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CHALLENGES IN eDNA DETECTION OF THE INVASIVE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*

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Abstract

The early detection of invasive species is essential to cease the spread of the species before it can cause irreversible damage to the environment. The analysis of environmental DNA (eDNA) has emerged as a non-harmful method to detect the presence of a species before visual detection and is a promising approach to monitor invasive species. Few studies have investigated the use of eDNA for arthropods, as their exoskeleton is expected to limit the release of eDNA into the environment. We tested published primers for the invasive European green crab, *Carcinus maenas*, in the Gulf of Maine and found them not species-specific enough for reliable use outside of the area for which they were designed for. We then designed new primers, tested them against a broad range of local faunal species, and validated these primers in a field study. We demonstrate that eDNA analyses can be used for crustaceans with an exoskeleton and suggest that primers and probe sequences must be tested on local fauna at each location of use to ensure no positive amplification of these other species.

Running head: eDNA challenges

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Introduction

Invasive species, introduced intentionally or by accident, can cause irreversible damage to the environment, threaten marine and freshwater ecosystems by outcompeting native species, thus decreasing biodiversity, and even threatening and impacting human health (Darling and Mahon 2011). 20-30% of all introduced species have caused major damages to their new environments, leading to over $120 billion of damages each year, and potential solutions to identify the most cost-effective way to repair and prevent these damages are still being investigated (Pimentel et al. 2001; Epanchin-Niell, 2017). Early detection of an invasive species is crucial to preserve biodiversity and prevent environmental damages as many eradication methods can be costly and cause harm to native wildlife. Therefore, a reliable method of identifying and tracking of an invasive species is necessary (Harvey, et al. 2009; Gherardi et al. 2011; Jerde et al. 2011; Simberloff et al. 2012).

Monitoring marine species, particularly in fisheries management, is often accomplished through catch and release techniques (Cooke et al. 2006; Pollock and Pine, 2007). However, these observations are sometimes inaccurate due to limited access to the respective areas (e.g., marine protected areas), required taxonomic expertise, limited time or funding for the respective detailed surveys, or morphological similarities between species, for example due to phenotypic plasticity (Cooke et al. 2006; Polonco Fernández et al. 2021; Thomsen and Willerslev, 2015). Capture detections often rely on bottom trawling, which can cause habitat destruction and possible bycatch of unrelated species. Underwater visual censuses and photography or video surveys can be problematic due to environmental conditions (e.g., light levels) and spatial coverage of these surveys, thus suffering from biases toward particular species. Furthermore, particular habitats (e.g., rocky areas with changing benthic characteristics) may be too costly to access with traditional visualization gear (Afzali et al. 2021; Danielsen et al. 2005; Danovaro et al. 2014).

The detection of environmental DNA (eDNA) has reliably been applied in terrestrial and aquatic environments to detect native and invasive species (Pilloid et al. 2013; Rusche et al. 2007; Taberlet et al. 2012). DNA is continuously released by organisms into their respective environment (Lawson- Handley, 2015), and can be isolated from water, soil, or air, and then amplified by quantitative real-time polymerase chain reaction (qPCR) which allows for quantification of DNA through the use of a fluorescent probe. The DNA is shed by living or deceased organisms (e.g., from skin and bodily excretions), as well as extracellular DNA from cell death or destruction (Deiner and Altermatt, 2014; Thomsen and Willerslev, 2015; Strickler et al. 2015; Taberlet et al. 2012). Once exposed to the environment, eDNA begins to degrade due to chemical hydrolysis and microbial activity. Additional abiotic factors,

including temperature, pH, UV radiation, and salinity can adjust this degradation rate, specifically by altering enzymatic activity which degrades DNA (Strickler et al. 2015). eDNA can be preserved in water from hours to weeks, or even years in ice, depending on the abiotic factors and the aggregate eDNA released (Baker et al. 2018; Balasingham et al. 2017; Dejean et al. 2011; Willerslev et al. 2014).

Species-specific detection by eDNA and qPCR has been attempted for multiple invasive species like *Carcinus maenas*, the European Green crab (Bott et al. 2010; Crane et al. 2021; Roux et al. 2020). This species is a prime example for an invader that has caused significant damage worldwide. Native to Europe and North Africa, it is now established throughout the coasts of North America, Australia, South Africa, and Asia (Ahyong 2005; Klassen and Locke 2007) and is predicted to even invade Antarctica (Aronson et al. 2015). *C. maenas* populations have been increasing along the coasts of the United States and other countries (Gharouni et al. 2017; Yamada et al. 2005) which has led to destruction of eelgrass beds and an increase in coastal erosion (Garbary et al. 2014; Infantes et al. 2016). Due to *C. maenas*' high adaptability, this crab quickly becomes the dominant species, outcompeting native species in the intertidal once it is established, and thus impacting the distribution and abundance of a multitude of species, specifically bivalves and other crabs (Bott et al. 2010).

Using eDNA analysis can aid in the early detection and tracking of *C. maenas* before it has become established in a new location. Methods of trapping the species are still possible, however this relies on the species being present in the exact location of the traps and actually being caught. eDNA analyses provide an additional method of tracking the species without relying on successful capture of the species itself. Furthermore, it could be easily implemented into other tracking methods, and can be used in conjunction with eDNA detections of other species without the need for additional equipment.

