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Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds

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Summary

1. The introduction of non-native species is a major threat to biodiversity. While eradication programs of well-established invaders are costly and hazardous for non-target species, the early detection of a non-native species at low density is critical for preventing biological invasions in recipient ecosystems. Recent studies reveal that environmental DNA (eDNA) is a powerful tool for detecting target species in aquatic ecosystems, but these studies focus mostly on fish and amphibians.

2. We examine the reliability of using eDNA to detect the presence of an invasive freshwater crustacean species, the red swamp crayfish *Procambarus clarkii*. Species-specific primers and probes were designed; their specificity was tested using *in silico* PCR simulations and against tissues of other crayfish species. Limits of detection and quantification were specified for the target DNA sequence by means of quantitative PCR amplifications on dilution series of known amount of *P. clarkii* DNA.

3. The method was applied to water samples collected in 158 ponds in a French Nature Park, and results were compared to a traditional method using food-baited funnel traps. Environmental DNA had a better detection efficiency but predominantly led to divergent results compared with the trapping method. While habitat features partly explained the failure of crayfish detection by trapping, detection by eDNA was problematic at low crayfish abundances. When *P. clarkii* was detected, the estimated concentrations of crayfish DNA in water samples were always below the limit of quantification for the target DNA sequence.

4. *Synthesis and applications*. The combination of environmental DNA (eDNA) and conventional trapping methods is recommended to monitor the invasion by *P. clarkii* in small waterbodies such as ponds. However, the risk of mortality for non-target species, notably amphibians, has to be carefully evaluated before large-scale deployment of traps. Contrary to fish and amphibians, a low amount of extracellular DNA in water is suspected to be the major limitation for crayfish detection by molecular approaches. Current advancements in PCR technology, together with optimization of the water sampling method, promise upcoming developments of eDNA detection for aquatic invertebrate species.

Key-words: biological invasions, eDNA, efficiency of detection, funnel traps, limit of detection, limit of quantification, qPCR

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Introduction

The accidental or intentional introduction of non-native species is a major threat to biodiversity, damaging native species and modifying ecosystem processes (Clavero & García-Berthou 2005; Ehrenfeld 2010). Freshwater ecosystems are hotspots of biodiversity as they support almost 6% of all described species although occupying <1% of the earth's surface (Dudgeon *et al.* 2006). In developed countries, freshwater communities and food webs have been profoundly transformed by cumulative invasions in connection with human activities and habitat alterations (Leprieur *et al.* 2008; Cucherousset, Blanchet & Olden 2012). At a regional scale, the ecological network of small waterbodies such as ponds is known to contribute to aquatic biodiversity (Dudgeon *et al.* 2006; Céréghino *et al.* 2008), but it can also facilitate the spread of invasive species. Consequently, anticipating biological invasion threat to small waterbodies should be a priority for management authorities.

The process of biological invasion consists of three consecutive phases: initial dispersal, establishment of self-sustaining populations and spread into the recipient ecosystems (Davis 2009). While eradication programs of well-established invaders are costly and hazardous for non-target species (Gherardi *et al.* 2011; Simberloff *et al.* 2012), a range of measures is recommended to prevent the initial dispersal of species into new recipient habitats (e.g. Davis 2009; Gherardi *et al.* 2011). When prevention fails, however, early detection of new species at low densities is essential to quickly extirpate them. Unfortunately, the detection of target species at low densities is particularly difficult in aquatic ecosystems (Harvey, Qureshi & MacIsaac 2009; Jerde *et al.* 2011). Thus, any field method that improves detectability at low densities will be of great interest, especially if it can be used in large-scale monitoring programs while minimizing impacts on native species.

Environmental DNA (eDNA) has recently been developed to detect molecular evidence of species in aquatic ecosystems (Ficetola *et al.* 2008). Using water samples as the DNA source, several studies have shown that eDNA can improve aquatic species detectability relative to traditional methods, particularly in freshwater ecosystems (Ficetola *et al.* 2008; Goldberg *et al.* 2011; Jerde *et al.* 2011; Dejean *et al.* 2012). Recently, quantitative molecular approaches succeeded in estimating population abundances in controlled experiments as well as in the field (Takahara *et al.* 2012; Thomsen *et al.* 2012a; Pilliod *et al.* 2013a). However, eDNA studies in aquatic environment mostly focused on amphibians and fish, which are known to produce abundant extracellular DNA *via* body mucus secretion (e.g. Livia *et al.* 2006). The applicability of this method for other aquatic species, notably invertebrates with an exoskeleton, has received less attention (Thomsen *et al.* 2012a).

