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Short Communication

Using eDNA to detect freshwater invasive non-native species under controlled conditions

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Abstract

Invasive Non-Native Species (INNS) are a principal threat to global biodiversity and the early detection of new introductions is key to facilitate a rapid response to limit the risk of establishment and spread. Related to this, there is growing interest in using environmental DNA (eDNA) for INNS detection. The applied use of eDNA in ecology is, however, in its relative infancy with few species for which regulator-approved methods are available. Here, laboratory trials were conducted to investigate the use of commercially available eDNA assays for detecting two high priority INNS in the UK, killer shrimp (*Dikerogammarus villosus*) and signal crayfish (*Pacifastacus leniusculus*), at different population densities. For killer shrimp, DNA of the expected fragment size was detected from all three trials where animals were maintained at a density of 1 per 1 L but species confirmation by Sanger sequencing was only possible for one of these replicates. Whilst capillary electrophoresis detected DNA of the expected fragment size in serially diluted samples down to a density of 1 shrimp in 100 L, this could not be confirmed by sequencing. Signal crayfish DNA was detected by qPCR in all four trials where animals were housed at a density of 1 per 10 L. Using serial dilutions, it was possible to detect signal crayfish in samples representing a density of 1 per 1,000 L, however this was unreliable. These results demonstrate the potential for using eDNA as a detection method for killer shrimp and signal crayfish but also highlights that detectability is species/assay specific and dependant on population density. The insights gained will help inform decisions surrounding the appropriateness of using these eDNA assays, and improve interpretation of any results generated.

Key words: signal crayfish, *Pacifastacus leniusculus*, killer shrimp, *Dikerogammarus villosus*, INNS, IAS

Introduction

Invasive non-native species (INNS) are considered one of the greatest causes of biodiversity loss worldwide (Gurevitch and Padilla 2004; Didenham et al. 2005; Jaureguiberry et al. 2022). Therefore, there is a growing demand for effective and efficient INNS detection methods. Recently, there has been increasing interest in the use of environmental DNA (eDNA) as a means of species monitoring and early detection, as genetic material can be obtained from samples such as water, soil or sediment, originating from a

variety of sources including urine, faeces, shed cells and gametes (Rees et al. 2014; Thomsen and Willerslev 2015). Current approaches use PCR-based methods or metabarcoding analysis of eDNA to either detect a single species/taxon or conduct a community biodiversity assessment for a given taxonomic group, respectively.

One of the key benefits of using eDNA as opposed to traditional survey methods, such as electrofishing, kick-sampling or trapping to detect aquatic INNS, is that eDNA is generally more sensitive, making it possible to detect species at lower population densities and thus capture a wider range of biodiversity (Dejean et al. 2011; Takahara et al. 2012; Rees et al. 2014; McDevitt et al. 2019; Blackman et al. 2020; Fediajevaite et al. 2020). This is important for INNS monitoring, especially for recently introduced species which may only be present at low densities, where early detection is critical to minimise the impacts on the recipient ecosystem (Hulme 2006). However, as the applied use of eDNA in ecology is still relatively in its infancy, there are few taxa for which standardised testing methods are available. Therefore, there remains significant uncertainty around the reliability of eDNA as a detection method and to what extent this varies between species, habitats, and environmental conditions.

Here, laboratory trials were conducted to investigate the efficacy of commercially available eDNA assays for detecting, at different population densities, two freshwater INNS considered as high priority within the UK (Water Framework Directive UK Technical Advisory Group 2021), killer shrimp (*Dikerogammarus villosus* Sowinsky, 1894) and signal crayfish (*Pacifastacus leniusculus* Dana, 1852). The assays used were based on the methods published by Blackman et al. (2018) and Harper et al. (2018) for killer shrimp and signal crayfish respectively. The first aim of the study was to determine whether the positive detections achieved by Blackman et al. (2018) and Harper et al. (2018) could be replicated at comparable population densities. Secondly, related to the interest in using eDNA as an early warning system for INNS introductions, assays were tested at lower population densities than in the original studies. Overall, this study aims to improve understanding of the reliability of existing commercially available eDNA assays for killer shrimp and signal crayfish detection in applied scenarios.