C. maenas' wide range and genetic variability between populations of *C. maenas* may require different primers for the detection of each *C. maenas* population (Darling et al. 2008; Darling, 2011; Jeffery et al. 2017; Roman and Palumbi 2004). Primers and probes for qPCR specific to *C. maenas* have previously been designed and implemented in detection methods in Australia and have proven to amplify only *C. maenas* DNA and not that of other local Australian species (Bott et al. 2010; Louise-Marie et al. 2020). However, it is still unknown whether these primers can be used in other locations for the detection of *C. maenas* due to the genetic variability and the presence of diverse local species. The recently published primers for *C. maenas* in the Gulf of Maine (Crane et al. 2021*)* have not been validated with local species and were only tested *in silico*.

The goal of this study was to develop and validate qPCR assays for use in detecting *C. maenas* in the Gulf of Maine, USA. Furthermore, we determined whether primers and probes for *C. maenas* in the Gulf of Maine can also be used in other, genetically different populations of this species.

Materials and Methods

2.1 Animal collection

Multiple species were collected by hand in the intertidal zone in Biddeford Pool, Maine, USA 43.442292° N, 70.341244° W, *Carcinus maenas* (European green crab), *Hemigrapsus sanguineus* (Asian shore crab), *Pagurus longicarpus* (Longwrist hermit crab), *Balanus improvisus* (Bay barnacle), *Asterias forbesi* (Forbes sea star), *Modiolus modiolus* (Northern horse mussel), *Mytilus edulis* (Blue mussel), *Littorina littorea* (Common periwinkle), and *Nucella lapillus* (Atlantic dog whelk). Specimen of *Homarus americanus* (American lobster), *Cancer irroratus* (Rock crab), and *Cancer borealis* (Jonah crab) were obtained from local lobster fishermen in Biddeford, Maine. Male *C. maenas* were also collected by hand and by traps in Kejimkujik Seaside National Park, Nova Scotia, Canada (43°50'32.3"N 64°50'04.1"W); Placentia Bay, Newfoundland, Canada (47°49'07.1"N 54°01'08.8"W); and Sandgerdi, Iceland (64°2'17.2284"N 22°43'16.8096"W) and transported in coolers by car (crabs from Maine and Canada) or air cargo (crabs from Iceland) to the Marine Science Center of the University of New England in Biddeford, Maine. Crabs from locations other than Maine were held in separate 300 l tanks in a flow through sea water system and fed an assortment of fish *ad libitum* once a week. Effluent from the system was filtered through 1 μm filters and sterilized by 2 UV filters (QL-40 Lifegard Ultraviolet Sterilizer) prior to discharge. The entire system was housed in a 4-meter diameter pool as a secondary containment. The system was permitted and inspected by the Maine Department of Marine Resources (permit numbers S2013-007, S2014-007, S2015-008, S2016-008, S2017-007). Hemolymph samples were taken from decapod crustaceans by inserting a needle into the arthropodial membrane of the 4th walking leg. In molluscs, echinoderms, and the barnacle a piece of tissue was excised with bleach - sterilized scissors. DNA was extracted from the hemolymph or tissue using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol.

Carcinus maenas-specific primers and probe published by Bott et al. (2010) were tested with the extracted DNA from multiple crustacean species by qPCR on a Stratagene Mx3005x thermocycler following the parameters specified in Bott et al. (2010): 95ºC 15 min, 40 cycles of 95ºC 15 seconds, 60ºC 1 minute.

2.2 New Primer Design

Sequences of the COI gene of *Carcinus maenas* (GenBank Accession number JQ306003.1), *Hemigrapsus sanguineus* (KT209545.1), *Homarus americanus* (KU564525.1), *Cancer irroratus* (MG320501.1), *Cancer borealis* (KY250734.1), *Callinectes sapidus* (MH235922.1), and *Crangon crangon* (KT209555.1) were aligned using the online Multalign tool (http://multalin.toulouse.inra.fr/multalin/) and regions of higher inter-specific nucleotide variability in the alignments were identified. Organisms for nucleotide alignment were chosen based on genetic relativity to *C. maenas* and population in the Gulf of Maine (i.e., organisms that are commonly found in the same location as *C. maenas* were chosen). GenBank sequences were compared to at least two other GenBank sequences to ensure no major nucleotide differences. Multiple degenerate forward and reverse primers were designed manually, along with TaqMan MGB-FAM qPCR probes. Primers were added as 1 μl of 100 nM stock solution in 40 μl GoTaq qPCR Master Mixture (Promega, Madison, WI, USA), TaqMan MGB-FAM probe was added as 1 μl of 100 μM stock solution. qPCR conditions were 15 minutes at 95˚C; then 40 cycles of 15 seconds at 95˚C and 1 minute at 62˚C. The last segment was one cycle for 1 minute at 95˚C, 30 seconds at 62˚C, and 30 seconds at 95˚C. The primers and probe which yielded amplification for *C. maenas* were then tested for specificity on a broad range of other local species, as well as validity of these primers on *C. maenas* from other populations (Table 1.1). If DNA amplification occurred before 40 cycles within qPCR, the sample was deemed to have positive amplification of DNA, if amplification curves did not reach the threshold (CT) at cycle 40 this was considered negative amplification and interpreted as no DNA present in the sample. For every qPCR, negative (no DNA added) and positive controls (DNA isolated from green crab tissue) were run along with the isolated DNA from the collected samples.

To quantify eDNA concentration, a standard curve with cycle thresholds (CT) from a dilution series (1x, 10x, 100x, 1000x) of isolated DNA from the species was used. DNA for these methods was isolated from the liver of *C. maenas* using the Qiagen DNeasy Blood and Tissue Kit and the concentration was determined using a NanoDrop 2000 spectrophotometer.

