No evidence that crayfish carcasses produce detectable environmental DNA (eDNA) in a stream enclosure experiment

Amanda N. Curtis[1] and Eric R. Larson[1,2]

[1] Program in Ecology, Evolution, and Conservation Biology, University of Illinois at Urbana-Champaign, Urbana, IL, United States of America

[2] Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, United States of America

Corresponding author
Eric R. Larson, erlarson@illinois.edu, erlarson36@gmail.com

ABSTRACT

Environmental DNA (eDNA) is an emerging tool for monitoring invasive and imperiled species, particularly at low densities. However, the factors that control eDNA production, transport, and persistence in aquatic systems remain poorly understood. For example, the extent to which carcasses produce detectable eDNA is unknown. If positive detections are associated with dead organisms, this could confound monitoring for imperiled or invasive species. Here, we present results from one of the first studies to examine carcass eDNA in situ by deploying carcasses of the invasive red swamp crayfish (*Procambarus clarkii*) in a stream enclosure experiment for 28 days. We predicted that carcasses would initially produce eDNA that would decline over time as carcasses decayed. Unsurprisingly, crayfish carcasses lost biomass over time, but at the conclusion of our experiment much of the carapace and chelae remained. However, no eDNA of *P. clarkii* was detected in any of our samples at the crayfish density (15 *P. clarkii* carcasses at ∼615 g of biomass initially), stream flow (520–20,319 L/s), or temperature (∼14–25 °C) at our site. Subsequent analyses demonstrated that these results were not the consequence of PCR inhibition in our field samples, poor performance of the eDNA assay for intraspecific genetic diversity within *P. clarkii*, or due to the preservation and extraction procedure used. Therefore, our results suggest that when crayfish are relatively rare, such as in cases of new invasive populations or endangered species, carcasses may not produce detectable eDNA. In such scenarios, positive detections from field studies may be more confidently attributed to the presence of live organisms. We recommend that future studies should explore how biomass, flow, and differences in system (lentic vs. lotic) influence the ability to detect eDNA from carcasses.

INTRODUCTION

Since the first application of environmental DNA (eDNA) to macrobiota (*Ficetola et al., 2008*), this methodology has emerged as an important conservation tool capable of detecting invasive and imperiled species at low abundances (e.g., *Foote et al., 2012*; *Dougherty et al.,*

2016). Although eDNA has successfully been applied to a variety of environments (soil, freshwater, marine) and organisms (e.g., *Jerde et al., 2011*; *Yoccoz et al., 2012*), factors that control eDNA production, transport, and persistence remain poorly understood (*Barnes & Turner, 2016*). Therefore, it is imperative to understand which factors are important to eDNA detection to use this tool reliably for conservation and management.

A positive detection of eDNA does not necessarily indicate that the target organism is currently present in the system at the time of sampling. Detection may indicate the organism was present in the past (buried eDNA), eDNA may have been transported from elsewhere, or detection may have been caused by field or laboratory contamination. Moreover, eDNA could have been produced by a carcass and thus not indicate the presence of a live target organism (*Roussel et al., 2015*). False positives arising from a variety of sources can be problematic for management of either rare native or invasive species. Thus, it is important to know whether positive detections indicate that the target species is currently present, or whether the organism is no longer in that area or dead. In instances where eDNA is used to monitor reintroductions or translocations of imperiled species (e.g., *Cowart et al., 2018a*), it is necessary to know whether positive detections truly indicate the presence of live organisms, or whether carcasses from a failed management action are producing detectable eDNA. Alternatively, if eDNA is used to assess the success of an invasive species removal effort, false positives from carcasses could result in unnecessary and costly additional removal efforts (e.g., *Merkes et al., 2014*; *Carim et al., 2020*). Therefore, understanding whether carcasses produce eDNA, and under what contexts, is necessary to successfully employ eDNA as a management tool.

Research examining whether carcasses can produce detectable eDNA has been limited (*Merkes et al., 2014*; *Kamoroff & Goldberg, 2018*; *Tillotson et al., 2018*). *Tillotson et al. (2018)* found that decaying fish carcasses released more eDNA than living fish during sockeye salmon (*Oncorhynchus nerka*) spawning. Similarly, using a chamber experiment with silver carp (*Hypophthalmichthys molitrix*), *Merkes et al. (2014)* recovered high levels (22 million copies/L) of eDNA from carcasses for up to 28 days and found that carcass biomass was positively related to eDNA in water samples. Alternatively, *Kamoroff & Goldberg (2018)* found that goldfish (*Carassius auratus*) carcasses produced detectable eDNA for weeks post-mortality, but positive detections only came from water samples collected at the bottom of the water column near carcasses (here in a 2 L container). While previous research indicates that carcasses are capable of producing detectable eDNA, these studies (*Merkes et al., 2014*; *Kamoroff & Goldberg, 2018*) have been largely been confined to microcosm or laboratory studies that represent lentic (standing water) conditions, and consequently there is a need to examine these relationships in situ, in lotic (flowing water) environments, and at low abundances.

Our purpose was to examine whether decaying crayfish carcasses in a stream under natural conditions produce detectable eDNA. We chose to use crayfish because there are a number of invasive and imperiled crayfish that are currently targets of management actions (removal of invasive species and reintroductions of rare species), many of which use eDNA to monitor populations (e.g., *Cai et al., 2017*; *Larson et al., 2017*; *Cowart et al., 2018a*; *Rice, Larson & Taylor, 2018*; *Robinson, De Leaniz & Consuegra, 2019*). Our study

organism, red swamp crayfish (*Procambarus clarkii*), is native to the southern United States of America (USA) and northeastern Mexico, but is globally invasive (*Huner, 2002*; *Oficialdegui et al., 2019*). *Procambarus clarkii* has been associated with declines in macrophytes, macroinvertebrates, amphibians, fish, and native crayfish (*Gherardi, 2006*; *Twardochleb, Olden & Larson, 2013*). A population of *P. clarkii* has recently established in the Chicago River in Illinois, USA (*Taylor & Tucker, 2005*; *Peters et al., 2014*), where it has been a target of research and eradication efforts (*O'Shaughnessey & Keller, 2019*). In this study, we deployed dead *P. clarkii* (removed from the Chicago River) in a stream enclosure experiment and collected water samples to examine relationships between the decay of crayfish carcasses and detection of eDNA. We expected that crayfish carcasses would release eDNA, and that the amount of eDNA detected would decline over time in response to the loss of carcass biomass. Because eDNA can persist in water ≤44 days after organisms have been removed (*Dejean et al., 2011*; *Thomsen et al., 2012*; *Goldberg et al., 2013*), we also examined if eDNA could be detected after crayfish carcasses were removed from the stream. Our study has important implications for monitoring of both invasive and imperiled crayfish using eDNA, as well as for other organisms in which carcasses could contribute to positive eDNA detections.

MATERIALS & METHODS

Study site

We conducted our study in the Saline Branch of the Vermilion River (40°07′44″N, 88°09′05″W) located within the Phillips Tract Natural Area of the University of Illinois at Urbana-Champaign (UIUC; permission granted by J Ellis, UIUC Natural Area Coordinator) in Champaign County, Illinois, USA. At this location, the Saline Branch drains approximately 121 km^2 and upstream land cover is primarily agriculture with some urban area and sparse forest. Additionally, this site has a United States Geological Survey (USGS) flow gage (USGS gage #03337570) that allowed us to measure discharge during our experiment. Importantly, there have been no records of *P. clarkii* in Champaign County (*Taylor & Tucker, 2005*), and consequently there should not have been any background eDNA at our study stream to confound our results.

