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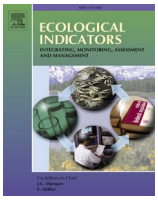

Effects of life stage on eDNA detection of the invasive European green crab (*Carcinus maenas*) in estuarine systems

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ABSTRACT

Early and efficient detection of rare and invasive species is critical for the effective management of their populations. Environmental DNA (eDNA) detection techniques have been used for monitoring soft-bodied organisms (e.g., fishes) and some invertebrates, primarily in freshwater systems, but there are limited examples of eDNA as a method for monitoring marine crustaceans. The present study evaluates the efficacy of applying eDNA methods for detecting the invasive European green crab (*Carcinus maenas*) in a dynamic estuarine environment, and the effect of crab life stage (sex, molt stage, ovigery, abundance) on eDNA detection rates. An initial field experiment conducted in a local salt marsh system detected no *C. maenas* eDNA in sediment samples associated with traps containing *C. maenas*. In subsequent laboratory trials, aquaria containing one or two *C. maenas* at different life stages (soft-shell, hard-shell, male, female, ovigerous) were evaluated in replicated treatments to test the hypothesis that *C. maenas* exudes eDNA at higher levels when ovigerous, soft-shell, or at higher abundances. Duplicate sediment slurry and water samples were collected from each aquarium (n = 23) prior to crab addition (T-0), and after 24 h (T-1), 4 days (T-2), and 7 days (T-3). Sediment slurry and water samples were filtered, extracted, and analyzed using a species-specific droplet digital PCR (ddPCR) assay. In all non-control aquaria, *C. maenas* eDNA was detected, but concentrations were low (<10 copies/μL) in non-ovigerous treatments. eDNA concentrations were significantly higher in sediment slurry versus water samples for male and ovigerous treatments. Overall, concentrations increased from T-0 to T-1 but did not significantly change from T-1 through T-3. The findings from this study indicate that during most of their life cycle, *C. maenas* shed low levels of DNA, highlighting the importance of considering life stage and sampling methodology when using eDNA to monitor crustaceans in estuarine environments.

1. Introduction

Efficient and effective monitoring of invasive species is critical for detecting their arrival, minimizing population growth and spread, and mitigating their impacts (Bax et al., 2003, 2002). Traditional methods of monitoring in marine coastal systems (e.g., trawl surveys, trapping, visual surveys) are often expensive, time- and labor-intensive, and ecologically invasive (Pilliod et al., 2013; Sigsgaard et al., 2015). Furthermore, rare species and early invaders occur at low abundances, making detection challenging (Gu and Swihart, 2004). To effectively detect the presence of invasive species at low abundances (i.e., at the beginning of an invasion), other monitoring methods may be more effective, more cost efficient, and yield greater detection rates.

An emerging approach to monitoring the distribution of species in aquatic systems involves the detection of environmental DNA (eDNA) in environmental samples. Because aquatic organisms typically exude DNA into their environment (Tréguier et al., 2014; York, 2016), their presence in a particular habitat or area can be determined by detecting their DNA signature in water or sediment samples (Tsuji et al., 2019). eDNA methods have been extensively tested and applied for fishes, amphibians, and mammals (Berry et al., 2019; Goldberg et al., 2016; Thomsen and Willerslev, 2015; Tsuji et al., 2019) and many studies have shown that eDNA monitoring techniques can be more efficient and cost-effective than traditional surveys, particularly when species

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abundance is low (Janosik and Johnston, 2015; Pilliod et al., 2013; Sigsgaard et al., 2015). Both eDNA and traditional approaches have strengths and weaknesses, and the appropriate application of each method depends on species biology, project goals, and available resources. In some cases, a combination of these methods may provide the most meaningful results.

While the benefits of eDNA monitoring methods have been extensively demonstrated for soft-bodied aquatic vertebrates (Levi et al., 2019; Thomsen et al., 2016; Ushio et al., 2018), few studies have investigated the efficacy of this approach for detecting benthic crustaceans in estuarine systems. Invertebrate eDNA studies have focused on a range of freshwater and marine species, including crayfish, bivalves, amphipods, isopods, snails, shrimp, insects, and Cladocera, and have had mixed results (Bayer et al., 2019; Geerts et al., 2018; Goldberg et al., 2016; Mächler et al., 2014; Thomsen and Willerslev, 2015; Tsuji et al., 2019). Many early eDNA detection studies were conducted in freshwater environments (Goldberg et al., 2016; Jerde et al., 2011), but more recent work is exploring the use of eDNA for assessing benthic invertebrate communities in coastal systems. One such study successfully detected eDNA from a benthic invasive mud crab (*Rhithropanopeus harrisii*) in a natural brackish environment; however, detection rates were very low (Forsström and Vasemägi, 2016). More information is needed on the efficacy of eDNA for detecting crustacean decapods such as invasive crab species in dynamic environments like estuaries. The present study tests the application of eDNA for monitoring the invasive and widespread European green crab, *Carcinus maenas*, in estuarine systems.

Carcinus maenas arrived in the mid-Atlantic region of the United States in the early 1800s and has since spread south to Virginia, USA and north to Newfoundland, Canada, arriving in southern Maine in the 1890s (Carlton and Cohen, 2003). Breeding populations are virtually ubiquitous and are now established on the Pacific coast of the United States and Canada as well as the coasts of South Africa, Australia, Japan, and Argentina (Carlton and Cohen, 2003; Compton et al., 2010; Hidalgo et al., 2005). Many other regions are at risk of future invasion by *C. maenas* (e.g. Alaska, Brazil, Chile, Uruguay, New Zealand, Russia, the Yellow Sea, China, Namibia; Compton et al., 2010). Within these invaded ranges, *C. maenas* populations have readily increased, impacting ecologically and economically important native species (reviewed in Young and Elliott, 2019). This species typically resides in sheltered estuarine and lagoonal environments in a variety of intertidal and shallow subtidal habitats including cobble, sand, mudflat, and salt marsh (Amaral et al., 2009; Crothers, 1968). A voracious predator, *C. maenas* has contributed substantially to declines in multiple fisheries in the northwestern Atlantic including soft-shell clams (*Mya arenaria*), Manila clams (*Venerupis philippinarum*), blue mussels (*Mytilus edulis*), bay scallops (*Argopecten irradians*), and hard-shell clams (*Mercenaria mercenaria*) (Beal and Kraus, 2002; Grosholz et al., 2011; Tan and Beal, 2015), and poses a predatory threat to other species including the commercially important American oyster (*Crassostrea virginica*) and American lobster (*Homarus americanus*) (Goldstein et al., 2017; Poirier et al., 2017; Rossong et al., 2006; Sigurdsson and Rochette, 2013).

