# Validation of a species-specific eDNA-based test system for detecting non-indigenous American lobster *Homarus americanus*


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## Abstract

Introduced species have the potential of being a major threat to local biodiversity. Intentionally or unintentionally release of live specimens across physical barriers limiting the natural distribution of species, may lead to the establishment of invasive populations. Challenges from invasive species calls for effective monitoring methods to detect early establishment of non-indigenous populations. During the last decade, the number of studies using environmental DNA (eDNA) for bio monitoring has increased significantly. Organisms release eDNA into the environment and has the potential of being detected by analysing environmental samples with species-specific or universal PCR primers. The aim of the present study was to investigate whether eDNA can be used to detect the introduced American lobster (*Homarus americanus*). *Homarus americanus* pose a potential threat to the Norwegian coastal ecosystem as it may establish populations and function as a competitor, predator, spreader of pathogens, in addition to crossbred with the European lobster (*Homarus gammarus*) and producing hybrids. Objectives to reach this aim was designing and testing species-specific primes and probes, in addition to optimising ddPCR assays for detecting *H. americanus* and *H. gammarus* eDNA. A laboratory experiment was also conducted to assess the amount of *H. americanus* eDNA shed and its degradation rate. In addition, field seawater samples were collected from a location with a known *H. gammarus* population and a location where a *H. americanus* female with remains of hybrid eggs was observed in 2016. Field samples were analysed for both *H. americanus* and *H. gammarus* eDNA. The concentration of *H. americanus* eDNA was low in all samples collected from laboratory experiment, and no eDNA from either *H. americanus* or *H. gammarus* was detected in the field samples. As no *H. gammarus* eDNA was detected in an area where a population is known to be present, results indicate that the tested eDNA approach as applied in current study is not suitable for detection of lobsters. This result is relevant and useful for conservation management, as it indicates that although eDNA has proved useful as a monitoring tool for many taxa, it may currently not be suitable for detecting all taxonomic groups present in a coastal environment. Further studies should focus on measuring the quantity of eDNA shed by individuals in different life stages, under varying environmental conditions, and should additionally include samples from different ecological compartments.

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# Terms

| | |
|---|---|
| **Non-indigenous species** | A species that is not native to an ecosystem |
| **Introduced species** | Species that is non-indigenous to an ecosystem |
| **Invasive species** | A non-indigenous species that is introduced to an ecosystem and causes harm |
| **Crustacea** | Subphylum consisting of animals with segmented exoskeleton, jointed limbs, gills and two pairs of antennas |
| **Decapoda** | Order of crustacea where ten of the appendages are considered as legs |
| **Hybridization** | The process of producing offspring by mating of parents from different species |
| **PCR primer** | A short, single-stranded DNA sequence used in the polymerase chain reaction (PCR) technique |
| **Poisson statistics** | A discrete probability distribution that expresses the probability of a given number of events occurring in a fixed interval of time or space |
| **Oligos** | Short single strands of synthetic DNA |
| **Moulting** | Shedding of exoskeleton |
| **Droplet rain** | Droplets with intermediate amount of amplified DNA/mid-level amplitude droplets which are difficult to distinguish between positive or negative |
| **Fluorescence amplitude** | The amount of fluorescens emitted when probe is attached to target DNA. The fewer amplicons in each positive droplet the lower the fluorescent amplitude |

# 1 Introduction

## 1.1 Invasive crustaceans

Introduced species have the potential of being a major threat to local biodiversity (Aschim et al., 2019). Intentional or unintentional release of live specimens across physical barriers limiting the natural distribution of species may lead to the establishment of invasive populations. Invasive species may affect native ecosystems by competition of resources, predation, hybridization or as vectors for parasites and pathogens to which native species are not evolutionary adapted (Smith, 2015).

Crustaceans are one of the most successful group to invade aquatic environments (Karatayev et al., 2009). The two most common ways of introduction into new areas are by ship transport mainly through ballast water, and by intentional introductions for aquaculture. Reasons for their invading success include factors such as high tolerance to different environmental conditions, diverse omnivorous diets and possessing certain r-selected life history traits, such as short generation time, long reproductive period, early sexual maturity, high growth rates and large brood sizes (Hänfling et al., 2011). These characters causes disruption in ecosystems as the invader outperform native species by rapid population growth and domination in communities (Engelkes et al., 2011).

Examples of decapod species that have increased its abundance and expanded their range following translocation, is the pacific red king crab *Paralithodes camtschaticus* in the northeast Atlantic (Nilssen et al., 2006), the Chinese mitten crab *Eriocheir sinensis* in Continental Europe, Southern France, USA and the UK (Herborg et al., 2003), and the European green crab *Carcinus maenas* in USA (Colnar et al., 2007), South Africa (Le Roux et al., 1990) and Australia (Walton et al., 2002). All these species have impacted the ecosystems they were introduced to. Introduction of *P. camtschaticus* has led to loss of biodiversity and biomass in invaded areas (Falk-Petersen et al., 2011). *Eriocheir sinensis* alters the environments by burrow-digging, which in some cases leads to river bank erosion (Herborg et al., 2003). *Carcinus maenas* has served as an intermediate parasite host that caused mass mortalities of a native species in the invaded area (Young et al., 2020).

## 1.2 Environmental DNA (eDNA)

The potential challenges caused by invasive species calls for effective monitoring methods to detect presence in an early phase of population establishment. During the last decade the number of studies using environmental DNA (eDNA) for biodiversity monitoring has increased significantly (Sepulveda et al., 2020). eDNA from macrobial organisms is defined by Thomsen et al. (2015) as "genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material". Sources of DNA to the environment can be substances such as urine, faeces, hair, mucous, spawning products, skin, or tissue cells.

Organisms shed eDNA when interacting with the surrounding environment, and by taking samples from the environment for eDNA analyses, presence of target species can be detected. Samples can either be analysed for specific species, by using species-specific PCR primers, or be analysed for a group of species or taxa using universal primers (Ruppert et al., 2019). No detection of target species' eDNA in environmental sample can mean that the species is not present. It may however also be a false negative result, meaning that the species is present but eDNA is not present in detectable quantity or quality in the sample. Reasons for this can be that the species is not releasing detectable amounts of eDNA, that shed DNA is transported away from the sample site, degradation of eDNA in the environment or use of suboptimal sampling method, DNA extraction method or PCR assay. False positive results may also occur as a result of contamination, transport or settling of eDNA where species is no longer present (Barnes et al., 2016). Acknowledging factors causing false results (negative or positive) will help interpreting results and prevent that wrong conservation management decisions are made.