2.3 Validation in the Field

To test whether the designed primers can be used to detect eDNA of *C. maenas* in the field we collected sea water samples from the dock in the harbor of Wells, Maine, USA (43.320093° N, 70.563395° W). A remote operated vehicle (BlueROV2, BlueRobotics, Torrance, CA) with an attached GoPro camera was deployed to record bottom fauna and confirm that *C. maenas* was present at the sample site before beginning the study. Ninety minutes before

the high tide, 1000 mL water samples were collected using a Niskin bottle every 0.6 meters starting at the surface and moving towards the bottom at 5 meters. Water samples were stored in sterile Nalgene bottles on ice and filtered in the lab within 2 hours (see methods 2.4). As a control, a cooler blank with 200 mL of deionized water was brough to the field to check for contamination. This, along with 200 mL of deionized water as a blank sample from the lab was filtered before the collected water samples to ensure there was no DNA contamination from the equipment used. The water collection with depth and negative control water samples were collected on 5 different days during a one-month period.

2.4 Filtration Methods and DNA Extraction

To extract eDNA from seawater a system of four 300 mL filter funnels with sterile 47 mm 0.45 cellulose nitrate filters connected to a vacuum pump (Gast DOA-P7004-AA) was constructed. This system was contained in a light proof box and sterilized by 7-Watt UV light for ten minutes before each water filtration (Ravanat et al. 2001). Sea water samples were filtered, and filters were stored in individual sterile 2 mL Eppendorf tubes at -80˚C until DNA isolation, if isolations could not be performed immediately.

DNA from filters was isolated using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer instructions, with minor modifications. 180 μL of buffer ATL and 20 μL Proteinase K were added to the filter and incubated at 56˚C for 10 minutes. 200 μL of buffer AL was added to the samples and they were incubated again at 56˚C for 10 minutes. After incubation, 200 μL of ethanol was added and the tubes were centrifuged for 1 minute at 8000 rpm to separate the liquid from the filter. The liquid was pipetted to a DNeasy Mini spin column and the column was washed with 500 μL buffer AW1 and 500 μL of buffer AW2. DNA was eluted with 200 μL buffer AE. The eluted DNA was then stored at -80˚C.

2.5 Quality Assurance/ Quality Control (QA/ QC)

The following quality control steps were performed to ensure no contamination of samples: Before sampling, all collection Nalgene bottles were sterilized with 10% bleach for 10 minutes and rinsed with deionized water. Once sterilized, Nalgene bottles were tightly closed and placed in a sealed, bleach-sterilized container for transport to the field. In the field, Nalgene bottles were removed from storage as needed using fresh gloves. After collection of samples, Nalgene bottles were immediately placed on ice to slow down the degradation rate of collected eDNA. All samples were filtered immediately after returning to the laboratory, within 2 hours of sample collection.

The filtration system set up within the laboratory was contained in a box to minimize contamination from other sources. The system was soaked with 10% bleach for 10 minutes and rinsed with DI water after filtering each sample. It was then further sterilized with a UV light for an additional 10 minutes. All filters and collection tubes used were individually packaged and sterile. After filtration, filters were either directly used for DNA isolation or stored at -80˚C until DNA isolation. Following DNA isolations, samples were stored at -80˚C until qPCR analysis. All samples were amplified in duplicates. Control samples of deionized water, as well as artificial sea water, were filtered before experimental samples and analyzed through qPCR to corroborate that the filtration system was not contaminated.

2.6 Statistics

Field test data was analyzed through Type III ANOVA and Kruskal Wallis tests to test for differences in eDNA by sample depth. All data are shown as mean +/- standard deviation. All analyses were performed using R Studio v. 1.3.1093 (R Core Development Team, 2020).

Results

3.1 New Primer design

Primers and probes within the COI gene published by Bott et al. (2010) tested on *C. maenas,* and related species including *H. sanguineus, C. irroratus,* and *H. americanus* yielded positive amplification of all tested species, rather than solely *C. maenas* (Fig 1). Positive amplification of *C. maenas* occurred at a CT of 25, the other crustaceans tested did have positive amplification as well (CT 33). Dissociation curves showed clean peaks, indicating that the primers amplified only one DNA product, as intended.

New primers had to be designed for the amplification of solely *C. maenas* through the alignment of the COI gene of four crustaceans. A total of 5 forward primers, 4 reverse primers, and 2 probes for qPCR were designed (Supplemental Material Table 2) and tested in varying combinations. The primers and probe that yielded robust amplification of *C. maenas* DNA were:

forward primer 5'-AAT ATT GGG AGG GCC AGA TAT AG-3'

reverse primer 5'-AGG ATC GAA GAA TGA GGT GTT TAG-3'

TaqMan probe 5'-6FAM-GGT TCT GAT TAC TTC CTCC GTC TTT AAC CT-MGB-3'.

These sequences are not identical to their corresponding sections of the COI gene of *C. maenas* to account for differences in annealing temperature between the primer and probe (Fig 5 in supplemental material).

Amplification of DNA of only *C. maenas* occurred early in the qPCR, at a CT of 23.05 ± 0.5 (n=5; Fig 2; Table 1.1). No amplification occurred for non-target species. The final qPCR conditions were 15 minutes at 95˚C; then 40 cycles of 15 seconds at 95˚C and 1 minute at 62˚C. The last segment was one cycle for 1 minute at 95˚C, 30 seconds at 62˚C, and 30 seconds at 95˚. CT values lower than 40 were considered positive amplification of *C. maenas*. For calculating average CT values, if no detection was found, this was considered a CT of 40.

