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PRIMARY RESEARCH PAPER

Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds

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Abstract Environmental DNA (eDNA) is a powerful method for assessing the presence and distribution of invasive aquatic species. We used this tool to detect and monitor several invasive crayfishes *Procambarus clarkii*, *Orconectes limosus* and *Pacifastacus leniusculus* present in, or likely to invade, the ponds of the Brenne Regional Natural Park. A previous study showed that the eDNA method was not very efficient in detecting *P. clarkii*. In the present study, we explored new improvements in the detection of invasive crayfish. We designed specific primers for each crayfish species, and set up an experimental mesocosm approach to confirm the specificity of the primers and the sampling protocol. We analysed samples taken from ponds in 2014 and 2015. We compared two qPCR protocols involving either SybrGreen or TaqMan assays. Using these same primers, we were able to detect crayfish eDNA with both assays during the mesocosm experiment. However, crayfish from field samples could only be detected by performing qPCR with a SybrGreen assay. We successfully monitored the presence of three invasive species of crayfish using eDNA. This method is a powerful tool for establishing the presence or absence of invasive species in various freshwater environments.

Keywords Biological invasions · *Procambarus clarkii* · *Orconectes limosus* · *Pacifastacus leniusculus* · Ponds · France · eDNA detection

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Introduction

All living organisms leave traces consisting of cell debris and extracellular DNA in their environment (Taberlet et al., 2012). These traces, also known as environmental DNA (eDNA), are present in many forms such as faeces, urine, pieces of skin or hair, living or dead and degraded cells, mucus, eggs and sperm (Laramie, 2013; Wilson & Wright, 2014) and can be sampled from aquatic environments, soil, sediment, or permafrost (Pilliod et al., 2013; Jerde & Mahon, 2015; Thomsen & Willerslev, 2015). The persistence of eDNA varies depending on the environment and various factors such as the temperature, the acidity or the presence of endonucleases (Dejean et al., 2011; Strickler et al., 2015; Thomsen & Willerslev, 2015). Environmental DNA may persist for up to 21 days in aquatic ecosystems (Dejean et al.,

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2011) and the amount of DNA detected depends on the number of living organisms present in the sampled area (Lodge et al., 2012). The eDNA method is recognized as an effective non-invasive method for detecting species that are present in very low abundance (Dejean et al., 2011; Jerde et al., 2011; Fukumoto et al., 2015), and go undetected by other conventional methods (Jerde et al., 2011; Janosik & Johnston, 2015; Smart et al., 2015). Consequently, this can be used as a complementary approach to traditional methods of species detection, such as electric fishing or trapping (Ficetola et al., 2008; Blanchet, 2012). For example, the eDNA extracted from freshwater samples has major applications for detecting not only heritage and endangered species but also invasive alien species and more generally as part of biodiversity inventories (Darling & Mahon, 2011; Thomsen et al., 2012a; Piaggio et al., 2014). This method has been successfully applied to several aquatic species (Jerde et al., 2011, 2013; Thomsen et al., 2012a, b), mainly for detecting amphibians and fish (Evans et al., 2015; Thomsen & Willerslev, 2015), aquatic plants (Epp et al., 2015; Scriver et al., 2015; Fujiwara et al., 2016) and reptiles (Piaggio et al., 2014; Davy et al., 2015; Hunter et al., 2015).

Applications to invertebrates in freshwater systems are just emerging, and few species of crustaceans have been investigated, such as the branchiopod *Daphnia longispina* (Deiner & Altermatt, 2014), the amphipod *Gammarus pulex* (Deiner et al., 2015; Mächler et al., 2015), the crayfish *Orconectes rusticus* (Dougherty et al., 2016) and the crayfish *Procambarus zonangulus* (Figiel & Bohn, 2015). Several studies have used the eDNA method for monitoring invasive or endangered species of crayfish (Dougherty et al., 2016; Ikeda et al., 2016). Only one study has been conducted on the invasive crayfish species *Procambarus clarkii* (Girard) (Tréguier et al., 2014) in ponds in the marshes located in Brière (France). This study showed low detection rates for *P. clarkii*. The authors concluded that it was necessary to improve the PCR technology methods used and to optimise the water sampling method.