The red swamp crayfish *Procambarus clarkii* (Girard) originates from north-eastern Mexico and South-Central USA. This freshwater crustacean species is qualified as one of the 100 worst invaders in Europe (European Environment Agency 2007). It has detrimental impacts on invaded freshwater ecosystems through predation or competition with native species, or the alteration of habitat characteristics, water quality and other ecosystems services (Geiger *et al.* 2005; Lodge *et al.* 2012). Numerous introductions have caused *P. clarkii* to become a prevailing freshwater crayfish world-wide (Henttonen & Huner 1999). Notably, the ability to disperse overland allows the species to reach and persist in isolated and temporary aquatic habitats and to quickly colonize large areas (Arrignon *et al.* 1999; Cruz & Rebelo 2007; Tréguier *et al.* 2011). For these reasons, it is perceived as a major risk for freshwater ecosystems and biodiversity (Geiger *et al.* 2005; Lodge *et al.* 2012).

The aim of this study was to evaluate the reliability of using eDNA method for detecting red swamp crayfish. We designed primers and probes for *P. clarkii*, tested their specificity and estimated their limits of detection and quantification for the target DNA sequence by means of quantitative PCR amplifications. We applied the method to samples of water collected in the field, and compared eDNA method to a traditional method using funnel traps. The circumstances under which each method performs best were explored, and potential limitations were pointed out. Finally, we discussed advantages and limitations of eDNA and trapping methods for large-scale monitoring of the invasion by *P. clarkii*.

Materials and methods

STUDY AREA

The study was conducted in the Regional Nature Park of Brière (490 km^2), in northwest France (47°23′N, 02°12′W, Fig. 1), where *P. clarkii* is the only crayfish species. It has been present in the marshes of Brière since 1981 when individuals escaped from a nearby crayfish farm (Arrignon *et al.* 1999). Crayfish were observed for the first time in the marshes of Mès in the early 2000s, and abundance is now high in the marshes of Brière (Tréguier *et al.* 2011). A sampling window of 7 km^2 was selected between the marshes of Brière and an inlet of the saltmarshes of Mès (Fig. 1). The study area included a large number of ponds and watercourses in a rural landscape. A total of 158 ponds were monitored with landowners' permission, from 9 May to 26 June 2012. Ponds were located in various surroundings including arable lands, pastures and residential properties, offering contrasting habitat features. Ponds were first sampled with the eDNA method and then using funnel traps within 24 h. This sampling order was chosen because funnel traps might provide a source of crayfish eDNA and thus contaminate subsequent eDNA water samples.

eDNA SURVEY AND ANALYSIS

The sampling protocol was modified from Ficetola *et al.* (2008) to optimize the detectability of *P. clarkii*. At each pond, 40 mL water samples were collected *c.* 1 m from the shoreline at 20

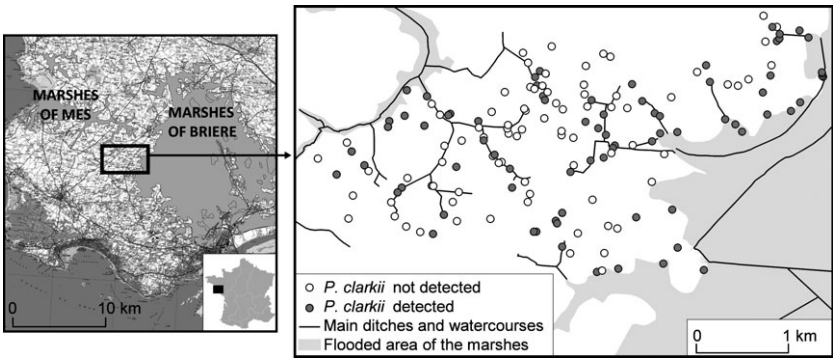

Fig. 1. Map of the study site showing spatial location of the 158 ponds (circles) sampled using eDNA and trapping methods.

