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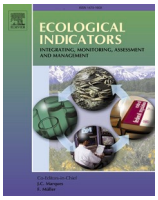

Mapping a super-invader in a biodiversity hotspot, an eDNA-based success story

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ABSTRACT

The lesser Antilles archipelago in the Caribbean is known as a biodiversity hotspot, hosting many endemic species. However, recent introduction of a highly invasive species, the Australian redclaw crayfish (*Cherax quadricarinatus*), has led to significant threats to this fragile ecosystem. Here we developed, validated, and optimized a species-specific eDNA-based detection protocol targeting the 16S region of the mitochondrial gene of *C. quadricarinatus*. Our aim was to assess the crayfish distribution across Martinique Island. Our developed assay was species-specific and showed high sensitivity in laboratory, mesocosm and field conditions. A significant and positive correlation was found between species biomass, detection probability and efficiency through mesocosm experiments. Moreover, we found eDNA persisted up to 23 days in tropical freshwaters. We investigated a total of 83 locations, spread over 53 rivers and two closed water basins using our novel eDNA assay and traditional trapping, the latter, undertaken to confirm the reliability of the molecular-based detection method. Overall, we detected *C. quadricarinatus* at 47 locations using eDNA and 28 using traditional trapping, all positive trapping sites were positive for eDNA. We found that eDNA-based monitoring was less time-consuming and less influenced by the crayfishes often patchy distributions, proving a more reliable tool for future large-scale surveys. The clear threat and worrying distribution of this invasive species is particularly alarming as the archipelago belongs to one of the 25 identified biodiversity hotspots on Earth.

1. Introduction

Introduction of invasive alien species in an environment often lead to an ecological disaster, caused by their significant negative impacts on indigenous communities and ecosystems (Dunn et al., 2017; Robinson et al., 2018). These effects are often worse in an island environment (Myers et al., 2000). Indeed, these areas are very sensitive due to their isolation, and any disturbance can have rapid and irreversible negative effects. Diffusion of invasive species often leads to a decrease in biodiversity, a result due to predation, food competition (Momot, 1995; Šidagytė et al., 2017) or spread of associated pathogens (Grandjean et al., 2017; Robinson et al., 2018). As an example, numerous crayfish species have been translocated in freshwater systems throughout the world, mainly for aquaculture purpose or as a results of recreational aquarium activities (Souty-Grosset et al., 2006; Lodge et al., 2012). Often associated with an omnivore regime, these ecosystem engineer species have profound negative impacts on biodiversity where they are introduced and the ecosystems in general (Reynolds et al., 2013).

Among them, *Cherax quadricarinatus* also known as the 'Australian redclaw crayfish' or 'tropical blue' are showing an increasing trend, regarding geographical spread (Mendoza-Alfaro et al., 2011). Native from Australia and Papua New-Guinea, *C. quadricarinatus* was introduced in a number of tropical and subtropical regions for aquaculture purposes (Mendoza-Alfaro et al., 2011). Further this species is highly appreciated by aquarists due to its blue colour. Following escape from ponds or via direct human releases, *C. quadricarinatus* is now known to

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quickly establish itself due to its ease of breeding, fast growth rates and high tolerance regarding water quality (Tropea et al., 2010). This is particularly worrying, as invasive crayfish are known to affect the trophic chain and modify their environment due to their opportunistic and omnivorous behaviour (Jackson et al., 2017; Lang et al., 2020). Indeed, Mendoza-Alfaro et al. (2011) highlighted a strong impact on local macroinvertebrates by direct predation after the introduction of *C. quadricarinatus* in Thailand. These effects are even more exacerbated on tropical islands, which are often regarded as biodiversity hotspots, hosting small and fragile native populations (Vitousek, 1988; Myers et al., 2000).

On Martinique Island, the freshwater shrimp species *Macrobrachium rosenbergii* was originally farmed in the North-Atlantic part of the island but a tense ecotoxicological context led to the closure of many aquaculture farms. The area suitable (non-contaminated) for the aquaculture was the North-Caribbean part, in smaller ponds, making the farming of *M. rosenbergii* unprofitable. As an alternative, *C. quadricarinatus* was imported from Cuba in 2004, aimed at reinvigorating aquaculture practices (Baudry et al., 2020). In 2015, the presence of this crayfish was observed in three rivers and one closed water system. In 2018, a larger survey, using baited-traps, confirmed the presence of 10 'new' populations (Baudry et al., 2020). However, these precedent surveys were relying on traditional monitoring methods such as electrofishing and baited-traps. Therefore, the species distribution was thought to be underestimated. Indeed, conventional methods for monitoring freshwater biodiversity often rely on electrofishing, gillnetting, snorkelling, trapping, or kick-sampling (Manfrin et al., 2019). These techniques require large sampling effort, therefore are time consuming, labour intensive, and expensive in addition to often being ecologically invasive (Hänfling et al., 2016; Harper et al., 2018; Robinson et al., 2018). As a result, traditional sampling methods have often been shown to lead to false negative detection of the monitored species, in particular when a species is present at relatively low density (Keller and Kumschick, 2017).