Materials and methods

Study animal collection and maintenance

All animal work in the aquatic laboratories followed Home Office licence PPL 8167141 guidelines and therefore as part of routine maintenance, all experimental animals were assessed daily for health status. Any moribund, and all experimental animals, were humanely euthanised by freezing at −80 °C at the end of the study period.

Killer shrimp

Killer shrimp were collected from an established population in Cardiff Bay, Wales (NGR: ST 19213 73491) using a combination of manual removal from mooring lines and sweep sampling with a long-handled dip net in September 2021. Within 2 hrs, animals were transported in secure containers to the Cable Aquatic Laboratories within Cardiff University School of Biosciences where, they were maintained for the duration of the trials.

In the laboratory, species identity was confirmed using a standard dissecting microscope. At this stage, overall health of the animals was also checked. Outwardly healthy adult specimens (size range: 6.6–9.7 mm) were transferred into 70 L glass housing tanks within a temperature-controlled room (15 ± 1 °C) at a density of < 2 shrimps per 1 L. Tanks were filled with 35 L dechlorinated water, which was continuously aerated. Refugia in the form of artificial plants and plastic mesh were added to the housing tanks for welfare purposes. Animals were checked daily, fed with a mixture of Spirulina powder (*Arthrospira* sp.) and water fleas (*Daphnia* sp.), with any deceased animals and excess food removed immediately. Shrimp were acclimated under these conditions for at least 1 week prior to trials commencing.

Signal crayfish

Signal crayfish were collected in September 2021 from two established populations in Wales: Dderw Farm Pools, Llyswen, Brecon (NGR: SO 13913 37537) and Rhydlydan Pools, Painscastle, Glasbury (NGR: SO 16832 45675). All animals were collected using "trappy traps" and under license from Natural Resources Wales. Traps were baited with commercial cat food and left *in situ* for 24 hrs. Upon collection, all specimens were confirmed as *P. leniusculus* before being transported in secure containers to the Cable Aquatic Laboratories where they were maintained for the duration of the trials. Crayfish were transported and housed under the appropriate Invasive Alien Species (Enforcement and Permitting) Order (2019) licence.

In the laboratory, all crayfish were visually inspected for health and outwardly healthy adult specimens were measured (carapace length range: 30.0–53.0 mm) and transferred into 350 L plastic housing tanks within a temperature-controlled room (15 ± 1 °C) at an approximate density of 1 crayfish per 7 L. Where possible, animals were maintained at a higher female to male sex ratio and smaller animals were housed separately. Tanks were filled with 200 L dechlorinated water, which was continuously aerated. Refugia in the form of plant pots and artificial plants were added to the housing tanks for welfare purposes. Animals were fed daily with frozen peas and any deceased animals, moulted exoskeletons or dropped limbs were removed. Crayfish were acclimated under these conditions for at least 1 week prior to trials commencing.

eDNA trials and sample collection

To minimise contamination risks, experimental tanks were set up in a separate laboratory to the housing tanks. Experimental tanks and all work surfaces were disinfected with a 10% bleach solution and thoroughly rinsed with distilled water before use. No substrate or refugia were added to the experimental tanks.

Trials were conducted separately for each species and comprised 10 size-matched killer shrimp or signal crayfish, which were not in moult or, except for one crayfish replicate, carrying eggs. Killer shrimp were housed in an experimental tank containing 10 L of dechlorinated water (i.e., at a density of 1 shrimp per 1 L of water) whereas signal crayfish were housed in an experimental tank containing 100 L of dechlorinated water (i.e., at a density of 1 crayfish per 10 L water). For crayfish trials, an even number of male and female crayfish was used, except for one trial which was conducted using berried females (i.e., females carrying eggs) only. Trials were replicated using different batches of shrimp three times and crayfish four times. For each species, a single control trial was also conducted (i.e., there were two negative control trials in total) using a tank set up in the same manner as described but containing only water (i.e., no killer shrimp/signal crayfish).