locations evenly distributed around the pond. If pond perimeter exceeded 120 m, the 20 samples were taken at *c*. 6-m intervals within the area where trapping was subsequently performed. As *P. clarkii* is a benthic species, samples were collected close to the bottom of the water column after a gentle circular movement to resuspend eDNA fragments and uppermost surface sediments. Adsorption to mineral or organic matters is thought to enhance DNA preservation against degradation by nucleases (Levy-Booth *et al*. 2007), and DNA could be well preserved once absorbed (e.g. Yoccoz *et al*. 2012). For this reason, attention was paid to avoid sampling subsurface sediments that could lead to the detection of ancient DNA fragments (leading to false positive results) in ponds. Samples from the same pond were placed into a common sterile bag and mixed. Six subsamples of 15 mL were extracted and added to 1·5 mL sodium acetate 3 M solution and 33 mL absolute ethanol. All samples were stored at −20 °C. In the laboratory, DNA was extracted following the protocol proposed by Ficetola *et al*. (2008) after slight modifications. The six subsamples per site were centrifuged at 14 000 ***g***, 30 min, 6 °C, and the supernatant was discarded. After this step, 360 μL ATL buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen GmbH, Hilden, Germany) was added to one subsample, the tube was then vortexed and the supernatant was transferred to a second subsample tube. This operation was repeated for all the six tubes. The supernatant in the 6th tube was transferred to a 2-mL tube, and the DNA extraction was performed following the manufacturer's instructions. Finally, owing the invasiveness of *P. clarkii*, it was nearly impossible to be sure that a pond was not colonized in the Nature Park of Brière. For negative controls, the same procedure was run on water samples collected in three ponds of another region of France (Sologne), where *P. clarkii* was unlikely to be present. As expected, crayfish eDNA was not detected in these negative controls.

To design and test primers and probes, DNA of several crayfish species was obtained from tissue collections maintained by the Laboratoire d'Ecologie Alpine (France). First, DNA was extracted from 10 mg of leg tissue samples preserved in ethanol. Then, *P. clarkii* DNA was amplified using primers and probes designed with Geneious 5 (created by Biomatters, available from http://www.geneious.com/) to amplify a 65 bp fragment of the COI region (SPY_ProCla_F 5′-AACTAGGGGTATAGTTGAGAG-3′, SPY_ProCla_R 5′-CAGAAGCTAAAGGAGGATAA-3′ and SPY_ProCla_Probe 5′-FAM-AGGAGTTGGAACAGGATGGACT-MBG-3′). *In silico* PCR was performed using the ECOPCR software (Taberlet *et al*. 2007, available at http://www.grenoble.prabi.fr/trac/ecoPCR) on the EMBL-Bank release 111 (released the 20th March 2012), and the primer pair and probe shown 100% specificity. Additionally, primers and probes were tested *in vitro* against tissues of the five other crayfish species present in western France [one sample of *Astacus astacus* (Linnaeus), one of *A. leptodactylus* (Eschscholtz), two of *Austropotamobius pallipes* (Lereboullet), two of *Orconectes limosus* (Rafinesque) and two of *Pacifastacus leniusculus* (Dana)], and none of these samples was amplified, showing the specificity of the primer pair and probe.

Quantitative PCR was performed in a final volume of 25 μL, using 3 μL of template DNA, 12·5 μL TaqMan® Environmental Master Mix 2.0 (Life Technologies®, Carlsbad, California, USA), 6·5 μL ddH2O, 1 μL of each primer (10 μM) and 1 μL of probe (2·5 μM) under thermal cycling 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. Samples were run in 12 replicates on a BIO-RAD® CFX96 Touch Real-Time PCR detection system (Hercules, California, USA). DNA of *P. clarkii* was extracted from a tissue sample, using DNA blood and Tissue kit (Qiagen®) following the manufacturer's instructions. The DNA extracted was quantified using a NanoDrop (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A dilution series of *P. clarkii* DNA from tissues, ranging from 10^{-2} to 10^{-4} ng μL^{-1}, was used as qPCR standard, and six negative controls (double-distilled H_2O) were performed for each PCR plate. The limit of detection (LOD, the minimum amount of target DNA sequence that can be detected in the sample) and the limit of quantification (LOQ, the lowest level of amount of target DNA that yield an acceptable level of precision and accuracy) were calculated running a dilution series of a known amount of *P. clarkii* DNA, ranging from 10^{-2} to 10^{-8} ng μL^{-1} (10^8 and 100 molecules, respectively) with eight replicates per concentration.

TRAPPING SURVEY

Trapping is the traditional method for sampling active *P. clarkii* (Geiger *et al*. 2005). Two types of funnel traps were used; their design and efficiency were previously tested in a companion study (Paillisson, Soudieux & Damien 2011). The first was a semi-cylindrical funnel trap (length 50 cm, width 29 cm, height 21 cm) of galvanized steel wire (5·5 mm mesh) with two side entrances (inner opening diameter of 4 cm). The second trap design was a collapsible cylindrical funnel trap (length 55 cm, width 17 cm, height 17 cm) of polyamide wire (5-mm mesh) with only one side entrance (inner opening diameter of 5 cm). Traps were baited with a small amount of sausage. The number of funnel traps was standardized according to the perimeter of ponds: 2, 4, 6, 8, 10 or 12 traps were, respectively, used in ponds with perimeters of 0–20, 20–40, 40–60, 60–80, 80–100 and >100 m. When the

perimeter exceeded 120 m, a maximum of 12 traps were set on the stretch that was previously sampled for eDNA. The two funnel trap types were set alternately within each pond. Traps were placed on the bottom of the pond *c.* 1 m from the shoreline and, whenever possible, the top of the trap extended above the water surface to allow non-target trapped species to breathe (aquatic insects and amphibians). Funnel traps were left in the water for 24 h. All captured individuals were counted and identified. The total length of each individual of *P. clarkii* was measured from the tip of the rostrum to the end of the telson.