3.2 *Carcinus maenas* Populations

The optimized primer and probe sequences were tested on *C. maenas* from the Gulf of Maine, Newfoundland, Nova Scotia, and Iceland. The COI gene was aligned between populations to note any nucleotide differences between them (Figure 1.3). DNA isolated from hemolymph of crabs from each of these populations yielded a positive signal in the qPCR and a clean single peak in the respective dissociation curve. *C. maenas* from the Gulf of Maine yielded a CT of 23.05 ±0.5 and Nova Scotia crabs yielded a CT of 23.34 ± 1.4, while samples of *C. maenas* from Newfoundland and Iceland had CT's of 36.17 ± 3.7 and 38.8 ± 1.68 (Table 1.1). CT values from *C. maenas* of Newfoundland and Iceland were significantly different from Maine and Nova Scotia (ANOVA $F_{3,10}$= 50.47, p= 2.41E-6).

3.3 Validation in the Field

The remote operated vehicle (BlueRov2) diving at the sampling site in the harbor of Wells, ME, showed a small number of *C. maenas* at our testing site. This site was chosen due to the easy access and anecdotal information on a large *C. maenas* population presence. Within a 15-minute dive covering about 100 m^2 of benthos we spotted 5 crabs and 2 lobsters walking on sandy ground. Despite this relatively low local abundance, their eDNA was detected in the water column through qPCR amplification (surface to 5 meters deep) of the water column. However, amplification throughout the depth of the water column was variable depending on day of sample collection (Fig 4). The only depth which had continuous amplification was the deepest depth of 5 meters. Despite this, no other clear trend of eDNA detection (measured in qPCR cycle threshold) through the water column could be detected as water conditions (temperature, salinity, currents) might have varied between sampling days in this highly dynamic harbor estuary system. Negative control samples of filtered DI water did not amplify, demonstrating no cross contamination of DNA samples due to the filtration equipment, and all positive control samples successfully amplified.

Discussion

The emerging prevalence of eDNA as a method of choice in conservation biology and other fields requires confidence in the species-specificity of the used primers and an understanding of the persistence of the eDNA in the

environment (Collins et al. 2018). Detecting eDNA is frequently used for the detection of rare and invasive species (Dejean et al. 2011; Hinlo et al. 2018; Jerde et al. 2011). For instance, eDNA analysis was used to determine the distribution of the endangered Chinook salmon (*Oncorhynchus tshawtscha*) (Laramie et al. 2015). This type of analysis was also used for the detection of bull trout (*Salvelinus confluentus*) and brook trout (*Salvelinus fontinalis)* to better understand their population dynamics and showed that especially when distinguishing between species of the same genus specificity of the primers is of most importance to avoid false positives (Wilcox et al. 2013). Similarly, to detecting rare species, invasive species can be easily detected by eDNA, as shown for the invasive oriental weather loach (*Misgurnus anguillicaudus*) in Australia (Hinlo et al. 2018), the invasive Burmese python (*Python bivittatus)* (Piaggio et al. 2013), the cane toad *Rhinella marina* (Tingley et al. 2019), the invasive Northern Pacific seastar, *Asterias amurensis*, and the invasive European green crab, *Carcinus maenas,* in Australia (Bott et al. 2010).

In the Gulf of Maine, USA, *C. maenas* has become a destructive invasive species, leading to environmental and biodiversity damages. Eelgrass (*Zostera marina* L.) destruction in southern Maine has been linked to increased bioturbation by this species (reference). This destruction of local eelgrass is of utmost concern due to the importance of this community on the local habitat. Eelgrass is one of the most productive plant communities in the area and is a necessary habitat for fish and shellfish species, thus providing an abundant food source for local marine, including avian, species, and the economic food industry of southern Maine (Neckles, 2015; Orth et al. 2006). Eelgrass also provides oceanographic importance by stabilizing bottom sediment and regulating wave action, prolonging the process of coastal erosion (Orth et al. 2006a). With the increase in devastation of this plant community due to *C. maenas* activity has come the increase in costal erosion and loss of habitat for numerous species (Neckles, 2015). *C. maenas* has also impacted native species due to predation. Juvenile rock crabs (*Caner irroratus)* and soft-shell clam (*Mya arenaria*) are highly consumed by green crabs and have decreased in population due to this predation. This not only harms the biodiversity of local habitats, but also the economy (Tan and Beal, 2015; Griffen and Riley, 2015). Efforts to monitor the arrival of this destructive species in new areas is thus of utmost importance to aid early eradication of *C. maenas* before causing irreversible damage to coastal ecosystems. eDNA analyses can be a helpful tool for this if used with the appropriate methods and species-specific primers.

Bott et al. (2010) developed a series of species-specific primers for eDNA detection of multiple species found in Australia, including *Carcinus maenas*. The *C. maenas*-specific primers were based on the COI gene, a standard target for DNA barcoding (for review see e.g., Krishnamurthy and Francis 2012; Kress et al. 2014). However, there

is considerable variability of the COI gene sequence among the *C. maenas* populations worldwide (Roman and Palumbi 2004; Brian et al. 2006). We tested the primers and probe published by Bott et al. (2011) on *C. maenas* caught in Maine, USA, as well as on multiple other species found in the Gulf of Maine. The described *C. maenas*-specific primers lead to gene amplification by qPCR not only in *C. maenas*, but also in the Asian shore crab, *Hemigrapsus sanguineus*, the Rock crab, *Cancer borealis*, and the Jonah crab, *Cancer irroratus*. *H. sanguineus* is also an invasive crab which competes with *C. maenas* in the rocky intertidal (Griffen, 2016). We did not test these primers on further local species as it was clear that these primers do not provide the species-specificity required in our test area.