The present paper aims to improve the method for detecting invasive crayfish present (*P. clarkii* and *Orconectes limosus* Rafinesque) or likely to be present (*Pacifastacus leniusculus* Dana) in ponds in La Brenne (France). The main species encountered is *P. clarkii*, one of the 100 most invasive species in Europe (Gherardi & Panov, 2009). *P. clarkii* is a successful colonizer that can quickly settle into new environments (Souty-Grosset et al., 2006; Reynolds & Souty-Grosset, 2012). Overviews of its worldwide occurrence (Loureiro et al., 2015), as well as its ecology and invasion in Europe (Souty-Grosset et al., 2016), have recently been published. In 2007, this invasive crayfish was identified for the first time in the Brenne Regional Natural Park (Coignet et al., 2012). In 2011, ten infestation sites were found in the park, with 62 ponds already colonized (Coignet et al., 2012). The appearance of this species is a major threat to biodiversity in the park (Holdich et al., 2009). Farmers and fish farmers have raised serious concerns due to the extensive agricultural damage inflicted by them burrowing and destroying plants (Reynolds & Souty-Grosset, 2012). It seems likely that *P. clarkii* can successfully survive in dried-up ponds in La Brenne so long as there is some form of water supply such as a small rivulet or rainwater (Souty-Grosset et al., 2014). Today this crayfish is invading more and more ponds in the park (personal communication from Catherine Souty-Grosset and Aurore Coignet) and controlling the species is crucial. Another invasive crayfish, *O. limosus* (Rafinesque), is present in small numbers, even coexisting with *P. clarkia,* in a few ponds and can be abundant when a pond has no *P. clarkii* individuals. Furthermore, a third invasive crayfish, *P. leniusculus* (Dana) is likely to invade ponds in the Brenne Regional Natural Park. Individuals of this species were recently found by park staff members in the south of the Regional Natural Park.

Monitoring invasive species using non-invasive methods is a key issue for the conservation of endangered species. The aim is this study is to demonstrate the reliable detection of several invasive crayfishes using the eDNA method. In order to get a better picture of the presence of the three invasive crayfish species in the park and to improve the control of *P. clarkii*, specific primers were designed for the three species. An experimental approach in mesocosm aimed to confirm the specificity of the primers before using qPCR with either a SybrGreen protocol (Wilcox et al., 2015; Mauvisseau et al., 2017) or the TaqMan qPCR protocol as used by Tréguier et al. (2014). Variation in the detection of invasive crayfishes using these two qPCR protocols shows the potential impacts of PCR inhibitors (Rees et al., 2014; Dougherty et al., 2016). As specified in Dougherty et al. (2016), water

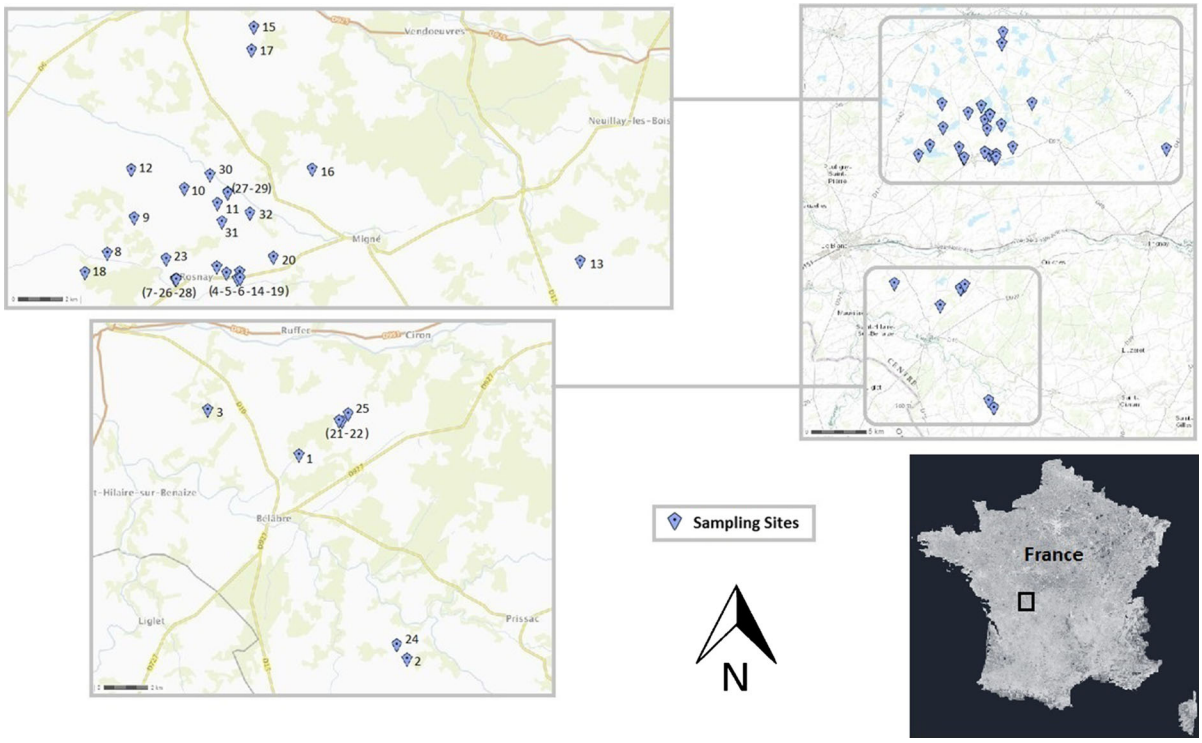

Fig. 1 Location of ponds and ditches sampled in the National Regional Park of Brenne. As some samples were geographically far away from others the sampling area is separated in two boxes for allowing a better zoom

