvasive nature of the method. Combined with citizen science, they could pave the way to larger-scale conservation programs, along with improving management decisions associated with already existing programs, as we had in this study. Although new assays require a high level of validation to ensure reliability and confidence in the results, the advent of novel eDNA approaches offers optimistic perspectives as complementary tools for assessing species distribution.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

Q.M, E.K, B.Z, R.B, and M.S designed the experiment and methodology. Q.M, E.K, and B.Z collected eDNA samples. Q.M performed DNA extraction and qPCR. Q.M and M.B analyzed the data. The manuscript was written by Q.M and M.S and reviewed by all authors.

DATA AVAILABILITY STATEMENT

Data are provided in supplementary materials.

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REFERENCES


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