While the primers designed by Bott *et al*. (2010) show great species specificity in the Australian fauna, using the same primers the Gulf of Maine may cause false positives when testing for the presence of *C. maenas* in the presence of other crustaceans. Our results indicate that species-specific eDNA primers for species that are distributed world-wide, and coexists with different other species, need to be assessed carefully in the respective context. It is thus suggested that primer and probe sequences be tested on related local species when using them for eDNA analysis in different locations. *C. maenas* has successfully invaded South Africa, Japan, Pacific Coast of USA, Canada, Tasmania, Argentina, and is predicted to invade Antarctica (Aronson, 2015; Carlton and Cohen, 2003; Hidalgo et al. 2005; Roman 2006). With this vast range comes variability in genetics and haplotypes. For example, between Canada and New York, USA there are 6 haplotypes alone and the differences in genetic makeup are consistent with minimal gene flow between particular populations (Roman and Palumbi, 2004; Williams et al. 2015). Thus, it is necessary to test the primer and probe sequences on each population of *C. maenas* as well to ensure species specificity of primers.

Our newly designed primer and probe sequences were additionally tested on *C. maenas* of three other populations found in Nova Scotia, Newfoundland, and Iceland. The population from Nova Scotia showed amplification of DNA at a CT of 23.34 ± 1.4, similar to that of *C. maenas* in the Gulf of Maine with a CT of 23.05 ±0.5. Green crabs from Newfoundland and Iceland, however, yielded amplifications at a CT of 36.17 ± 3.7 and 38.8 ± 1.68, respectively. This wide range of CT values is possibly due to DNA degradation attributed to age of the samples, and not the difference in genetic makeup due to a lack of nucleotide differences within the location of base pairs of primers and probes. These samples were stored in a -80ºC freezer for approximately three years prior to this test, while the samples of crabs from Nova Scotia and the Gulf of Maine were new (samples taken immediately before DNA isolations and qPCR). Comparing samples from crabs from Nova Scotia that were stored for 3 years with freshly sampled DNA from Nova Scotian crabs showed CTs of 32.27±1.25 vs 23.34 ± 1.4, respectively. Consequently, we

interpret the lower CT values in crabs from Iceland, Newfoundland and Nova Scotia not as a lower performance of the new primers, but as caused by deterioration of the DNA in the freezer.

We conclude that we do get positive amplification of DNA from all four populations of *C. maenas* tested with our new primer and probe combination. However, based on our findings the primer and probe sequences need to be tested against the local fauna in Iceland, Newfoundland and Nova Scotia, to make sure that the primers don't detect a species that is not located and already tested in Maine.

Exoskeletons of arthropods are expected to hinder the release of eDNA, thus making eDNA analyses in the filed challenging (Dougherty et al. 2016; Tréguier et al. 2014). Therefore, field tests were used to determine whether *C. maenas* eDNA could be detected in the water with a low abundance of the species present. Primer and probe sequences for eDNA detection of *C. maenas* in Southern Maine designed by Crane et al. (2021) easily detected even life stage differences in a laboratory study, but failed to detect eDNA in sediment samples in the field from below baited traps. In that study, no eDNA from the species was found in the sediment, despite the positive presence of *C. maenas*. While we did not test our primers and probe on sediment samples, in water samples *C. maenas* eDNA was easily detected along the depth profile.

Depth profiles additionally showed variability of eDNA detection per day, with detection ranging from qPCR CT values of approximately 28 to 40, with 40 corresponding to no detection of the species. The most consistent eDNA amplification of *C. maenas* occurred closest to the benthos at 5 meters. However, positive amplification was not consistently shown at the depths preceding this at 4.4 meters and 3.8 meters. The sampling location in the Wells harbor is a highly dynamic estuarine system with 4 rivers (Webhannet River, Pope Creek, Depot Brook, and Blacksmith Brook) draining through a marsh area into a harbor. The estuary is protected by a peninsula and drains into the ocean through a narrow channel. The average tidal range at this harbor is about 2 m (Monserrat et al. 2014). This dynamic system leads to significant water mixing, potentially either leading to an omnipresence of *C. maenas* eDNA, or a dilution below detection level, or any scenario in between, dependent on currents, tides and other parameters. Whether a more pronounced stratification of the eDNA signal can be found in deeper more stratified water with less mixing remains to be shown and should be addressed in future studies.

In conclusion, we have developed and validated qPCR primers for species-specific eDNA detection and shown that they need to be tested against the local fauna to prevent false positives. It was questioned earlier whether crustaceans leave a detectable eDNA signal in the water, due to their exoskeleton and subsequent potentially reduced

eDNA release (Dougherty et al. 2016; Tréguier et al. 2014). Our field data show that green crab eDNA can be detected in the field and this method therefore provides an additional tool to detect and monitor the spread of this highly invasive species. This is especially important in invasive species with a nearly world-wide distribution. Furthermore, for species with genetically distinct populations the species-specific primers need to be tested on all the respective populations. In our case, the developed primers could be used on green crabs from Maine, Nova Scotia, Newfoundland and Iceland. However, whether the same primers would work on green crabs from other locales requires further testing.

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Figure captions

Fig. 1 qPCR amplification curves using published primer and probe combination (Bott et al. 2010) for four crustacean species of the Gulf of Maine (*Carcinus maenas, Hemigrapsus sanguineus, Homarus americanus,* and *Cancer irroratus*). DNA amplified in all species, demonstrating that the primer/probe combination is not species-specific. Insert: The melting curve shows two peaks, indicating some non-specific binding of the primers.