samples could contain substances such as humic acids that could inhibit PCR and qPCR reactions leading to incorrect results (Smith & Osborn, 2009). In a second step, water sampling was performed in ponds of the Brenne Regional Natural Park during different periods of activity of *P. clarkii* (i.e. spring and summer) and the reliability of the detection was tested by comparing the results obtained with the data obtained from trapping. Different sampling periods were chosen in order to maximize the detection of crayfishes. Our results showed that eDNA method is a very promising tool for detecting invasive species in aquatic ecosystems.

Methods

Study area and sampling scheme

The Brenne Regional Natural Park is located in the south-west of the department of Indre, in the Centre region of France. It covers an area of 183,000 hectares and the natural region of La Brenne comprises one of the most important wetlands in France with a mosaic of landscapes that include ponds, grasslands, ponds, moors, forests and valleys. Its exceptional wealth of fauna, flora and habitats led to it being recognized as an International RAMSAR (International Convention on Wetlands) wetland zone since 1991 (Souty-Grosset et al., 2014). Indeed, this territory is well conserved since the park is a very important place for the reproduction of migratory bird species. It also hosts heritage or protected species, for example it is the location of the largest population of the European pond turtle *Emys orbicularis* (Servan & Roy, 2004). Recently introduced invasive species are reducing biodiversity at different sites (Dejean et al., 2012). Since 2007, the crayfish *P. clarkii* has been identified in ponds in La Brenne (Coignet et al., 2012).

Water samples were collected from ponds in the Brenne Regional Natural Park pond in summer 2013 (July and August) (Fig. 1; Table 1). Further samples from these ponds and other new ponds and ditches lying in the path of *P. clarkii,* were collected in spring 2015 (March and April) (Fig. 1; Table 1). We sampled 19 ponds during summer 2013. We sampled 10 of

Table 1 Table showing the location of ponds and ditches sampled in the National Regional Park of the Brenne by GPS coordinates, the sampling date and the estimated surface (Ha) of each sampled area

Site	GPS coordinates	Sampling date (s)	Type of area sampled	Surface (Ha)
1	1°10′25.4″E/46°34′30.0″N	08/2013 and 05/2015	Pond	1.7
2	1°14′18.9″E/46°29′26.5″N	08/2013 and 04/2015	Pond	12
3	1°07′07.5″E/46°35′37.1″N	08/2013 and 042015	Pond	1.7
4	1°14′27.4″E/46°41′59.7″N	08/2013 and 03/2015 and 05/2013	Pond	1
5	1°13′58.9″E/46°41′58.3″N	08/2013 and 03/2015 and 05/2013	Pond	1.7
6	1°13′37.4″E/46°42′07.4″N	08/2013 and 04/2015	Pond	1.8
7	1°12′06.9″E/46°41′48.9″N	08/2013 and 04/2015	Pond	0.07
8	1°09′39.8″E/46°42′27.7″N	08/2013 and 04/2015	Pond	16
9	1°10′38.3″E/46°43′20.3″N	07/2013 and 04/2015	Pond	5
10	1°12′26.9″E/46°44′03.6″N	08/2013 and 042015	Pond	5
11	1°13′39.1″E/46°43′41.9″N	08/2013	Pond	5.4
12	1°11′28.2″E/46°43′49.4″N	08/2013	Pond	4
13	1°26′44.9″E /46°42′16.0″N	07/2013	Pond	0.4
14	1°14′22.6″E/46°41′49.9″N	08/2013	Pond	1
15	1°14′58.2″E/46°48′02.9″N	07/2013	Pond	11.5
16	1°17′04.2″E/46°44′32.5″N	08/2013	Pond	19.5
17	1°14′53.1″E/46°47′28.8″N	07/2013	Pond	1
18	1°08′52.0″E/46°41′58.8″N	07/2013	Pond	1
19	1°14′28.4″E/46°41′51.0″N	03/2013	Pond	2
20	1°15′39.9″E/46°42′22.3″N	03/2015 and 04/2015	Pond	7
21	1°11′58.0″E/46°35′20.6″N	03/2015 and 04/2015	Pond	4
22	1°11′51.9″E/46°35′21.0″N	03/2015	Pond	1
23	1°11′47.4″E/46°42′19.6″N	04/2015	Pond	3
24	1°13′56.4″E/46°29′48.0″N	04/2015	Pond	1.6
25	1°12′10.7″E/46°35′31.6″N	04/2015	Pond	1.5
26	1°12′09.5″E/46°41′49.5″N	04/2015	Pool	0.01
27	1°14′00.9″E/46°43′58.0″N	04/2015	Pool	0.001
28	1°12′09.5″E/46°41′49.5″N	04/2015	Pool	0.001
29	1°14′00.9″E/46°43′58.0″N	04/2015	Ditch	0.001
30	1°13′22.8″E/46°44′23.7″N	04/2015	Pond	1.2
31	1°13′49.2″E/46°43′14.9″N	04/2015	Pond	2.8
32	1°14′03.5″E/46°43′27.2″N	04/2015	Ditch	0.001