Experimental set-up

Crayfish used in this study were collected from the North Branch of the Chicago River by baited trapping during the summer of 2018, frozen at −20 °C, and then transported to UIUC where they remained in a −20 °C freezer until use. On 14 September 2018, we allocated three frozen crayfish to each stream enclosure for our experiment. First, we measured crayfish to size match them between enclosures (range: 47.2−61.3 mm total carapace length). Then, we weighed crayfish prior to placement in enclosures (mean ± SE: 42.62 ± 1.73 g per crayfish; mean ± SE: 122.87 ± 5.77 g per enclosure). Crayfish in enclosures were contained individually within labelled polyester mesh bags (30.5 cm × 20.3 cm; irregular mesh approximately 413 μm × 341 μm × 277 μm, purifyou^{TM}, USA) to restrict decomposition to microbial action rather than effects of invertebrate or vertebrate detritivores (see discussion). Mesh bags were then placed in crayfish traps (∼43.5 cm ×
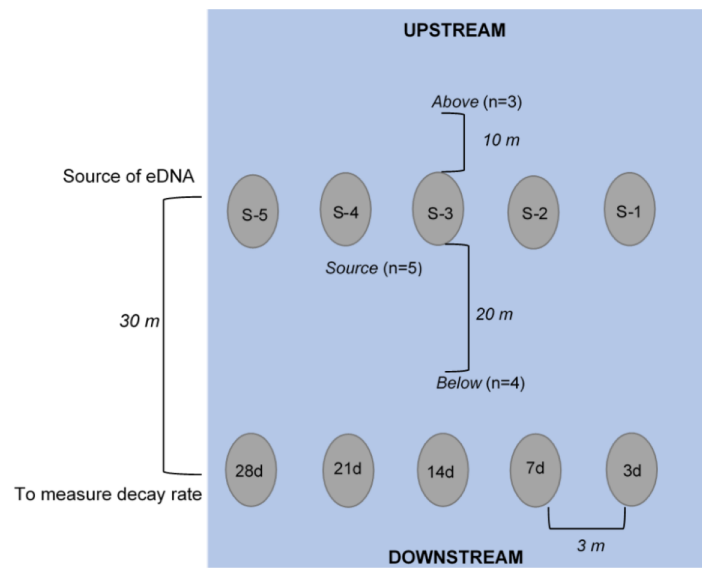
Figure 1 Schematic of experimental set-up (not to scale), where enclosures downstream (labeled 3d, 7d, etc.) held crayfish ($n = 3$ each) used to measure the rate of decay over the 28-day experiment. The enclosures upstream (labeled S-1, S-2, etc.) held crayfish ($n = 3$) used as the source of eDNA. Water was collected at "below" ($n = 4$, 250 mL bottles at 20 m downstream of the "enclosures"), at "source" ($n = 5$, 250 mL bottles directly in front of enclosure), and "above" ($n = 3$, 250 mL bottles at 10 m upstream of enclosures) locations. All enclosures were deployed approximately 3-m apart from one another longitudinally across the width of the stream.

17.5 cm; Frabill®, USA) with their openings (two at each end of ∼5.5 cm each) closed to restrict access of larger consumers to carcasses. Thus, every enclosure held three crayfish, in which each crayfish was individually contained in its own mesh bag.

Ten crayfish enclosures were attached to rebar stakes that were hammered into the streambed. Five enclosures were used as sources of eDNA and were placed 30 m upstream from the remaining five enclosures, which were used to determine the loss of crayfish biomass over time (Fig. 1). We placed all enclosures approximately 3 m apart from each other laterally across the width of the stream. The five enclosures used as eDNA sources were left in the stream for the entirety of our study and were removed after water collection on the last day. One of the five enclosures used to determine the rate of crayfish decay was removed at each of five eDNA water sampling events (days 3−28; below) to measure crayfish biomass remaining over time. After water samples for eDNA had been collected and placed in sealed bags, we removed the enclosure containing *P. clarkii* used to determine decay rate from the stream and placed the mesh bags in a plastic bag. We froze these bags at −20 °C until the conclusion of the experiment, when we then recorded wet weight of *P. clarkii* carcasses. We anticipated comparing crayfish decay rate, or estimated biomass remaining, over 28 days to eDNA copy number from our water samples.

eDNA sampling
Water samples were collected in 250 mL clean (previously washed with 50% bleach) Nalgene® (USA) bottles (*Goldberg et al., 2016*). Bottles were triple-rinsed on-site with
stream water and then the bottle was submerged underwater until full, capped, and placed in a sealed bag. We collected water samples at day 1 (~1.5 h after enclosure deployment), 3, 7, 14, 21, 28 after deployment, and 3 and 7 days after removal of enclosures. At each of these sampling dates, samples were collected at the following three locations: "above", "source", and "below" (Fig. 1). To assess whether there was background *P. clarkii* eDNA in the stream, we collected three water samples across the width of the stream approximately 10 m upstream of enclosures. Next, to examine if detectable eDNA was released from carcasses, one water sample was collected directly at the base of each of the five source enclosures ($n = 5$ per sampling date). Lastly, to assess whether carcass eDNA was transported downstream, we collected four water samples across the width of the stream 20 m below the source enclosures but 10 m above the biomass loss enclosures (Fig. 1). We expected that eDNA detections would be highest at the source enclosures, less likely downstream (below enclosures), and particularly unlikely upstream (above enclosures); therefore, we adjusted replication accordingly to save time and materials. Due to high flows (~20,319 L/s on 8 October 2018 (day 24), one source enclosure (S#2; Fig. 1) was displaced and lost downstream; hence the remaining eDNA samples were only collected at the remaining four source enclosures.

At each sampling date, two bottles (250 mL) filled with distilled water were used as field blanks to test background contamination. Field blanks were transported to the field and handled in the same way as field samples for eDNA. Additionally, on every sampling date, we recorded temperature, pH, conductivity, total dissolved solids, and salinity using a hand-held probe (Oakton®, USA), and turbidity was recorded with a portable meter (Sper Scientific©, USA; Table 1). To monitor temperature during our experiment, we attached a HOBO Pendant® Temperature/Light 8K logger (Onset®, USA) to one enclosure on 15 September 2018 (the day after deployment) and logged data every two hours until day 28 when all crayfish enclosures were removed (Figs. 2A & 2B). We do not report light data here because periphyton growth on the logger affected these results. We anticipated that water chemistry (*Strickler, Fremier & Goldberg, 2015*), temperature (*Eichmiller, Best & Sorensen, 2016*), and stream flow (*Jane et al., 2015*; *Stoeckle et al., 2017*) could influence eDNA persistence and detection in our system. Thus, we provide these site characteristics to facilitate comparison of our experiment to other study systems.

After all eDNA samples were collected on a date, each enclosure was cleared of debris (garbage, leaf litter, etc.) to ensure water flow was similar across all enclosures. Water samples were placed on ice in a cooler and transported back to UIUC, where they were filtered within two hours of collection. To avoid carry-over contamination from handling *P. clarkii* carcasses, we showered and changed clothes prior to filtration. In addition, we wore disposable nitrile gloves and used frequent glove changes during all field collection and subsequent lab work (e.g., filtration, extraction, and qPCR).

Prior to filtration, the entire work surface was cleaned with a 50% bleach solution and all materials used (e.g., funnels, forceps) had been previously washed with a 50% bleach solution (*Goldberg et al., 2016*) and subsequently rinsed with deionized water. To minimize any potential contamination during filtration, we filtered water samples in the following order: field blanks, above, below, and source samples. Water samples were vacuum filtered

Table 1 Water chemistry values during the experiment from carcass deployment (day 0) to removal (day 28) to one-week post-removal.