Despite challenges as outlined above, the use of eDNA is often more cost- and time efficient monitoring approach than many traditional methods that depend on catch or other types of invasive observations (Lugg et al., 2018; Rees et al., 2014). eDNA may be exploited to detect rare species or low-density populations, typically present just after introduction. Several studies have demonstrated higher detection rates using eDNA relative to traditional monitoring (Fraija-Fernández et al., 2020; Lugg et al., 2018). The need for morphology based taxonomy expertise also decreases (Ruppert et al., 2019).

eDNA has been used for detection of marine species including fish (Lacoursière-Roussel et al., 2016; Thomsen et al., 2012), whales (Baker et al., 2018), corals (Kutti et al., 2020) and echinoderms (Uthicke et al., 2018), but only a few studies have examined eDNA of marine

crustaceans. Forsström et al. (2016) showed that eDNA signal was low in a crab species (*Rhithropanopeus harrisii*) compared to fish and amphibians. The same authors indicated that it may be more challenging to develop sufficiently sensitive eDNA methods for the detection of species in the marine environments. Allan et al. (2021) compared DNA shedding and decay in the fish mummichog (*Fundulus heteroclitus)* and grass shrimp (*Palaemon* spp.) and concluded that shedding rates for the decapod was lower than for the fish species. It has been suggested that the chitin exoskeleton present in most crustacean limits the release of eDNA (Harper et al., 2018). Studies also indicates that crustaceans may shed different levels of eDNA throughout their life cycle (Crane et al., 2021).

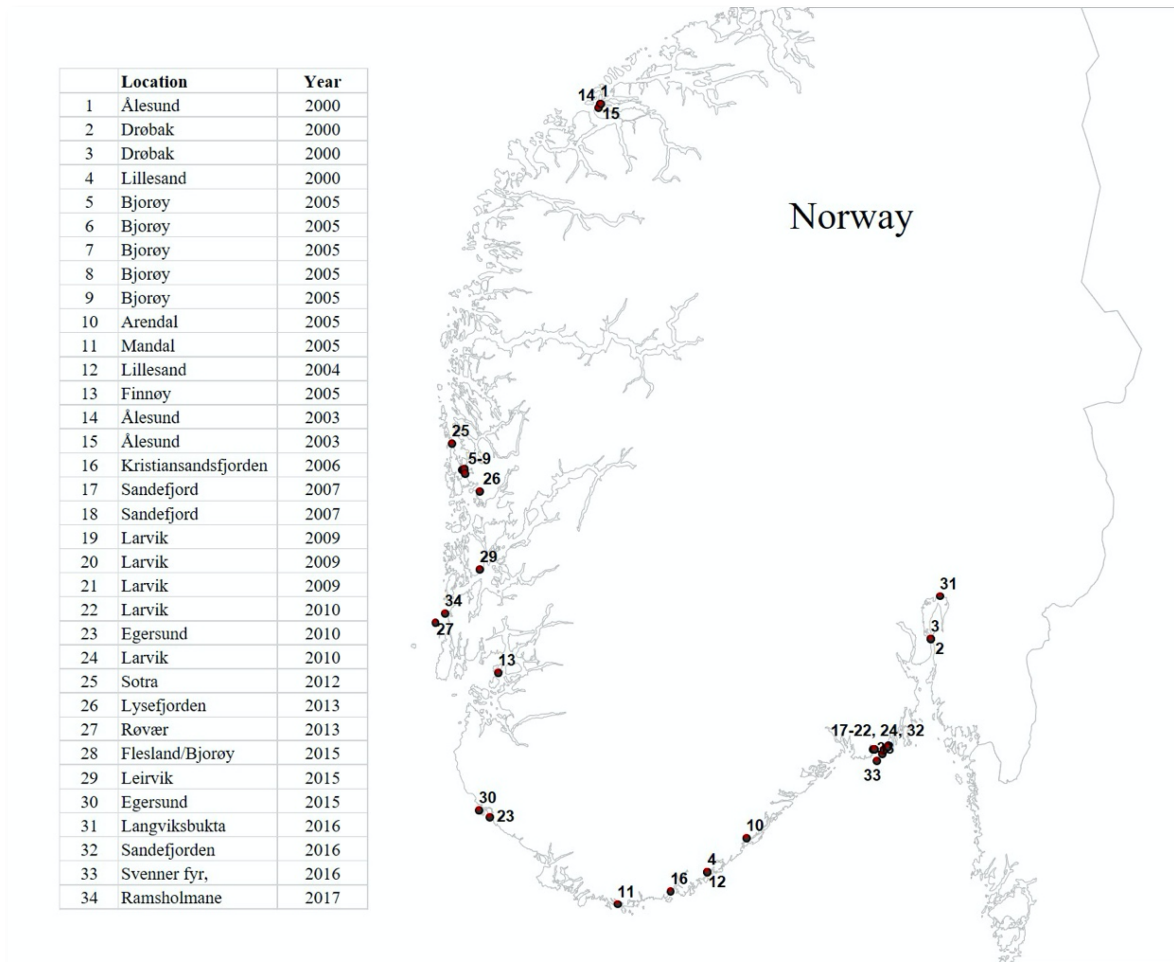
### 1.2.1 Digital droplet PCR (ddPCR)

Polymerase chain reaction (PCR) is a technique used to amplify short segments of DNA from a low starting concentration to analysable quantities. Digital droplet PCR (ddPCR) can be used to quantify eDNA in a sample. The technique is dependent on the PCR-reaction master mix (template and PCR reagents) being divided in up to 20.000 droplets by using an oil emulsion, where a separate PCR amplification takes place in each droplet. For droplets containing target template, a fluorescent component (incorporated in the master mix) will emit fluorescence during capillary droplet reading (Miotke et al., 2014). Fluorescence intensity decides whether a droplet is positive or negative (i.e., droplet contains the target DNA or not). The ratio between negative and positive droplets is used to estimate the number of copies of the target DNA in the sample, under the assumptions that the target molecules are distributed among the droplets according to a Poisson function (Baker et al., 2018). Quantitative PCR (qPCR) is also used to quantify eDNA in environmental samples. However, ddPCR has proven to be more suitable as the partitioning of the PCR reaction makes it less affected by PCR inhibition (Basu, 2017), providing sensitive amplification and hence appropriate for detecting low copy targets in environmental samples (Kokkoris et al., 2021).

## 1.3 American lobster – Introduced to European waters

The American lobster (*Homarus americanus* Milne-Edwards, 1837*)* native to the North East Atlantic shallow waters is geographically isolated from the European lobster (*Homarus gammarus* Linnaeus, 1758), by the deep Atlantic Ocean (Barrett et al., 2020). However, significant amounts of live American lobsters are being imported to Europe through sea food trade. In 2015 Canada and USA exported approximately 75500 tonnes of *H. americanus* (FAO, 2020) and about 1700 tonnes live specimens of these were imported to the UK (Barrett et al., 2020). Import of live specimens has led to escaped individuals and intentional release into the European marine environment. Presence of *H. americanus* in European waters has been confirmed by observations in Iceland (Skúladóttir, 1968), Denmark (Jørstad et al., 2011), Sweden (Øresland et al., 2017), UK (Stebbing et al., 2012a), Ireland (Minchin, 2007) and in Norway (Jørstad et al., 2011; Sandlund et al., 2011; van der Meeren et al., 2000). Some of the observations were confirmed by molecular genetic techniques.