POND DESCRIPTION

The following habitat features were measured or calculated for each of the 158 ponds (Table 1): perimeter, surface area, volume, maximum water depth, mean shoreline slope and occurrence of leaf litter. Pond perimeter and surface area were calculated using aerial maps with ARCGIS (version 9.2, ESRI, Redlands, California, USA). Water depth 1 m from the shoreline was measured at each funnel trap location, and these values were used to calculate the mean shoreline slope for each pond. The maximum water depth was measured by wading in most cases (129 ponds) or estimated assuming regular sloping banks and symmetric profiles. Pond volume was estimated using maximum water depth and surface area by considering ponds as reverse cones. The presence of leaf litter was checked by a single 30 cm stroke from the pond bottom using a rake at each funnel trap position. The occurrence of litter was calculated as the ratio of strokes with leaf litter present for each pond. In addition to habitat features, complementary data on water quality (water temperature, turbidity, conductivity, pH and dissolved oxygen) collected in July 2011 in a subsample of 82 ponds were integrated into the subsequent statistical analyses. Measurements were performed using portable electronic probes (models Orion 5-STar; Thermo Fisher Scientific Inc. and TN100; Eutech Instruments, Nijkerk, the Netherlands) in the morning to avoid unexpected daily variations in the water quality data set.

DATA ANALYSIS

A series of preliminary analyses based on Spearman rank correlation tests was conducted to calculate the degree of multicollinearity among measured and calculated habitat variables. The perimeter, the surface area, the maximum water depth and the estimated volume of ponds were strongly and positively correlated ($0{\cdot}41 < \rho < 0{\cdot}95$, $P < 0{\cdot}001$, $n = 158$). Similarly, the mean shoreline slope positively correlated with the maximum water depth ($\rho = 0{\cdot}43$, $P < 0{\cdot}001$, $n = 158$) and the estimated volume of the ponds ($\rho = 0{\cdot}24$, $P = 0{\cdot}002$, $n = 158$). Therefore, only three habitat variables were kept in the subsequent models: perimeter, mean shoreline slope and occurrence of litter. Catch-per-unit effort (CPUE, the number of crayfish caught per trap per day) was calculated for each pond where crayfish were trapped. FISAT II software package (version 1.2.2 FAO-ICLARM, FAO, Rome, Italy) was used to apply a modal progression analysis to the total length-frequency distribution data and to classify each individual into body size classes.

Table 1. Description of main habitat features and water quality in the sampled ponds

Variables	Mean	Range	*n*
Habitat features			
Perimeter (m)	72	10–425	158
Surface area (m^2)	476	7–8951	158
Maximum water depth (m)	1·2	0·2–5·8	157
Estimated volume (m^3)	333	1–16 811	157
Mean shoreline slope (°)	19	7–43	158
Occurrence of litter	0·73	0–1	158
Water quality			
Temperature (°C)	13	8–20	82
Turbidity (NTU)	44	3–224	82
pH	7·1	5·3–9·5	82
Conductivity ($\mu S\ cm^{-1}$)	317	81–992	82
Dissolved O_2 ($mg\ L^{-1}$)	5·9	1·1–12·0	82