Sampling Point (day)	Salinity (PSU)	pH	TDS (mg/L)	Conductivity (µS/cm)	Turbidity (NTU)
0 (Deployment)	0.48	8.53	657	926	0.16
3	0.48	8.43	654	922	0
7	0.49	8.42	654	923	5.34
14	0.52	8.42	710	1,000	0.97
21	0.56	8.30	763	1,077	4.67
28 (Removal)	0.44	8.61	613	864	6.32
3-post removal	0.44	8.64	604	851	4.70
7-post removal	0.46	8.74	651	890	0.64

onto 1.0 µm cellulose nitrate filters (Whatman[TM], General Electric Healthcare, USA) and then placed into two mL centrifuge tubes filled with one mL of cetyl trimethylammonium bromide (CTAB; Teknova©, USA). Filters remained in the dark at room temperature (∼20−22 °C) for approximately two months, to reduce degradation by UV-B, to increase cell lysis over time (*Renshaw et al., 2015*; *Wegleitner et al., 2015*), and so that all filters would be in CTAB for the same length of time and be handled in the same manner. After two months, filters in CTAB were frozen at −80 °C until extraction. We then extracted DNA using a chloroform isoamyl alcohol extraction procedure (*Renshaw et al., 2015*), which can increase DNA yield relative to other eDNA methods and is resistant to many common PCR inhibitors (*Schrader et al., 2012*). One extraction blank for every ∼12 eDNA samples was used to test the level of lab contamination.

qPCR assay

We used the *P. clarkii* primer-probe assay developed by *Tréguier et al. (2014)* to amplify a 65 bp fragment of the COI region:

    F-primer: 5′-AACTAGGGGTATAGTTGAGAG-3′

    R-primer: 5′-CAGAAGCTAAAGGAGGATAA-3′

    Probe: 5′-FAM-AGGAGTTGGAACAGGATGGACT-MBG-3′.

We conducted initial assay optimization by running different primer and probe concentrations and modifying the annealing temperature and selected the conditions that produced the best results (e.g., earliest Cq values, highest R^2 values, and greatest % efficiency; Fig. S1). A 20 µL qPCR reaction was run on a QuantStudio 3 Real-Time PCR (Applied Biosystems®, USA) composed of: 10 µL iTaq[TM] Universal Probes Supermix (Bio-Rad Laboratories, USA), 5.2 µL of sterile water, 0.8 µL of each primer (10 µm), 0.2 µL probe (10 µm), and 3 µL of eDNA. The following qPCR procedure was used to amplify DNA: initial denaturation at 50 °C for 5 min and 95 °C for 10 min, and then 95 °C for 30 s and 60 °C for 1 min for 40 cycles (adapted from *Tréguier et al., 2014*).

In a clean room, we prepared all plates, added all eDNA samples, and then transported sealed strip tubes to our physically separate qPCR lab and added standards. All samples were run in triplicate with negative controls (3 µL of the master mix replaced DNA).
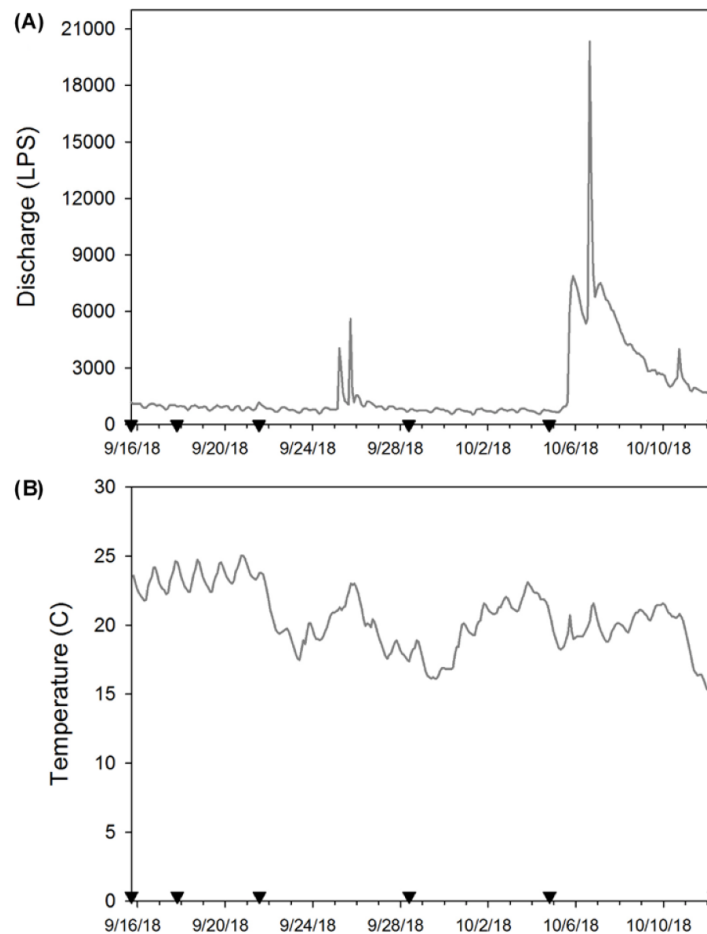
Figure 2 **(A) Stream discharge (L/s) and (B) temperature (°C) during the enclosure experiment (9/16/18 to 10/12/18).** Here the inverted black triangles indicate when eDNA samples were collected and enclosures used as the source of eDNA were removed to estimate crayfish decay. We provide stream discharge as L/s so that our results are more comparable with other published eDNA studies.

Using *P. clarkii* from the same source as our enclosure experiment, we extracted DNA from muscle tissue using a DNeasy® Tissue and Blood Kit (Qiagen©, Germany). We amplified this DNA using the primer-probe assay developed by *Tréguier et al. (2014)* to concentrate a 65 bp gene fragment of only *P. clarkii* DNA. Then we quantified this DNA (Qubit® Fluorometer, ThermoFisher, USA) and used it to run standards (1:10 dilution) from 1. 4×10^7 copies/µL (1×10^{-3} ng/µL gene fragment) to 1.4 copies/µL (1×10^{-10} ng/µL gene fragment) on each plate and to create a standard curve. All standard curve analyses (R^2, % efficiency, slope, etc.) and Cq values were calculated using the Thermo Fisher Connect™ Cloud (Applied Biosystems®, USA) interface. Here we define our limit of detection (LOD) as the lowest concentration standard that amplified in 1/3 wells (*Hunter et al., 2016*) and our limit of quantification (LOQ) as the lowest concentration standard that always produced replication in 3/3 wells (*Tréguier et al., 2014*). Context on *P. clarkii* COI

mtDNA haplotypes present at our Chicago River collection site is provided in *Oficialdegui et al. (2019)*.

Crayfish biomass loss over time

At the conclusion of the experiment, frozen crayfish were rinsed of sediment and debris, patted dry, and then weighed ($\pm$ 0.01 g; Proster®, United Kingdom) to obtain a final wet weight. Then to calculate the loss of biomass over time for each individual, we took the difference between the initial weight (before deployment) and the weight after removal. We did this for the crayfish enclosures that were removed on day 3, 7, 14, 21, 28, as well as for the crayfish used in the eDNA source enclosures upon their removal.

Statistical analyses

We fit a Michaelis–Menten curve to the loss in *P. clarkii* biomass over time, from the biomass enclosures removed at each sampling date, using the *drc* package (*Ritz et al., 2015*) in R (v. 3.6.2). We used a Michaelis–Menten model to represent a plateau in which crayfish carcass decay slowed in our experiment after soft tissues had been lost but the exoskeleton of the carapace or chelae remained. In addition, we compared the initial mass of the individual crayfish used in the source enclosures to the final mass at the conclusion of the experiment using a paired *t*-test. Data met parametric assumptions and no transformations were used. Lastly, we anticipated using multiple linear regression models with AIC model competition (*Burnham & Anderson, 2004*) to evaluate whether crayfish biomass, discharge, temperature, or other water chemistry variables explained eDNA copy number and detectability over time.