From year 2000 and up till 2017, 35 out of 120 "unusual-looking" lobsters delivered to the Institute of Marine Research (IMR) was identified as *H. americanus*, using microsatellite markers (developed by Jørstad et al. (2007)). In Norway individuals of *H. americanus* has been observed at several locations along the coast as far North as Ålesund (Figure 1). (Jørstad et al., 2011). Most likely only a small fraction of the *H. americanus* caught has been recorded, as it may be difficult to distinguish *H. americanus* from *H. gammarus* based on morphology (Agnalt A-L, pers. comm). Norway banned import of live *H. americanus* in 2016 (Jelmert et al., 2019). Despite this, continued illegal import, establishment of previously escaped individuals and migrating escapees from other European waters, can potentially still pose a threat to the Norwegian native ecosystems.



|  | Location | Year |
|---|---|---|
| 1 | Ålesund | 2000 |
| 2 | Drøbak | 2000 |
| 3 | Drøbak | 2000 |
| 4 | Lillesand | 2000 |
| 5 | Bjorøy | 2005 |
| 6 | Bjorøy | 2005 |
| 7 | Bjorøy | 2005 |
| 8 | Bjorøy | 2005 |
| 9 | Bjorøy | 2005 |
| 10 | Arendal | 2005 |
| 11 | Mandal | 2005 |
| 12 | Lillesand | 2004 |
| 13 | Finnøy | 2005 |
| 14 | Ålesund | 2003 |
| 15 | Ålesund | 2003 |
| 16 | Kristiansandsfjorden | 2006 |
| 17 | Sandefjord | 2007 |
| 18 | Sandefjord | 2007 |
| 19 | Larvik | 2009 |
| 20 | Larvik | 2009 |
| 21 | Larvik | 2009 |
| 22 | Larvik | 2010 |
| 23 | Egersund | 2010 |
| 24 | Larvik | 2010 |
| 25 | Sotra | 2012 |
| 26 | Lysefjorden | 2013 |
| 27 | Røvær | 2013 |
| 28 | Flesland/Bjorøy | 2015 |
| 29 | Leirvik | 2015 |
| 30 | Egersund | 2015 |
| 31 | Langviksbukta | 2016 |
| 32 | Sandefjorden | 2016 |
| 33 | Svenner fyr, | 2016 |
| 34 | Ramsholmane | 2017 |



**Figure 1.** Observations of American lobster (*Homarus americanus*) in Norwegian waters confirmed by genetic identification (modified from Jørstad et al., 2011, Agnalt A-L, pers. comm.)

The species' potential to form invasive populations in Europe is unknown, but several potential impacts are possible on native *H. gammarus* if establishment of a population should occur. *Homarus americanus* and *H. gammarus* share the same habitat and food preferences. Presence of both species in the same areas could lead to interspecific competition, where one of the species could outcompete the other. Hybrids can also function as competitors with similar preferences (van der Meeren et al., 2000).

Predation on the *H. gammarus* population by the introduced counterpart may also be an issue. Both species has been described as aggressive when defending their territory and as *H. americanus* can grow much larger than *H. gammarus* and in their native range occur in denser populations, it may have an advantage in a predatory situation. Predation on a *H. gammarus* with hard shell by *H. americanus* has been observed in Sweden (Øresland et al., 2017).

Furthermore, transfer of disease is a potentially negative outcome of a possible establishment of *H. americanus.* Gaffkemia, a bacterial disease caused by *Aerococcus viridans* (var.) *homari* with origins in North America, has been introduced to European waters by the import of *H. americanus*. High mortalities caused by gaffkemia has been reported in both *H. americanus* and *H. gammarus* held at high density (Stebbing et al., 2012b). However, a study conducted in the UK indicated low prevalence of gaffkemia in wild *H. gammarus*. Other diseases that may be a threat to *H. gammarus* include shell disease which presents itself in at least three forms (Cawthorn, 2011). One of them is epizootic shell disease, an epidemic disease in North-America which can cause severe damage to *H. americanus* (Castro et al., 2012). Infected individuals have been observed in Norway (Sandlund N, pers. comm). Studies comparing damage among infected *H. gammarus* and *H. americanus*, indicate difference in susceptibility for shell diseases (Davies et al., 2014). Although the presence of interspecies disease transmission is currently unknown, one cannot exclude this as a potential issue (Agnalt A-L, pers. comm.). Some of the "unusual-looking" lobsters delivered to IMR, showed symptoms of shell disease.

Another potential impact is the effects of interspecific mating and hybridization, which may lead to unproductive use of energy in mating due to infertile or no offspring. This can lead to decreased reproduction in the pure-bred species (Rhymer et al., 1996). Changes in morphology, behaviour, dilution of genetic integrity and reduction in breeding capacity are other potential consequences, but are so far poorly understood (Stebbing et al., 2012a). *Homarus americanus* female x *H. gammarus* male hybrids have proven their ability to grow to adults in culture (e.g., specimens used for method validation in the present study). Whether these hybrids are fertile or not is yet not known (Agnalt A-L, pers. comm.). *Homarus americanus* male x *H. gammarus* female hybrids has also been produced by artificial insemination, but the offspring were infertile (Talbot et al., 1984). Hybrids has also been observed in the wild represented by two *H. americanus* females in Sweden (Øresland et al., 2017) and five females in Norwegian waters (Agnalt A-L, pers. comm.). These were observed with hybrid eggs, but no data on hybrid eggs among *H. gammarus* females is available (catch of berried females is forbidden so detection from catches is not likely to occur).

## 1.4 Knowledge requirements

Even though *H. americanus* have been found in Norwegian waters since 2000, it is not clear if the species has established viable populations. The specimens observed have been adults and may all be escapees. Six out of 26 lobsters identified as *H. americanus* in UK waters were banded (Stebbing et al., 2012a), confirming that they were escapees that had not moulted after being released. Due to mandatory 6 cm escape openings in lobster pots and a minimum legal size (MLS) of 25 cm from rostrum to tail in Norway, catches will not provide information about individuals smaller than MLS.

Due to its potential harm to native biodiversity, it is important with a better overview over the presence of *H. americanus* in Norwegian waters. To do so there is a need for a reliable and cost-efficient monitoring method, independent of current fishing practices.

## 1.5 Aims and objectives

The main aim of the present study was to investigate whether eDNA can be used to detect presence of *H. americanus*. Objectives to reach this aim was designing and testing species-specific primes and probes, in addition to optimising ddPCR assays for detecting both *H. americanus* and *H. gammarus* eDNA partly based on assays for *H. americanus* suggested by Knudsen et al. (2020). A laboratory experiment was conducted to assess quantity of shed *H. americanus* x *H. gammarus* hybrid eDNA and the degradation rate after removal of individuals from tanks. In addition, seawater samples were collected from locations with documented presence of lobster and analysed for both *H. americanus* and *H. gammarus* eDNA. Analysis of field samples for *H. gammarus* eDNA was included to test the detection potential of the approach for species with known presence in field.

# 2 Materials and methods

## 2.1 Target DNA region

Mitochondrial genes are typically used in animal eDNA studies as they have more copies per cell than nuclear DNA, and therefore more likely to be detected in eDNA samples (Geerts et al., 2018). In addition, it is expected to be more resistant than nuclear DNA to degradation in environmental samples (Turner et al., 2014). Due to low intraspecific (with-in species) sequence variability and high interspecific (between species) sequence variability, the mitochondrial DNA cytochrome b (mtDNA-cytb) gene is often used for species identification (Linacre, 2012). Using mtDNA enabled use of hybrids (with *H. americanus* mother) in the laboratory experiments as all mtDNA is inherited from mother (Harrison, 1989). The targeted DNA sequence used in the present study could therefore be amplified if eDNA of both *H. americanus* and hybrids with *H. americanus* mother were present in the sample.

