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Research Article

Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems

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Abstract

Procambarus virginalis, also known as the Marmorkrebs is a highly invasive crayfish species characterized by parthenogenetic reproduction. As conservation management plans rely on the accuracy of the presence and distribution information of invasive species, a reliable method is needed for detecting such species in aquatic systems. We developed and validated a qPCR-based assay for monitoring *P. virginalis* at low abundance, by detecting their eDNA traces left in freshwater systems. We were able to implement this new assay *in-situ* at two separate lakes in Germany, where the crayfish were known to be present. Furthermore, we did not detect the pathogenic fungus *Aphanomyces astaci* in the locations where the Marmorkrebs were detected. We conclude that the use of eDNA is therefore a reliable tool for the early detection of this "perfect invader".

Key words: eDNA detection, lakes, Marmorkrebs, crayfish plague, qPCR

Introduction

Non-indigenous crayfish species are starting to outnumber indigenous crayfish species throughout much of Europe (Holdich et al. 2009). Only five indigenous species were originally found to exist in various freshwater systems across Europe and now eleven non-indigenous species are spreading at alarming rates across this eco-region (Holdich et al. 2009; Kouba et al. 2014). Three of these species are from North America, *Procambarus clarkii* (Girard, 1852), *Faxonius limosus* (Rafinesque, 1817) and *Pacifastacus leniusculus* (Dana, 1852) and have been classified as the most problematic of the invasive crayfish (Holdich et al. 2009). Several other species, also from North America include; *Faxonius immunis* (Hagen, 1870), *Faxonius juvenilis* (Hagen, 1870), *Faxonius virilis* (Hagen, 1870), *Procambarus virginalis* (Lyko, 2017), *Procambarus alleni* (Faxon, 1884) and *Procambarus acutus* (Girard, 1852), introduced after 1980 and therefore have a considerably more restricted range (Holdich et al. 2009).

This is the same for the two Australian species; *Cherax destructor* (Clark, 1936) and *Cherax quadricarinatus* (von Martens, 1868). In addition to these already established species, there is the substantial risk of further introductions, especially through the aquarium trade where many American or Australian species are still readily available (Holdich et al. 2009). There are a number of well documented examples, whereby certain non-indigenous crayfish have been discarded as they outgrow their tanks (such was the case for *Cherax* or *Faxonius* species) or reproduce excessively (such as *P. virginalis*) (Holdich et al. 2009). Due to these introductions (or at least in part), there has been a global decline of indigenous crayfish (Holdich et al. 2009). This is due largely to increased and direct competition for habitat space and resources, along with many (of these American invasive species) carrying the crayfish plague, *Aphanomyces astaci* (Schikora, 1906), a lethal pathogen affecting native species (Bramard et al. 2006; Schrimpf et al. 2013; Keller et al. 2014; Lipták et al. 2016; Ludányi et al. 2016). As a result of these combined threats, conservation programs and "ark" sanctuary sites are being established in various countries with the goal of protecting the local crayfish biodiversity (Holdich et al. 2009; Reynolds and Souty-Grosset 2012). Native crayfish are keystone species in freshwater systems and are also useful as bioindicators of pollution (Reynolds and Souty-Grosset 2012). They have also been proposed as umbrella species, from which the protection is expected to benefit a large range of co-occurring species, for ecosystem conservation (Reynolds et al. 2013).

Although, many of these invasive species currently have restricted ranges, at least one has the potential to be of major concern (Keller et al. 2014). *P. virginalis*, also known as the Marmorkrebs has been labelled as a "perfect invader" due to its recent speciation and, more specifically, its parthenogenetic reproduction mode (Jones et al. 2009; Vogt et al. 2015; Gutekunst et al. 2018). Distribution via the pet trade and anthropogenic releases have led to an increasing spread of the species in various countries, inside and outside Europe (Chucholl et al. 2012; Vojkovská et al. 2014; Lipták et al. 2016; Ludányi et al. 2016; Pârvulescu et al. 2017; Gutekunst et al. 2018). The species is also remarkably tolerant to changes in habitat parameters and can adapt to temperatures as low as 2 to 3 °C (Veselý et al. 2015; Lipták et al. 2016; Ludányi et al. 2016; Andriantsoa et al. 2019). Furthermore, *P. virginalis* has also been cited as carrying *A. astaci* (or crayfish plague) (Keller et al. 2014; Lipták et al. 2016).