Sensitivity tests of results

Given our initial results, we subsequently sought to examine whether some non-detections of *P. clarkii* eDNA from carcasses could be attributable to either poor performance of the *Tréguier et al. (2014)* assay across intraspecific genetic diversity within our species (*Oficialdegui et al., 2019*) or PCR inhibition of our field samples (*Schrader et al., 2012*). First, we collected nine additional *P. clarkii* individuals from the North Branch of the Chicago River in August 2019, and then extracted DNA from muscle tissue using a DNeasy® Tissue and Blood Kit (Qiagen©, Germany). Then we used a random number generator to select eDNA water samples from our preceding experiment. To these eDNA water samples, we followed the same procedure as above and added a 1:10 serial dilution from 1. 4×10^7 copies/µL (1×10^{-3} ng/µL gene fragment) to 1.4 copies/µL (1×10^{-10} ng/µL gene fragment) using the nine additional crayfish and DNA from the individual *P. clarkii* originally used to create standard curves (10 total). This allowed us to examine if samples across more *P. clarkii* individuals reliably amplified and if amplifications were delayed (e.g., inhibited) relative to the original serial dilution series from assay testing. We followed the same qPCR recipe as above but added 3 µL of extracted *P. clarkii* serial dilution to each well and ran plates following the same qPCR procedure.

Confirmation of DNA in samples

To ensure that non-detections of *P. clarkii* carcass eDNA were not due to methodological decisions (e.g., CTAB storage, chloroform extraction) and that eDNA was present in our

samples, we first measured the concentration of all samples on a Qubit™ 2.0 using a dsDNA HS Assay Kit (Invitrogen, U.S.A). We then ran a subset of eDNA samples (those collected on days 0, 3, and 7) on qPCR for the invasive Asian clam (*Corbicula fluminea*) common in our study stream (~6.8 *C. fluminea*/m^2; A Curtis, 2018, unpublished data). We used an existing *C. fluminea* eDNA assay (*Cowart et al., 2018b*) with a new genus-specific probe to amplify a 208 bp COI fragment:

F-primer: 5′-TTTATTAGATGATGGGCAGCTGTA-3′

R-primer: 5′-TGATCTAACCAACAAAAGCATAGC-3′

Probe: 5′-FAM-AGTGATGCCAATAATAATGGGTGGTTTTGG-MGB-NFQ -3′.

All samples were prepared in a dedicated clean room using the following: 10 µL iTaq™ Universal Probes Supermix (Bio-Rad Laboratories, USA), 6.15 µL of sterile water, 0.35 µL of each primer (10 µm), 0.15 µL probe (10 µm), and 3 µL of eDNA. Samples were run in triplicate, including non-target controls on each plate on a QuantStudio 3 Real-Time PCR (Applied Biosystems®, Foster City, CA) with the following parameters: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 62 °C for 1 min. We used a gBlock fragment (Integrated DNA Technologies, USA) to run serial dilutions (1:10) from 4.4×10^6 copies/µL (1×10^{-3} ng/µL) to 0.44 copies/µL (1×10^{-10} ng/µL), and to generate standard curves for both plates (*Cowart et al., 2018b*).

RESULTS

In the biomass enclosures, *P. clarkii* biomass declined over time (Fig. 3A; $R^2 = 0.85$, $P < 0.001$), from an average of ~71% biomass remaining on day 3 to a plateau of ~38% remaining on day 28 (Fig. 3A). Further, in the source enclosures that were deployed for the full 28 days, total crayfish biomass was significantly lower at the conclusion of the experiment relative to the start (Fig. 3B, $t = 14.32$, $df = 11$, $P < 0.001$). However, even at day 28 much of the crayfish carapace and chelae remained. More specifically, the total initial biomass of the source cages was 614.7 g (mean ± SE: 122.93 ± 8.99 g per cage) and 98.67 g (mean ± SE: 31.04 ± 2.26 g per cage) remained on day 28 (one enclosure holding three crayfish was lost due to high flows and thus the final biomass estimate is lower than might be expected). On average, crayfish from the eDNA source enclosures lost ~74% of biomass over the 28-day deployment.

No field blanks, extraction blanks, or negative plate controls amplified. From the standard curves, run using the serial dilutions of *P. clarkii* DNA on every plate, slopes ranged between −3.317 and −3.447, y-intercepts ranged between 34.010−37.344, R^2 values were between 0.997−1.000, and assay efficiency ranged between 95−103%. Our LOD was 0.14 copies/µL (1×10^{-11} ng/µL gene fragment) and our LOQ was 14 copies/µL (1×10^{-9} ng/µL gene fragment). In our sensitivity tests following enclosure experiment results, the assay successfully amplified DNA from all ten *P. clarkii* individuals used without apparent differences in assay performance by crayfish. We found no evidence that our samples were inhibited, as curves from the serial dilutions added into field eDNA samples amplified at similar or earlier Cq values relative to the serial dilutions run on the initial plates (Fig. 4). Therefore, the assay used here worked well and amplified *P. clarkii* tissue from crayfish that
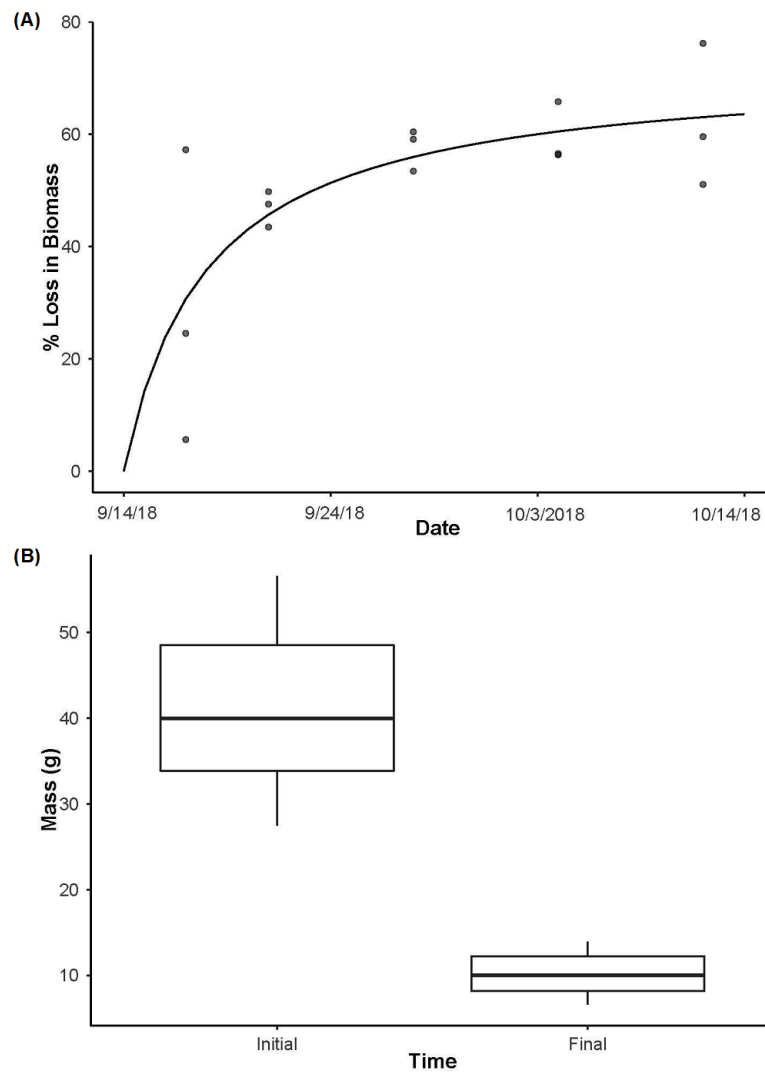
Figure 3 Crayfish decay. (A) The loss in biomass (wet weight loss) over time from day 3 to day 28 with a Michaelis-Menten model fit ($R^2 = 0.85$, $P < 0.001$) to the data for crayfish removed from decay cages. (B) The initial mass of individual crayfish used as the source of eDNA ($n = 15$) and then the final mass of crayfish after being deployed for 28 days ($n = 12$, as one enclosure was displaced and lost during high flows) highlighting the loss of crayfish biomass over time ($t_{11} = 14.32, P < 0.001$). Boxplots represent the median value and interquartile range, and whiskers indicate the minimum and maximum values.

were collected at the same location as those used in our experiment and without evidence of inhibition. However, none of our field eDNA samples amplified from any of the sample locations or dates, indicating that crayfish carcasses were not producing detectable eDNA in this system.