## 2.2 PCR primers and probes

Primers and probe selected for detecting *H. americanus* were developed and tested *in vitro* by Knudsen et al. (2020). These were designed to specifically amplify a fragment (193 base pairs) of the cytb gene of *H. americanus*. The oligos for the *H. americanus* specific mtDNA-cytb assay is given in Table 1. Knudsen et al. (2020) tested the oligos on DNA from *H. gammarus* and 4 other relevant decapod species found in Northern Europe. None of the species resulted in false positive detection.

**Table 1**. PCR primers and probe – American lobster (*Homarus americanus*).

| Oligo name | Oligo sequence in 5'->3'' direction |
|---|---|
| Homame_cytb_F02 | TTTTAGTAGCAGCAGCGACTCTT |
| Homame_cytb_R14 | CCAAGAAGGTAGGGATTTAGAAGA |
| Homame_cytb_P12 | FAM- TGCAAGACATATTGATAAAGTTCCATTCCA -BHQ |

Primers and probe selected for *H. gammarus* were developed *in silico* using GenBank and tested in this study. These were also designed for detecting a fragment (179 bp) of the cytb gene (Dahlgren, pers. comm.). The oligos for the *H. gammarus* specific mtDNA-cytb assay is given in Table 2.

**Table 2.** PCR primers and probe – European lobster (*Homarus gammarus*).

| Oligo name | Oligo sequence in 5'->3'' direction |
|---|---|
| H.gam CytB F2 | CAGCTGCAACTCTGATCCATATC |
| H.gam CytB R | GAGGTAGGGATTCAGAAGAGTT |
| H.gam CytB P | FAM-TGCAAGACATATCGATAAAGTTCCATTTCA-BHQ |

## 2.3 ddPCR assay

To optimise detection of targeted amplicons, different assays were tested for both *H. americanus* and *H. gammarus*. Amount of the different components, concentration of primer/probe, type of fluorescent dye (ddPCR EvaGreen Supermix or ddPCR Supermix for Probes), annealing- and stabilization temperature and number of amplification cycles were changed in order to optimise the assay (see Table A1-A4, Appendix A).

The chosen assays and protocol had a ddPCR master mix volume of 22 µL which included 11 µL Bio-Rad ddPCR Supermix for probes (no dUTP), 1.98 (10 µM) µl of each primer, 1.1 (5 µM) µL of probe, 1.94 µL of PCR-grade water and 4 µL PCR template. Triplicate PCR reactions were performed for each sample. Reagents was mixed in a template free pre-PCR room, and template was added in another room. The BioRad QX200 droplet generator portioned the master mix into nanodroplets by combining 20 µL of the master mix and 70 µL of *BioRad* droplet oil in a droplet generator cartridge. 40 µL of the droplet mix were then transferred to a 96-well PCR plate before the plate was put in a C1000 Touch™ Thermal Cycler for PCR amplification. For amplification an annealing temperature of 60°C was used for the *H. americanus* assay, and 56.4°C for the *H. gammarus* assay, with a PCR program consisting of 40 amplification cycles after an initial denaturation at 95°C for 10 min. The 40 amplification cycles each consisted of 94°C for 30 sec and 60/56.4°C for 1 min. Finishing steps were 98°C for 10 sec and a hold on 4°C min. Ramp rate was set to +2°C $s^{-1}$for all steps. The PCR plate was placed in a BioRad QX200 Droplet reader, where individual droplets were analysed. The *QuantaSoft™* software for the droplet reader uses Poisson statistics for calculating the number of copies per microliter in each sample. For each analysis positive controls (1:100 diluted tissue template) and negative controls (Milli-Q water) were included.

## 2.4 Longevity of lobster eDNA – laboratory experiment

To investigate if *Homarus* shed detectable levels of eDNA, and the rate at which DNA degrades in a tank environment, a laboratory experiment was conducted. The experiment took place in a laboratory facility at Institute of Marine research (IMR) and was carried out by IMR personnel from September 17$^{th}$-23$^{rd}$ 2018 in a climate-controlled room kept at 10°C (see Figure 2 for summary).

For the experiment three lobster hybrids (males weighing between 653 to 702 g), offspring of a *H. americanus* female and a *H. gammarus* male were transferred to three 32 L tanks filled with 20 L of sand filtered deep water (from Byfjorden outside IMR) and kept there for 24 hours, before being removed. Some stones (rinsed with bleach and tap water) were added to the tanks to trigger movement of the lobster. In addition, 32 L tanks were filled with 6 L of sand filter deep water and 10 L distilled water respectively, for negative controls. All tanks were fitted with air stones providing circulation and oxygen to the water. The climate room did not contain

other animals than the individuals used in the experiment, and these were only kept in the laboratory for the first 24 hours of the experiment.

All lobsters were removed after 24 hours ($18^{th}$ of September, at 08:15) and 1 L of water was sampled from each of the three tanks. The filtration took longer than expected (>3h), due to clogging by organic matter originating from the lobster (e.g., faecal pellets). Hence the air stones were switched off after the first filtration to allow some sedimentation. The air stones were kept off during rest of the experiment.

6 hours after removal of the lobsters, samples of 1 L of water were collected from each of the three tanks. One litre of tap water was also filtered. Samples were filtered at times given in Table B2 (Appendix B), from lobster tanks, water supply, distilled- and tap water, following the same procedure. See Table B3 (Appendix B) for detailed information about the sampling.

All water samples were filtered through Whatman nitrocellulose membrane filters (pore size =0.45 µM, diameter=47 mm), using a vacuum pump. Filters were folded and put in a 2 ml sterile cryogenic vial, to which 1.96 ml of tissue lysis buffer (Qiagen) was added. The sample was subsequently stored at -20°C.

Between each filtration the filtration kit and forceps were cleaned, first with bleach (10%) and then with water from the tank for the upcoming sample.

Lobsters were weighed and measured (see Table B1, Appendix B), and tissue samples were collected from one of the $4^{th}$ walking legs and placed in 0.98 ml tissue lysis buffer (Qiagen) in a 2 mL cryovial. Samples were stored at -20°C until analysis and used as positive controls.