Effective management plans rely on the knowledge of species presence and the use of molecular-based detection has recently been shown as a reliable alternative for monitoring species in aquatic habitats (Rees et al., 2014). Environmental DNA detection (or eDNA), relies on the detection of DNA in pieces of skin, eggs, mucus left by organisms in their environments, without the need to physically observe the targeted species, at any stage of life (Ficetola et al., 2008; Thomsen and Willerslev, 2015). eDNA-detection based techniques have been shown to be very effective, making them promising tools for the early detection of both invasive and/or rare, endangered endemic species (Hänfling et al., 2016; Harper et al., 2018). eDNA has now been used extensively, to track the presence of invasive crayfish for example; *Procambarus clarkii*, *Pacifastacus leniusculus* and *P. virginalis* (Tréguier et al., 2014; Harper et al., 2018; Mauvisseau et al., 2019b) and for endangered crayfish species such as *Austropotamobius pallipes* (Atkinson et al., 2019; Troth et al., 2020).

Here, we aimed to evaluate the potential of eDNA-based detection for mapping the distribution of *C. quadricarinatus* throughout the island of Martinique. We developed and validated a species-specific real-time quantitative PCR (qPCR) assay amplifying a 109 bp fragment of the 16S region of *C. quadricarinatus*. We further investigated its reliability and efficiency under controlled laboratory conditions and within mesocosms with variable biomass treatments. Finally, a large field survey (combining both eDNA detection and traditional monitoring assessment) was performed on a total of 83 locations across Martinique. We now present a precise distribution map of *C. quadricarinatus* presence and discuss its impacts on autochthones species. This led to an optimised eDNA protocol being established for the detection of *C. quadricarinatus* in Martinique ecosystems. Finally, we assessed the efficiency of DNA-based detection as reliable monitoring tool compared to traditional methods and provide recommendations for future surveys.

2. Material and methods

2.1. Study area

Martinique is a French volcanic island of 1128 km^2 belonging to the Lesser Antilles, in the eastern Caribbean Sea/Atlantic Ocean. This area, identified as one of the 25 biodiversity hotspots, harbour a large number of endemic species (Anadón-Irizarry et al., 2012).

The Martinique hydrographic network is vast, and encompass 70 main rivers, fed by numerous tributaries, most of which are permanent (Martinique, 2018).

2.2. Sampling sites

In this study, we sampled a total of 83 locations spread over 53 rivers, one ornamental pond and one water retention dam, from September 2019 to December 2019. To allow an exhaustive validation of our assay, we sampled locations known to be positive, negative, or unknown for the presence of *C. quadricarinatus*. All sampled locations were further investigated using traditional crayfish trapping to compare the efficiency between eDNA-based detection and previously established methods. All locations were investigated twice: each time, cassava-baited traps were set at the end of the day and raised in the morning.

Filtration occurred on-site, after water collection from the river. A hand-operated vacuum pump (Nalgene™ Repairable Hand-Operated PVC Vacuum Pumps with Gauge) was utilised, together with a 1L-filtration unit (Nalgene™) (Lawson Handley et al., 2019) and nitrocellulose filters (Sartorius® 47 mm diameter and 0.45 μm size pore). Water samples were collected from the middle of the water body, along a transect originating from the margin outwards, using a decontaminated plastic bottle and non-powdered gloves (Cowart et al., 2018). This was immediately poured in the funnel of the unit. Filtration occurred until the filter was clogged, and the volume filtered was recorded at each sampled location. The filter was then removed and placed (folded in quarters) into 1.5 mL tube using sterile forceps and preserved with 1 mL of absolute molecular-grade ethanol. Three natural replicates (i.e. independent eDNA water samples) were collected at each sampling location.

To avoid potential field cross-contamination, filtration units and sampling bottles were decontaminated using 50% bleach after each sampling site and thoroughly rinsed using tap water. Furthermore, 1000 mL of distilled water (blank control sample) was also filtered at each site, before conducting eDNA sampling in order to account for potential field contamination (following the protocol above). All samples were stored in a cooling bag until return to the laboratory and stored at −20 °C until DNA extraction.

2.3. Mesocosm

Controlled mesocosm experiments were conducted to: (i) assess a potential correlation between eDNA detection and quantification related to various abundance and biomass and (ii) determine eDNA persistence in water under controlled conditions.

Five treatments, consisting of: (A) 1.33 g/L of biomass (corresponding to one individual of 40 g in a 30 L aquarium); (B) 3.33 g/L of biomass (corresponding to two individuals of 50 g each in a 30 L aquarium); (C) 33.33 g/L of biomass (corresponding to 15 individuals of 1000 g of total biomass in a 30 L aquarium); (D) approximately 50 g/L of biomass (corresponding to one ton of crayfish biomass in a 20 m^2 tank) and (E) approximately 3.75 g/L of biomass (corresponding to three tons of crayfish biomass in a farming pond of 1000 m^2). Water samples were collected and filtered as described above two days after crayfish introduction. For the three 30 L aquarium conditions, water temperature and pH were maintained at 27 °C and 7.5 respectively, corresponding to optimal values for *C. quadricarinatus* development (Tropea et al., 2010). This was modulated using a 12 h light/dark photoperiod. Each

treatment was conducted in duplicate (i.e. two identical 20 m^2 mesocosms), two independent water samples filtered (two biological replicates per mesocosm) and two qPCR replicates per biological replicate (i.e. a total of eight qPCR replicates per treatment).

To test persistence of eDNA in the water over time, samples from treatments A and B were collected again after crayfish removal. Water collection and filtration occurred three, eight, 13, 17, 23 and 28 days after crayfish removal, following the methods described above.

2.4. DNA extraction protocol from filters

DNA extractions were performed using Qiagen DNeasy® Blood & Tissue Kit following manufacturers guidelines with minor modifications (Appendix A).