As European countries have a mandate to prevent the deliberate introduction of exotic species under the Habitats Directive (European Commission 1992, 2000, 2014; Souty-Grosset et al. 2004), the ability to screen for this particular invasive species would be useful for management of aquatic ecosystems. However, early detection of aquatic organisms, especially when they occur at low densities, has been challenging and often

ineffective using currently established methods (Forsström and Vasemägi 2016). For marbled crayfish, this is further compounded by the nocturnal or crepuscular activity patterns.

In recent years, interest has increased for methods which detect DNA traces within any given environment (known as environmental DNA or eDNA) (Hinlo et al. 2017). This non-invasive method (which targets DNA from skin, blood, mucus or gametes for example) allows a reliable and cost-effective tool for monitoring many different organisms within a wide variety of aquatic habitats, especially when populations are low in abundance (Forsström and Vasemägi 2016; Eiler et al. 2018). Indeed, the method has already been used to detect many of the current indigenous and native crayfish species mentioned earlier (Tréguier et al. 2014; Figiel and Bohn 2015; Dougherty et al. 2016; Larson et al. 2017; Cai et al. 2017; Agersnap et al. 2017; Mauvisseau et al. 2018; Dunn et al. 2017; Harper et al. 2018; Robinson et al. 2018; Geerts et al. 2018; Riascos et al. 2018; Ikeda et al. 2016; Cowart et al. 2018; Rice et al. 2018).

The aim of this study is to design and validate a qPCR (i.e. quantitative Polymerase Chain Reaction) assay for the detection of the newly described, yet highly invasive, crayfish species *P. virginalis*. The assay was tested *in-vitro* and *in-silico* against various indigenous and non-indigenous crayfish species known to occur throughout Europe. Further, we assessed the reliability of the developed assay i.e. the Limit of Detection (LOD) and Limit of Quantification (LOQ) by following the MIQE Guidelines (Bustin et al. 2009). 15 locations were then sampled in Germany (which included sites with known presence of *P. virginalis*) (Figure 1). Finally, at all locations shown to contain *P. virginalis* (either via eDNA sampling and/or direct searching) we tested for the presence of *A. astaci* (Table S1).

Materials and methods

Assay development

Primers and a probe targeting the Cytochrome *C* oxidase subunit 1 (i.e. COI) mitochondrial gene of *P. virginalis* were designed using the Geneious Pro R10 Software https://www.geneious.com (Kearse et al. 2012) following the method outlined in (Tréguier et al. 2014). In addition to the target species, DNA was extracted from one individual of each of the following crayfish species (present or likely to be present in European freshwater systems): *P. clarkii*, *F. limosus*, *P. leniusculus*, *Astacus astacus (Linnaeus, 1758)*, *A. leptodactylus* (Eschscholtz, 1823) and *Austropotamobius pallipes* (from a UK population) (Lereboullet, 1858). DNA from each individual was sequenced using Eurofins Genomics (Wolverhampton, UK), as identification of controls using primers and methods described in Folmer et al. (1994). When designing the assay, we obtained COI sequences from *P. virginalis* (GenBank Accession No. KJ690261.1), *P. clarkii* (JN000901.1, JF438002.1),