Quantification of our eDNA samples indicated that DNA was present (Data S1). When a subset of eDNA samples were run on qPCR for *C. fluminea*, none of the field blanks, extraction blanks, or negative plate controls amplified. From the standard curves, the slopes ranged between -3.426 to -3.474, y-intercepts ranged between $35.416-36.461$, R^2 values
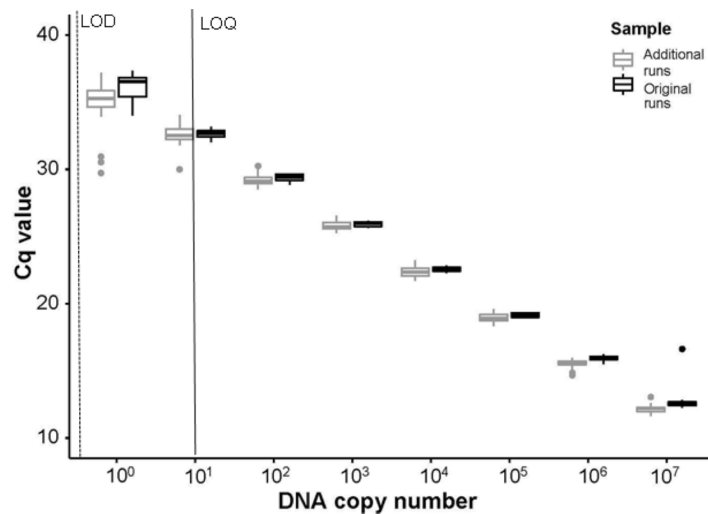
Figure 4 Comparisons of Cq values between initial and additional qPCR runs. The Cq values from serial dilutions (10^7–10^0 DNA copy number or 1×10^{-3} ng/µ L –1×10^{-10} ng/µ L gene fragment) run on the initial experimental plates, denoted as "Original runs." In comparison, the Cq values from the combination of serial dilutions run using extracted DNA from ten *P. clarkii* (nine additional crayfish and the initial DNA used to create the standard curves in our experimental plates) with randomly selected eDNA samples, denoted as "Additional runs." Here we show that the *Tréguier et al. (2014)* assay amplified intraspecific genetic variation in *P. clarkii* collected from the North Branch of the Chicago River, USA and that non-detections in eDNA samples were not caused by inhibition. From the initial standard curve, LOD here is shown in a dashed line at ~0.14 copies/µ L (1×10^{-11} ng/ µ L gene fragment); LOQ is signified with a black line at ~14 copies/µ L (1×10^{-9} ng/µ L gene fragment). Boxplots show the median and interquartile ranges, whiskers show the highest and lowest values without outliers, and points extending past whisker denote outliers.

were between 0.996–0.999, and assay efficiency was 94–95.8% (Data S1). Our LOD was ~0.44 copies/µL (1×10^{-10} ng/µL) and our LOQ was ~4.4 copies/µL (1×10^{-9} ng/µL). All samples amplified in at least 1/3 wells and most amplified in 3/3 wells (Data S1), further indicating that methodological choices cannot account for lack of amplification of *P. clarkii* eDNA.

DISCUSSION

Here we demonstrated that crayfish carcasses did not release detectable eDNA at the biomass used (15 *P. clarkii*) or under the abiotic conditions (temperature, flow, pH) of this stream enclosure experiment. Although research on this topic has been limited, some past studies have found carcasses can produce detectable eDNA (*Merkes et al., 2014*; *Dunker et al., 2016*; *Kamoroff & Goldberg, 2018*; *Tillotson et al., 2018*; *Strand, 2019*). However, these previous studies have largely been restricted to lab experiments (*Merkes et al., 2014*), mesocosms (*Kamoroff & Goldberg, 2018*), or in field locations (*Strand, 2019*) where living target individuals may confound results (*Dunker et al., 2016*) or have recently spawned (*Tillotson et al., 2018*). We speculate that our results differ from previous research largely due to differences in system-specific conditions (lentic vs. lotic and lab vs. in situ) and taxa.

Due to the chitinous exoskeleton of crayfish and their benthic habitat use, some researchers have suggested that crayfish eDNA may be more difficult to detect than other taxa that occur in the water column or shed more mucous, like fish or amphibians (*Tréguier et al., 2014*; *Rice, Larson & Taylor, 2018*). However, successful detection of low abundances of crayfish using eDNA has been documented (*Dougherty et al., 2016*; *Cai et al., 2017*). In the original *P. clarkii* eDNA study, *Tréguier et al. (2014)* were successful in detecting *P. clarkii* in 57 out of the 78 ponds known to have *P. clarkii* present (~73% success rate) using eDNA. *Tréguier et al. (2014)* conducted their research in lentic environments, collected smaller sample volumes, and used different laboratory methods relative to our study. Two additional *P. clarkii* assays (*Geerts et al., 2018*; *Mauvisseau et al., 2018*) have been developed and implemented since *Tréguier et al. (2014)*, and both were reported to possibly work better to amplify *P. clarkii* eDNA. However, *Geerts et al. (2018)* only used the primers developed by *Tréguier et al. (2014)* and may have decreased the specificity and success of the assay by neglecting to include the probe. *Mauvisseau et al. (2018)* found that the initial assay (*Tréguier et al., 2014*) could detect *P. clarkii* eDNA, but did not work well using TaqMan^{TM} master mix. Here we demonstrated that the *Tréguier et al. (2014)* assay was excellent (good efficiency and low LOD) in amplifying tissue extracted from 10 *P. clarkii* individuals collected from the North Branch of the Chicago River, USA. In addition, others have found the assay developed by *Tréguier et al. (2014)* to be successful (100% success rate) in detecting *P. clarkii* eDNA from water samples (*Cai et al., 2017*). Per our LOD estimates, we should have been able to detect eDNA from *P. clarkii* down to ~0.14 copies/μL (10^{-11} ng/μL gene fragment), yet we were unable to do so from carcasses.

The *P. clarkii* biomass used here might have been too low relative to stream discharge to produce detectable eDNA. However, eDNA can detect organisms with low abundance and low biomass (e.g., *Goldberg et al., 2013*; *Dougherty et al., 2016*; *Cowart et al., 2018a*). For example, *Cai et al. (2017)* were able to detect eDNA at ~1 live *P. clarkii* in 100,000 L of water compared to the 15 crayfish in ~515 L (~1 crayfish/34 L) at the lowest flow and ~20,319 L (1 crayfish/ 1,355 L) at the highest flow used in this experiment. Additionally, the biomass used in this experiment (initially ~616 g or ~1.2 g/L) is comparable to the 1 g/L used by *Dunn et al. (2017)* that produced detectable eDNA from signal crayfish (*Pacifastacus leniusculus*) in a laboratory setting. Moreover, the sample volume or replication used here might have been insufficient to detect eDNA. Previous research has demonstrated that increasing sample volume and the number of qPCR replicates can improve detection (e.g., *Harper et al., 2018*; *Doi et al., 2019*; *Sepulveda et al., 2019*). However, other in situ eDNA studies have used similar or smaller volumes of water and sample replicates and were able to detect crayfish at low abundances (e.g., *Dougherty et al., 2016*; *Rice, Larson & Taylor, 2018*; *Robinson et al., 2018*) and our use of 250 mL sample volumes was sufficient to amplify *C. fluminea* eDNA in these samples. Therefore, we believe that the biomass, sample volume, and number of replicates used in our study should have resulted in detectable eDNA, and we conclude that crayfish carcasses likely produce less eDNA than living organisms of this taxa.