2.5. qPCR assays

Species-specific primers and probe, targeting a 109 bp fragment within the 16S region of *C. quadricarinatus* (Forward: 5′- TGG AGG CTG GAA TGA ATG G-3′, Reverse: 5′- GGT CTT ATC GTC CCT CTA A-3′ and specific 6-FAM MGB labelled probe 5′- TGG ACG AGA AGG AAG CTG TC-3′) were developed. Primer design, *in-silico* and *in-vitro* tests were performed following Troth et al. (2020) (Appendix B). Optimization of primers and probe concentration was processed using different concentrations ranging from 0.5 μM to 10 μM. Annealing temperature for qPCR was also optimized by testing a gradient of temperature ranging from 55 °C to 65 °C.

2.6. qPCR treatments

Each natural replicate (i.e. water sample) was analysed in four technical replicates (qPCR replicates) to obtain a final number of 12 replicates per sampling 'station' following methods outlined in Appendix C.

Standard curves were generated using DNA extracted from *C. quadricarinatus* (20 ng/μL, quantified using a NanoDrop® 1000 Spectrophotometer) following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al., 2009), and concentrations ranged from 5 $ng.\mu L^{-1}$ to 2×10^{-8} $ng.\mu L^{-1}$. According to Agersnap et al. (2017), positive signals were considered when a Ct value < 42 (i.e. considered 'false positive' if above). A site was identified as 'invaded by *C. quadricarinatus*' if at least one replicate of the 12 technical replicates (per station) was positive (Bedwell and Goldberg, 2020).

2.7. Limit of detection (LOD) and Limit of Quantification (LOQ)

In order to align with the MIQE Guidelines (Bustin et al., 2009), Limit of Detection (LOD) and Limit of Quantification (LOQ) must be determined. For that, the serial dilutions used for standard curves generation (16 dilutions in total), were treated in 10 replicates by qPCR. LOD corresponds to the lowest concentration at which one replicate of a dilution can be amplified by qPCR at a threshold cycle (Ct) < 45. LOQ corresponds to the lowest concentration at which targeted DNA can be quantified in a minimum of nine replicates of qPCR with Ct < 45 (Mauvisseau et al., 2019a).

2.8. Optimal protocol

From our data, which consisted of three natural replicates (i.e. water samples) and four technical replicates (i.e. qPCR reactions) per natural replicate, we explored the optimal level of sampling and replication needed to detect *C. quadricarinatus* in Martinique. For this, we tested the detection efficiency with one, two and three natural replicates and between two and four technical replicate per natural replicate.

2.9. Analyses

Maps were generated using QGIS 2.18 (Las Palmas) software (QGIS Development Team, 2016): Martinique map was imported from the database ©IGN and the streams from BD Carthage® and BD Topo®. Watersheds were created from Field Numeric Model (BD Alti®) performing the "r.watershed" function in Grass Gis 7 software and the six main invaded watersheds were extracted.

Statistical analyses were all performed using RStudio v1.1.463 (Core Team R Development, 2019), all field data are averaged by station in Appendix D. Two hypotheses were tested:

(i) Do environmental parameters have an influence on the detection probability of *C. quadricarinatus*?
(ii) Does water volume filtered have an influence on the detection probability of *C. quadricarinatus*?

Shapiro-Wilk normality test used to verify the normal distribution of the data. We ran site occupancy modelling approaches to assess effects of environmental covariates on presence of *C. quadricarinatus* eDNA and to estimate detection probability of our assay (MacKenzie et al., 2002; Royle and Dorazio, 2009). Covariates tested included: (i) volume of water filtered (potentially leading to an increase of target DNA or inhibition), (ii) pH, (iii) Oxygen concentration, (iv) Oxygen saturation, (v) Temperature, and (vi) Conductivity. Analyses were performed using the 'eDNAoccupancy' package in R (Core Team R Development, 2019). Model selection, and interpretation, followed procedures given in Dorazio and Erickson (2018). Our model was fitted using the 'occModel' function from the described package. MCMC chains ran for 10,000 iterations for obtaining parameter estimates and credible intervals.

The model developed by Griffin et al. (2019) allowed us to estimate false positive and false negative errors, and was applied to our data following instructions under the defaults settings. Analyses were performed using the website (https://seak.shinyapps.io/eDNA/), with the presence/absence of crayfish as covariate. Probability of species presence (ψ), probability of eDNA presence given species presence ($\theta 11$ or true positive field sample), probability of eDNA presence given species absence ($\theta 10$ or false positive field sample), probability of eDNA detection given eDNA presence ($p11$ or true positive qPCR detection), probability of eDNA detection given eDNA absence ($p10$ or false positive qPCR detection) were investigated. To investigate the influence of crayfish biomass on detection efficiency, Student t-tests were performed under RStudio v1.1.463 (Core Team R Development, 2019).

3. Results

3.1. qPCR assays and protocol optimization

The primers and probe successfully amplified *C. quadricarinatus* extracted DNA and were found to be species-specific (i.e. no amplification was observed using DNA extracted from the other freshwater crayfish species tested: *A. pallipes, P. leniusculus, P. clarkii* and *C. destructor*). After qPCR optimization, optimum yields were observed using a final concentration of 5 μM for both primers and probe with an annealing temperature of 58 °C.