these ponds again in spring 2015, along with 12 new ponds, pools and ditches. Water samples were also collected from different locations in a pond where *O. limosus* and *P. clarkii* had been detected by trapping or visual detection. We followed the method used by Ficetola et al. (2008): for each pond, at least ten samples of 15 ml of surface water were collected. For large ponds, water samples were collected every hundred metres along the bank. Then, after homogenization, a single sample of 15 ml was preserved for the analysis. This sampling method increases the probability of detecting the targeted invasive species. Moreover, a method that homogenizes a layer of water (Reynolds & Walsby, 1975) also avoids the degradation of DNA by nucleases (Tréguier et al., 2014). Pools and ditches were sampled in the same way. We also collected a sample from a pond known to be invaded by *P. leniusculus* in the same park, as well as from a pond in Saint Benoit (Vienne Department, France) (Gherardi et al., 2013). A total of 32 ponds were sampled in this study. Data on the presence of *P. clarkii* and *O. limosus* detected by trapping or visual

Table 2 Species-specific primers targeting the mitochondrial cytochrome oxidase subunit I (COI) gene in three species of crayfish, showing fragment length (in base pairs; bp) and annealing temperature for PCR (Tm) whether eDNA was detected through PCR or qPCR (Y = yes for all replicates)

Species	Primer	Sequence (5′-3′)	bp	Tm	eDNA detected	
					PCR	qPCR
Procambarus clarkii	CO1-Pc-03-F	GGAGTTGGAACAGGATGGACT	73	59°C	Y	Y
	CO1-Pc-03-R	AATCTACAGATGCTCCCGCA				
	Probe	CCTCCTTTAGCTTCTGCTATTGCTC				
Orconectes limosus	CO1-Ol-01-F	CCTCCTCTCGCTTCTGCAAT	78	59°C	Y	Y
	CO1-Ol-01-R	AACCCCTGCTAAATGCAACG				
	Probe	CTCATGCAGGGGCATCAGTGG				
Pacifastacus leniusculus	CO1-Pl-02-F	TGAGCTGGTATAGTGGGAACT	114	59°C	Y	Y
	CO1-Pl-02-R	AGCATGTGCCGTGACTACAA				
	Probe	CGGGTTGAATTAGGTCAACCTGGAAG				

observations were provided by A. Coignet with the help of park staff.

Control PCR assay

We designed three specific primer pairs (Table 2) targeting the mitochondrial cytochrome oxidase subunit (COI) gene of the three invasive species of crayfish according to the following procedure: for each species, the coding sequences of the COI region were retrieved from the GenBank database (6 February 2015) (http://www.ncbi.nlm.nih.gov/genbank/) then aligned using the Bio Edit software (Hall, 1999) and MEGA (Tamura et al., 2013).

The primers were then drawn using the Primer3 website (Koressaar & Remm, 2007; Untergasser et al., 2012) and then tested in silico using the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). For each species studied, a short specific COI gene fragment was chosen (Herder et al., 2014). The specific primer set to *P. clarkii* amplified a 73 bp fragment, the specific primer set to *O. limosus* amplified a 78 bp fragment and the specific primer set to *P. leniusculus* a 114 bp fragment (Table 2). We tested the specificity of each primer set using total DNA extracted from the legs of live individuals of *P. clarkii*, *O. limosus* and *P. leniusculus*, and the native crayfish species *Astacus astacus* and *Austropotamobius pallipes* using standard methods of phenol dichloromethane-isoamyl alcohol (24:24:1) and ethanol precipitation (Sambrook et al., 1989). DNA was also extracted from one leg of the crayfish *Astacus leptodactylus* preserved in ethanol. Concerning protected native species, individuals were captured by hand or using small aquarium nets, and then returned to the place of capture immediately after removing the tissue sample, which was placed in a vial containing 95% ethanol.