Fig. 2 Example of a qPCR amplification curve using newly designed primer and probe combination for four crustacean species (*Carcinus maenas, Hemigrapsus sanguineus*, *Homarus americanus,* and *Cancer irroratus*) of the Gulf of Maine. DNA amplified only *C. maenas* DNA, demonstrating that the new primer/probe combination is species specific. Insert: The melting curve shows a single peak, indicating specific binding of the primers.

Fig. 3 Alignment of partial DNA sequences of the COI gene in *Carcinus maenas* for crabs from Maine (ME), Newfoundland (NF), Iceland (Ice), and Nova Scotia (NS) with the newly designed forward and reverse primers and the TaqMan probe. COI sequences vary between populations at the positions highlighted with blue boxes. See text for details.

Fig. 4 Amplification of *C. maenas* eDNA from water samples collected for a total of 5 meters. Each day is plotted separately, n=2 per depth for 10/16/2020, 10/19/2020, and 10/20/20. Temperatures were recorded at the surface and might differ at depth. X represent datapoints that include one or more replicates with no amplification.

Fig. 5 Partial COI gene comparison of *Carcinus maenas* (GenBank Accession number JQ306003.1), *Hemigrapsus sanguineus* (KT209545.1), *Cancer irroratus* (MG320501.1), *Homarus americanus* (KU564525.1). The *Carcinus*-specific primers and probe are highlighted in blue.

Figure 1

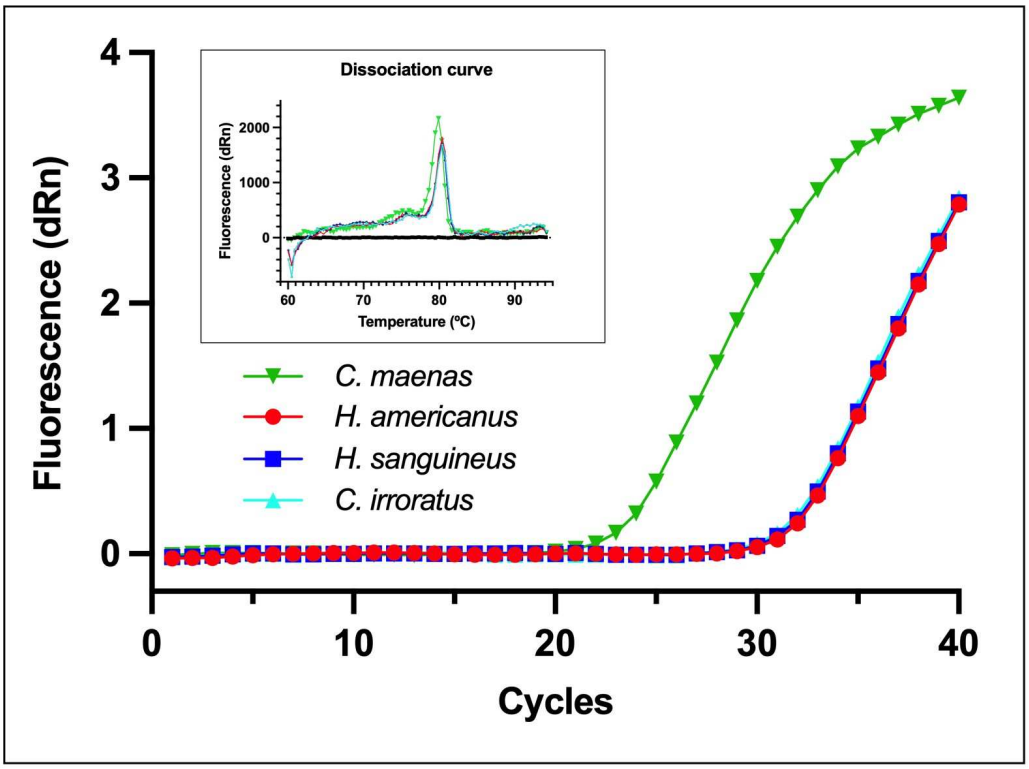
Dissociation curve
Fluorescence (dRn)
2000
1000
0
60
70
80
90
Temperature (ºC)
C. maenas
H. americanus
H. sanguineus
C. irroratus
Fluorescence (dRn)
4
3
2
1
0
0
10
20
30
40
Cycles