The mean amplification value (Ct Value) for DNA extracted from *C. quadricarinatus* tissue was 15.63 for a concentration of 60 $ng.\mu L^{-1}$ (measured with NanoDrop® 1000 Spectrophotometer). Limit of Detection was found to be 1.19×10^{-6} $ng.\mu L^{-1}$ (2/10 replicates) and Limit of Quantification 1.91×10^{-5} $ng.\mu L^{-1}$ (9/10 replicates). Concentration of *C. quadricarinatus* DNA in a sample can be correlated to the crossing point of amplification with the equation: $y = 21.004–3.3525x$.

3.2. Mesocosm

Mesocosm experiments revealed a significant influence of crayfish

biomass on the detection efficiency by qPCR, with mean Ct values ranging from 29.52 ± 1.55 (Treatment C, 1000 g of crayfish) to 34.24 ± 0.76 for treatment A (one crayfish or 40 g) (t = 8.100, df = 10.171, p-value < 0.001). Further there was significant differences between the 40 g aquarium mesocosm vs one ton pond (t = 5.381, df = 8.496, p-value < 0.001) and the 40 g vs three tons raised pond (t = 11.064, df = 13.641, p-value < 0.001) (Fig. 1). There were no significant differences between the 40 g aquarium mesocosm vs the 100 g aquarium mesocosm or between the 1000 g aquarium mesocosm and one ton pond or three tons raised pond (Fig. 1).

There was a decrease in the detection probability over time, once the crayfish were removed across both treatments. For treatment A this started at 100% at Day 0, followed by 50% after 13 days, 12.5% after 23 days and 0% after 28 days (Fig. 2A). For treatment B, DNA was successfully amplified during all 28 days of the experiment: detection probability reached 100% up to 8 days and decreased to 50% after 17 days, then 12.5% after 28 days. (Fig. 2B). These results were mirrored by an increase of Mean Ct value (starting from 37.32 ± 2.49 to 40.47 ± 0 for treatment A and from 38.25 ± 0.88 to 40.91 ± 0 for treatment B) (Fig. 2).

3.3. eDNA detection in situ and comparison with traditional method

Among the 83 sampled field sites, *C. quadricarinatus* eDNA was detected in 47 locations (i.e. 56.6% of sites surveyed). This corresponds to 23 rivers and 2 closed water basins. Crayfish were caught with traps only in 28 sites (33.7% of those surveyed), and all these were positive using the eDNA-based survey (Fig. 3). Six watersheds were therefore classed as being 'invaded', with detection at most of the prospected sites: 'Lezarde', 'Case-Navire', 'Rivière Salée-Coulisses', 'Capot', 'Galion' and 'Rivière Pilote' (Fig. 3). None of these stations had previously shown to be positive using traditional methods. No amplification occurred in control samples (distilled water filtered on the field) and in negative samples, showing no cross-contamination occurred. The total volume filtered by station varied from 300 mL to 7500 m.

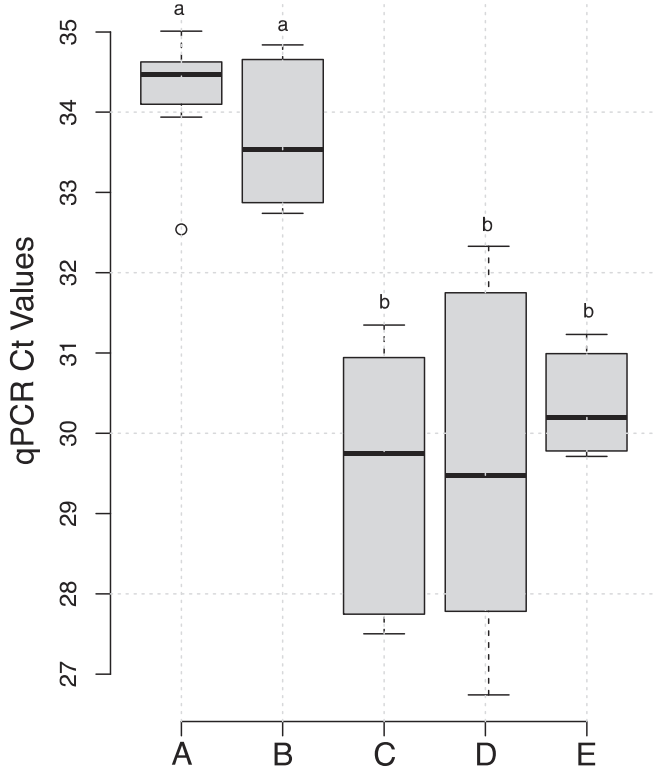

Fig. 1. Influence of crayfish biomass, under mesocosm conditions, on qPCR detection efficiency. Treatment (A) correspond to 40 g of total biomass (one crayfish) in 30 L aquarium, (B) 100 g of total biomass (two individuals) in 30 L aquarium, (C) 1000 g of total biomass in 30 L aquarium, (D) Approximately one ton of total biomass in a 20 m^2-decontamination pond and (E) Approximately three tons of total biomass in a farming pond of 1000 m^2. Symbols a and b showed the significant statistical difference between each treatment.

3.4. Covariates influence on detection probability

A significant effect of the total volume filtered per station on the probability of eDNA occurrence was shown (F = 9.879; p-value: 0.002). Detection probability decreased when the total volume of water filtered increased (see Fig. 4A). Optimal detection probability was observed when between 300 and 2000 mL water was filtered (Fig. 4A). A decrease of detection, up to 50%, was observed with a total volume of 4000 mL and the eDNA occurrence was < 10% when filtering up to 7500 mL. Furthermore, we also found that the variability of total eDNA measured increased with the total volume of water filtered. Other parameters recorded and investigated (pH, conductivity, oxygen and temperature) were not found to have an impact on the species presence, highlighting its high tolerance regarding water quality in freshwater habitats (Fig. 4).