As *C. maenas* continues to expand its range in coastal habitats, efficient and effective monitoring techniques are critical for tracking and understanding changes in this invasive species' populations. In locations with established breeding populations, monitoring efforts can improve our understanding of local population dynamics and inform mitigation strategies accordingly. In areas at risk of invasion, monitoring for *C. maenas* is critical for the early detection of their arrival so that mitigation and management efforts can begin before their population becomes too large to be eradicated.

The overall objective of this study was to determine whether eDNA can be used to detect the presence of *C. maenas* in estuarine environments. A field experiment was conducted to determine if eDNA could be detected in sediment samples from the vicinity of crab traps containing *C. maenas*, while a series of complementary laboratory experiments were conducted to determine (a) if *C. maenas* eDNA could be detected in enclosed aquaria, (b) whether water or sediment samples contain more *C. maenas* eDNA, and (c) whether crab condition or life history stage (sex, ovigery, molt stage, abundance) affect the detection rate of *C. maenas* eDNA. It was hypothesized that eDNA would be detected in aquaria containing *C. maenas* and detection rates would be higher in sediment samples than water samples and in tanks containing: (1) ovigerous females due to the presence of an exposed egg mass; (2) soft-shell crabs due to the lack of a hard exoskeleton; and (3) tanks containing multiple crabs due to larger crab surface area and the potential for agonistic behavior resulting in injury.

2. Materials and methods

2.1. Field trials

A field study was conducted in June-July 2018 to determine whether *C. maenas* eDNA could be detected in sediment adjacent to and beneath baited crab traps.

2.1.1. Study site

The study was conducted in the Little River estuary marsh (43°20′10.70″ N 70°32′32.94″ W, Wells, ME), which is a small (84 km^2 drainage area) back-barrier salt marsh with strong tidal currents, deep tidal channels (Dionne et al., 2006), and a substantially established *C. maenas* population (Aman and Grimes, 2016; Beal et al., 2018; Raposa et al., 2019).

2.1.2. Study design and sampling

Prior to trap deployments, all equipment (traps, line, bait bags, etc.) were sterilized with a 1:10 Clorox® germicidal bleach solution (8.25% sodium hypochlorite), thoroughly rinsed, and air dried. Equipment was transported to the site in sterilized containers. Samplers wore disposable nitrile gloves throughout sampling activities and sterilized boots with bleach solution.

Deployment and retrieval of traps occurred within one hour of daytime low tides. At the start of each of three deployment events (5, 18 June, and 9 July 2018), three sterilized standard crab traps (62 cm L × 31 cm W × 27 cm H) were baited with herring (*Clupea harengus)* and arranged in a triangular formation (6–8 m apart), with two traps in a tidal creek and a third trap on the adjacent marsh platform. A water blank was collected by uncapping a 500 mL Nestle Pure Life® water bottle for 15 s of air exposure. A sediment blank was collected by placing a sterile tongue depressor in a Ziploc® bag containing 5 g of sterile aquarium sand (see lab methods for sand sterilization methods) and sealing the bag. At each trap, a sterile tongue depressor was used to collect a sediment sample 0.5 m outside each side of the trap; these four samples were combined into one composite "OUT" sample. A sterile tongue depressor was used to collect two additional sediment samples from underneath the trap, which were combined into one composite "IN" sample. Each composite sample or blank was placed in a new Ziploc® bag, temporarily stored on ice, and frozen upon return to the lab. After 36 hrs of deployment, crabs were removed from each trap, identified, sexed, weighed, and measured (carapace width, CW). Water blanks, sediment blanks, and "IN" and "OUT" samples were collected again, repeating the methods used during deployment. All samples were frozen and transferred to the University of New Hampshire (UNH) Hubbard Center for Genome Studies (HCGS).

2.2. Laboratory trials

A series of laboratory experiments were conducted to measure DNA concentrations in aquaria containing *C. maenas* to evaluate the effect of life stage (ovigerous, soft-shelled) and number of crabs on DNA concentrations in sediment and water samples.

2.2.1. Specimen collection and aquarium setup

Hard-shell and ovigerous *C. maenas* were collected in June 2019 from the Webhannet River estuary (43°19′4.10″ N 70°33′44.67″ W, Wells, ME) using standard baited crab traps and transported in coolers. Crabs were held in aquaria (75 L) containing aerated ambient seawater until use in experiments. Soft-shell (newly molted) *C. maenas* were collected by hand in October 2019 during low tide from the rocky intertidal in the nearby Piscataqua River estuary, Rye, NH (43°3′10.99″ N 70°43′45.07″ W). Soft-shell crabs were wrapped in seawater-soaked paper towels and stored on ice for 24 hrs prior to use to slow metabolic activity and discourage shell hardening (G. Bradt, pers. obs.).

All equipment and workspaces were sterilized with a 1:10 Clorox® bleach solution, thoroughly rinsed, and air dried. A stock solution of artificial seawater at a salinity of 30–32 psu was prepared using Instant Ocean® (Aquarium Systems, Inc., Mentor, OH) with de-chlorinated tap water (API Tap Water Conditioner). Aquarium sand (Nature's Ocean® Marine White Sand #0) was sterilized by soaking it in a 1:10 bleach solution, rinsing it thoroughly with tap water, and placing it in a drying oven for 12–14 hrs at 100 °C (repeated twice). Disposable nitrile gloves were worn while handling materials and changed between samples.

A series of 7.5 L aquarium tanks were arranged on shelving in an environmental chamber at the Wells National Estuarine Research Reserve (NERR) Coastal Ecology Center (Wells, ME) with cardboard dividers to minimize splash and visual cues between tanks. Each tank contained 5.3 L of artificial seawater, 0.4–0.6 L of sand covering the tank bottom, and a low-power aeration stone. Trials were run at 16.4 °C and a 12:12 hr (light:dark) cycle using a full spectrum 40 W bulb.