For each trial, animals were kept within the experimental tank for 24 hrs to allow release of DNA (previous studies indicate that this is sufficient time to achieve positive detection; Davison et al. 2016; Blackman et al. 2018; Harper et al. 2018). After 24 hrs, all animals were removed and the water in the experimental tanks was mixed using a sterile spatula. Samples for eDNA analysis were then collected immediately after mixing. As per the guidelines of the molecular analysis provider, each sample was prepared by filtering 1 L of water from the experimental tanks through Sartorious Sartolab P20 Plus with prefilter and a 0.45 μm polyethersulfone filter membrane using an ezDNA pump sampler.

Immediately after the initial samples were filtered, an additional 1 L of water was collected from each experimental tank, including the killer shrimp but not signal crayfish control, and added to 9 L of molecular grade water in a sterilised container to create a 1 in 10 dilution. For killer shrimp this represented a density of 1 animal in 10 L whereas for crayfish it was 1 animal in 100 L. This process was repeated 10 or 9 times for killer shrimp and signal crayfish respectively to achieve a final dilution density reflective of 1 animal in 10,000 million L. From this final dilution, 7.5 L of water was removed and diluted with 2.5 L of molecular grade water to achieve a dilution density reflective of 1 animal in 25,000 million L, which is representative of the size of a large reservoir in the UK. No serial dilution was created for the signal crayfish control replicate as it was decided that a single control (i.e., the killer shrimp control replicate) would be sufficient to detect potential contamination introduced through the dilution process.

eDNA assays

All DNA analyses were performed by a reputable commercial provider based in the UK. DNA extraction was conducted using QIAGEN DNEasy Blood and Tissue kits in accordance with manufacturer instructions. All analyses included eDNA extraction blanks and appropriate negative PCR controls. Further details of the PCR assays used for each species are provided in the sections below.

Killer shrimp

The collected eDNA samples were tested for killer shrimp using an end point PCR method developed by Blackman et al. (2018), which was the only published assay for the targeted detection of killer shrimp using eDNA-based methods at the time of testing. PCR was performed in a total reaction volume of 25 µl: 13.4 µl H_20, 3 µl buffer (5X), 2.5 µl $MgCl_2$ (25 mM), 2 µl dNTPs (4 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.1 µl ALLin™ Taq DNA polymerase and 2 µl template DNA. Primer sequences were as follows, forward: DV1_F 5'-TCTTGGCAGGTGCCAT TACG-3', reverse: DV1_R 5'-GAATAGGATCACCCCCGCCT-3'. Cycling conditions included an initial denaturation step at 95 °C for 5 min followed by 45 cycles at 95 °C for 30 s, 65 °C for 60 s and 72 °C for 60 s, with a final extension time of 10 min at 72 °C.

PCR products were initially investigated by capillary electrophoresis using a QIAGEN QIAxcel Advanced system to determine whether target DNA had been amplified. Samples positive for target DNA were then cleaned using a 2-step process in preparation for Sanger sequencing. Given the small size of the amplicons from this assay (~ 87 bp), amplicons were extended using Illumina adapters to initiate Sanger sequencing. The gene sequences obtained were characterised using the Basic Local Alignment Search Tool of the target amplicon sequence against the NCBI GenBank (Sayers et al. 2018) non-redundant nucleotide database.

Signal crayfish

The collected eDNA samples were tested for signal crayfish through qPCR run using a Bio-Rad CFX Connect real-time PCR system. The assay used was the TaqMan fluorescent probe-based method developed by Harper et al. (2018) which has been validated *in silico, in vitro* and *in situ.* qPCR was performed in a total reaction volume of 25 µl: 7 µl H_20, 12.75 µl TaqMan environmental master mix (2x), 0.25 µl probe (10 µM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 3 µl template DNA. Primer sequences were as follows, forward: qPlCOIF 5´-ATAGTTGAAAGAGGA GTGGGTACT-3´, reverse: qPlCOIR 5´-TAAATCAACAGAAGCCCCTG CA-3´, probe: FAM-5´-CCTC CTCTAGCAGCGGCTATTGCTCATGC-3´-BHQ1. Cycling conditions included an initial denaturation step at 95.3 °C

for 5 min followed by 60 cycles at 95 °C for 20 s and 60 °C for 20 s at which point fluorescence was measured. The limit of detection for the assay is 0.00167 pg/µL in water samples and the limit of quantification is 0.167 pg/µL.