Ponds where crayfish were not detected by any method could be false negatives (i.e. crayfish present but not detected) or true negatives (i.e. crayfish not detected because absent). To avoid incorporating uncertainty into the evaluation of eDNA and funnel traps reliability for detecting crayfish, data analysis was restricted to the subset of ponds where *P. clarkii* was detected by at least one of the two methods. For eDNA method, a positive detection corresponded to the detection of *P. clarkii* DNA in at least one of the 12 replicates. Generalized linear models (GLMs, binomial error family) were produced to explore the effect of (i) water quality and (ii) habitat variables on crayfish detection by trapping and eDNA methods, successively. Subsequently, GLMs were used on the subset of ponds where crayfish were trapped to explore (iii) the effect of crayfish abundance (CPUE data), body size classes and habitat variables on crayfish detection by eDNA specifically. For each of these three groups of GLMs, all combinations of variables were tested, and the model with best fit was selected according to Akaike's information criterion. AIC was corrected for small sample sizes relative to the number of estimated parameters (AIC_c), which protects against over-fitting models due to small sample size (Burnham & Anderson 2002). The ΔAIC_c was calculated as the difference between the AIC_c values for the focal model and the model with the best fit. As suggested by Richards (2008), a model was selected only if $\Delta AIC_c \leq 6$, and its AIC_c was less than the AIC_c of all the simpler models within which it was nested. AIC_c weights ($wAIC_c$), that is an estimate of the probability that the focal model is chosen as being the best fit model if the study is repeated (Burnham & Anderson 2002), were also calculated for each model. The percentage of deviance explained was calculated based on residual deviance in each focal model and the deviance of the null model. Finally, the Kappa coefficients, that is the proportions of ponds correctly classified as either present or absent after accounting for chance (Manel, Williams & Ormerod 2001), were calculated to evaluate the degree of concordance of each model, following the scale proposed by Landis & Koch (1977): 0·81–1·00: almost perfect, 0·61–0·80: substantial, 0·41–0·60: moderate, 0·21–0·40: fair, <0·20: fail. All analyses were conducted using R (version 3.0.1, R Foundation for Statistical Computing, Vienna, Austria).

Results

Laboratory tests with a known amount of *P. clarkii* DNA demonstrated that the LOQ in this study was 10^{-4} ng μL^{-1}. *Procambarus clarkii* DNA can still be detected at a concentration of 10^{-8} ng μL^{-1}, with one qPCR replicate up to eight showing a positive result. This concentration was set as the LOD (Fig. 2).

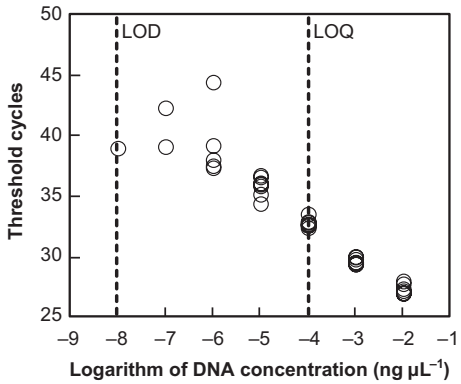

Fig. 2. Limit of quantification (LOQ) and limit of detection (LOD) of the crayfish *Procambarus clarkii*. These limits were calculated from a dilution series of known amounts of *P. clarkii* DNA and eight replicates per concentration. Threshold cycles represent the minimum number of PCR amplifications leading to positive detection.

A total of 1066 funnel traps were set, and 948 water samples of 15 mL were collected *in situ*. A total of 1285 crayfish were trapped, including 628 small (21–76 mm), 626 large (77–129 mm) and 31 dead individuals that were partly consumed. The abundance of *P. clarkii* was 2·9 crayfish per trap per day on average, but varied greatly between ponds (from 0·1 to 13·1 crayfish per trap per day). By combining results of trapping and eDNA analyses, crayfish were detected in 78 of the 158 ponds (49·4%, Fig. 1). Among these 78 ponds, crayfish were trapped in 51 ponds (65%), and eDNA analyses were positive in 57 ponds (73%). However, only 30 ponds (38·5%) were positive for both methods, meaning that trapping and eDNA methods predominantly led to divergent results. Crayfish DNA was detected in 59% of the ponds where crayfish were trapped, and conversely, crayfish were trapped in 53% of the ponds where crayfish DNA was detected. In ponds where *P. clarkii* was detected by eDNA method, the estimated concentrations of crayfish DNA were always below the LOQ, meaning that eDNA quantification was not possible.

For *P. clarkii* detection efficiency by trapping, the best model indicated that the probability of trapping significantly increased with turbidity, but this result was not further considered since the concordance of the model failed ($\kappa < 0{\cdot}20$, Table 2). Conversely, model that used habitat variables explained 19·9% of the total deviance, and its concordance was fair (Table 2). This model revealed higher probability of trapping when the pond perimeter increased and the occurrence of litter decreased (Fig. 3a, b). Notably, the probability of *P. clarkii* detection by trapping was below 50% when the pond perimeter was less 40 m (Fig. 4a). By contrast, the probability of crayfish detection by eDNA was not explained by any water quality or habitat variables (Table 2). Using CPUE data, the best model for crayfish detection by eDNA explained 39·2% of the total deviance of data, and its concordance was moderate (Table 2). The probability of detecting *P. clarkii* was higher when the abundance of crayfish increased (Fig. 3c). In particular, the probability of *P. clarkii* detection by eDNA was below 50% when its abundance was <2 crayfish per trap per day (Fig. 4b). Similarly, correct eDNA detection of *P. clarkii* was consistently observed in ponds where small individuals were trapped (Fig. 3d).