Crayfish biomass loss occurred rapidly, with only ~26% of the biomass remaining at the end of the experiment in our source enclosures. As such, crayfish tissue and DNA were

being released into the stream, yet we did not detect eDNA from our sampling. Others have reported that open (i.e., not enclosed) fish carcasses experience rapid decay with complete biomass loss in as few as four days in a stream (*Minshall, Hitchcock & Barnes, 1991*), but *Elliott (1997)* reported ∼45% biomass loss after 20 days when carcasses were enclosed (protected from scavengers). If the crayfish used here were not contained in mesh bags within the enclosures, it is plausible that no or minimal biomass would remain at the end of the experiment due to consumption of the carcasses by vertebrate or invertebrate scavengers. Notably, both live crayfish and fish were often found in our enclosures (mesh bags remained closed), despite our attempts to close the entrances of these crayfish traps. Further, crayfish carcasses would likely have been transported downstream, particularly during high flows, thereby further decreasing the likelihood of eDNA detection at our sampling site. Therefore, our enclosure of carcasses in mesh bags likely resulted in slower decay rates that should have made eDNA persist in the stream for longer. Accordingly, we expect that our experimental design should have increased the likelihood of detecting eDNA from crayfish carcasses relative to more natural conditions.

A variety of choices made during any eDNA study can ultimately affect the results (e.g., *Goldberg et al., 2016*; *Tsuji et al., 2019*). Despite our attempts to control for a number of these factors, various abiotic (i.e., UV-B, inhibition) or biotic (i.e., use of frozen carcasses) conditions may have influenced our study. For example, UV-B radiation can reduce the persistence of eDNA (*Strickler, Fremier & Goldberg, 2015*) and was not measured in our study, although our enclosures were well-shaded by a dense riparian forest canopy. Additionally, UV-B radiation may alter long-term eDNA persistence and detectability (*Strickler, Fremier & Goldberg, 2015*), but it should not have influenced our samples collected immediately following enclosure deployment. Next, selection of filter or extraction methods may have impacted our results. Our use of 1.0 μm filters was consistent with past results that have found 0.4 μm to 1.2 μm filters to work well for carcass eDNA relative to larger pore sizes (*Kamoroff & Goldberg, 2018*). Inhibition often caused by tannins and humic acids can reduce the potential of qPCR reactions and the overall ability to detect eDNA (i.e., increase false negatives). However, the combined used of CTAB and subsequent chloroform isoamyl alcohol DNA extraction procedure has been repeatedly documented to reduce inhibition and result in higher DNA yields than other eDNA methods (e.g., *Schrader et al., 2012*; *Renshaw et al., 2015*; *Williams, Huyvaert & Piaggio, 2017*; *Hunter et al., 2019*). Here we directly assessed whether inhibition in our eDNA water samples may have affected our results. We re-ran our eDNA samples with serial dilutions of *P. clarkii* DNA added and then compared the Cq values to those estimated from our original standard curves. The Cq values were similar across the dilution series regardless of whether they were in environmental samples or not, showing no shift to delayed Cq values associated with PCR inhibition.

Additionally, it seems unlikely that our decision to preserve filters in CTAB at room temperature and then use a chloroform extraction protocol degraded DNA and resulted in non-detections. Filters stored in CTAB at room temperature produce more eDNA (due to cell lysis) than CTAB filters stored in a −20 °C freezer, and this protocol produces degrees of magnitude more eDNA than other methods like ethanol preservation followed

by extraction with Qiagen kits (*Renshaw et al., 2015*; *Tsuji et al., 2019*). All of our field samples amplified in at least 1/3 of plate replicates when we re-ran a subset for a different species that occurs at our study site (*C. fluminea*), indicating that our storage and extraction protocols do not explain non-detections of eDNA from *P. clarkii* carcasses. In addition, past work by our laboratory using this method to detect rare endangered and invasive organisms (e.g., *de Souza et al., 2016*; *Cowart et al., 2018a*; *Cowart et al., 2018b*; *Kessler et al., 2020*) further demonstrates its consistent sensitivity to low abundances of eDNA. Thus, we conclude that it is unlikely that our non-detections can be attributed to effects of UV-B, inhibition, or methodological decisions around sample handling and extraction.

The decision to use frozen carcasses was a byproduct of ease in storage and transportation from the Chicago River to UIUC and could have reduced or delayed natural microbial decomposition, affecting our results. For example, *Micozzi (1986)* noted that in the short-term (days) aerobic decomposition drove the terrestrial decay of frozen lab rats (*Rattus norvegicus domestica*), while fresh rat carcasses had largely anaerobic decomposition. Information on decay and decomposition in aquatic systems is somewhat limited (*Elliott, 1997*; *Minshall, Hitchcock & Barnes, 1991*), and we are not aware of any published studies that have directly examined crayfish decay to compare with our results. Nevertheless, other comparable eDNA studies have used frozen carcasses and reported detectable levels of eDNA (*Merkes et al., 2014*; *Dunker et al., 2016*). In summary, we acknowledge that a number of unmeasured factors may have contributed to our inability to detect eDNA but conclude that crayfish carcasses at low densities in lotic systems do not produce detectable eDNA. Further, we speculate that our non-detections are likely due to the combined dilution and transport of eDNA in a lotic system coupled with the overall lower concentrations of eDNA released by carcasses compared to live organisms.

Our results are encouraging for the use of eDNA to monitor outcomes of management actions for either invasive or imperiled crayfish and potentially other taxa. Eradication efforts for invasive crayfishes are common (e.g., *Freeman et al., 2010*; *Gherardi et al., 2011*; *Hansen et al., 2013*; *Manfrin et al., 2019*), but eDNA could produce misleading results if positive detections are produced by carcasses that remain after the management effort (*Dunker et al., 2016*; *Kamoroff & Goldberg, 2018*; *Carim et al., 2020*). These false positives could be costly if they prompt further eradication or monitoring that is not warranted (*Darling & Mahon, 2011*; *Jerde et al., 2013*; *Carim et al., 2020*). Similarly, reintroduction efforts of endangered species could be inaccurately interpreted as successful if eDNA from a species is detected only from carcasses and not from living individuals (*Laramie, Pilliod & Goldberg, 2015*; *Cowart et al., 2018a*). Our study suggests that when crayfish biomass is low, eDNA detections from carcasses are unlikely. However, we caution that management actions should not be based on single or short-term eDNA results and recommend that other more traditional methods or repeated long-term eDNA sampling be used to confirm initial eDNA results (*Darling & Mahon, 2011*; *Dunker et al., 2016*; *Cowart et al., 2018a*). We propose that future studies replicate our work in-situ in other ecosystems examining additional taxonomic groups. In addition, future research should examine the effects of field sample volume and replication on detection probabilities for eDNA from carcasses (*Doi et al., 2019*). Further, laboratory, mesocosm, or in situ experiments that compare

eDNA production and detectability between live organisms and carcasses would be useful. Future studies might also examine the effect biomass, stream size, and abiotic conditions, such as temperature or seasonality, on carcass eDNA.

CONCLUSION

Positive eDNA detections were not produced by *P. clarkii* carcasses enclosed in our study stream during the 28-day experiment or seven days after carcasses were removed from the stream. Therefore, our results suggest that eDNA detections from carcasses are unlikely when crayfish are rare, such as in cases of new invasive populations or endangered species, and that positive detections may indicate the presence of live organisms. However, future studies are necessary to examine how biomass, flow, and differences in system (lentic vs. lotic) or taxa (fish vs. invertebrate) influence the ability to detect eDNA from carcasses.

ACKNOWLEDGEMENTS

We thank Rachel Egly and Dr. Reuben Keller of Loyola University, Chicago for an ample supply of *P. clarkii* carcasses, Dr. Mark Davis and Dr. Lynsey Harper for assistance with assay optimization and interpreting serial dilutions, and Dr. Robert Schooley for comments that improved this manuscript. We are grateful to Mohamed Sam from purifyou™ for providing technical assistance in measuring the mesh size of the bags used in this experiment. Lastly, we are grateful to Dr. Xavier Pochon, Dr. Hideyuki Doi, and two anonymous reviewers for comments and suggestions that greatly improved this manuscript.