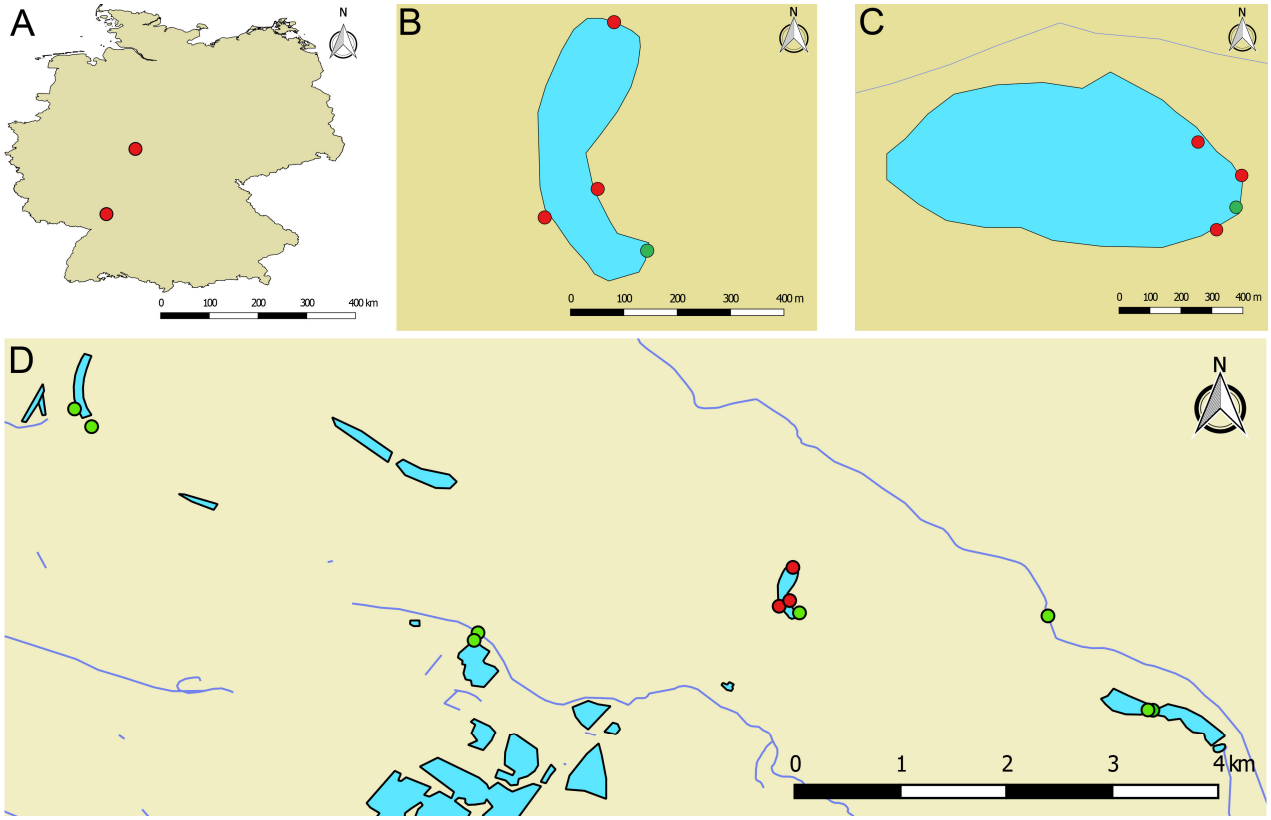

Figure 1. Detection of *P. virginalis* eDNA in established marbled crayfish populations in Germany. (A) Location of Reilinger See (lower left red circle) and Singliser See (upper right red circle) in Germany. (B) Map of Reilinger See with locations of the four sampling sites. Sampling sites with *P. virginalis* eDNA detection are indicated by red circles. (C) Map of Singliser See with locations of the four sampling sites. (D) Map of Reilinger See and surrounding water bodies with locations of eleven sampling sites.

F. limosus (JF437991.1, KT959387.1, KT959445.1), *P. leniusculus* (KU603472.1, JF437998.1, JF437996.1), *A. astacus* (JN254661.1, JN254666.1, JN254672.1), *A. leptodactylus* (KU571456.1, KU571460.1, KU571463.1), *A. pallipes* (AB443446.1, AB443448.1) and *P. alleni* (HQ171452.1, HQ171450.1). Specificity of the set of primers and probe was assessed *in-silico* using the Basic Local Alignment Search Tool (BLASTn) and Primer-BLAST tools from the National Centre for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/. *In-silico* validation was performed before *in-vitro* tests.