Figure 2

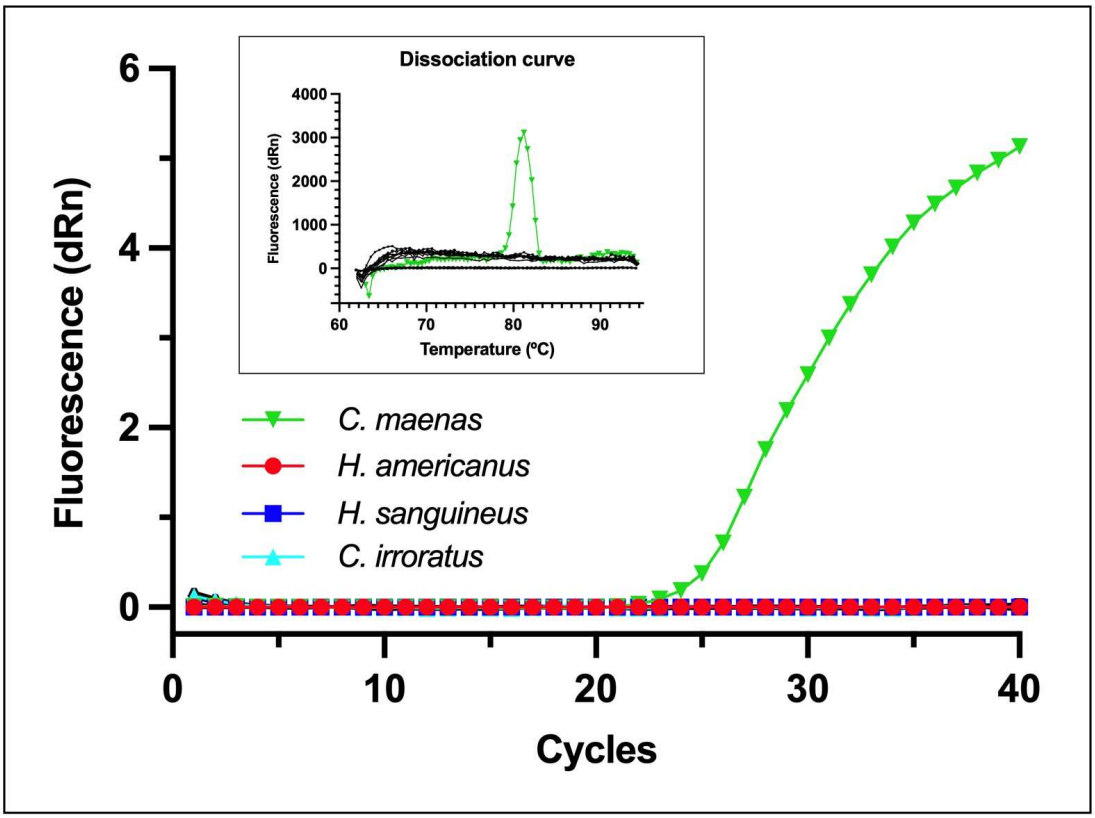
Dissociation curve
Fluorescence (dRn)
4000
3000
2000
1000
0
60
70
80
90
Temperature (ºC)
Fluorescence (dRn)
6
4
2
0
0
10
20
30
40
Cycles
C. maenas
H. americanus
H. sanguineus
C. irroratus