Discussion

The present study examines the reliability of using environmental DNA to detect the presence of an invasive crustacean, *P. clarkii*, in ponds of the Regional Nature

Table 2. Generalized linear models (binomial error family) testing the probability of *Procambarus clarkii* detection by trapping and eDNA analysis using water quality variables, habitat variables, crayfish abundance and body size classes

	n	*K*	AIC_c	ΔAIC_c	$wAIC_c$	DEV (%)	κ
Detection by trapping							
Water quality variables							
Turbidity	39	2	41·4	0·0	0·25	12·1	<0·20
Null model	39	1	44·2	2·8	0·06	0·0	<0·20
Habitat variables							
Perimeter + Occurrence of litter	78	3	86·9	0·0	0·65	19·9	0·24
Perimeter	78	2	90·8	3·9	0·09	13·9	0·37
Detection by eDNA							
Water quality variables							
Null model	39	1	51·8	0·0	0·15	0·0	<0·20
Habitat variables							
Null model	78	1	92·9	0·0	0·21	0·0	<0·20
Crayfish abundance							
Abundance + Proportion of small crayfish	51	3	48·5	0·0	0·34	39·2	0·55
Abundance	51	2	52·9	4·4	0·04	29·6	0·56

K is the number of estimated parameters in the model; AIC_c is the Akaïke's information criterion corrected for small sample sizes; ΔAIC_c is the difference between the AIC_c values for the model and the model with the best fit; $wAIC_c$ is an estimate of the probability that the focal model is chosen if the study is repeated; DEV is the percentage of total deviance explained by the model; Kappa coefficient (κ) represent the proportions of ponds correctly classified as either present or absent after accounting for chance. See text for details.

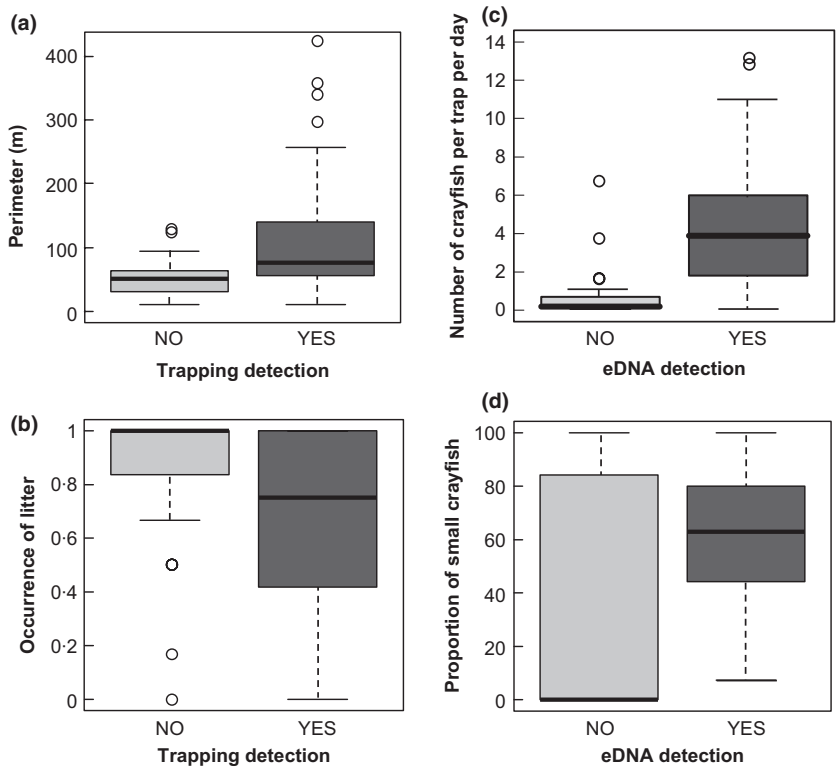

Fig. 3. Efficiency of crayfish detection by trapping (a, b) and eDNA (c, d) according to significant variables in the generalized linear models.

Park of Brière (France) where it was introduced 30 years ago. Detection efficiency was slightly better than conventional trapping method (73% vs. 65% of the 78 ponds in which *P. clarkii* was detected); however, crayfish DNA in water samples was detected in only 59% of the ponds where crayfish presence was observed by trapping. We found that trapping was less efficient in small ponds with abundant leaf litter and that detection efficiency by eDNA was significantly reduced in ponds where crayfish abundance was low. Moreover, the estimated concentrations of crayfish DNA in water samples were always below the limit of quantification for the target DNA sequence. To the best of our knowledge, our study is the first to report such limitations of eDNA to detect an invertebrate species in aquatic environments.