Laboratory experiment: validation of the primers and probes

We ran a mesocosm experiment to verify the specificity of our primers. One individual of each of the following invasive species *P. clarkii*, *O. limosus* and *P. leniusculus* was placed in a previously unused mesocosm filled with 3 l of tap water, each oxygenated using a brand-new bubbler. Each individual was kept alone in a dedicated mesocosm. As a control, a PCR assay was performed on water samples from each mesocosm before the introduction of the crayfish in order to verify the absence of crayfish eDNA. Individuals were previously fed ad libitum before the experiment and were maintained for 21 days without any input in the trays. Single water samples of 15 ml were collected after 24, 48, 72, 96 h and 10, 17, 21 days before removing the individuals from the mesocosm. After the removal of individuals, water samples were collected once a week over five weeks to establish the persistence of eDNA in mesocosm. The last samples were collected while agitating the water in the mesocosm to ensure maximum homogenization. In order to track potential fluctuations in water temperature, the temperature was recorded every half

hour in each mesocosm with StowAway TidbiT temperature data loggers.

eDNA extraction from water samples

DNA was extracted from the 15 ml water samples using the QIAGEN Blood and DNA tissues kit according to the manufacturer's instructions with slight modifications. Centrifugation of the 50 ml Falcon tube was carried out for 30 min at 5°C and 5,500×*g* to recover the DNA and cells at the bottom of the tube. The tube was then drained and the remaining supernatant discarded. The pellet was filled with 360 µl of Buffer ATL instead of 180 µl to maximize DNA recovery. The wall of the tube was rinsed with Buffer ATL to recover any DNA traces. The Buffer ATL containing the DNA and cells was then transferred to a 1.5 ml tube. 40 µl of proteinase K instead of 20 µl was then added in order to maximize cell lysis. The sample was spun for a few seconds and then incubated in a water bath at 56°C for 4 h to lyse the cells. The sample was then vortexed for 15 s. 400 µl of Buffer AL and 400 µl of 100% ethanol (instead of 200 µl for each of these products) were then added in order to ensure optimal DNA binding in the column. The sample was vortexed several times in a DNeasy Mini Spin Column 2 ml tube and centrifuged for 1 min at 6,000×*g*. The liquid obtained in the collection tube was discarded. 500 µl of Buffer AW1 was added and centrifuged for 1 min at 6,000×*g*. The volume obtained in the collection tube was again discarded and a new collection tube was placed in the column. 500 µl of Buffer AW2 was added and centrifuged at 20,000×*g* for 3 min. The liquid obtained in the collection tube was discarded. The column was then transferred into a 1.5 ml tube and 40 µl of Buffer AE was added for the elution. The tube was incubated at room temperature for 1 min and then centrifuged 1 min at 6,000×*g*. The column was then discarded and the DNA solution was kept at −20°C.

Quantitative PCR (qPCR) assays

TaqMan protocol: Quantitative PCR was performed in a final volume of 25 µl using 6.5 µl of ddH2O, 3 µl of template DNA, 1 µl of each primer (10 µM), 1 µl of the corresponding probe (2.5 µM) and 12.5 µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems) under thermal cycling at 50°C for 5 min and 95°C for 10 min followed by 55 cycles of 95°C for 30 s and 56°C for 1 min. Each sample was run in 6 replicates on a LightCycler 480 (Roche). We used DNA extracted from the tissue of each species in order to obtain dilution series ranging from 10^{-2} to 10^{-9} - ng μl^{-1} as a qPCR standard. Eight negative controls (ddH2O) were used for each PCR plate.

SybrGreen protocol: Quantitative PCR was performed in a final volume of 10 µl using 3 µl of ddH2O, 1 µl of template DNA, 0.5 µl of each primer (10 µM), and 5 µl SybrGreen (LightCycler 480 SYBR Green I Master) (Roche) under thermal cycling at 95°C for 10 min followed by 55 cycles of 95°C for 10 s and 56°C for 10 s and finally 72°C for 20 s. Then followed a melting cycle wherein the temperature rises from 65 to 95°C. Samples were run in 6 replicates on a LightCycler 480 (Roche). We used DNA extracted from the tissue of each species in order to obtain dilution series ranging from 10^{-2} to 10^{-9} ng μl^{-1} and we calculated the detection limit using the same method as Tréguier et al., 2014. Eight negative controls (ddH2O) were used for each PCR plate.

A sample was deemed to be positive when a sigmoidal amplification curve was detected in at least one qPCR replicate. We assessed the species identity of qPCR products by comparing melt curves against species-specific standards.

Statistical analyses

Analyses were conducted using R 3.3.0 software (R Core Team, 2016). Differences in the detection rate were tested using Fisher's exact test. The inter-rater reliability of observational data was tested using unweighted Cohen's Kappa coefficient (Fleiss et al., 1969).