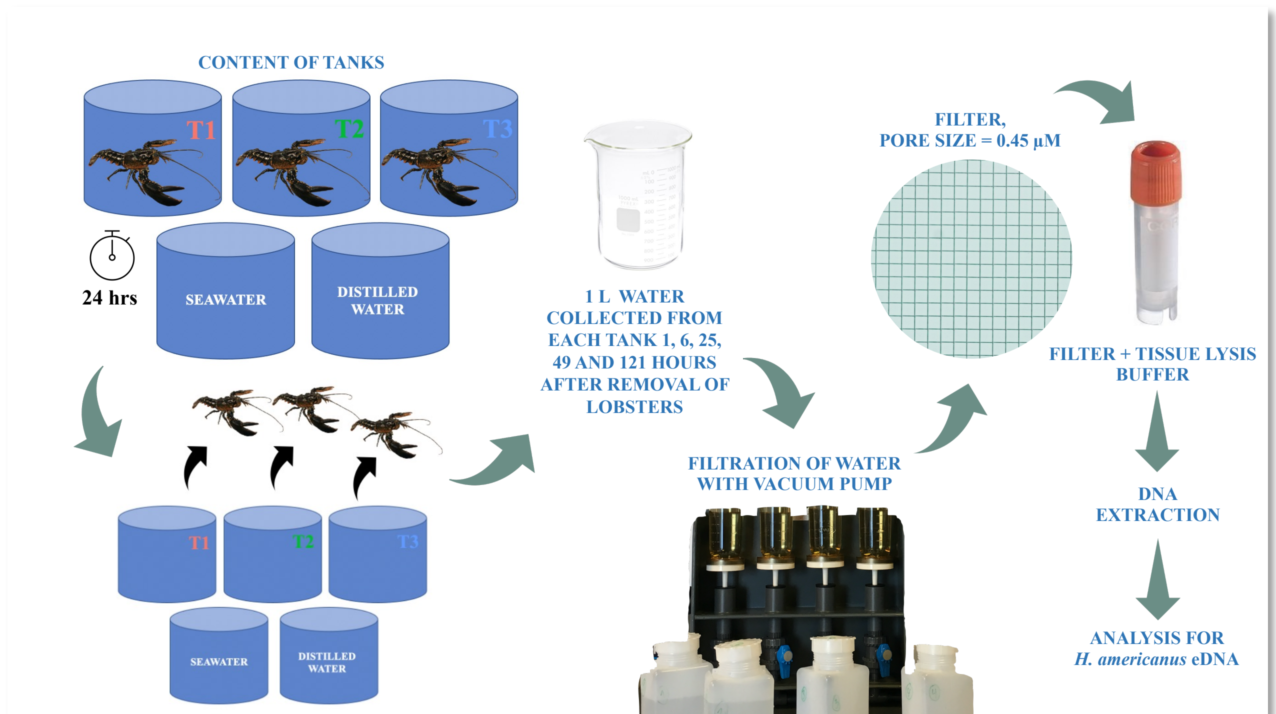



**Figure 2.** Summary of laboratory experiment. Tank 1, 2 and 3 had lobsters for 24 hrs before removal. 1 L of water was collected from the tanks (in addition to control tanks with sea- and distilled water) at given times and filtrated with a vacuum pump, before filter was put in tissue lysis for DNA extraction and eDNA analyses.

## 2.5 Field sampling

Water samples were collected at two different locations: Ramsholmane and Vinnes (Figure 3). At Ramsholmane a female hybrid with remains of eggs were found in 2016 (Agnalt A-L, pers. comm.). It was therefore relevant to collect samples from the area as recruitment potentially could have taken place. Vinnes is a known location for a well-established and large *H. gammarus* population (Agnalt A-L, pers. comm.). This served as a control area, as one could assume that *H. gammarus'* eDNA would be detected by the *H. gammarus* assay if the method provided sufficient sensitivity.

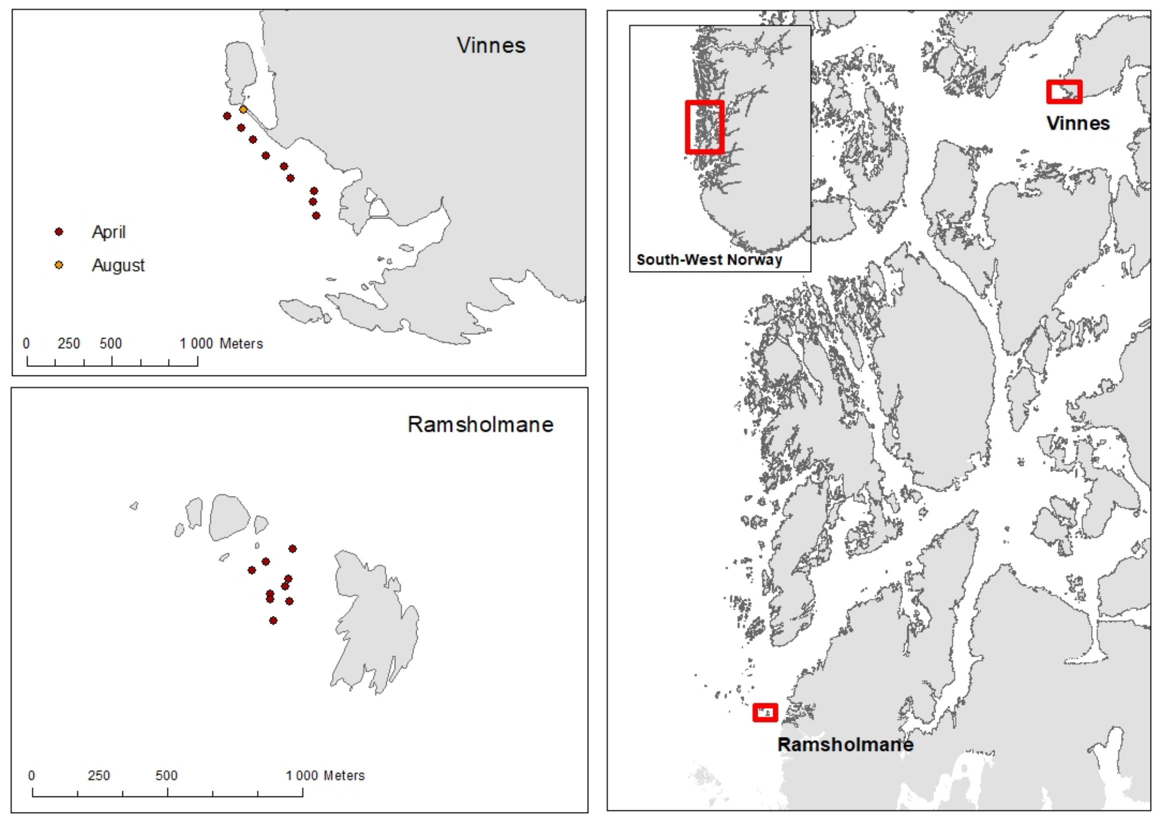



**Figure 3.** Map over sampling areas in April (red points) and August (yellow point) at Vinnes and Ramsholmane, in South-West Norway.

### 2.5.1 Field sampling – April

Water was collected with Niskin bottles at different depths (Table C1, Appendix C). As lobsters are benthic species, samples were collected with a CTD rosette as close to the bottom as the sampling technique allowed, i.e., about two meters above the bottom at all stations. Samples of 3-5 litres of water were individually filtered using a vacuum pump, through a Whatman nitrocellulose membrane filter (pore size 0.45 µm, diameter 47 mm). Control samples with distilled water were filtered simultaneously as filtration from three stations. Water from each station was filtrated for an hour. The filters were folded and put in a 1.8 mL cryogenic vial, to which 1.6 mL of tissue lysis buffer (Buffer ATL, 19076, Qiagen, Norway) was added, right after filtration. The samples were subsequently stored at -20˚C. All equipment were rinsed with 10% bleach and distilled water before and in between each sampling to prevent cross contamination.

Sampling was conducted from the research vessel *Hans Brattström* the April 16th 2021 at Ramsholmane and April 17th 2021 at Vinnes. The samples from Ramsholmane were collected from between 16 to 34 meters depth. The samples from Vinnes were collected from between 6 to 11 meters depth. The water temperature at Vinnes were ~8˚C at the sampling depths.