Figure 3

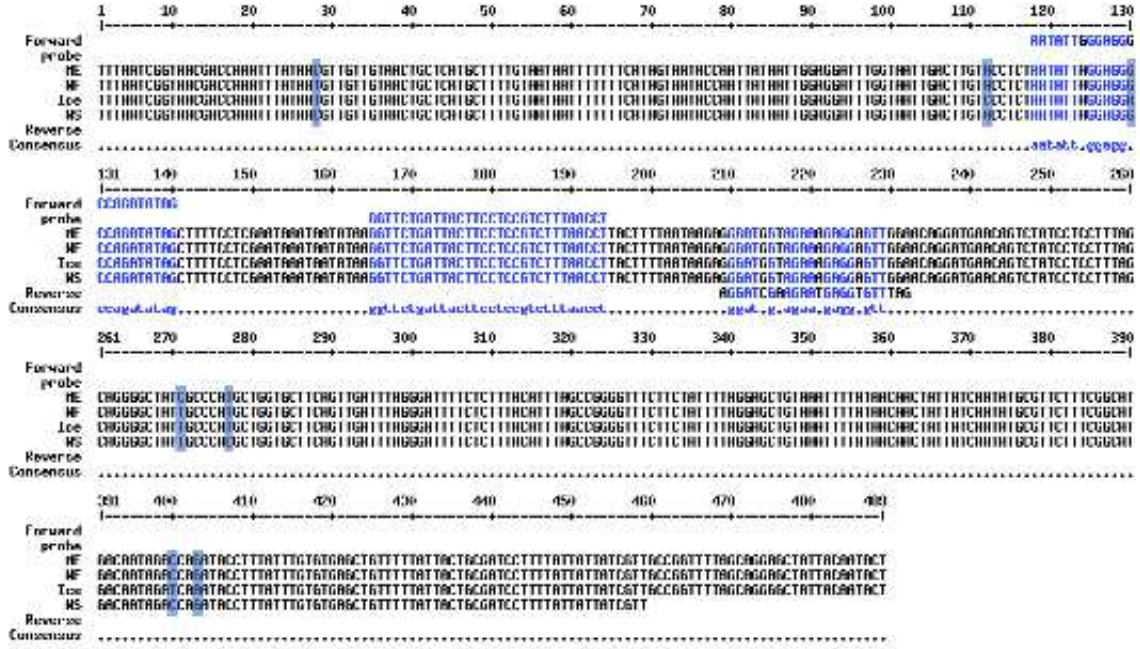

Figure 4

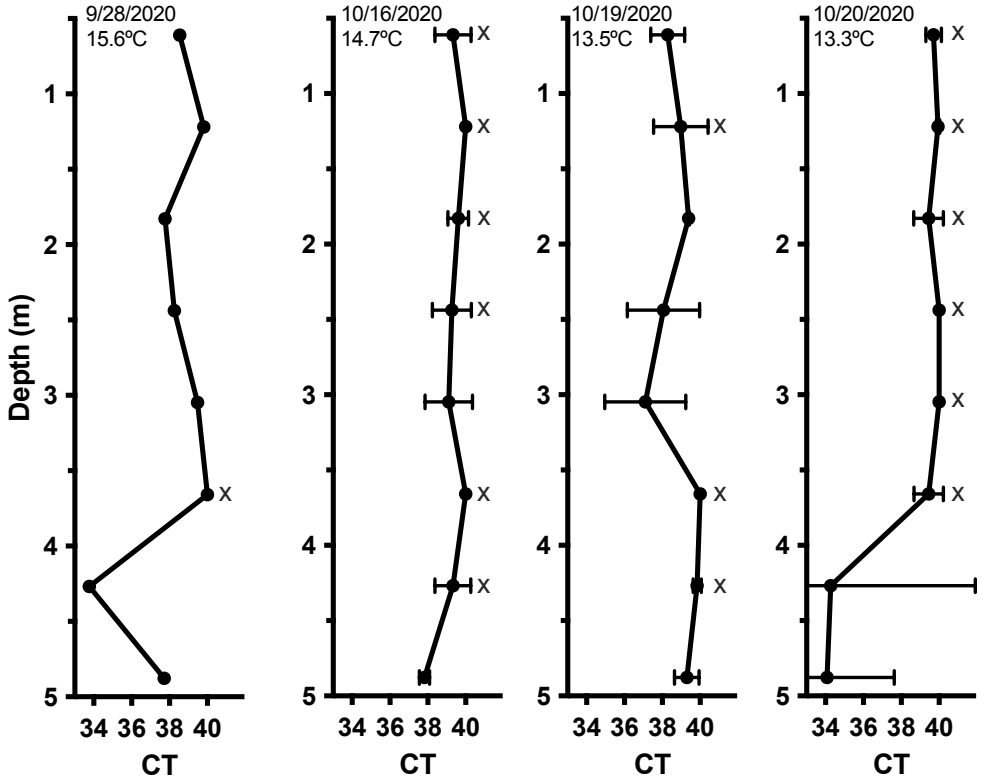
9/28/2020
15.6ºC
10/16/2020
14.7ºC
10/19/2020
13.5ºC
10/20/2020
13.3ºC
Depth (m)
1
2
3
4
5
34 36 38 40
CT

Figure 5 (supplemental figure)

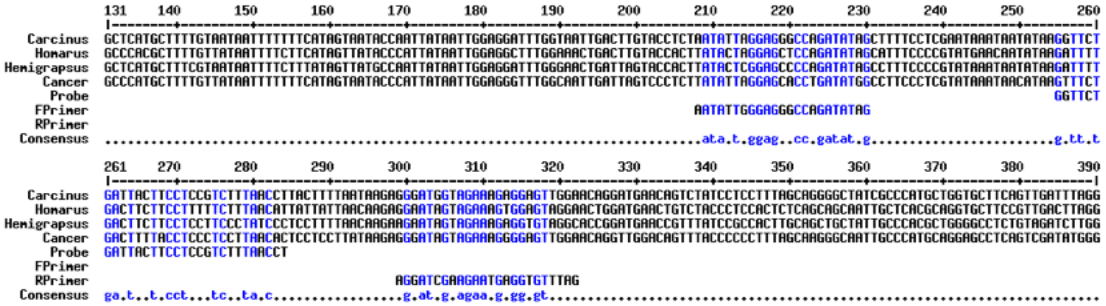

Table 1 Amplification of DNA of local native and invasive species in the Gulf of Maine through qPCR assays using designed primer and probe sequences (Ct: cycle threshold; UD: undetected)

Sub-Phylum	**Species name**	**Common name**	**Ct Value**
Bryozoa	*Bugula neritina*	Brown bryozoan	UD
	Membranipora membranacea	Lacy crust bryozoan	UD
Crustacea	*Balanus improvisus*	Bay barnacle	UD
	Cancer borealis	Rock crab	UD
	Cancer irroratus	Jonah crab	UD
	Caprella mutica	Japanese skeleton shrimp	UD
	Carcinus maenas	**European green crab, Gulf of Maine, USA**	**23.05 ±0.5**
	Carcinus maenas	**European green crab, Iceland**	**38.8 ± 1.68**
	Carcinus maenas	**European green crab, Newfoundland**	**36.17 ± 3.7**
	Carcinus maenas	**European green crab, Nova Scotia, Canada**	**23.34 ± 1.4**
	Hemigrapsus sanguineus	Asian shore crab	UD
	Homarus americanus	American lobster	UD
	Pagurus longicarpus	Long-clawed hermit crab	UD
	Palaemon elegans	Rockpool shrimp	UD
Echinodermata	*Asterias forbesi*	Forbes' seastar	UD

Mollusca	*Littorina littorea*	Common periwinkle	UD
	Modiolus modiolus	Northern horse mussel	UD
	Mytilus edulis	Blue mussel	UD
	Nucella lapillus	Dog whelk	UD
	Ostrea edulis	European oyster	UD
Tunicata	*Ascidiella aspersa*	European sea squirt	UD
	Botrylloides violaceus	Orange sheath tunicate	UD
	Botryllus schlosseri	Star tunicate	UD
	Didemnum vexillium	Carpet sea squirt	UD
	Diplosoma listerianum	Compound sea squirt	UD
	Styela clava	Club tunicate	UD

Table 2 (supplemental material)

Table 2 All primers and probes designed for amplification of only *C. maenas*. Primers and probes used in future applications are bolded

Forward Primers	5'-GCT GGT GCT TCA GTT GAT TTA G-3'
	5'-AGA GGG ATG GTA GAA AGA GGA G-3'
	5'-**AAT ATT GGG AGG GCC AGA TAT AG**-3'
	5'-ATT GGA GGA TTT GGT AAT TGA CTT G-3'
	5'-GGC ATA GTA GGG ACT TCT TTG AG-3'
Reverse Primers	5'-TCA TTC TTC GAT CCT GCA GG-3'
	5'-**AGG ATC GAA GAA TGA GGT GTT TAG**-3'
	5'-CTG CTA AAG GAG GAT AGA CTG TTC-3'
	5'-AAA GAG GAG TTG GAA CAG GAT G-3'
TaqMan Probes	6FAM-TGC GTT CTT TCG GCA TGA CAA TAG AC-MGB
	6FAM-**GGT TCT GAT TAC TTC CTC CGT CTT TAA CCT** -MGB