The forward primer Pv-COI-F 5' – GTATAGTTGAGAGGGGAGTA –3', reverse primer Pv-COI-R 5' – CCATAGTTATACCAGCTGCC – 3' and probe 6-FAM – AGGTATTTTTTCCTTGCA – BHQ-1 were developed to amplify a 189 bp fragment. Primers and the probe were tested via both conventional PCR and qPCR with DNA extracted from the crayfish species mentioned above.

eDNA samples

15 locations including rivers, lakes and one pond were sampled between the 10th May 2018 and the 15th June 2018 in Baden-Württemberg, south-west Germany and in Hessen, central Germany (Figure 1). Eight of the

sampled locations were previously known for the presence of *P. virginalis* (Table S1) (Dümpelmann and Bonacker 2012; Lyko 2017). At each location, two independent 1 L water samples (hereafter referred as "natural replicates") were collected using a sterile polypropylene ladle and placed into a sterile plastic bag (Whirl-Pak® 1242 ml Stand-Up Bag Merck®, Darmstadt, Germany). Samples from rivers consisted of combining water subsamples, regularly sampled from across the width of the rivers, by moving downstream to upstream, in order to avoid disturbing the sediments (Mauvisseau et al. 2019a). Samples from the lakes and pond consisted of combining surface-water subsamples, sampled across a ten-meter-wide strip, approximately 1 meter away from the bank. Subsamples across the entirety of the two lakes were not possible, as the size, anthropogenic activities, and vegetation cover did not always allow for complete access to all lake side locations. Samples from each location were then filtered with a 50 mL syringe (sterile Luer-Lock™ BD Plastipak™, Ireland) through a sterile 0.45 µm Sterivex™ HV filter (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Millipore®, Germany). Sterivex filters were then immediately fixed with 2 mL of absolute ethanol and stored at room temperature until the end of the fieldtrip (Spens et al. 2016). All filters were then stored at −80 °C in the laboratory before further analysis. Sterile equipment and disposable nitrile gloves were used during the sampling process and replaced at each location to avoid contamination.

DNA extraction

DNA was extracted from both tissue samples and Sterivex filters using the Qiagen DNeasy® Blood and Tissue Kit. DNA was extracted from tissue samples following manufacturers' guidelines. eDNA was extracted from Sterivex filters following the methods described in (Spens et al. 2016). Three control samples consisting of ddH_2O were extracted as above with the Sterivex filters for assessing the absence of cross-contamination. Pipettes and tube holders were disinfected and decontaminated under UV-treatment throughout the whole process. All other laboratory equipment and surfaces were regularly disinfected using 10% bleach solution and ethanol before the analysis.

PCR and qPCR protocols

Procambarus virginalis

PCR amplifications were performed on a Gen Amp PCR System 9700 (Applied Biosystem) with the set of species-specific primers described above. PCR reactions were performed in a 25 µL reaction, with 12.5 µL of PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10 µM), 9.5 µL of ddH_2O and 1 µL of template DNA. Optimal PCR conditions were performed under thermal cycling 50 °C for 2 min and 95 °C for 10 min,

followed by 45 cycles of 95 °C for 15 s and 62 °C for 1 min. For each PCR (with DNA from tissue samples), at least one positive and one negative control were included.

qPCR reactions were performed in final volumes of 25 µL, using 12.5 µL of PrecisionPlus qPCR Master Mix with ROX (Primer Design, UK), 1 µL of each primer (10 µM), 1 µL of probe (2.5 µM), 6.5 µL of ddH_2O and 3 µL of extracted DNA on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems). Optimal qPCR conditions were performed under thermal cycling 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 56 °C for 1 min.

Aphanomyces astaci

For samples which showed the presence of *P. virginalis*, we also assessed the presence or absence of *A. astaci*. Detection of *A. astaci* was performed using the method (including the primers and probe), developed by (Vrålstad et al. 2009; Strand et al. 2011). In brief, qPCR reactions were performed using the forward primer AphAstITS-39F (5' – AAGGCTTGT GCTGGGATGTT – 3'), reverse primer AphAstITS-97R (5' – CTTCTTGC GAAACCTTCTGCTA – 3') and a MGB probe AphAstITS-60T (5'– 6 – FAM – TTCGGGACGACCCMGBNFQ – 3') in a final volume of 25 µl using 12.5 µl of TaqMan™ Environmental Master Mix 2.0 (ThermoFisher Scientific, UK), 1 µl of each primer (10 µM), 1 µl of the corresponding probe (2.5 µM), 4.5 µl of ddH2O and 5 µl of extracted DNA on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems). qPCR conditions were as follows; warm up at 50 °C for 5 min and denaturation at 95 °C for 8 min, followed by 50 cycles 95 °C for 15 s, 58 °C for 1 min. Negative controls were also collected and run as above.