3.5. False negative and false positive errors

Posterior mean values of the *C. quadricarinatus* presence probability Ψ (0.514) and the probability of false positive field sample θ_{10} (0.115) are in accordance with expected values (respectively 0.5 and 0.11) (Table 1). The probability of 'true positive' field samples (θ_{11}), is overestimated in comparison with expected value (0.986 vs 0.88) (Table 1). The probability of 'true positive' qPCR detection (p_{11}) and false positive qPCR detection (p_{10}) are lower than expected values given by the model (p_{11}: 0.778 vs 0.9 and p_{10}: 0.001 vs 0.1) (Table 1).

3.6. Optimal protocol

When only one field-filtered biological replicate was utilised with one technical replicate, we were unable to detect crayfish with any level of confidence. When two technical replicates were used, the assays efficiency was significantly higher (87.71%). This increased even further with four technical replicates (97.76%). Processing two biological replicates in contrast (each with two qPCR replicates) saw a detection efficiency as high as 98.88%. 100% efficiency was achieved when four qPCR replicates were used, each with two biological replicates and/or when three biological replicates were used with either two or four qPCR replicates (Fig. 5).

4. Discussion

Our study was conducted to explore the feasibility of using environmental DNA to detect *C. quadricarinatus* with high reliability. In order to ensure this reliability in this novel assay, we followed the validation steps outlined by Thalinger et al. (2020). The specificity of our primers was initially assessed *in-silico*, followed by *in-vitro* validation using DNA extracted from other closely related crayfish species. The designed assay showed high sensitivity and the Limit of Detection and Limit of Quantification were in accordance with other recently optimized studies on freshwater invertebrates (Mauvisseau et al., 2019a, 2019b).

Mesocosm experiments indicated that biomass had a positive effect on the detection efficiency and demonstrated that eDNA persisted (once the animal was removed) for at least 21 days (when one crayfish was present per 30 L) and 28 days (two crayfish present). Our results are in accordance with Minamoto et al. (2012) who showed a long persistence of fish eDNA in cold water (upwards of a month at 17 °C). This result can be surprising, as other studies shown an increase of the eDNA degradation with an increase of the water temperature (Robinson et al., 2018).

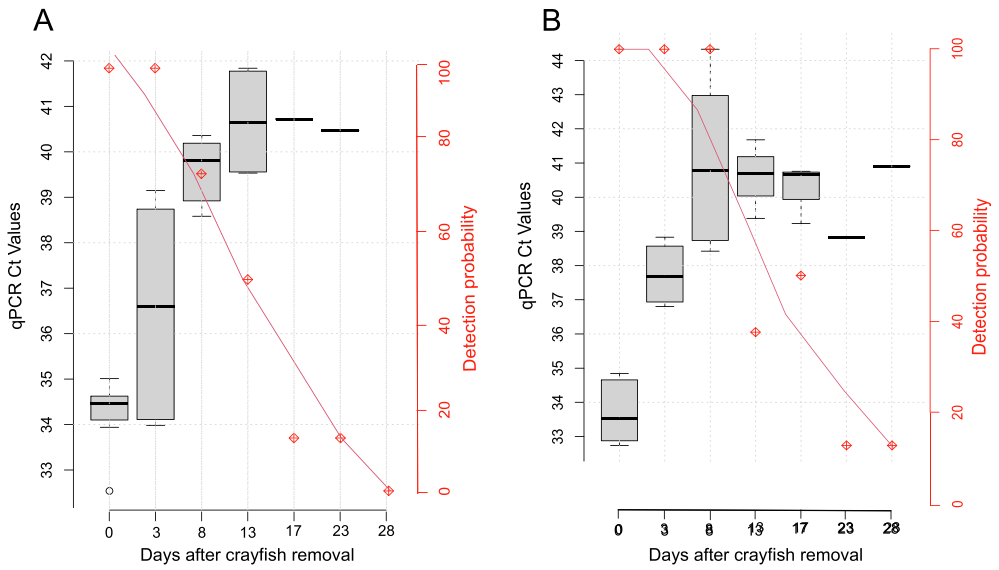

Fig. 2. Evolution of qPCR Ct values and proportion of qPCR positive replicates (in red) following removal of one crayfish (A) or two crayfish (B) in a 30L container after 28 days of experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