### 2.5.2 Field sampling – August

To examine if the amount of lobster eDNA present was affected by seawater temperatures or time of the year (and hence level of activity and preferred depth by the lobsters) new samples were collected at Vinnes August 25th 2021. In August, lobsters are typically more active and found at a shallower depths than in April (Moland et al., 2011). eDNA detection probabilities in general has also proven to be higher during summer months (Buxton et al., 2018; Salter, 2018).

Five samples were collected with buckets from the same locality (approx. 60°09’10.2’’N 5°34’37.9’’E) (Figure 3). Two of the samples were collected from the surface in the littoral zone. The other three were collected 2.5 meter further out from land, in a depth of roughly 1.5 meter. The water temperature at sampling depths was ~16°C. Samples were filtrated at the IMR laboratory in Bergen. Hence, the three first samples were filtrated two hours after collection, and the two last ones three hours after collection.

The filtration procedure was the same as in April and the amount of seawater filtered was 2 litres from each station. Two controls with tap water were filtrated the same time.

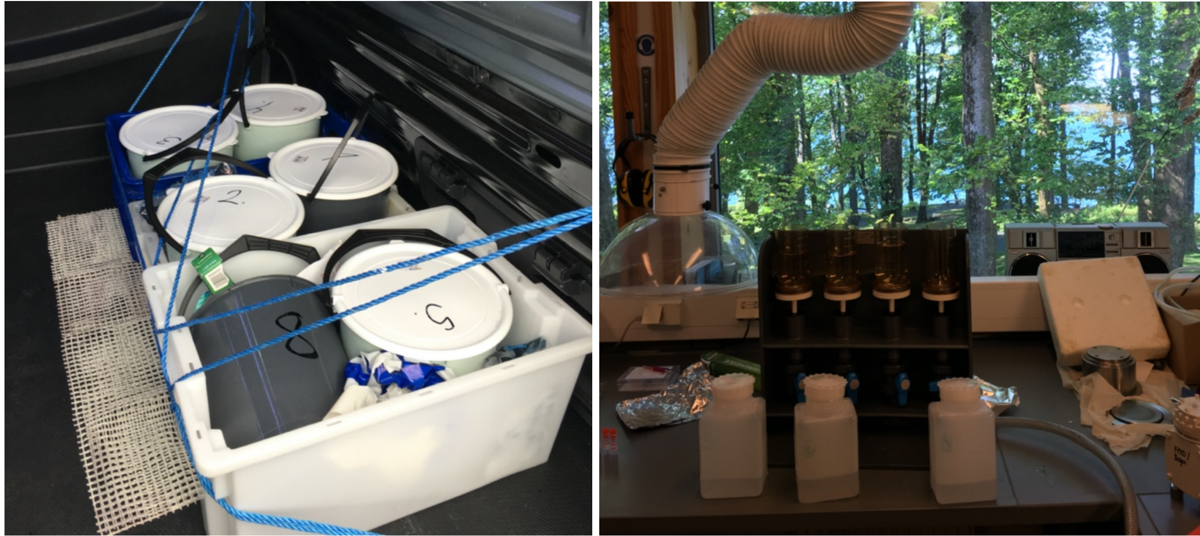


**Figure 4.** Pictures from field sampling in August showing buckets used for water collection (left) and the filter set up at the IMR laboratory (right).

## 2.6 DNA extraction protocols

DNA from both filter and tissue samples were extracted using DNeasy Blood and Tissue kit (Qiagen, Norway).

DNA from tissue samples was used as positive controls for the ddPCR. Extraction of DNA from *H. americanus* x *H. gammarus* hybrid tissue samples collected in the laboratory experiment, was conducted December 4$^{th}$ 2020. After thawing the samples were incubated at 56°C for 15 minutes and subsequently left at room temperature for 15 minutes. By use of scalpel and forceps, approximately 25 mg of muscle tissue were collected and mixed with 180 µL ATL buffer and 20 µL Proteinase K, vortexed and incubated at 56°C for 2.5 hours. 4 µL RNase was then added and the remaining extraction followed the standard manufacturer's protocol (Qiagen, 2020).

Eggs from live *H. gammarus* were collected from IMR laboratory April 21$^{st}$ 2021, Buffer ATL was added and placed in -20°C overnight. The extraction was conducted April 22$^{nd}$-23$^{rd}$ 2021. Sample with eggs were thawed in room temperature for 10 minutes. By use of scalpel and forceps, one egg was punctured and mixed with 180 µL Buffer ATL and 20 µL Proteinase K

and incubated at 56°C overnight. The next day 4 µL RNase was added and the remaining extraction followed the standard manufacturer's protocol (Qiagen, 2020).

The extractions from laboratory experiment samples were conducted December 8th-9th and 14th-15th 2020. The samples thawed in an incubation oven for 30 minutes. A 180 µL subsample of the total volume of 1.96 mL were added 20 µL Proteinase K, vortexed and incubated at 56°C overnight. The next day 4 µL RNase was added and the remaining extraction followed the standard manufacturer's protocol (Qiagen, 2020).

The extractions from field samples collected in April were conducted April 21st-22nd 2021 and the extractions from field samples collected from in August were conducted August 26th-27th 2021. April samples thawed in an incubation oven for 20 minutes at 56°C and August samples thawed in room temperature for 45 minutes, the rest of the protocol was identical: After thawing 20 µL Proteinase K was added, vortexed and incubated at 56°C overnight. The following day the samples were shaken and 200 µL subsample of the total volume of 1.8 mL were added with 4 µL RNase. The remaining extraction followed the standard manufacturer's protocol (Qiagen, 2020).

Elution volume for all extractions was 100 µL and samples were stored at -20°C.

## 2.7 Reanalysis of filter samples collected in August

According to Wilcox et al. (2018) the volume of water sampled in the field and volume of water analysed is critical to maximize the detection rate. That study defines effective quantity of DNA analysed as "the mean proportion of the DNA concentration in the environment (copies/L) that is tested in the laboratory". The effective quantity indicates how much DNA from a sample that will be available when using a certain volume of filtrated water, sample volume used in DNA extraction, volume eluted from DNA extraction and volume of PCR template.

Previously, 180 µL of the total sample was used in DNA extraction. To examine if an increased sample volume used in DNA extraction would lead to detection, a new DNA extraction from the rest of the sample volume (1.4 mL) of samples collected in August was conducted October 12th.

The DNA extraction protocol followed the same procedure as the other extractions with the exception that 28 µL RNase was added into the 1.4 mL sample, followed by vortexing and incubation in room temperature for 2 minutes. Next 1.4 mL ethanol and 1.4 mL Buffer AL were added to the sample. 600 µL of the mix centrifuged in a spin column at 8000 rpm for 1 min, seven times until the whole sample volume was centrifuged. The remaining extraction followed the protocol and the elution volume for the extractions was 100 µL and samples were stored at -20°C. For the eDNA analysis the same *H. gammarus* ddPCR assay was used, as for the field samples.