Results

Specificity of the primer sets and probes

The reliability and specificity of the primers must be assessed as the quality of primer design greatly influences the robustness of the data. The specificity of the designed primers and probes (Table 2) was confirmed by PCR on the DNA matrix from tissue from invasive and local crayfish and then tested by

qPCR on DNA extracted from water samples from the mesocosm containing invasive species (Table 3). All replicates for each species gave the same results with no false positives. Crayfish DNA could still be detected at a concentration of 10^{-9} ng μl^{-1}.

Mesocosm experiment

We detected the presence of eDNA from the three invasive species as of 24 h after their introduction in the mesocosm (Table 4). Thereafter these species were always detected in each round of sampling during their stay in the mesocosm. Detection of eDNA varied after the specimens were removed from the mesocosm. *P. clarkii* was detected 14 and 34 days after removal, *O. limosus* was detected 7, 21 and 34 days after removal and *P. leniusculus* was detected only 34 days after removal. However, after homogenization of the mesocosm water before the final collection, eDNA was detected in all cases. We did not detect any species that was not present in a given mesocosm (*i.e.* a false positive). These results were obtained by qPCR both with SybrGreen and TaqMan protocols using our specific primers and probes (Table 2). We also tested the primer set and probe used by Tréguier et al. (2014) with a TaqMan protocol: we did not detect eDNA at 17 days and 21 days. We detected the presence of eDNA only 21 days after the removal of the specimens. We did not find any statistical variation in the temperature during the mesocosm experiment (*t* test P value $= 2.2 \times 10^{-16}$, $t = 3{,}220.13$, $df = 13{,}439$, mean temperature 15.56°C).

Detection of invasive crayfish in field samples

All the results presented in Table 5 were obtained with the qPCR SybrGreen protocol using our specific primers. Indeed, the detection of *P. clarkii* using the TaqMan qPCR protocol with the primer set and probe used by Tréguier et al. (2014) was negative for all eDNA samples.

Table 3 Specificity of the primer sets on different species of crayfishes whether eDNA was detected through PCR assay or qPCR assay (+ yes for all replicates) (– no for all replicates) (*NT* not tested); the number of replicates PCR and qPCR is indicated in brackets

	Specific primer sets											
	P. clarkii		*O. limosus*		*P. leniusculus*		*A. astacus*		*A. leptodactylus*		*A. pallipes*	
	PCR	qPCR	PCR	qPCR	PCR	qPCR	PCR	qPCR	PCR	qPCR	PCR	qPCR
Procambarus clarkii	+(6)	+(6)	–(6)	–(6)	–(6)	–(6)	–(6)	NT	–(6)	NT	–(6)	NT
Orconectes limosus	–(8)	–(8)	+(8)	+(8)	–(8)	–(8)	–(6)	NT	–(6)	NT	–(6)	NT
Pacifastacus leniusculus	–(6)	–(6)	–(6)	–(6)	+(6)	+(6)	–(6)	NT	–(6)	NT	–(6)	NT

Table 4 Detection of eDNA from three species of crayfishes in the aquaria by qPCR. A qPCR using TaqMan protocol was performed with the primer set used in Tréguier et al. (2014)

		24 h	48 h	72 h	96 h	10 days	17 days	21 days	Removal of individuals				
									7 days	14 days	21 days	27 days	34 days
Primers designed for the present study	*P. clarkii*	+	+	+	+	+	+	+	–	+	–	–	+
	O. limosus	+	+	+	+	+	+	+	+	–	+	–	+
	P. leniusculus	+	+	+	+	+	+	+	–	–	–	–	+
Primers used in Tréguier et al. (2014)	*P. clarkii*	+	+	+	+	+	–	–	–	–	+	–	–

A qPCR using SybrGreen protocol was performed with the primers designed for this experiment (+ detection of the species) (– absence of detection of the species). All the analyses were conducted in six replicates

Table 5 Detection of eDNA of *P. clarkii* and *O. limosus* in the ponds and ditches in the National Regional Park of the Brenne in the sample taken in 2013 and 2015 (+ detection of the species) (– no detection of the species) (*Nd* no data about the presence or absence of the crayfish). All the analyses were conducted in six replicates