2.2.2. Sampling design

2.2.2.1. Hard-shell and ovigerous trials. Trials were conducted in July 2019 to compare eDNA detection rates for crabs representing different life stages and for samples collected from sand versus water. Treatments included: ovigerous females (n = 6; one crab per tank), hard-shell females (n = 3; one per tank), hard-shell males (n = 3; one per tank), and multiple crabs (n = 3; two hard-shell males per tank) (summarized in Table 1). Crabs averaged 55 ± 3.9 mm CW and 46 ± 9.9 g. Aquaria were arranged with six tanks per shelf, including one control tank (no crabs) on each shelf (n = 3). At the start of the trial, a lab blank was collected by opening a new 500 mL Nestle Pure Life® water bottle in the environmental chamber for 30 s of air exposure. A sediment blank was collected by placing 2 mL of dry sterilized aquarium sand in a microcentrifuge tube. Blanks were immediately stored in Ziploc® bags in a refrigerator. Duplicate sediment slurry and water samples were collected from each tank before crabs were added to tanks (T-0), as well as 24 h (T-1), 4 days (T-2), and 1 week (T-3) after crabs were added. Duplicate water samples were collected by submerging two sterile 50 mL vials in each tank. A sterilized turkey baster was used to transfer a sand-and-water slurry from the bottom of each tank into two additional sterile 50 mL vials so that each vial contained ~30 mL of sand and ~10 mL of water supernatant. Samples from a given tank were placed together in a Ziploc® bag and stored in a refrigerator. After T-0 samples were collected, crabs were added to each tank; weight, carapace width, missing limbs, deformities, and sex were recorded before rinsing the crab off under running tap water and placing it into its respective tank.

2.2.2.2. Soft-shell trials. Trials were conducted in October 2019 to compare eDNA detection rates for tanks containing soft-shell crabs with the previously described treatments. Five aquarium tanks were arranged on a single shelf, including one control tank (no crabs). A lab blank was collected by opening a sterile vial containing 50 mL of tap water in the environmental chamber for 30 s of air exposure. Sediment blanks and duplicate sediment slurry and water samples were collected as previously described for the hard-shell and ovigerous trials, except that no T-1 samples were collected. Prior to adding each crab to a tank, crabs were weighed, measured (CW), sexed, inspected for missing limbs and deformities, and rinsed under running tap water. Soft-shell crabs (42 ± 11.6 mm CW; 20 ± 15.8 g, n = 5 crabs) were allocated into four tanks as follows: one crab in each of three tanks ("Soft"; two males, one female), and two female crabs into one additional tank ("Soft × 2") (summarized in Table 1). Additionally, a control tank contained no crabs.

2.2.3. Sample processing and filtration

All samples were filtered at the Wells NERR the day of collection and stored on ice during this process. Prior to filtering, the vacuum filtration apparatus, magnetic filter cups, stoppers, and forceps were exposed to 25 W UV light for 15 min to sterilize all materials. All workspaces and materials were sterilized with 1:10 bleach solution and thoroughly rinsed with tap water. Disposable nitrile gloves were worn throughout the process and changed between samples. A 4.7 cm 1.5 μm glass microfiber filter (VWR International, LLC., Radnor, PA) was placed on each filtration dock, secured with a magnetic filter cup, and the vacuum pump was turned on. Prior to sample filtration, a lab blank was collected by filtering 500 mL of distilled water. Water samples were poured directly into filter cups, while sediment slurry samples were vortexed by hand until well mixed, then decanted into filter cups. At the completion of filtering, each filter was placed into a 2 mL microcentrifuge tube. Samples (filters) from the same tank were placed together in a Ziploc® bag and stored temporarily on ice. Filter cups, stoppers, and forceps were sterilized between samples. Samples (filters) were stored at −80 °C and transferred to the HCGS for DNA extraction and analysis.

2.3. DNA extraction and analysis

All samples were extracted at the HCGS in a dedicated laboratory space separate from PCR and raw sample handling. Sample filters were cut in half, with one half retained for storage and one half processed for DNA extraction. Filters were placed in a lyse and spin basket with 400 mL of buffer ATL and 20 μL of proteinase K and incubated at 56 °C for one hr, then centrifuged. The remainder of the filter extraction was performed on a QIAcube Connect system (QIAGEN®, Hilden, Germany) following the QIAamp DNA Mini Kit protocol. Sediment samples from the field study and sediment blanks from the laboratory study were extracted with QIAGEN® PowerSoil Pro, and crab tissue was extracted with DNeasy Blood and Tissue kits. One negative control sample was extracted with every 11 field samples. DNA concentration was measured using an Invitrogen Qubit 2.0 (Thermo Fisher®, Waltham, MA). The manufacturer's protocol for the Qubit dsDNA HS Assay kit was followed using 1 μL of sample. Samples were stored at −20 °C until further processing.

Field samples were analyzed by both metabarcoding and droplet

Table 1
Number of aquarium tanks sampled for each treatment at each time period. Tanks in which the crab(s) died were excluded from subsequent sampling. All T-0 and T-3 samples, and a subset of T-1 and T-2 samples, were processed for inclusion in analyses (see Table S1).

	Hard-Shell and Ovigerous Trial					Soft-Shell Trial		
	Control	Male	Male × 2	Female	Ovigerous	Control	Soft	Soft × 2
T-0	3	3	3	3	6	1	3	1
T-1	3	2	2	3	5	–	–	–
T-2	3	2	2	3	5	1	3	1
T-3	3	2	2	3	5	1	1	0

digital PCR (ddPCR). Lab methods are summarized below and described in more detail in the Supplementary Material section.

A CO1 metabarcoding assay designed for marine invertebrates (Lobo et al., 2017) was applied in a two-step process. The first reaction consisted of 10 μL Quantabio MasterMix, 1 μL forward primer, 1 μL reverse primer, 12 μL ultra-pure water, and 2 μL sample. The plate was sealed with the PX1 PCR Plate Sealer (Bio-Rad®) and the reaction was performed in a T100 Thermo Cycler (Bio-Rad®). The thermocycling profile is outlined in Table S4. Gel electrophoresis was completed using a 2% agarose gel with SybrSafe and TBE buffer. Additionally, 1.2 mL PCR product with 17 mL loading dye was run alongside a 100 base pair ladder. Gels were run for 1–1.5 hrs at 80–90 V and bands were visualized on a transilluminator to confirm the presence of the correct size product. The second PCR reaction included 6 μL Kapa ReadyMix, 5 μL mixed forward and reverse primers, and 2 μL step 1 product diluted 1:10. The plate was sealed with the PX1 PCR Plate Sealer (Bio-Rad®) and the reaction was performed in a T100 Thermo Cycler (Bio-Rad®) following the profile outlined below. The samples were sequenced on an Illumina HiSeq 2500 by the HCGS and the sequence data was returned for analysis. See supplementary material for details on PCR program cycles and primer sequence.