As an additional confirmatory step all samples determined as positive from the qPCR reaction were sequenced using Sanger sequencing and only samples that returned as a 97.44% or greater Pairwise Identity (this is the percentage of pairwise DNA residues that are identical in the alignment of the query and reference sequences) to signal crayfish were considered as positive. Prior to sequencing samples were cleaned using a 2-step process and amplicons were extended using Illumina adapters to initiate Sanger sequencing (given that the amplicon size for this assay is only ~ 87 bp). The gene sequences obtained were characterised using the Basic Local Alignment Search Tool of the target amplicon sequence against the NCBI GenBank (Sayers et al. 2018) non-redundant nucleotide database.

Results

Killer shrimp

Bands of the expected fragment size were detected via electrophoresis in all the samples collected from trials where animals were held at a density of 1 in 1 L inferring the presence of killer shrimp (Supplementary material Table S1). Killer shrimp presence was confirmed in one of these samples (DV25) by Sanger sequencing with 100 % Pairwise Identity. Useable sequences could not be obtained from the two other 1 in 1 L treatment group replicates (Table S1). As such, killer shrimp detection cannot be confirmed in these samples.

For the dilution series, bands of the expected fragment size were detected via electrophoresis in all three samples collected from animals housed at 1 in 10 L (DV02, DV14 and DV26) and in one sample collected from animals housed at 1 in 100 L (DV03; Table S2). Whilst these bands infer the presence of killer shrimp, no useable sequences could be obtained from any of these samples (Table S1), so killer shrimp presence could not be confirmed.

No bands of target DNA were observed from any of the samples collected from the negative control trial (Table S1).

Signal crayfish

Signal crayfish DNA was confirmed in all four trial replicates for animals held at a density of 1 in 10 L (Table S2). The mean (all samples were tested in triplicate) Cq value (the number of PCR cycles at which the sample amplification reaction reached a threshold level of detection) ranged from 37.5–44.9 where lower Cq values indicate higher initial copy numbers of target DNA (Table S2).

For the dilution series, signal crayfish were detected down to a density of 1 in 1,000 L however this was only for one treatment group replicate (PL03, mixed males and females; Table S2). The mean Cq value for this sample was 43.9. At the 1 in 100 L dilution, positive detection was only achieved in treatment groups one (mixed males and females), three (berried females) and four (mixed males and females) (Table S2). The mean Cq value for these samples ranged from 41.2–46.2 (Table S2).

No amplification above the baseline threshold was observed for the sample from the negative control trial (Table S2).

Discussion

For killer shrimp, it was only possible to confirm detection in a single 1 in 1 L density sample. This is congruent with the findings of Blackman et al. (2018) who failed to detect killer shrimp held at a density lower than 1 in 3 L using the same assay. In the current study, however, detection was inferred via electrophoresis in samples representative of a 1 animal in 100 L density. These findings suggest that, with assay refinement, there may be greater potential for detection of killer shrimp using eDNA at densities lower than 1 in 3 L. For signal crayfish, whilst detection was only reliable for the 1 crayfish in 10 L samples, it was possible to detect signal crayfish in one sample representative of a 1 animal in 1,000 L density using the eDNA assay developed by Harper et al. (2018). Previously, this assay had been used to successfully detect signal crayfish held in a laboratory at a density of 1 animal per 5.5 L but, to our knowledge, had never been tested at densities lower than this (Harper et al. 2018).