Site	Summer 2013		Summer 2013		Spring 2015		Spring 2015	
	Presence of *P. clarkii* known	eDNA detection	Presence of *O. limosus* known	eDNA detection	Presence of *P. clarkii* known	eDNA detection	Presence of *O. limosus* known	eDNA detection
1	+	+	–	–	+	+	–	–
2	+	–	+	–	+	+	+	+
3	+	+	+	+	+	–	+	–
4	+	+	–	–	+	–	–	–
5	+	+	–	+	+	+	–	–
6	+	+	–	+	+	+	–	–
7	+	+	–	–	+	+	–	–
8	+	–	+	+	+	+	+	+
9	–	–	+	+	–	+	+	+
10	–	–	+	+	–	+	+	–
11	–	–	+	+				
12	–	–	+	+				
13	+	+	–	–				
14	+	–	–	–				
15	–	–	+	+				
16	–	–	+	+				
17	+	–	+	–				
18	+	–	–	–				
19					+	+	–	+
20					+	+	+	–
21					+	+	–	–
22					+	+	–	–
23					+	+	+	+
24					+	+	+	–
25					+	–	Nd	–
26					Nd	+	Nd	–
27					Nd	+	Nd	–
28					Nd	+	Nd	+
29					Nd	+	Nd	–
30					Nd	+	Nd	–
31					Nd	–	Nd	–
32					Nd	–	Nd	–

For the water samples collected in 2013, crayfish eDNA was detected in 58% (7/12) of the ponds where the presence of *P. clarkii* has been confirmed. For *O. limosus*, a positive detection was obtained in 80% (8/10) of cases. For the water samples collected in 2015, we detected *P. clarkii* in 80% (12/15) of cases. For *O. limosus*, eDNA detection was obtained in 50% (4/8) of cases. There was no significant difference in the detection rate of *P. clarkii* and *O. limosus* between years (P value = 0.7628 and P value = 0.7086 respectively, Fisher's exact tests). The overall detection rate for *P. clarkii* was therefore 70% (19/27)

whereas it was 66% (12/18) for *O. limosus*. Significance of agreement between eDNA and conventional detections of invasive crayfish was evaluated using unweighted Cohen's kappa coefficient (Fleiss et al., 1969). The resulting kappa value was 0.5814 with a standard error of 0.0817 (95% Confidence Interval = 0.4212 to 0.7416) indicating a reasonable agreement between the two methods.

We also detected *P. clarkii* in 60% (6/10) and *O. limosus* in 50% (5/10) of the ponds known to harbour both *P. clarkii* and *O. limosus*. Concerning these invasive species, we did not observe statistical differences in eDNA detection between the samples collected in spring or in summer. The signal crayfish *P. leniusculus* was not detected in samples apart from those collected in the south of the Brenne Regional Natural Park and in the pond in Saint Benoit.

Discussion

In this study, we propose a method for detecting invasive crayfishes and particularly *P. clarkii* that is more reliable than the method used by Tréguier et al. (2014). The location of their experiments took place in the Brière Regional Natural Park in France, and did not involve a laboratory experiment as for other aquatic species (Dejean et al., 2011; Foote et al., 2012; Olson et al., 2013; Piaggio et al., 2014) and for the crayfish *Procambarus zonangulus* (Figiel and Bohn, 2015). Tréguier et al. (2014) concluded that the detection of *P. clarkii* by eDNA was not completely reliable. They observed improved detection of crayfish in ponds using eDNA (73%) compared to traditional surveys using traps (65%). However, their detection by eDNA was confirmed by trapping in only 38.5% of the ponds. Examining the literature on the detection of eDNA in aquatic environments, Roussel et al. (2015) concluded that the low detection of crayfish eDNA may be due to the dilution, degradation or transport of DNA. Insufficient primer specificity or qPCR inhibitors may also explain these results. Deiner & Altermatt (2014) have shown that species-specific transport distances can also exist for eDNA and may impact the detection rates.

We can observe that the detection rate of crayfishes in our study and in Tréguier et al. (2014) is quite low in comparison to other species (Goldberg et al., 2011, 2013, 2014; Deiner & Altermatt, 2014). However, recent studies on the invasive rusty crayfish *Orconectes rusticus* (Girard 1852) have shown promising results for detecting invasive crayfish (Dougherty et al., 2016). Furthermore, Ikeda et al. (2016) successfully detected endangered crayfish in streams. Our study highlights the need for improved assessment of the release of DNA by the species studied in future eDNA studies.