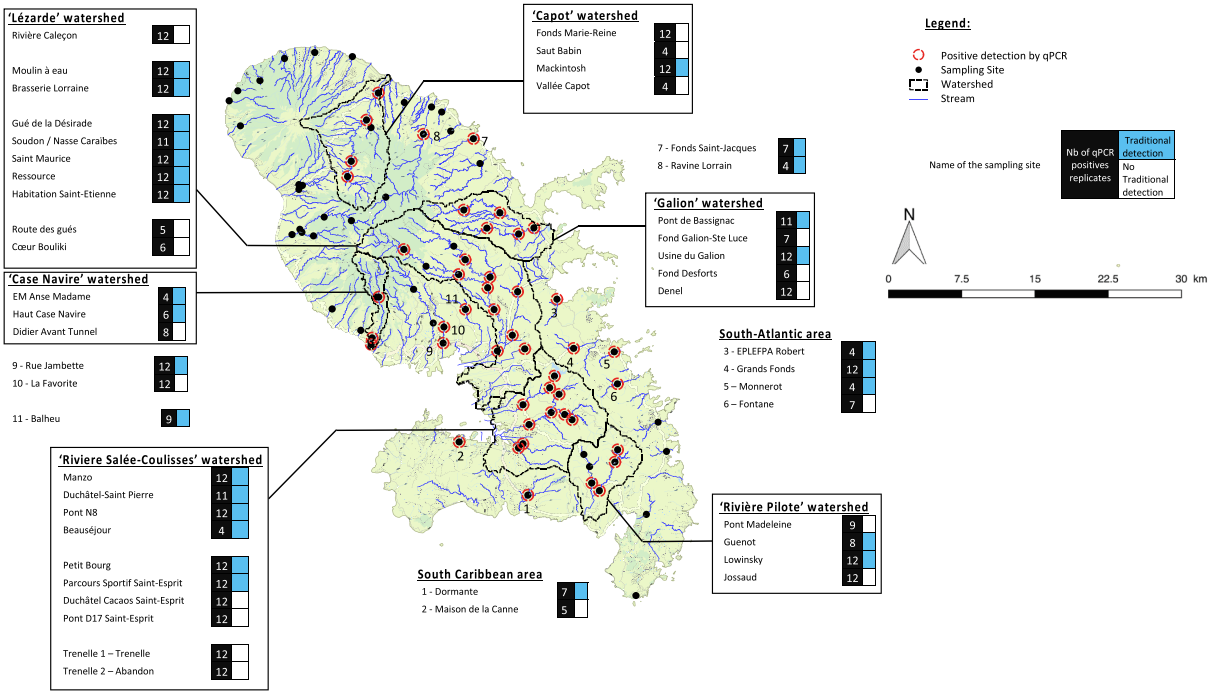

Fig. 3. Detection of *Cherax quadricarinatus* in Martinique. Black points on the map represent the 83 sampling sites while red-surrounded points show *Cherax quadricarinatus* presence. The black squares in legend represent *Cherax quadricarinatus*-specific qPCR positive amplification with the number of qPCR positive replicates (total of 12 technical replicates). All sites were surveyed by traditional trapping method and blue squares are the sites where *Cherax quadricarinatus* have been detected with this method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, with eDNA persistence being higher than 20 days, there is a decrease in the probability of false negatives results often associated with rapid eDNA degradation (Li et al., 2019). That said, other environmental parameters associated with false negatives may well still play important roles here, such as flow rates (Deiner and Altermatt, 2014). However, the long detection efficiency may result in false positives if (or when) the crayfish has left the surveyed site or died. That said, recent evidence suggests dead crayfish release little to non-detectable eDNA traces (Curtis and Larson, 2020), and so this should be of least concern with the reliability of the assay.

Our mesocosm experiments also showed a positive correlation between biomass and detection efficiency. This is in accordance with findings from Dougherty et al. (2016) who showed 95% detection probability, with low sampling effort, when sampling the rusty crayfish (*Faxonius rusticus*) at moderate-to-high densities (CPUE from 1.52 to > 10). Interestingly, in our study, eDNA detection remained successful at locations where the CPUE was relatively low, again this reflects other studies on *Pacifastacus fortis*, or *Astacus astacus* and *P. leniusculus* for example (Cowart et al., 2018; Strand et al., 2019).

As part of our survey data we were also able to further test the reliability of the assay by utilizing known presence/absence of *C. quadricarinatus* across a number of sites (Guan et al., 2019). From 83