Samples were then sequenced on an Illumina HiSeq 2500 (Illumina Inc., San Diego, CA), demultiplexed, and returned for bioinformatic analysis. The sequences were BLASTed (Altschul et al., 1990) against reference *C. maenas* COI sequences to detect *C. maenas*. Provisional taxonomy for other species was completed by blasting the sequences against the Barcode of Life (BoLD) COI database.

Droplet digital analyses were performed on a Bio-Rad® Droplet Digital PCR (ddPCR) system (Bio-Rad Laboratories, Inc., Hercules, CA) using a previously developed assay (Neigel and Germane, unpub.). The primer design was confirmed for this study by conducting in Silico validation. To test for sensitivity, a web BLAST alignment of the primers and probe was conducted using a reference *C. maenas* COI sequence (GenBank: JQ306003.1). We found a complete match of both primers and the probe to the reference sequence. To test for specificity, a BLAST search was performed, excluding *C. maenas* of the nucleotide database with the amplified region of our reference sequence as the query. The primers and probe were BLASTed against the top 100 BLAST results from the prior BLAST. The only sequences with a mismatch in the probe were some haplotypes of Mediterranean green crab (*Carcinus aestuarii*) which contained 1–2 mismatches but none near the 3′ ends, so it is possible that this assay would amplify some haplotypes of *C. aestuarii*. Secondary structure was checked with ThermoFisher Scientific Multiple Primer Analyzer with 0.3 μM primer concentration and 50 mM salt concentration; no secondary structure was reported.

A TaqMan probe (Applied Biosystems®) was added (Table 2) and assay performance was confirmed with tissue extraction from crab reference samples collected at the Wells NERR.

Samples were prepared for ddPCR according to the ddPCR Supermix for Probes protocol (Bio-Rad®) using 2 μL of undiluted sample extract. Reactions were scaled up to 24 mL for preparation, 20 mL were used for droplet generation, and the reaction was performed in a C1000 Touch Thermal Cycler (Bio-Rad®) (see Supplementary Material for detailed protocol).

Each set of reactions (for both metabarcoding and ddPCR) included a no template control (NTC) with reagent-grade water and positive controls of diluted DNA extracted from *C. maenas* tissue collected at the Wells NERR. PCR replicates were not run for the metabarcoding analysis, which was conducted as an initial method test, but the negative results were confirmed with ddPCR. The ddPCR method divides the sample aliquot into 20,000 micro droplets where each droplet is amplified separately to create thousands of technical replicates and a "count" of positive reactions (Hindson et al., 2011). Droplets with amplified target sequences fluoresce and the amplitude of the fluorescence is used to separate positive and negative reactions. In rare cases, reflection or other optical interference may result in a false positive reading, so samples with less than 3 positive droplets should be treated with caution. Results are reported as concentration (copies/μL). All ddPCR analyses were conducted with QuantaSoft™ Analysis Pro 1.0.596 (Bio-Rad Laboratories, Inc., Hercules, CA).

Table 2
Carcinus maenas CO1 assay (Neigel and Germane, unpub.).

Species	Gene	Description	Sequence (5′ – 3′)
Carcinus maenas	CO1	Forward	GTTGGAACAGGATGAACAGTCTATC
		Reverse	CGGCTAAATGTAAAGAGAAAATCCC
		Probe	TCAACTGAAGCACCAGCATGGGC

2.4. Data analyses

A series of Welch's (unequal variances) t-tests was conducted on log-transformed shifted DNA concentrations (copies/μL) (log10*(concentration + 0.04)) to test for statistical differences between comparable treatments (i.e., ovigerous-sediment vs. control-sediment; ovigerous-sediment vs. ovigerous-water). The shift of +0.04 was calculated as approximately half the single sample limit of detection, and so is a conservative bias to our measured concentrations; the shift is necessary for log transforming the samples with a concentration of zero. The exponential nature of the measured DNA concentrations was addressed by applying a log-normal model to perform the statistical tests. This analysis included samples collected at T-2 and T-3 combined (see Results for justification), from tanks shown in Table S1.

For each tank and sample type (sediment, water), the mean log change in concentration between timesteps (T-0 to T-1, T-1 to T-2, T-2 to T-3) was calculated as: mean[log(post-concentrations)]-mean[log(pre-concentrations)]. For each timestep and sample type, a single-sample two-sided *t*-test was used to determine if the mean log change in concentration (for all treatments combined) was significantly different from zero. This analysis included all tanks where samples were analyzed across the given timestep (see Table S1). All statistical analyses were conducted using Python 3.6 and SciPy 1.4.1 (Virtanen et al., 2020).

3. Results

3.1. Field study

The metabarcoding analysis did not detect *C. maenas* DNA in any of the field study samples. These samples yielded over 900 sequences with 114 species identified in the BLAST search, primarily marine oligochaete worms, copepods, and nematodes. These samples were re-analyzed with ddPCR which confirmed that detectable levels of *C. maenas* DNA were not present. These results indicated that the crabs, although present in traps, were not shedding detectable amounts of DNA into the environment. Based on these findings, laboratory trials were conducted to determine how much DNA is shed at different life stages.

3.2. Laboratory study

Sediment and water samples collected from tanks where live *C. maenas* were held for seven days showed that the mean concentration of DNA was similar for all life stages except for ovigerous crabs, where significantly higher DNA concentrations were present in sediment samples compared to the sediment samples of all other treatments (Fig. 1; Table S2; $p \leq 0.001$). In one of the ovigerous tanks, the eggs hatched between T-2 and T-3, with crab zoeae (larvae) swimming in the water column and near the sediment–water interface. Even without this tank, however, the ovigerous treatment still yielded significantly higher DNA concentrations compared to the other treatments. Since there was no significant difference between male and male x 2 ($p = 0.410$ water, 0.528 sediment), or between soft and soft x 2 ($p = 0.086$ water, 0.260 sediment), these were combined into "male" and "soft" treatments for