Funding

This research was supported by the United States Department of Agriculture National Institute of Food and Agriculture Hatch Project #1008988 to Eric R. Larson. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
United States Department of Agriculture National Institute of Food and Agriculture Hatch Project: #1008988.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Amanda N. Curtis conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Eric R. Larson conceived and designed the experiments, authored or reviewed drafts of the paper, contributed reagents/materials/analysis tools, and approved the final draft.

Data Availability

All associated data and the R code are available as Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.9333#supplemental-information.

REFERENCES

Barnes MA, Turner CM. 2016. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics* **17**:1–17 DOI 10.1007/s10592-015-0775-4.

Burnham KP, Anderson DR. 2004. Multimodel interference: understanding AIC and BIC in model selection. *Sociological Methods and Research* **33**:261–304 DOI 10.1177/0049124104268644.

Cai W, Ma Z, Yang C, Wang L, Wang W, Zhao G, Geng Y, Yu DG. 2017. Using eDNA to detect the distribution and density of invasive crayfish in the Honghe-Hani rice terrace World Heritage site. *PLOS ONE* **12**:e0177724 DOI 10.1371/journal.pone.0177724.

Carim KJ, Bean NJ, Connor JM, Baker WP, Jaeger M, Ruggles MP, McKelvey KS, Franklin TW, Young MK, Schwartz MK. 2020. Environmental DNA sampling informs fish eradication efforts: case studies and lessons learned. *North American Journal of Fisheries Management* **40**:488–508 DOI 10.1002/nafm.10428.

Cowart DA, Breedveld KGH, Ellis MJ, Hull JM, Larson ER. 2018a. Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (Decapoda: Astacidea) through monitoring of invasive species barriers and relocated populations. *Journal of Crustacean Biology* **38**:257–266 DOI 10.1093/jcbiol/ruy007.

Cowart DA, Renshaw MA, Gantz CA, Umek J, Chandra S, Egan SP, Lodge DM, Larson ER. 2018b. Development and field validation of an environmental DNA (eDNA) assay for invasive clams of the Genus *Corbicula*. *Management of Biological Invasions* **9**:27–37 DOI 10.3391/mbi.2018.9.1.03.

Darling JA, Mahon AR. 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research* **111**:978–988 DOI 10.1016/j.envres.2011.02.001.

de Souza LS, Godwin JC, Renshaw MA, Larson E. 2016. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLOS ONE* **11**:e0165273 DOI 10.1371/journal.pone.0165273.

Dejean T, Valentini A, Duparc A, Pellier-Cuit S, Pompanon F, Taberlet P, Miaud C. 2011. Persistence of environmental DNA in freshwater ecosystems. *PLOS ONE* **6**:e23398 DOI 10.1371/journal.pone.0023398.

Doi H, Fukaya K, Oka SI, Sato K, Kondoh M, Miya M. 2019. Evaluation of detection probabilities at the water-filtering and initial PCR steps in environmental DNA metabarcoding using a multispecies site occupancy model. *Scientific Reports* **9**:3581 DOI 10.1038/s41598-019-40233-1.

Dougherty MM, Larson ER, Renshaw MA, Gantz CA, Egan SP, Erickson DM, Lodge DM. 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *Journal of Applied Ecology* **53**:722–732 DOI 10.1111/1365-2664.12621.

Dunker KJ, Sepulveda AJ, Massengill RL, Olsen JB, Russ OL, Wenburg JK, Antonovich A. 2016. Potential of environmental DNA to evaluate Northern Pike (*Esox lucius*) eradication efforts: an experimental test and case study. *PLOS ONE* **11**: DOI 10.1371/journal.pone.0162277.

Dunn N, Priestley V, Herraiz A, Arnold R, Savolainen V. 2017. Behavior and season affect crayfish detection and density inference using environmental DNA. *Ecology and Evolution* **7**:7777–7785 DOI 10.1002/ece3.3316.

Eichmiller JJ, Best SE, Sorensen PW. 2016. Effects of temperature and trophic state on degradation of environmental DNA in lake water. *Environmental Science and Technology* **50**:1859–1867 DOI 10.1021/acs.est.5b05672.

Elliott JM. 1997. An experimental study on the natural removal of dead trout fry in a Lake District stream. *Journal of Fish Biology* **50**:870–877 DOI 10.1111/j.1095-8649.1997.tb01979.x.

Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. *Biology Letters* **4**:423–425 DOI 10.1098/rsbl.2008.0118.

Foote AD, Thomsen PF, Sveegaard S, Wahlberg M, Kielgast J, Kyhn LA, Salling AB, Galatius A, Orlando L, Gilbert MTP. 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLOS ONE* **7**:e41781 DOI 10.1371/journal.pone.0041781.

Freeman MA, Turnbull JF, Yeomans WE, Bean CW. 2010. Prospects for management strategies of invasive crayfish populations with an emphasis on biological control. *Aquatic Conservation: Marine and Freshwater Ecosystems* **20**:211–223 DOI 10.1002/aqc.1065.

Geerts AN, Boets P, Van den Heede S, Goethals P, Van der Heyden C. 2018. A search for standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): a lab and field evaluation. *Ecological Indicators* **84**:564–572 DOI 10.1016/j.ecolind.2017.08.068.

Gherardi F. 2006. Crayfish invading Europe: the case study of *Procambarus clarkii*. *Marine and Freshwater Behaviour and Physiology* **39**:175–191 DOI 10.1080/10236240600869702.

Gherardi F, Aquiloni L, Diéguez-Uribeondo J, Tricarico E. 2011. Managing invasive crayfish: is there a hope? *Aquatic Sciences* **73**:185–200 DOI 10.1007/s00027-011-0181-z.

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP. 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science* **32**:792–800 DOI 10.1899/13-046.1.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Strickler KM, Waits LP, Fremier AK, Takahara T, Herder JE,

Taberlet P. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution* **7**:1299–1307 DOI 10.1111/2041-210X.12595.

Hansen GJA, Hein CL, Roth BM, Vander Zanden MJ, Gaeta JW, Latzka AW, Carpenter SR. 2013. Food web consequences of long-term invasive crayfish control. *Canadian Journal of Fisheries and Aquatic Sciences* **70**:1109–1122 DOI 10.1139/cjfas-2012-0460.

Harper LR, Lawson Handley L, Hahn C, Boonham N, Rees HC, Gough KC, Lewis E, Adams IP, Brotherton P, Phillips S, Hänfling B. 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution* **8**:6330–6341 DOI 10.1002/ece3.4013.

Huner JV. 2002. Procambarus. In: Holdich D, ed. *Biology of freshwater crayfish.* Oxford: Blackwell Science, 541–584.

Hunter ME, Dorazio RM, Butterfield JSS, Meigs-Friend G, Nico LG, Ferrante JA. 2016. Detection limits of quantification and digital PCR assays and their influence in presence-absence surveys of environmental DNA. *Molecular Ecology Resources* **17**:221–229 DOI 10.1111/1755-0998.12619.

Hunter ME, Ferrante JA, Meigs-Friend G, Ulmer A. 2019. Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. *Scientific Reports* **9**:5259 DOI 10.1038/s41598-019-40977-w.

Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letchers BH, Whiteley AR. 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources* **15**:216–227 DOI 10.1111/1755-0998.12285.

Jerde CL, Chadderton WL, Mahon AR, Renshaw MA, Corush J, Budny M, Mysorekar S, Lodge DM. 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences* **70**:522–526 DOI 10.1139/cjfas-2012-0478.

Jerde CL, Mahon AR, Chadderton WL, Lodge DM. 2011. Sight-unseen detection of rare aquatic species using environmental DNA. *Conservation Letters* **4**:150–157 DOI 10.1111/j.1755-263X.2010.00158.x.

Kamoroff C, Goldberg CS. 2018. An issue of life or death: using eDNA to detect viable individuals in wilderness restoration. *Freshwater Science* **37**:685–696 DOI 10.1086/699203.

Kessler EJ, Ash KT, Barratt S, Larson ER, Davis MA. 2020. Radio telemetry reveals effects of upstream biomass and UV exposure on environmental DNA occupancy and detection for a large freshwater turtle. *Environmental DNA* **2**:13–23 DOI 10.1002/edn3.42.