Despite the single positive detection obtained for signal crayfish maintained at a density of 1 in 1,000 L, INNS detection using eDNA has been achieved at much lower densities in other studies. For instance, eDNA was used to successfully detect pumpkinseed sunfish (*Lepomis gibbosus*) at an approximate density of 1 in 4,290 L in laboratory and field conditions (Davison et al. 2016). There is also evidence from a dilution series conducted using a similar method to that of the current study that zebra mussels (*Dreissena polymorpha*) can be detected down to a density of 1 per 1 million L using eDNA (Stroud 2018). The reduced detectability reported within the current study could be due to species specificity. Compared to some other aquatic taxa, including fish, crustaceans release a relatively low amount of DNA into the environment (Agersnap et al. 2017) which is speculated to be as a result of their exoskeleton limiting release (Tréguier et al. 2014). There is also some evidence that the detection of crayfish by eDNA is restricted to periods of moulting and spawning, particularly for low density populations (Strand et al. 2019). Whilst here, the sample with the lowest Cq value (i.e., highest amount of target DNA in the initial sample) was from the berried female treatment group, this did not translate into detection at lower densities. The reason for this is unclear, but it demonstrates that it should

not be assumed that eDNA sampling during the breeding season will always yield a better detection probability. Overall, broader scale studies are recommended to investigate the link between the physical state of crayfish, including egg-carrying, and eDNA detection.

Despite the potential challenges, eDNA assays have been developed for a range of crustacean species including red swamp crayfish (*Procambarus clarkii*), marbled crayfish (*P. virginalis*), rusty crayfish (*Faxionus rusticus*), spiny-cheek crayfish (*F. limosus*) and demon shrimp (*Dikerogammarus haemobaphes*) as well as signal crayfish and killer shrimp (Tréguier et al. 2014; Dougherty et al. 2016; Agersnap et al. 2017; Larson et al. 2017; Blackman et al. 2018; Harper et al. 2018; Mauvisseau et al. 2018, 2019; Robinson et al. 2018; Rusch et al. 2020; Chucholl et al. 2021). Some of these assays have been used successfully to detect wild populations, in some cases where conventional methods have failed to do so (Dougherty et al. 2016; Agersnap et al. 2017). However, the ability of eDNA to confirm the presence of these species is variable with detection rates compared to traditional surveys ranging from 20–100% across these studies (Tréguier et al. 2014; Dougherty et al. 2016; Agersnap et al. 2017; Blackman et al. 2018). The lowest comparability to traditional methods was observed for killer shrimp (Blackman et al. 2018) which is consistent with the difficulties experienced in the current study in detecting this species using the same eDNA assay. In contrast, in a survey of nine Danish lakes, Agersnap et al. (2017) detected signal crayfish using eDNA in both of the two lakes where they were located using traditional methods as well as at one site where the traditional surveys were negative. It should be noted though that a positive detection via eDNA alone does not necessarily mean that the organism is present. Therefore, the results obtained by Agersnap et al. (2017) could also be the result of genetic material being introduced, for instance in predator faeces or on equipment used at another site, without crayfish being present.

Overall, the results of the current study support the potential value in using eDNA as a detection method for INNS but also highlight the potential limitation in using this technology specifically for detecting crustaceans. The population densities of killer shrimp and signal crayfish that were detectable in this study were not as low as studies on other taxonomic groups (Davison et al. 2016; Stroud 2018), but it should not be ignored that some studies have also reported crustacean INNS being detected using eDNA but not via traditional methods (Dougherty et al. 2016; Agersnap et al. 2017). Therefore, eDNA should be considered as complimentary survey method for invasive crustaceans but should not be used in isolation for ruling out or confirming species presence. Finally, the variability in the results obtained across the trials, even in a controlled environment, reinforces the importance of collecting multiple samples and in designing bespoke eDNA surveying programmes depending on the habitat, target species, environmental conditions and level of certainty required in the results.

Authors' contribution

JJ designed the study, collected the study animals, interpreted the data and wrote the manuscript; EMM wrote and reviewed the manuscript, RN conceptualised the research and designed the study, BA conceptualised the research and reviewed the manuscript, SJB reviewed the manuscript, NM carried out the laboratory experiments and reviewed the manuscript, JC oversaw the laboratory trials and reviewed the manuscript, PDS designed the study and reviewed the manuscript.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Results of end point PCR and sequencing on samples from killer shrimp (*Dikerogammarus villosus*) detection (1/1 L) and dilution (1/10–25,000 million L) trials.

Table S2. Results of qPCR on samples from signal crayfish (*Pacifasticus leniusculus*) detection (1/10 L) and dilution trials (1/100–25,000 million L).

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