Fish and amphibian species have received most of the attention for developing eDNA methods in aquatic environments. Successful detection is reported in more than 80% of cases when collecting only three samples of water (45 mL in total) per pond (Ficetola *et al.* 2008; Dejean *et al.* 2012; Thomsen *et al.* 2012a). PCR amplifications can be successfully used to quantify eDNA in water and provide reliable estimations of the abundance of amphibians and fish (Takahara *et al.* 2012; Thomsen *et al.* 2012a; Pilliod *et al.* 2013a). Moreover, aquaria experiments showed that DNA fragments disappear in water in <1 month after the species removal (Dejean *et al.* 2011; Thomsen *et al.* 2012a,b), meaning that eDNA detection provides information on the contemporary presence of the species. Conversely, adsorption of DNA to mineral or organic matters may enhance its preservation in bottom sediments (Levy-Booth *et al.* 2007), and attention was paid in our study to avoid sampling subsurface sediments that could have led to the detection of ancient DNA fragments (false positive) in ponds.

Despite our large sampling effort (20 samples of 40 mL in each pond), detection efficiency of crayfish by eDNA was poor (59%) in ponds where the presence of crayfish was confirmed by trapping. Compared with conventional trapping method, eDNA performed better in small and shallow ponds where sampling effort was higher than for large ponds, and where crayfish DNA was more concentrated in a smaller volume of water. With the eDNA method in such small ponds, however, it is possible that false positives marginally occurred from dead or transient individuals. To clarify this point, additional trapping was performed in six ponds where crayfish were initially detected by eDNA, but not by trapping, 1 month after the main experiment in July 2012. Number of traps was increased by five, and crayfish were trapped in three additional ponds. This suggests false positives are unlikely with the eDNA method. Similarly, the efficiency of eDNA detection was improved at high crayfish abundance, confirming that detection is probably more consistent when a sufficient concentration of crayfish DNA is reached in the water. Nevertheless, in all the samples, the amount of target DNA was lower than the DNA concentrations that yield an acceptable level of precision and accuracy for quantification (i.e. below the LOQ, 10^{-4} ng μL^{-1}), meaning that a proper estimation of crayfish abundances by PCR amplifications was not possible in our study.

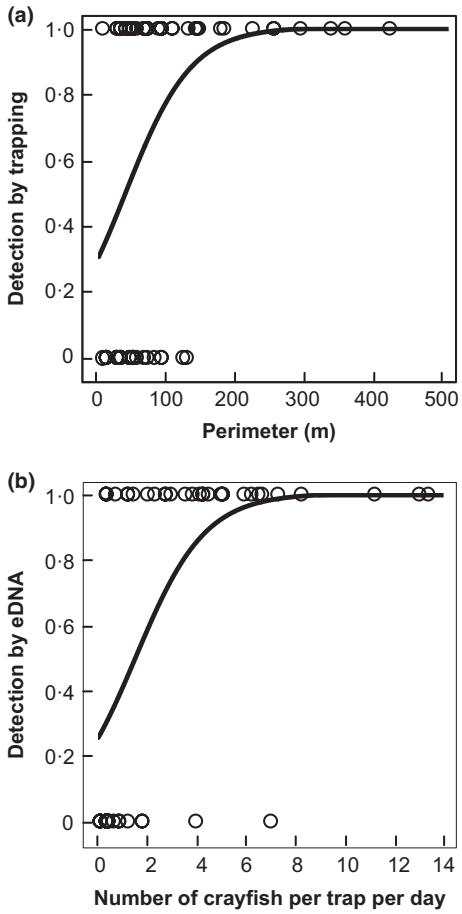

Fig. 4. Probability of crayfish detection by trapping (a) and eDNA (b) according to the most informative variables in the generalized linear models. White circles correspond to the observed success or failure of detection.