# 3 Results

## 3.1 Optimisation of ddPCR assays

The BioRad QX200 Droplet reader was used for quantifying the amount of amplified target DNA. The results are presented in tables and droplet plots in *QuantaSoft™* Software (Figure 5). The y-axis shows the fluorescence intensity (amplitude), and the x-axis shows the droplet number. Blue droplets are positive with amplified DNA, dark droplets are negative without DNA amplification. The positive droplets in Fig. 4 show the amplification of *H. americanus* target DNA in tissue samples and laboratory samples from tank 1, 2 and 3. Samples with less than 13.000 droplets are not accepted by the software. The pink line represents a manually set "high threshold" based on the positive and negative control. All droplets over this line are defined as positive and all under are defined negative. Threshold set for the *H. americanus* assay was around 3200 droplets, and for *H. gammarus* it was set for around 4500.

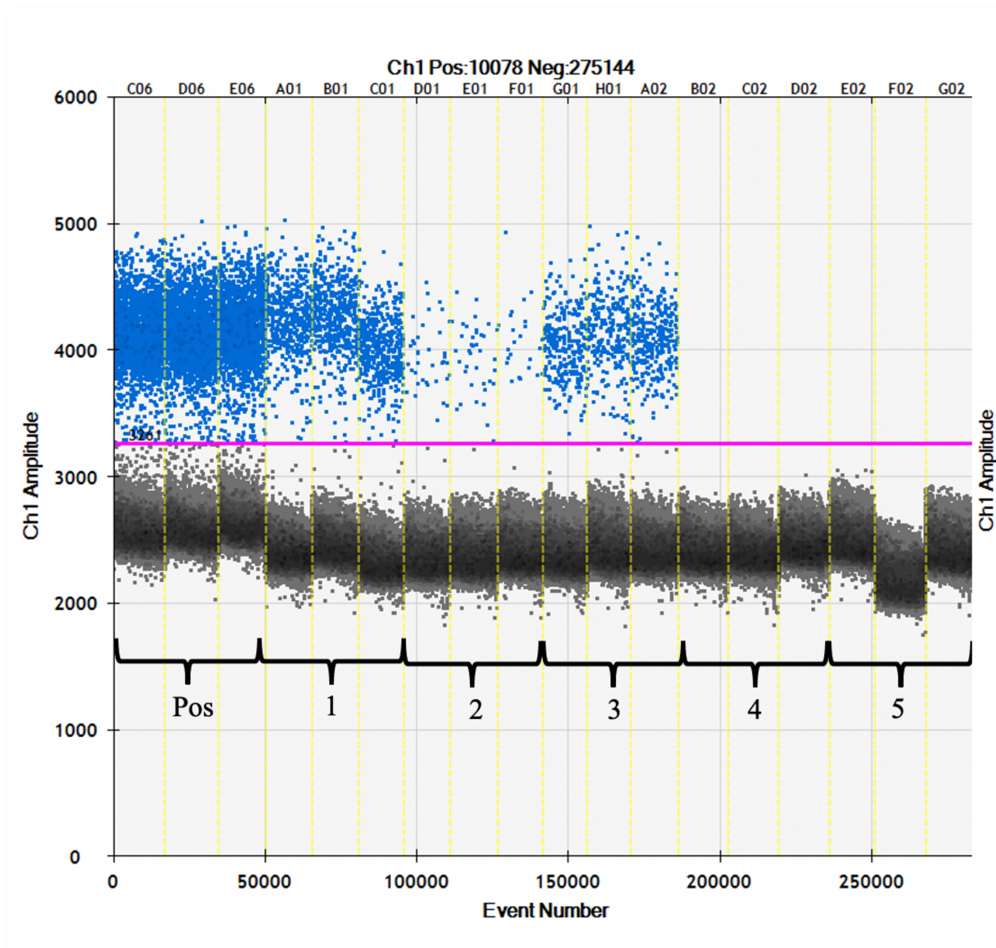



**Figure 5.** Representative samples of droplet distributions from the ddPCR. The figure presents results from the analysis of filter samples from the laboratory experiment. One positive control (pos), three filter samples (1, 2, 3) from tanks with lobsters and two negative controls; tap (4) and seawater (5). The "positive" droplets (in blue) indicate successful amplification of target amplicon.

The goal of optimizing the assays, is to have the correct ratio of reagents in the PCR master mix and optimal PCR conditions for successful amplification, in addition to clear separation between negative and positive droplets. For optimisation different fluorescent dyes, annealing temperatures and probe concentrations were tested. Decisions on how the different parameters should be changed to optimise the assays was done by observing droplet plots in *QuantaSoft*$^{TM}$ (Figure 6). High probe concentration improves separation of negative and positive droplets. Adjusting annealing temperature can improve reaction efficiency, which improves droplet clustering and fluorescence levels (Kokkoris et al., 2021). Other parameters in the assay were also adjusted, but these three were given main focus when observing the droplet plots.