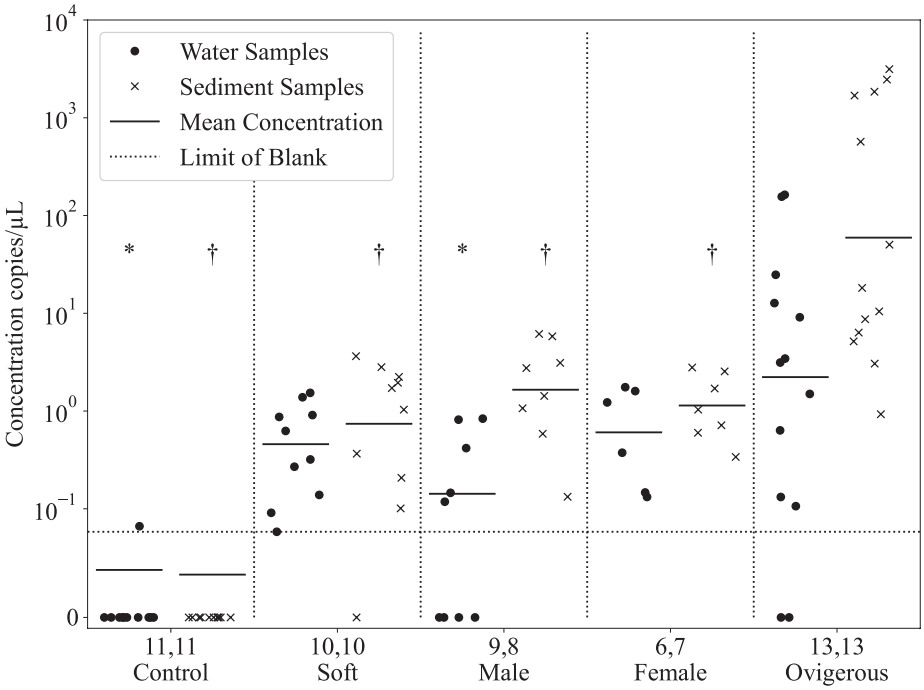

Fig. 1. DNA concentration (copies/µL) for each treatment (control, soft (including soft x 2), male (including male x 2), female, ovigerous) at times T-2 and T-3 combined, plotted on a symlog scale that transitions from linear to logarithmic at 0.08 copies/µL. Numbers on the x-axis indicate sample size (water, sediment). Symbols indicate statistical significance from ovigerous ($p < 0.05$; * for water, † for sediment) based on Welch's *t*-test pairwise comparisons on the log-transformed concentration (log10* (concentration + 0.04)). The limit of blank (dotted horizontal line) is drawn at the approximate concentration of a single positive droplet.

subsequent analyses to improve sample size symmetry across treatments. For all treatments, mean DNA concentrations were significantly higher than the controls (Table S2; $p \leq 0.031$) and most samples had concentrations greater than the limit of blank (Fig. 1). Mean DNA concentrations were higher for sediment compared to water samples for all treatments, but this difference was only significant for male ($p = 0.001$) and ovigerous ($p = 0.008$) treatments (Fig. 1; Table S2). All control tanks and all samples collected at T-0 had ≤1 positive droplet (Fig. 1), indicating a lack of contamination. The mean concentration of DNA in sediment and water samples was positively correlated ($r = 0.512$, $p < 0.001$).

DNA concentrations increased from T-0 to T-1 ($p = 0.075$ water, 0.026 sediment; two-sided *t*-test), but there was no significant change afterwards (Fig. 2). Outliers seen in Fig. 2 include tanks where DNA concentrations did not change from T-0 to T-1, and those that changed drastically from T-1 to T-2 or from T-2 to T-3; outliers primarily occurred in the ovigerous tanks. As there was no significant change in concentration from T-2 to T-3, we chose to combine those samples for the analysis in Fig. 1.

4. Discussion

The results from this study demonstrate the challenges associated with detecting *C. maenas* eDNA and confirm that crab life stage and sample collection method influence eDNA detection rates. Our field experiment did not detect *C. maenas* DNA in or around traps which contained hard-shelled green crabs in a natural estuarine environment. These findings were corroborated by subsequent laboratory trials that found low DNA concentrations (<10 copies/µL) in enclosed aquaria containing non-ovigerous hard- and soft-shelled crabs, regardless of crab abundance or sex. By comparison, Mizumoto et al. (2018) found eDNA concentrations magnitudes of order higher for aquaria containing comparable biomass of fish (Sakhalin taimen, *Parahucho perryi*). Meanwhile, a similar study to our own found relatively low concentrations of DNA in aquaria containing individual crayfish (*Procambarus clarkii*) (Geerts et al., 2018). In our study, eDNA detection rates were significantly higher for tanks containing ovigerous females, especially for the tank containing hatched zoeae. Across all treatments, eDNA detection rates were higher for sediment slurry samples compared to water samples, suggesting that the method of sample collection is important to consider when studying benthic organisms.

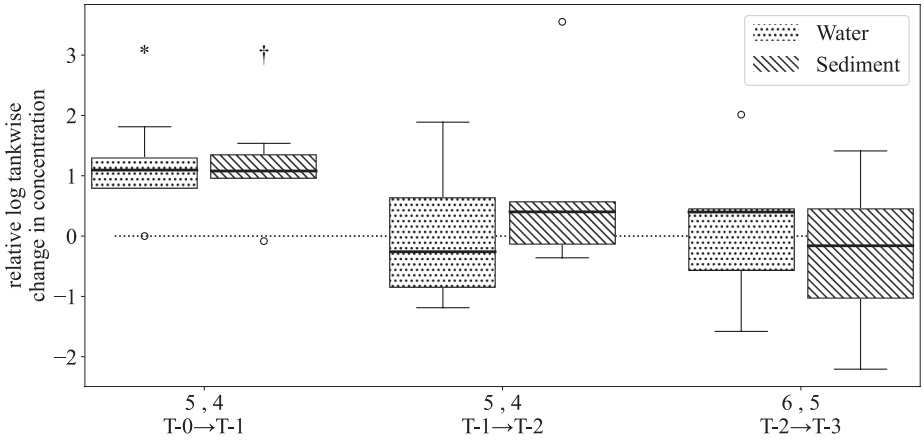

Fig. 2. Boxplot depicting the relative log change in DNA concentration between timesteps for sediment slurry and water samples (all treatments combined). Each data point represents a log change in concentration between timesteps for a given tank normalized by the average l1 norm of the log changes for that treatment. This includes all tanks where samples were analyzed across timesteps (see Table S1). Numbers on the x-axis indicate sample size (number of tanks). Symbols (* for water, † for sediment) indicate a significant difference from zero ($p < 0.05$) using a single sample *t*-test.

Our laboratory results indicate that the concentration of *C. maenas* eDNA in field conditions is likely below detection limits in many cases. DNA concentrations in an enclosed aquarium averaged 0.55 and 1.79 copies/μL in water and sediment samples, respectively, for all non-ovigerous crabs (at T-2 and T-3 combined). This is approximately an order of magnitude greater than the detectable level using common ddPCR analytic methods. In a natural environment, dilution and mixing are likely to reduce DNA concentrations to below detectable levels. In estuarine environments, hydrodynamics (e.g., currents, tides) may dilute DNA concentrations and transport DNA away from the source organism; natural processes (e.g., ultraviolet light) may also catalyze DNA degradation (Foote et al., 2012; Schmelzle and Kinziger, 2016; Thomsen et al., 2012), all of which may result in false negatives. False positives can result from DNA being transported from other waterways or from sources other than living crabs (e.g., predators, ballast water, sample contamination) (York, 2016). However, the present laboratory study suggests that consideration of crab life stage (ovigery in particular) and sample collection method (water vs. sediment) may facilitate green crab eDNA detection in the natural environment.