qPCR analysis

A standard curve was first established by analysing a 1:10 dilution series of DNA extracted from *P. virginalis* (55.2 ng/µL, Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific) following the MIQE Guidelines (see Supplementary material Appendix 1) (Bustin et al. 2009). A second standard curve was performed for the analysis targeting *A. astaci*. DNA for this was acquired from the reference isolate of *Aphanomyces astaci* 8866_2 (Department of Environmental and Biological Science, University of Eastern Finland) (13.1 ng/µL, Nanodrop 2000 Spectrophotometer, ThermoFisher Scientific). Again, this was conducted using a 1:10 dilution series similar to that for *P. virginalis* (see Appendix 2) (Bustin et al. 2009). For both targets, the dilution ranged from 10^{-1} to 10^{-9} with 10 "technical replicates" (i.e. qPCR replicates) used for each of the dilution steps in order to assess the LOD and LOQ (Figure 2 and Figure 3) (Bustin et al. 2009; Hunter et al. 2016). When running each assay (for *A. astaci* and *P. virginalis*) two positive and two negative controls were also included.

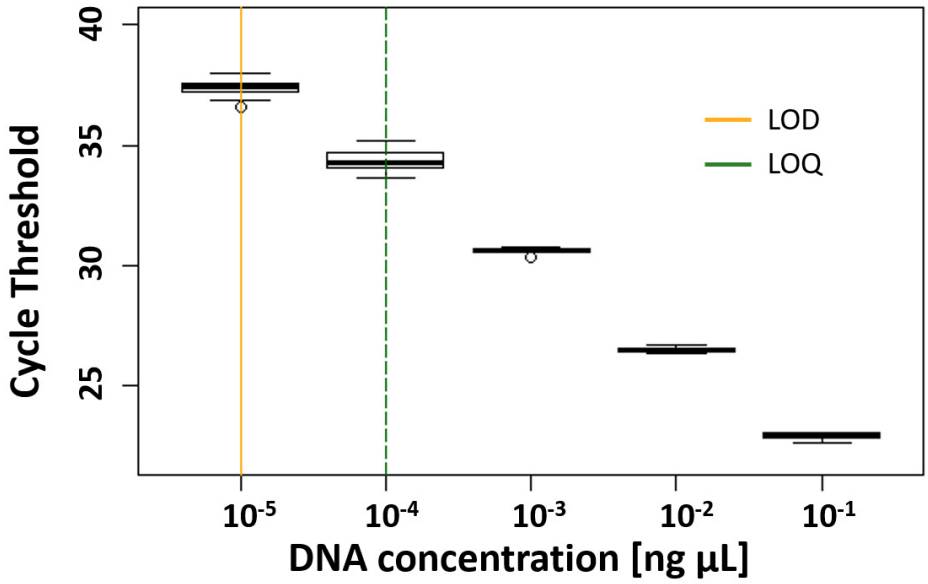

Figure 2. Standard curve established by the analysis of a 1:10 dilution series of DNA extracted from *P. virginalis* tissue (55.2 ng/ µL). The standard curve ranged from 10^{-1} to 10^{-9} with 10 technical replicates used for each dilution steps in order to assess the Limit of Detection (LOD) and Limit of Quantification (LOQ). The cycle threshold (i.e. Ct) represents the number of qPCR amplification cycles required for a positive amplification of the targeted DNA fragment. LOD was found to be 0.552 pg per μl^{-1} at 37.36 ± 0.40 Ct and the LOQ was indicated at 5.52 pg per μl^{-1} at 34.30 ± 0.44 Ct.