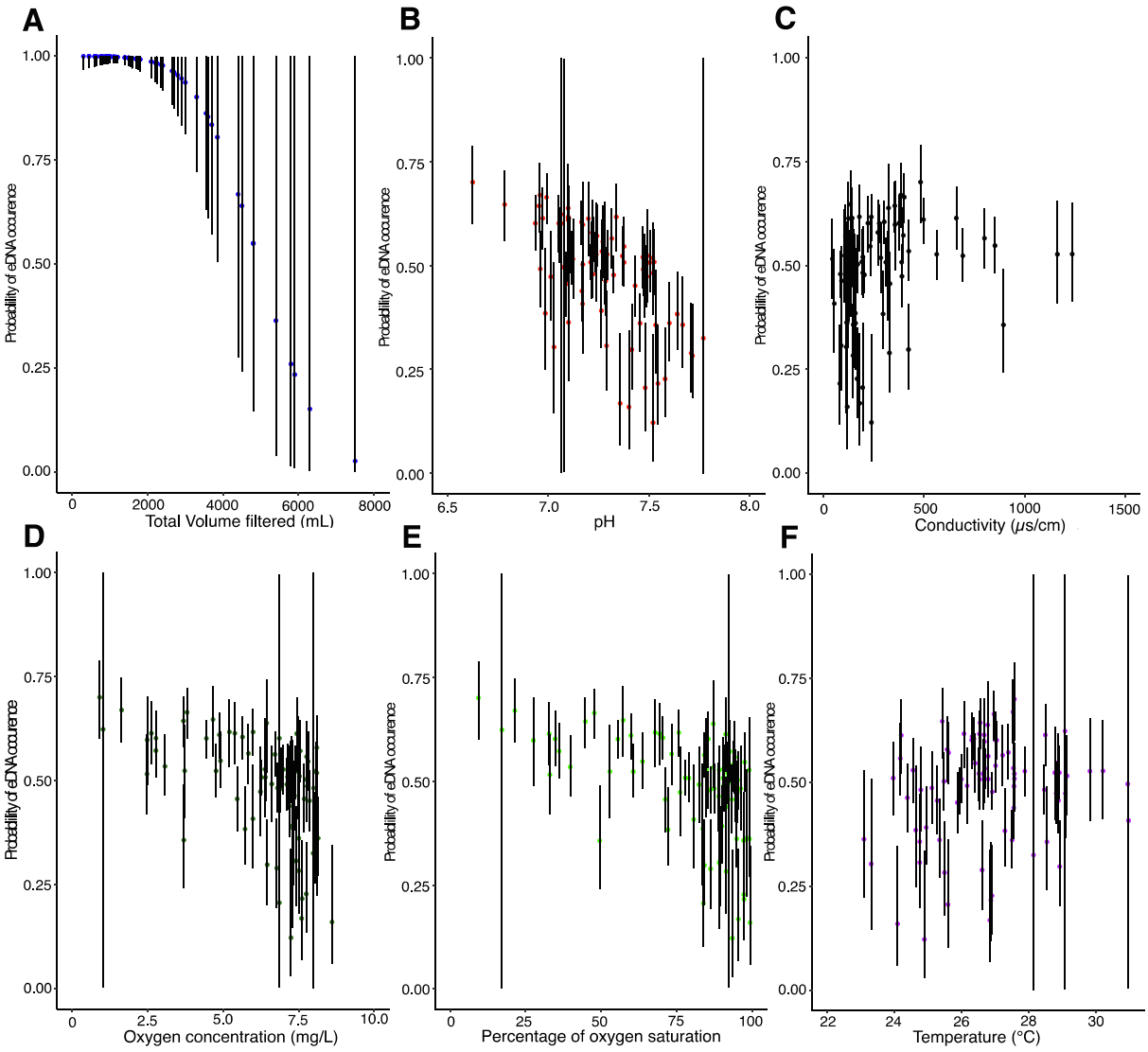

Fig. 4. Influence of covariates: (A) Total volume filtered, (B) pH (C) Conductivity, (D) oxygen concentration, (E) Percentage of oxygen saturation and (F) Temperature on the probability of *C. quadricarinatus* eDNA occurrence following site occupancy modelling.

Table 1
False negative and false positive rates in eDNA-based detection.

	2.5 Credible Interval	Posterior Mean	97.5 Credible Interval	Expected Value
Ψ: Probability of species presence	0.324	0.514	0.664	0.5
θ_{11}: Probability of true positive field sample	0.916	0.986	0.999	0.88
θ_{10}: Probability of false positive field sample	0.00002	0.115	0.372	0.11
p_{11}: Probability of true positive qPCR detection	0.741	0.778	0.811	0.9
p_{10}: Probability of false positive qPCR detection	6×10^{-5}	0.001	0.007	0.1

surveyed sites, 47 were positive for eDNA. Out of the 28 sites found positive using trapping, all were shown to be positive for eDNA indicating 100% efficiency of the assay when mapped to the traditional survey methods. This is similar to other studies who also focused on a number of different invertebrate species (Dougherty et al., 2016; Harper et al., 2018; Atkinson et al., 2019; Troth et al., 2020). However, interestingly, one study showed much lower efficiency values (59%) when detecting *P. clarkii* in freshwater ponds in France (Tréguier et al., 2014).

We were also interested in exploring what was the desired number of natural and/or technical replicates need to give optimal efficiency (Mauvisseau et al., 2019a, 2019b). This is particularly important when working with crayfish over fish for example, as crustaceans release less DNA (Dougherty et al., 2016). Here, we clearly showed that with only one field-filtered biological replicate, we were unable to detect any crayfish with any confidence. According to our results, the optimal protocol in terms of detection efficiency and lab experiments is three field-filtered biological replicates and two qPCR technical replicates per biological replicate.

Finally, we explored the possibilities of tackling the issues around potential false negatives and false positives using modelling (Griffin et al., 2019). Here, we were able to ascertain the influence of covariates (total volume filtered per station, oxygen saturation, oxygen

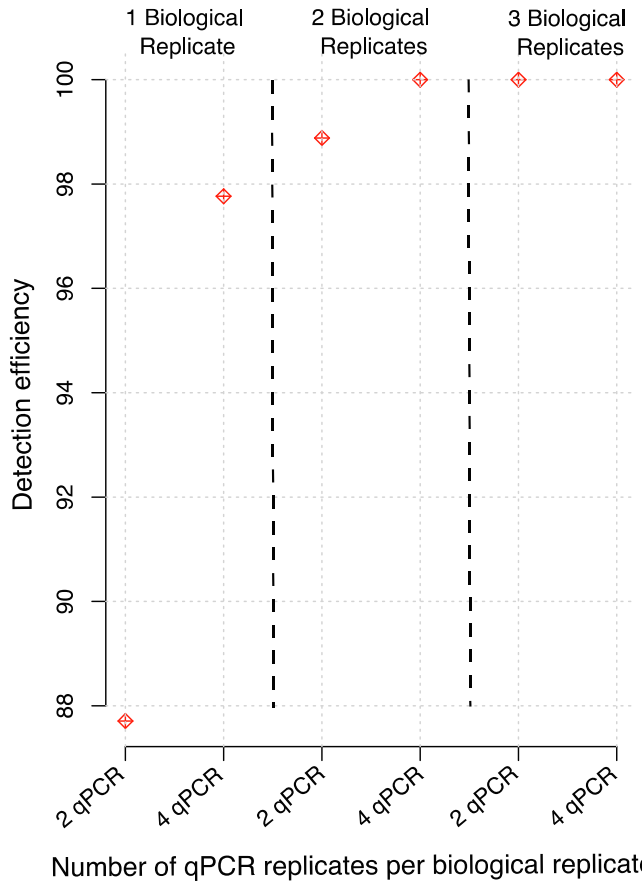

Fig. 5. Exploring the optimal protocol for eDNA detection of *Cherax quadricarinatus* in Martinique. *In-situ* detection efficiency of *C. quadricarinatus* with one, two or three biological replicates (filtrations on the field) followed by either two or four qPCR replicates per filter.