Laramie MB, Pilliod DS, Goldberg CS. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation* **183**:29–37 DOI 10.1016/j.biocon.2014.11.025.

Larson ER, Renshaw MA, Gantz CA, Umek J, Chandra S, Lodge DM, Egan SP. 2017. Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and

Pacifastacus leniusculus in large lakes of North America. *Hydrobiologia* **800**:173–185 DOI 10.1007/s10750-017-3210-7.

Manfrin C, Souty-Grosset C, Anastácio PM, Reynolds J, Giulianini PG. 2019. Detection and control of invasive freshwater crayfish: from traditional to innovative methods. *Diversity* **11(1)**:Article 5 DOI 10.3390/d11010005.

Mauvisseau Q, Coignet A, Dulaunay C, Pinet F, Bouchon D, Souty-Grosset C. 2018. Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. *Hydrobiologia* **805**:163–175 DOI 10.1007/s10750-017-3288-y.

Merkes CM, McCalla SG, Jensen NR, Gaikowski MP, Amberg JJ. 2014. Persistence of DNA carcasses slime and avian feces may affect interpretation of environmental DNA data. *PLOS ONE* **9**:e113346 DOI 10.1371/journal.pone.0113346.

Micozzi MS. 1986. Experimental study of postmortem change under field conditions: effects of freezing, thawing, and mechanical injury. *Journal of Forensic Sciences* **31**:953–961.

Minshall GW, Hitchcock E, Barnes JR. 1991. Decomposition of Rainbow Trout (*Oncorhynchus mykiss*) carcasses in a forest stream ecosystem inhabited only by nonanadromous fish populations. *Canadian Journal of Fisheries and Aquatic Sciences* **48**:191–195 DOI 10.1139/f91-026.

Oficialdegui FJ, Clavero M, Sanchez MI, Green AJ, Boyero L, Michot TC, Klose K, Kawai T, Lejeusne C. 2019. Unravelling the global invasion routes of a worldwide invader, the red swamp crayfish (*Procambarus clarkii*). *Freshwater Biology* **64**:1382–1400 DOI 10.1111/fwb.13312.

O'Shaughnessey EM, Keller RP. 2019. When invaders collide: competition, aggression, and predators affect outcomes in overlapping populations of red swamp (*Procambarus clarkii*) and rusty (*Faxonius rusticus*) crayfishes. *Biological Invasions* **21**:3671–3683 DOI 10.1007/s10530-019-02079-6.

Peters JA, Cooper MJ, Creque SM, Kornis MS, Maxted JT, Perry WL, Schueler FW, Simon TP, Taylor CA, Thoma RF, Uzarski DG, Lodge DM. 2014. Historical changes and current status of crayfish diversity and distribution in the Laurentian Great Lakes. *Journal of Great Lakes Research* **40**:35–46 DOI 10.1016/j.jglr.2014.01.003.

Renshaw MA, Olds BP, Jerde CL, McVeigh MM, Lodge DM. 2015. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. *Molecular Ecology Resources* **15**:168–176 DOI 10.1111/1755-0998.12281.

Rice CJ, Larson ER, Taylor CA. 2018. Environmental DNA detects a rare large river crayfish but with little relation to local abundance. *Freshwater Biology* **63**:443–455 DOI 10.1111/fwb.13081.

Ritz C, Baty F, Streibig JC, Gerhard D. 2015. Dose–response analysis using R. *PLOS ONE* **10**:e0146021 DOI 10.1371/journal.pone.0146021.

Robinson CV, De Leaniz CG, Consuegra S. 2019. Effect of artificial barriers on the distribution of the invasive signal crayfish and Chinese mitten crab. *Scientific Reports* **9**:7230 DOI 10.1038/s41598-019-43570-3.

Robinson CV, Uren Webster TM, Cable J, James J, Consuegra S. 2018. Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biological Conservation* **222**:241–252 DOI 10.1016/j.biocon.2018.04.009.

Roussel J-M, Paillisson J-M, Tréguier A, Petit E. 2015. The downside of eDNA as a survey tool in water bodies. *Journal of Applied Ecology* **52**:823–826 DOI 10.1111/1365-2664.12428.

Schrader C, Schielke A, Ellerbroek L, Johne R. 2012. PCR inhibitors-occurrence, properties and removal. *Journal of Applied Microbiology* **113**:1014–1026 DOI 10.1111/j.1365-2672.2012.05384.x.

Sepulveda AJ, Schabacker J, Smith S, Al-Chokhachy R, Luikhart G, Amish SJ. 2019. Improved detection of rare, endangered and invasive trout in a new large-volume sampling method for eDNA capture. *Environmental DNA* **1**:227–237 DOI 10.1002/edn3.23.

Strand DA, Johnsen ST, Rusch JC, Agersnap S, Larsen WB, Knudsen SW, Møller PR, Vrålstad T. 2019. Monitoring a Norwegian freshwater crayfish tragedy-eDNA snap-shots of invasion, infection and extinction. *Journal of Applied Ecology* **56**:1661–1673 DOI 10.1111/1365-2664.13404.

Stoeckle BC, Beggel S, Cerwenka AF, Motivans E, Kuehn R, Geist J. 2017. A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. *PLOS ONE* **12**:e0189119 DOI 10.1371/journal.pone.0189119.

Strickler KM, Fremier AK, Goldberg CS. 2015. Quantifying the effects of UV, temperature, and pH on degradation rates of eDNA in aquatic microcosms. *Biological Conservation* **183**:85–92 DOI 10.1016/j.biocon.2014.11.038.

Taylor CA, Tucker JK. 2005. New distributional records for Illinois crayfishes (Decapoda: Cambaridae) with comments on the continued spread of non-native species. *Transactions of the Illinois State Academy of Science* **98**:75–80.

Tillotson MD, Kelly RP, Duda JJ, Hoy M, Kralj J, Quinn TP. 2018. Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. *Biological Conservation* **220**:1–11 DOI 10.1016/j.biocon.2018.01.030.

Thomsen PF, Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert MTP, Orlando L, Willerslev E. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology* **21**:2565–2573.

Tréguier A, Paillisson J-M, Dejean T, Valentini A, Schlaepfer MA, Roussel J-M. 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology* **52**:871–879 DOI 10.1111/1365-2664.12262.

Tsuji S, Takahara T, Doi H, Shibata N, Yamanaka H. 2019. The detection of aquatic macroorganisms using environmental DNA analysis-a review of methods for collection, extractions, and detection. *Environmental DNA* **1**:99–108 DOI 10.1002/edn3.21.

Twardochleb LA, Olden JD, Larson ER. 2013. A global meta-analysis of the ecological impacts of nonnative crayfish. *Freshwater Science* **32**:1367–1382 DOI 10.1899/12-203.1.

Wegleitner BJ, Jerde CL, Tucker A, Chadderton WL, Mahon AR. 2015. Long duration, room temperature preservation of filtered eDNA samples. *Conservation Genetics Resources* **7**:789–791 DOI 10.1007/s12686-015-0483-x.

Williams KE, Huyvaert KP, Piaggio AJ. 2017. Clearing muddied waters: capture of environmental DNA from turbid waters. *PLOS ONE* **12**:e0179282 DOI 10.1371/journal.pone.0179282.

Yoccoz NG, Bråthen KA, Gielly L, Haile J, Edwards ME, Goslar T, Von Stedingk H, Brysting AK, Coissac E, Pompanon F, Sønstebø JH, Miquel C, Valentini A, De Bello F, Chave J, Thuiller W, Wincker P, Cruaud C, Gavory F, Rasmussen M, Gilbert MTP, Orlando L, Brochmann C, Willerslev E, Taberlet P. 2012. DNA from soil mirrors plant taxonomic and growth form diversity. *Molecular Ecology* **21**:3647–3655 DOI 10.1111/j.1365-294X.2012.05545.x.