In this study, detection rates were higher in sediment slurry samples compared to water samples which suggests that sampling from the sediment or at the sediment–water boundary may be more efficient for detecting green crabs and other benthic organisms, at least in enclosed aquaria. Other studies have found that larger free-floating DNA fragments and DNA in organic material tend to settle onto the sediment surface and that DNA is more persistent in sediment compared to water samples (Jane et al., 2015; Schmelzle and Kinziger, 2016; Turner et al., 2015). However, direct sediment sampling is often limited by sample size, as relatively small volumes of sediment (<1 g) can be processed via most soil extraction methods. By contrast, filtering allows concentration of DNA from a larger volume of water. In the present laboratory study, filtering a 40 mL sediment–water slurry sample incorporated DNA from a larger volume for increased detection capacity. This is a unique method of sediment collection that should be explored further, particularly in field settings. If sediment slurry sampling is shown to improve the detectability of *C. maenas* in field settings, this sampling method could help facilitate the early detection of benthic invasive species.

In addition to the type of sample being collected (sediment vs. water), timing of sampling and life history of the target species should be considered when designing an eDNA detection study. In the present laboratory trials, aquaria containing ovigerous females had significantly higher detection rates than those containing hard- or soft-shelled crabs. In general, eDNA sources can include epidermal cells, fecal matter, urine, sloughed tissues or cells, larvae, eggs, mucus, or extracellular DNA (Deiner and Altermatt, 2014; Egan et al., 2015; Tréguier et al., 2014; York, 2016). In hard-shelled crabs, the presence of an exoskeleton may hinder the release of DNA into the environment via many of these sources (Dougherty et al., 2016; Tréguier et al., 2014). However, green crab eggs (like most crustacean egg masses) are composed of soft tissue and held externally, thereby exposing these eggs to the surrounding environment. Additionally, ovigerous females regularly aerate their egg masses (Dunn et al., 2017; Wheatly, 1981), providing an additional opportunity for DNA to be exuded into the water column or onto the sediment. In the present study, it was observed that some eggs fell off the brood onto the sediment surface. Detection rates were especially high in the aquarium in which the brood hatched and there were thousands of crab zoeae dispersed throughout the tank. Similar results were found in laboratory experiments with the invasive crayfish, *Pacifastacus leniusculus*, where eDNA detection rates were higher for ovigerous females (Dunn et al., 2017). While these experiments were conducted in enclosed aquaria, hatched larvae and dropped eggs in the natural environment would normally be dispersed by currents, likely resulting in lower detection rates. Regardless, these findings suggest that eDNA surveys conducted during a spawning event may help facilitate the detection of *C. maenas*. Therefore, the successful application of eDNA for crustaceans may depend in large part on sampling timing when eggs or larvae may be present in the water column.

An unexpected finding from this study was that eDNA detection rates were not significantly greater for soft- versus hard-shelled crabs. The degree of carapace softness as well as activity levels varied between crabs used in the soft-shell trial. Therefore, further research is needed to better understand the effects of carapace hardness and crab activity on eDNA detection.

Collectively, our findings provide insight into how eDNA can be used for improved management of invasive crab species. Detecting the presence of an invasive species while population densities are low is critical for early implementation of management efforts to keep abundances low or eradicate the population. To increase the efficacy of eDNA for detecting green crabs, the present findings suggest that sampling should be conducted during spawning events due to the presence of eggs and hatched larvae that leave more robust eDNA signatures in water and sediment samples. Targeting ovigerous females with eDNA surveys for example may (1) facilitate their removal from the population and (2) provide an improved understanding of local green crab reproduction strategies to manage their populations more effectively. Traditional trapping methods tend to bias catch toward hard-shelled and non-ovigerous adults since ovigerous females and soft-shelled crabs are less active and less likely to enter traps (Baeta et al., 2005; Crothers, 1968; Ropes, 1968); therefore, traditional methods can be supplemented with eDNA to obtain a more complete picture of population dynamics. This study also suggests that large sample sizes are needed to detect *C. maenas* eDNA in the natural environment, particularly when at low abundances, and that the collection of sediment–water slurry samples may be more effective than water sampling. The collection of slurry samples rather than direct sampling of the sediment should be further explored as a possible way of collecting larger sediment samples, particularly its application and efficacy in the natural environment.

5. Conclusions

The findings from this study indicate that crabs, and likely other benthic crustaceans, shed relatively low concentrations of DNA during most life stages, and highlight the importance of considering life stage and sampling methodology when applying eDNA techniques. The lack of *C. maenas* eDNA detection in our field study and low detection rates for aquaria housing hard- and soft-shell *C. maenas* suggest that benthic crustaceans may be difficult to detect using current eDNA techniques in estuarine systems. However, our results also suggest that eDNA sampling timed during spawning events and collection of samples from the sediment-water interface may improve detection rates in the natural environment. This study provides valuable information needed to make eDNA methods more effective for detecting benthic crustaceans in estuaries, which could serve as a critical tool for more efficiently monitoring and managing invasive estuarine crustaceans.

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CRediT authorship contribution statement

Laura C. Crane: Methodology, Investigation, Data curation, Writing - original draft. **Jason S. Goldstein:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. **Devin W. Thomas:** Formal analysis, Investigation, Writing - original draft, Visualization. **Kayla S. Rexroth:** Methodology, Investigation, Writing -

original draft. **Alison W. Watts:** Conceptualization, Methodology, Writing - original draft, Supervision, 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.2021.107412.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Aman, J., Grimes, K.W., 2016. Measuring Impacts of Invasive European Green Crabs on Maine Salt Marshes: A Novel Approach (Report to the Maine Outdoor Heritage Fund). Wells National Estuarine Research Reserve.

Amaral, V., Cabral, H.N., Jenkins, S., Hawkins, S., Paula, J., 2009. Comparing quality of estuarine and nearshore intertidal habitats for Carcinus maenas. Estuar. Coast. Shelf Sci. 83, 219–226. https://doi.org/10.1016/j.ecss.2009.03.029.