concentration, pH, temperature and conductivity) on eDNA detection efficiency. Interestingly, we found that optimal detection yields were obtained after filtering a maximum of 2000 mL spread over three natural replicates at any given site. These results differ from other studies that often highlight an increase of species detection rates when processing larger volumes: up to 30 L by site for Strand et al. (2019), and 34 to 340 L for Cantera et al. (2019) for example. In our study, we used relatively large pore size filters (0.45 μm), to facilitate an increase of the total volume filtered. Smaller pore sizes lead to an increase of retrieved suspended matter, rapidly clogging them (Bedwell and Goldberg, 2020). However, increasing the volume filtered, could lead to a increase of the amount of total eDNA captured (targeted and non-targeted). In particular, increased columns often results in increases in particular matter captured on the filter and or may have a dilution effect on the target DNA. Indeed, various studies have already highlighted the influence of turbidity on the detection efficiency for example, potentially leading to false negative results in particular (Cowart et al., 2018; Strand et al., 2019). The other covariates (oxygen, pH, temperature and conductivity) were not correlated with the detection efficiency. However, when considering temperature, Moyer et al. (2014) showed a decrease of detection of 1.7 times when the temperature rises by 1.02 °C. In our case, this contradictory finding may be due to *C. quadricarinatus* high tolerance regarding these environmental parameters (Tropea et al., 2010). However, regardless of the lack of effect associated with these covariates we were able to show that the probability of detection could be overestimated (probability of true positive field sample θ_{11}: 0.986 while expected value is 0.88) or underestimated (probability of true positive qPCR detection p_{11} and false positive qPCR detection p_{10}: respectively 0.778 and 0.001 while expected values are 0.9 and 0.1).

Nevertheless, such over or under estimation would likely be consistent regardless of technique used to survey and we conclude by highlighting the usefulness of this novel eDNA based assay for surveying *C. quadricarinatus*. Worryingly, this study highlights that *C. quadricarinatus* is present across much of Martinique. However, the impacts of this invasive species remain unknown. Invasive crayfish in general are known to negatively affect the trophic chain at all levels through competition or predation (Loureiro et al., 2015; Jackson et al., 2017; Lang et al., 2020) and various studies have shown a strong decrease of invertebrates after the introduction of invasive crayfish (Jackson et al., 2014; Loureiro et al., 2015; Lang et al., 2020). In our study, crayfish seem highly abundant in some sites such as the Lezarde river for example. These high densities, coupled with an absence of predators able to control populations point to the likelihood of critical loss of the biodiversity in the very near future, unless active management and control of this species is undertaken. The first record of this species in Martinican streams was only in 2009 during electrofishing surveys (Pers. Com., ODE Martinique), another survey, targeting directly this crayfish species, described 10 sites where populations of *C. quadricarinatus* were found (six streams and four closed water bodies) (Baudry et al., 2020). Here, we presented 47 identified sites populated by *C. quadricarinatus* (23 rivers and two closed water basins), indicating the species is widely distributed throughout Martinique. Only 36 sites appear to be 'free' from the species. How long these will remain as such, is a difficult question to answer. Monitoring these crayfish-free stations, using the eDNA assay developed here will now enable rapid detection of newly colonised sites and therefore allow for a mitigation strategy to be implemented before populations get too large and out of control (Gherardi et al., 2011; Havel et al., 2015; Early et al., 2016). A citizen science approach could also be applied here, aiming to educate locals around the risk of release and escapes.

5. Conclusion

Here we have developed and validated (through *in-silico*, *in vitro*, mesocosm and *in-situ* experiments) a novel assay to detect the highly invasive crayfish species *C. quadricarinatus*. We utilised this assay to assess the current invasion of this species across Martinique, a biodiversity hotspot in the Caribbean tropical area. We found a positive relationship between crayfish biomass and eDNA detection probability and illustrated that eDNA persisted up to three weeks in the absence of the crayfish, even in the warm tropical waters of the island. This update, in the distribution of *C. quadricarinatus* across Martinique can now be used by management as a way to try and contain the invasion and to protect invasive-free watersheds.

CRediT authorship contribution statement

Thomas Baudry: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Validation, Project administration, Funding acquisition. **Quentin Mauvisseau:** Conceptualization, Methodology, Software, Formal analysis, Writing - review & editing, Visualization. **Jean-Pierre Goût:** Supervision, Writing - review & editing, Project administration, Funding acquisition. **Alexandre Arque:** Supervision, Writing - review & editing, Project administration, Funding acquisition. **Carine Delaunay:** Methodology, Validation. **Juliette Smith-Ravin:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **Michael Sweet:** Writing - review & editing, Supervision, Funding acquisition. **Frédéric Grandjean:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2021.107637.

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