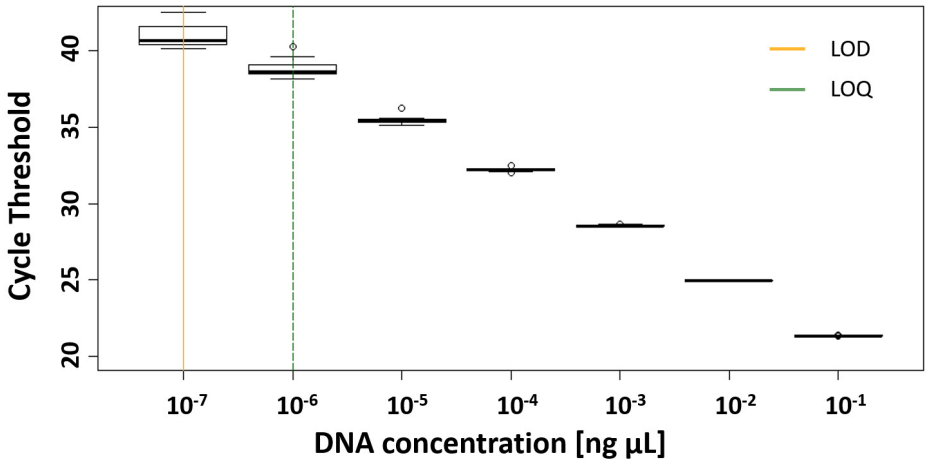

Figure 3. Standard curve established by the analysis of a 1:10 dilution series of DNA extracted from a pure *A. astaci* culture isolate (13.1 ng/ µL). The standard curve ranged from 10^{-1} to 10^{-9} with 10 technical replicates used for each dilution steps in order to assess the Limit of Detection (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 the targeted DNA fragment. LOD was found to be 1.31×10^{-3} pg per μl^{-1} at 41.09 ± 1.02 Ct and the LOQ was indicated at 1.31×10^{-2} pg per μl^{-1} at 38.83 ± 0.61 Ct.

For each of the eDNA samples (i.e. the Sterivex filters) six "technical replicates" were ran, at the same time two further replicates of the dilution series (see above - ranging from 10^{-1} to 10^{-5} for *P. virginalis* and from 10^{-2} to 10^{-6} for *A. astaci*), and six negative control (i.e. blanks) were also run. The negative controls consisted of water free of both *A. astaci* and *P. virginalis* DNA which was collected at the same time as the eDNA samples and in the same way.

Results

Primers and probes designed in this study were found to be species-specific to *P. virginalis* using PCR and qPCR against DNA from the six other crayfish species mentioned above in the assay development section. All negative controls were found to be negative for *P. virginalis* DNA. The standard dilution obtained for the set of primers/probe targeting the COI gene was used for determining the LOD and the LOQ (Bustin et al. 2009; Tréguier et al. 2014; Hunter et al. 2016) (Figure 2). The LOD was identified as the last dilution of the standard curve in which the DNA from the targeted gene is amplified with a cycle threshold (Ct) below 45 (Bustin et al. 2009; Mauvisseau et al. 2019b). The LOQ was identified as the last dilution of the standard curve in which the DNA from the targeted gene is detected, amplified and quantified in at least 90% of the qPCR replicates with a cycle threshold below 45 (Mauvisseau et al. 2019a, b). After the standard curve analysis (Slope = −3.68, Y inter = 19.27, R^2 = 0.99, Eff% = 86.82), the LOD was found to be 0.552 pg per μl^{-1} at 37.36 ± 0.40 Ct and the LOQ was indicated at 5.52 pg per μl^{-1} at 34.30 ± 0.44 Ct (Figure 2).

In order to detect *P. virginalis* eDNA, water samples were obtained from two lakes in Germany with known stable populations (Figure 1A) and from rivers, lakes and one pond surrounding one of the positive sites (Reilinger See, Figure 1B) with unknown status about the presence of this invasive crayfish. At both Reilinger See, and Singliser See, *P. virginalis* eDNA was detected in three of the four sampled locations (Figure 1B and C respectively). The mean Ct values ranged from 34.86 ± 1.9 to 29.86 ± 0.12 (Slope = −3.68 / −4.29 (range), Y inter = 17.57 / 18.95 (range), R^2 = 0.98 / 0.99 (range), Eff% = 70.10 / 86.92 (range)) (Table S1). These results show that our eDNA assay can detect *P. virginalis* in the majority of samples from established populations.