Epidermal cells of fish and amphibians produce abundant mucus that is known to be a significant source of DNA (Livia *et al.* 2006). Contrary to vertebrates, aquatic arthropods have an exoskeleton of chitin, sometimes combined with calcium carbonate to produce a stronger composite. This exoskeleton may reduce the release of extracellular DNA in water and hamper the detection of these species using molecular approaches. Information on the use of eDNA to detect aquatic arthropod species is rare in the literature. Thomsen *et al.* (2012a) reported that the dragonfly *Leucorrhinia pectoralis* and the tadpole shrimp *Lepidurus apus* were detected in more than 80% of the freshwater ponds with 100% occurrence of species confirmed by dipnetting. However, species abundance was not reported, and it is possible that ponds with low population densities were not included in the survey. Moreover, the quantity of DNA released into the water may vary not only among aquatic arthropod species, but also with the age of individuals in the pond. We observed that eDNA detection was more efficient in ponds containing a large proportion of small crayfish. *Procambarus clarkii* has a rapid growth rate, and moulting is frequent for small individuals (Reynolds 2002). Moulting possibly enhances the release of DNA in water by arthropods. Similarly, exuviae are known to be a source of DNA (Watts *et al.* 2005), and numerous juvenile exuviae in a pond may increase DNA concentration in water. Additional experiments would be needed to further identify the mechanisms of DNA release by aquatic arthropods, and to quantify possible differences between species. Finally, the effect of sampling methods (e.g. sampling location, time of day, volume of water sampled) on eDNA detection efficiency should also be investigated for invertebrate species as it has recently been done for amphibians (Pilliod *et al.* 2013a).

The main limitation of the eDNA approach for crayfish detection was the low amount of DNA retrieved in the samples. Most of the protocols for eDNA sampling in aquatic ecosystems that have been proposed until now are based on point sampling (review by Pilliod *et al.* 2013b). In our study, we collected water samples from 20 different locations in the same pond (800 mL in total), but the optimal solution would probably be to sample several litres of water to maximize the chance of detecting rare extracellular DNA molecules. One promising improvement has recently been proposed by Goldberg *et al.* (2011) for detecting amphibians in headwater streams: 5–10 L of water were sampled using a flow-through filter with a peristaltic pump, each filter being preserved in ethanol for subsequent molecular analyses at laboratory. Moreover, the DNA detection and quantification should be improved in the near future thanks to a new technology that has been commercialized recently, the digital PCR or dPCR (Vogelstein & Kinzler 1999; Hindson *et al.* 2011). This dPCR technology allows the absolute quantification of DNA. dPCR is more appropriate than classical qPCR to the detection of small amounts of DNA molecules, and it is also more tolerant to inhibitors and does not need to rely on references or standards (Whale *et al.* 2012). For the moment, dPCR has only been used for medical application (e.g. Hindson *et al.* 2011; Whale *et al.* 2012), but its ability to detect rare alleles could be translated for the detection of rare species.

Looking beyond technical difficulty associated with the quantity of DNA released into water, eDNA surveillance has advantages over conventional trapping methods for detecting the presence of invasive crayfish *P. clarkii* in aquatic environments. Trapping surveys may require decontamination of the equipment to limit possible transmission of pathogens between studied sites, especially the crayfish plague *Aphanomyces astaci* and the chytrid fungus *Batrachochytrium dendrobatidis* (Phillott *et al.* 2010; McMahon *et al.* 2013). Trapping provides biological information on sex, age and body size while eDNA does not, but non-target species can be accidentally trapped too. In our study, unwanted catches were mostly insects (diving beetles, *Dytiscidae*), but some amphibians were occasionally also caught, notably the agile frog tadpole *Rana dalmatina* (Fitzinger), the palmate newt *Lissotriton helveticus* (Razoumowsky) and the marbled newt *Triturus*

marmoratus (Latreille). They were returned to water after trapping, but drowning deaths and evidence of predation by crayfish were observed in funnel traps. In contrast, eDNA surveillance is a totally non-invasive method, easy to implement in the field, and allows for large-scale surveys without collateral damage to aquatic fauna.

In conclusion, our study reveals that eDNA performs better for detecting crayfish in small and shallow ponds where trapping is not optimal. However, food-baited funnel traps appear to be more efficient in deeper ponds and when crayfish abundance is low. Consequently, the combination of these two methods appears to be a good option to monitor the invasion by *P. clarkii* in small waterbodies such as ponds. However, the risk of mortality for non-targeted species, notably amphibians, has to be carefully evaluated before using conventional trapping methods, particularly in deep ponds. If eDNA was to be developed to survey *P. clarkii* colonization at a large scale, collecting water samples during the growing period is recommended, that is when crayfish juveniles and subadults moult and release more DNA in the water. Finally, current advancements in PCR technology, together with optimization of water sampling methods, promise upcoming developments of eDNA surveillance for aquatic invertebrate species.

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Data accessibility

Detection of *Procambarus clarkii* by funnel traps and environmental DNA depends on pond characteristics and crayfish abundances: DRYAD entry doi: 10.5061/dryad.0ck3q (Tréguier *et al.* 2014).

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