In the present study, we set up a preliminary laboratory experiment in order to establish the possibility of specifically detecting the eDNA of the three invasive crayfishes *P. clarkii*, *O. limosus* and *P. leniusculus* after one day's presence in the mesocosm. We also established that the three species could be detected in the mesocosm after 1–21 days of presence with a high reproducibility between replicates. We also show that the detection of eDNA from invasive crayfishes varies after the individuals are removed from the mesocosm (Table 4). Only a few eDNA studies have set up a mesocosm experiment before conducting a field experiment: Dejean et al. 2011 (fish and amphibians), Foote et al. 2012 (marine mammals), Olson et al. 2013 (eastern hellbenders), Piaggio et al. 2014 (reptiles), Figiel & Bohn 2015 (crustaceans). Our study is the only one that has set up a mesocosm experiment with *P. clarkii, O. limosus* and *P. leniusculus* for an eDNA study. A study by Dejean et al. (2011) showed that eDNA can persist 21 days in freshwater ecosystems. Another study by Maruyama et al. (2014) shows that the half-life of fish eDNA is only 6.3 h in freshwater ecosystems. Nevertheless, it was possible to detect the targeted species until 21 days after their removal from the mesocosm. The results of eDNA studies can be biased when positive detections are observed even when the target species are no longer present. Better comprehension of eDNA degradation rates for the target species should be the first step before starting monitoring with the eDNA method.

The eDNA method is very useful for the detection and monitoring invasive species (Nathan et al., 2014; Comtet et al., 2015) but still not effective for monitoring all animal species (Rees et al., 2015). Concerning the invasive crayfish species *P. clarkii* and *O. limosus,* we did not find statistical differences in eDNA detection between samples collected in summer 2013 and spring 2015 (P value = 0.7628 and P value = 0.7086 respectively, Fisher's exact tests). Furthermore, we obtained an overall detection rate of

70% for *P. clarkii* and 66% for *O. limosus*. Overall trapping or visual detection confirms that our protocol, modified from Tréguier et al. (2014) with the SybrGreen assay, gives better results for the detection of invasive crayfish. Although we could not be totally sure of the absence of any individuals in the ponds studied, these highly congruent results reinforced the validity of our method. Reasons are threefold: (i) Primer specificity has a major impact on species detection and insufficient specificity can result in false negative detections (Wilcox et al., 2013). (ii) We maximized the recovery of eDNA by making slight modifications to the protocol described by Tréguier et al. (2014). The same QIAGEN kit was used, but we increased the volume of ATL Buffer, proteinase K, AL Buffer and ethanol. Many other studies have successfully used this kit (Goldberg et al., 2011; Rees et al., 2014; Davy et al., 2015; Deiner et al., 2015; Fukumoto et al., 2015; Sigsgaard et al., 2015; Spear et al., 2015; Thomsen & Willerslev, 2015; Eichmiller et al., 2016). (iii) The eDNA detection method used by Tréguier et al. (2014) can be improved for crayfish detection in ponds, as we showed that qPCR using SybrGreen protocol should be preferred to the TaqMan protocol. Both assays were able to detect crayfish during the mesocosm experiment. However, the fact that qPCR with TaqMan assay failed to detect target species as opposed to qPCR with SybrGreen assay highlights the potential effects of inhibition in the field experiment. The most common method of avoiding contamination is to use dilution. Nevertheless, dilution is not adapted to eDNA studies. As specified by Sigsgaard et al. (2015) and Takahara et al. (2015), dilution will most likely decrease the probability of detection. The TaqMan protocol appears to be more affected by the presence of inhibitors than SybrGreen protocol. Several studies show strong and reliable detection using qPCR with SybrGreen assay (Davy et al., 2015; Libert et al., 2016; Mauvisseau et al., 2017). Our study shows that we can reliably detect invasive crayfish using eDNA detection. This non-invasive method has a promising future for assessing the distribution of various species in freshwater ecosystems.

However, in the future, eDNA detection with droplet digital PCR (ddPCR) could lead to higher and reliable detection rates, as promising results have recently been obtained, but at a high cost (Nathan et al., 2014; Doi et al., 2015a, b; Simmons et al., 2015). It could also be a powerful tool for managing the endangered species. Crayfish monitoring could also be improved through a citizen science scheme (as proposed by Biggs et al., 2014) encouraging water sampling by pond owners. This could increase the sampling area and encourage better communication between pond owners and the staff of the Brenne Regional Natural Park responsible for invasive species control. Involving volunteers could also increase the range of species monitoring using eDNA methods (Biggs et al., 2014). As the Brenne Regional Natural Park contains a huge number of ponds (more than 2000), our method represents a promising avenue for investigating quickly and at reasonable cost the present distribution of the invasive crayfish and for detecting where intensive trapping and integrated management should be focused (Souty-Grosset et al., 2016). Using the Natural Park as a proxy for the detection of invasive crayfish in freshwater ecosystems, we are confident that this method can be successfully used for their detection in ponds located in Western Europe.

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Author contributions Conceived and designed the experiments: Quentin Mauvisseau (QM) and Catherine Souty-Grosset (CSG); assisted with water sampling: Aurore Coignet (AC) and François Pinet (FP). Performed the molecular biology: QM and Carine Delaunay (CD). Analysed the data: QM and Didier Bouchon (DB). Wrote the paper: QM, CSG and DB.

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