Baeta, A., Cabral, H.N., Neto, J.M., Marques, J.C., Pardal, M.A., 2005. Biology, population dynamics and secondary production of the green crab *Carcinus maenas* (L.) in a temperate estuary. Estuar. Coast. Shelf Sci. 65, 43–52. https://doi.org/10.1016/j.ecss.2005.05.004.

Bax, N., Hayes, K., Marshall, A., Parry, D., Thresher, R., 2002. Man-made marinas as sheltered islands for alien marine organisms: Establishment and eradication of an alien invasive marine species. In: Veitch, C.R., Clout, M.N. (Eds.), Turning the tide: the eradication of invasive species. Invasive Species Specialist Group of The World Conservation Union (IUCN), Aukland, pp. 26–39.

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., Geeves, W., 2003. Marine invasive alien species: a threat to global biodiversity. Marine Policy 27, 313–323. https://doi.org/10.1016/S0308-597X(03)00041-1.

Bayer, S.R., Countway, P.D., Wahle, R.A., 2019. Developing an eDNA toolkit to quantify broadcast spawning events of the sea scallop *Placopecten magellanicus*: moving beyond fertilization assays. Mar. Ecol. Prog. Ser. 621, 127–141. https://doi.org/10.3354/meps12991.

Beal, B.F., Coffin, C.R., Randall, S.F., Goodenow, C.A., Pepperman, K.E., Ellis, B.W., Jourdet, C.B., Protopopescu, G.C., 2018. Spatial variability in recruitment of an infaunal bivalve: experimental effects of predator exclusion on the softshell clam (*Mya arenaria* L.) along three tidal estuaries in Southern Maine, USA. J. Shellfish Res. 37, 1–27. https://doi.org/10.2983/035.037.0101.

Beal, B.F., Kraus, M.G., 2002. Interactive effects of initial size, stocking density, and type of predator deterrent netting on survival and growth of cultured juveniles of the softshell clam, *Mya arenaria* L., in eastern Maine. Aquaculture 208, 81–111. https://doi.org/10.1016/S0044-8486(01)00900-0.

Berry, T.E., Saunders, B.J., Coghlan, M.L., Stat, M., Jarman, S., Richardson, A.J., Davies, C.H., Berry, O., Harvey, E.S., Bunce, M., 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. PLoS Genet. 15, e1007943 https://doi.org/10.1371/journal.pgen.1007943.

Carlton, J.T., Cohen, A.N., 2003. Episodic global dispersal in shallow water marine organisms: the case history of the European shore crabs *Carcinus maenas* and *C. aestuarii*. J. Biogeogr. 30, 1809–1820. https://doi.org/10.1111/j.1365-2699.2003.00962.x.

Compton, T.J., Leathwick, J.R., Inglis, G.J., 2010. Thermogeography predicts the potential global range of the invasive European green crab (*Carcinus maenas*). Divers. Distrib. 16, 243–255. https://doi.org/10.1111/j.1472-4642.2010.00644.x.

Crothers, J.H., 1968. The biology of the shore crab *Carcinus maenas* (L.) 2. The life of the adult crab. Field Studies 2, 579–614.

Deiner, K., Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS One 9, e88786. https://doi.org/10.1371/journal.pone.0088786.

Dionne, M., Dalton, C., Wilhelm, H. (Eds.), 2006. Site Profile of the Wells National Estuarine Research Reserve. Wells National Estuarine Research Reserve, Wells, ME.

Dougherty, M.M., Larson, E.R., Renshaw, M.A., Gantz, C.A., Egan, S.P., Erickson, D.M., Lodge, D.M., 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. J. Appl. Ecol. 53, 722–732. https://doi.org/10.1111/1365-2664.12621.

Dunn, N., Priestley, V., Herraiz, A., Arnold, R., Savolainen, V., 2017. Behavior and season affect crayfish detection and density inference using environmental DNA. Ecol. Evol. 7, 7777–7785. https://doi.org/10.1002/ece3.3316.

Egan, S.P., Grey, E., Olds, B., Feder, J.L., Ruggiero, S.T., Tanner, C.E., Lodge, D.M., 2015. Rapid molecular detection of invasive species in ballast and harbor water by integrating environmental DNA and light transmission spectroscopy. Environ. Sci. Technol. 49, 4113–4121. https://doi.org/10.1021/es5058659.

Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A., Salling, A.B., Galatius, A., Orlando, L., Gilbert, M.T.P., 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLoS One 7, e41781. https://doi.org/10.1371/journal.pone.0041781.

Forsström, T., Vasemägi, A., 2016. Can environmental DNA (eDNA) be used for detection and monitoring of introduced crab species in the Baltic Sea? Mar. Pollut. Bull. 109, 350–355. https://doi.org/10.1016/j.marpolbul.2016.05.054.

Geerts, A.N., 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. Ecol. Indic. 84, 564–572. doi:10.1016/j.ecolind.2017.08.068.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. doi: 10.1111/2041-210X.12595.

Goldstein, J.S., Morrissey, E.M., Moretti, E.D., Watson, W.H., 2017. A comparison of the distribution and abundance of European green crabs and American lobsters in the Great Bay Estuary, New Hampshire, USA. Fish. Res 189, 10–17. https://doi.org/10.1016/j.fishres.2017.01.002.

Grosholz, E., Lovell, S., Besedin, E., Katz, M., 2011. Modeling the impacts of the European green crab on commercial shellfisheries. Ecol. Appl. 21, 915–924. https://doi.org/10.1890/09-1657.1.

Gu, W., Swihart, R.K., 2004. Absent or undetected? Effects of non-detection of species occurrence on wildlife–habitat models. Biol. Conserv. 116, 195–203. https://doi.org/10.1016/S0006-3207(03)00190-3.

Hidalgo, F.J., Barón, P.J., Orensanz, J.M. (Lobo), 2005. A prediction come true: the green crab invades the Patagonian coast. Biol. Invasions 7, 547–552. doi:10.1007/s10530-004-5452-3.

Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., Kitano, T.K., Hodel, M.R., Petersen, J.F., Wyatt, P.W., Steenblock, E.R., Shah, P.H., Bousse, L.J., Troup, C.B., Mellen, J.C., Wittmann, D.K., Erndt, N.G., Cauley, T.H., Koehler, R.T., So, A.P., Dube, S., Rose, K.A., Montesclaros, L., Wang, S., Stumbo, D.P., Hodges, S.P., Romine, S., Milanovich, F.P., White, H.E., Regan, J.F., Karlin-Neumann, G.A., Hindson, C.M., Saxonov, S., Colston, B.W., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal. Chem. 83, 8604–8610. https://doi.org/10.1021/ac202028g.

Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., Letcher, B.H., Whiteley, A.R., 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol. Ecol. Resour. 15, 216–227. https://doi.org/10.1111/1755-0998.12285.

Janosik, A.M., Johnston, C.E., 2015. Environmental DNA as an effective tool for detection of imperiled fishes. Environ. Biol. Fishes 98, 1889–1893. https://doi.org/10.1007/s10641-015-0405-5.

Jerde, C.L., Mahon, A.R., Chadderton, W.L., Lodge, D.M., 2011. Sight-unseen“ detection of rare aquatic species using environmental DNA. Conserv. Lett. 4, 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x.

Levi, T., Allen, J.M., Bell, D., Joyce, J., Russell, J.R., Tallmon, D.A., Vulstek, S.C., Yang, C., Yu, D.W., 2019. Environmental DNA for the enumeration and management of Pacific salmon. Mol. Ecol. Resour. 19, 597–608. https://doi.org/10.1111/1755-0998.12987.

Lobo, J., Shokralla, S., Costa, M.H., Hajibabaei, M., Costa, F.O., 2017. DNA metabarcoding for high-throughput monitoring of estuarine macrobenthic communities. Sci. Rep. 7, 15618. https://doi.org/10.1038/s41598-017-15823-6.

Mächler, E., Deiner, K., Steinmann, P., Altermatt, F., 2014. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshwater Sci. 33, 1174–1183. https://doi.org/10.1086/678128.

Mizumoto, H., Urabe, H., Kanbe, T., Fukushima, M., Araki, H., 2018. Establishing an environmental DNA method to detect and estimate the biomass of Sakhalin taimen, a critically endangered Asian salmonid. Limnology 19, 219–227. https://doi.org/10.1007/s10201-017-0535-x.

Pilliod, D.S., Goldberg, C.S., Arkle, R.S., Waits, L.P., Richardson, J., 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Can. J. Fish. Aquat. Sci. 70, 1123–1130. https://doi.org/10.1139/cjfas-2013-0047.

Poirier, L.A., Symington, L.A., Davidson, J., St-Hilaire, S., Quijón, P.A., 2017. Exploring the decline of oyster beds in Atlantic Canada shorelines: potential effects of crab predation on American oysters (*Crassostrea virginica*). Helgol. Mar. Res. 71 https://doi.org/10.1186/s10152-017-0493-z.

Raposa, K.B., Goldstein, J.S., Grimes, K.W., Mora, J., Stacey, P.E., Mckinney, R.A., 2019. A comparative assessment of salt marsh crabs (Decapoda: Brachyura) across the National Estuarine Research Reserves in New England, USA. J. Crustacean Biol. https://doi.org/10.1093/jcbiol/ruz083.

Ropes, J.W., 1968. The feeding habits of the green crab, *Carcinus maenas* (L.). Fishery Bull. 67, 183–203.

Rossong, M.A., Williams, P.J., Comeau, M., Mitchell, S.C., Apaloo, J., 2006. Agonistic interactions between the invasive green crab, *Carcinus maenas* (Linnaeus) and juvenile American lobster, *Homarus americanus* (Milne Edwards). J. Exp. Mar. Bio. Ecol. 329, 281–288. https://doi.org/10.1016/j.jembe.2005.09.007.

Schmelzle, M.C., Kinziger, A.P., 2016. Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. Mol. Ecol. Resour. 16, 895–908. https://doi.org/10.1111/1755-0998.12501.

Sigsgaard, E.E., Carl, H., Møller, P.R., Thomsen, P.F., 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biol. Conserv. 183, 46–52. https://doi.org/10.1016/j.biocon.2014.11.023.

Sigurdsson, G.M., Rochette, R., 2013. Predation by Green Crab and Sand Shrimp on Settling and Recently Settled American Lobster Postlarvae. J. Crustacean Biol. 33, 10–14.

Tan, E.B.P., Beal, B.F., 2015. Interactions between the invasive European green crab, *Carcinus maenas* (L.), and juveniles of the soft-shell clam, *Mya arenaria* L., in eastern Maine. USA. J. Exp. Mar. Bio. Ecol. 462, 62–73.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x.

Thomsen, P.F., Møller, P.R., Sigsgaard, E.E., Knudsen, S.W., Jørgensen, O.A., Willerslev, E., 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PLoS One 11, e0165252. https://doi.org/10.1371/journal.pone.0165252.

Thomsen, P.F., Willerslev, E., 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biol. Conserv. 183, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019.

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M.A., Roussel, J.-M., 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. J. Appl. Ecol. 51, 871–879. https://doi.org/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, extraction, and detection. Environ. DNA 1, 99–108. https://doi.org/10.1002/edn3.21.

Turner, C.R., Uy, K.L., Everhart, R.C., 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biol. Conserv. 183, 93–102. https://doi.org/10.1016/j.biocon.2014.11.017.

Ushio, M., Murakami, H., Masuda, R., Sado, T., Miya, M., Sakurai, S., Yamanaka, H., Minamoto, T., Kondoh, M., 2018. Quantitative monitoring of multispecies fish environmental DNA using high-throughput sequencing. MBMG 2, e23297.

Virtanen, P., Gommers, R., Oliphant, T.E., Haberland, M., Reddy, T., Cournapeau, D., Burovski, E., Peterson, P., Weckesser, W., Bright, J., van der Walt, S.J., Brett, M., Wilson, J., Millman, K.J., Mayorov, N., Nelson, A.R.J., Jones, E., Kern, R., Larson, E., Carey, C.J., Polat, I., Feng, Y., Moore, E.W., VanderPlas, J., Laxalde, D., Perktold, J., Cimrman, R., Henriksen, I., Quintero, E.A., Harris, C.R., Archibald, A.M., Ribeiro, A. H., Pedregosa, F., van Mulbregt, P., SciPy 1.0 Contributors, 2020. SciPy 1.0: Fundamental algorithms for scientific computing in Python. Nat. Methods 17, 261–272.

Wheatly, M.G., 1981. The provision of oxygen to developing eggs by female shore crabs *(Carcinus maenas)*. J. Mar. Biol. Ass. 61, 117–128. https://doi.org/10.1017/S0025315400045951.

York, G., 2016. Environmental DNA Detection of Invasive Species (Master thesis). University of Maine.

Young, A.M., Elliott, J.A., 2019. Life history and population dynamics of green crabs (*Carcinus maenas*). Fishes 5, 4. https://doi.org/10.3390/fishes5010004.