A qPCR analysis targeting *A. astaci* was conducted on all the natural replicates from the same six locations as those tested for *P. virginalis*. After the standard curve analysis targeting *A. astaci* (Slope = −3.34, Y inter = 11.76, R^2 = 0.99, Eff% = 99.33), the LOD was found to be 1.31×10^{-3} pg per μl^{-1} at 41.09 ± 1.02 Ct and the LOQ was indicated at 1.31×10^{-2} pg per μl^{-1} at 38.83 ± 0.61 Ct (Figure 3). All the natural replicates of the locations positive to *P. virginalis* DNA were found to be negative to the presence of *A. astaci.* All negative controls were found to be negative for *A. astaci.* These results show that the pathogen is not present at the sites surveyed and at the time of sampling, despite the invasion of *P. virginalis.*

Discussion

Our study is the first to highlight the use of a bespoke eDNA assay for the detection of a highly invasive and parthenogenetic crayfish species (*P. virginalis*) which is spreading throughout Europe and other areas of the

globe (including Madagascar) (Gutekunst et al. 2018). In addition to validating the assay *ex situ* (under controlled laboratory settings) we tested the feasibility *in situ* (at 8 locations in two lakes in Germany – the epicentre for the invasion of this newly identified species, and 7 other locations surrounding Reilinger See (Figure 1B)). Interestingly, when sub-sampling the same lake (i.e. sampling from multiple sites within the lake), we were only able to detect an eDNA cycle threshold from three of the four sites. We were not able to detect any DNA traces from *P. virginalis* in the 7 other sampled locations (lakes, rivers and pond). Therefore, although these results illustrate the efficiency of the assay, it also identifies the need for taking multiple "environmental replicates" from any given location. In large freshwater systems (a pond or lake for example), sub-sampling across the entire banks circumference, then merging and homogenising these sub-samples would allow a more reliable analysis of the entire habitat/ecosystem. However, this is not always practical and in our study the lakes were too large or had areas which were inaccessible for such a sample strategy to be undertaken. If only one site at any given location had been sampled, the negative eDNA read would have indicated no *P. virginalis* populations in either of the two lakes sampled (despite knowing to the contrary) i.e. this would have been a false negative. Reasons why such a result may have occurred are likely related to the behaviour of the organism in question. Many crayfish species are known to have patchy distribution (Kershner and Lodge 1995) and even when populations are high, the eDNA detection rate may not increase in correlation (Rice et al. 2018). Further, the flow or movement of eDNA may not be even across the system. Indeed, although in this study the mean Ct and the number of samples indicating positive eDNA detection varied, we were unable to correlate this with numbers/density of *P. virginalis*. Further work should therefore focus on assessing if this eDNA assay can be used for quantifying *P. virginalis* populations.

P. virginalis has also been shown to be a vector of the pathogenic agent *Aphanomyces astaci* (Lipták et al. 2016). As this pathogen results in the dramatic decline of native species including *A. pallipes*, early detection of the pathogen and the vectors would therefore be invaluable. Interestingly, although we were able to detect *P. virginalis* in six locations, none of these showed a positive signal for *A. astaci*. This is encouraging and if populations spread from these two main locations it may be the case that *A. astaci* does not spread with them. However, it should be noted that we only sampled for the presence or absence of *A. astaci* at one time point and a more detailed seasonal study should be completed before we can say without any doubt that these populations are pathogen free.

In conclusion, the newly developed eDNA assay (presented here) has been shown to be species-specific to *P. virginalis* and can be used *in-situ* to test for unidentified populations of *P. virginalis* across Europe. Such surveying may highlight areas where active management such as physical

removal can be concentrated to minimise the spread of this potentially dangerous species. Preliminary data suggests a quantitative approach may be possible with further assessment of known populations in any given environment. Furthermore, the appearance of a false negative highlights the need of multiple "natural replicate" samples when undertaking eDNA research – particularly for this assay but most likely for all assays developed to date. Finally, we did not detect the presence of crayfish plague in these populations. Although this is a promising finding, we urge caution as season could play a yet unknown role in the detection of *A. astaci* and further work should be undertaken to assess if this is the case.

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Author contributions

Q.M., M.S., S.T., and F.L., designed the experiment and methodology; Q.M., S.T. and R.A. collected field samples, Q.M., performed extraction and qPCR. The manuscript was written by Q.M, S.T. and M.S. and reviewed by all other authors.

Competing interest

The authors declare no competing interests.

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Supplementary material

The following supplementary material is available for this article:

Appendix 1. *Procambarus virginalis* MIQE Guidelines.
Appendix 2. *Aphanomyces astaci* MIQE Guidelines.
Table S1. Table showing various results and environmental variables of each sampled sites.

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