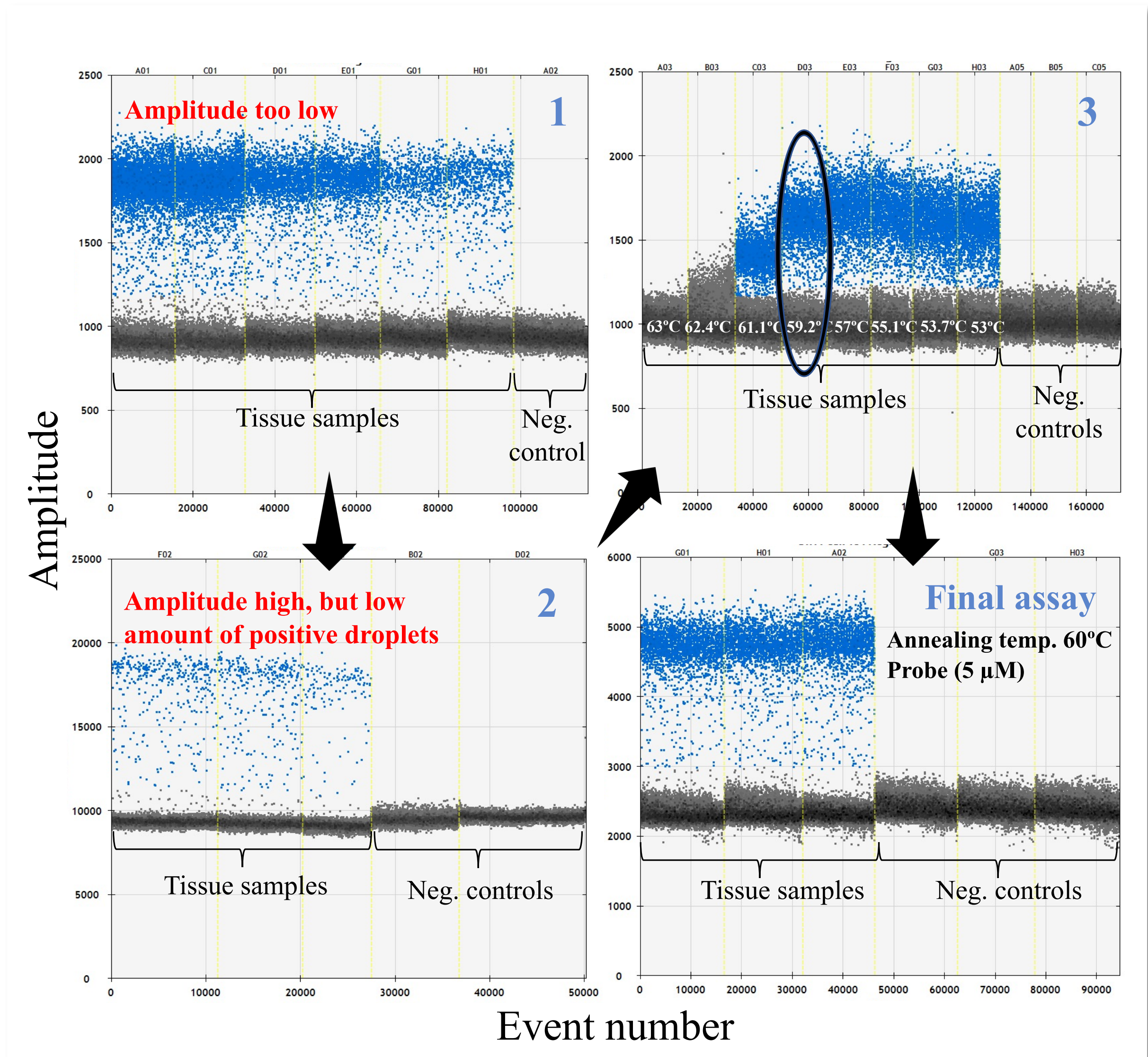



**Figure 6.** Droplet plots from *QuantaSoft™* showing results from different *H. americanus* ddPCR assay optimalization. The panels illustrate representative results, to show why different parameters were adjusted. The assay conditions are given in Appendix A. (Same approach was also used for optimising *H. gammarus* assay). **1)** 2.5 µM probe resulted in high amplification, but positive droplet with low amplitude. **2)** EvaGreen was used to increase amplitude, but amplification was reduced. **3)** An annealing gradient was tested, with a 2.5 µM probe. **Final assay)** Optimal annealing temperature found in test 3 was used, in addition to higher probe concentration (5 µM). Droplet rain was present, but the amplification and amplitude were high.

## 3.2 *H. americanus* eDNA concentration and degradation in laboratory experiment

eDNA from water collected in tanks with lobsters was successfully amplified (Figure 7). Sampling over time after removal of the lobsters showed that degradation of eDNA can be described using an exponential regression. The eDNA quantity measured 1 hour after the removal of lobsters varied among tanks, ranging from about 2.6 copies/µL to 38.9 copies/µL. After 6 hours the concentration had decreased to between 2.4 and 9 copies/µL. After 25 hours the concentration increased in two of the tanks, with a concentration from 1.9 to 25 copies/µL. 49 hours after removal the concentration was 0.8-15.4 copies/µL. Amplification of DNA was still detected 121 hours after removal, but the signal was very low (0.3-2.8 copies/µL).

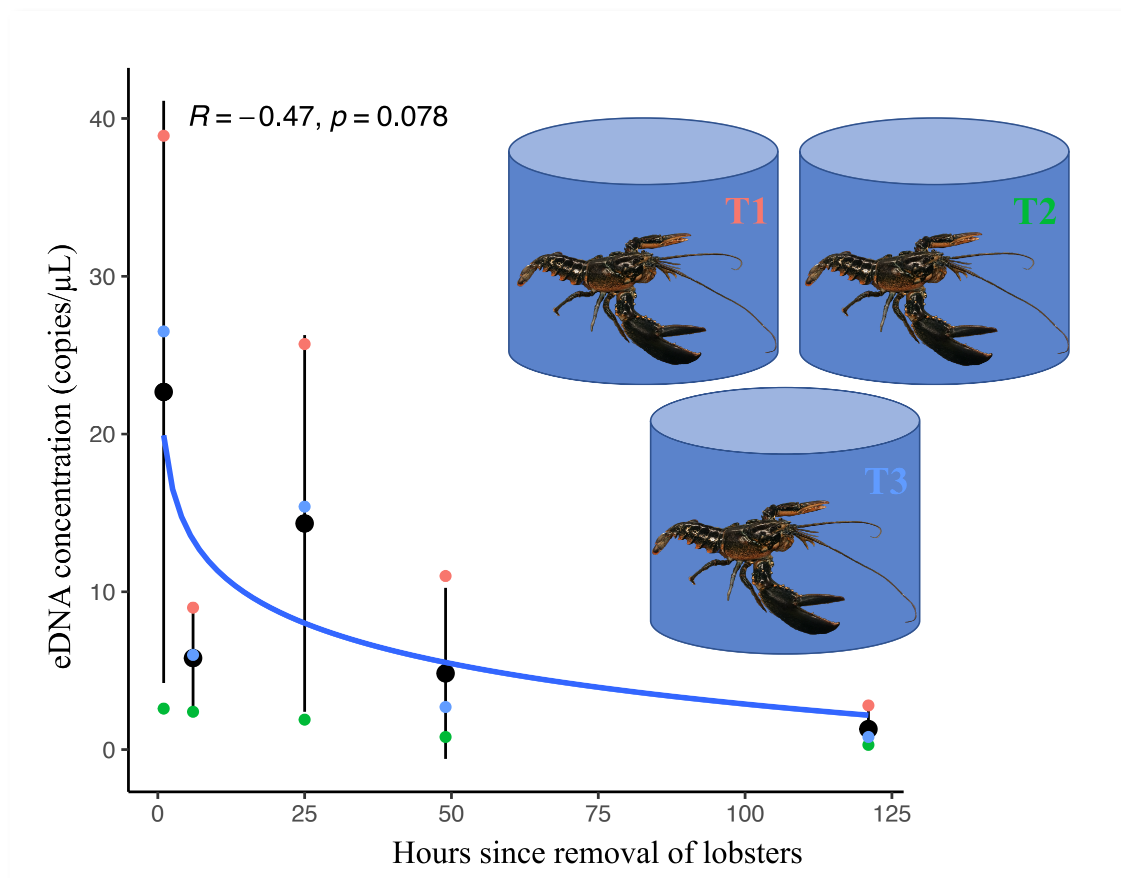



**Figure 7.** Concentration and decay rate of detectable lobster eDNA in tanks as a function of time after removal. Three individual lobsters indicated by green, blue and pink dots. Mean concentration and standard deviation is indicated in black, the exponential regression is given by the blue line. A Pearson correlation was used to investigate the relationship between concentration and time (R=-0.47, p=0.078). Plot made in RStudio (Version 1.3.1073 © 2009-2020 RStudio, PBC).

In previous studies (Barnes et al., 2014; Kutti et al., 2020; Maruyama et al., 2014) the decay rate and half-life of target eDNA was calculated using:

$$C(t) = C_0 e^{-\alpha t}$$

Where C(t) is the concentration of DNA measured at time t, $C_0$ the initial concentration and α=0.03 (calculations in Appendix E) being the degradation constant. Equation from the half-life of target DNA was calculated using:

$$0.5C_0 = C_0 e^{-\alpha t}$$

The eDNA half-life, defined as time in which half of the eDNA copies are degraded (Maruyama et al., 2014), was 27 hours.

## 3.3 eDNA detection in field samples

None of the 23 field samples, filtering between 2-5 L, collected at two locations during two different seasons, contained detectable levels of eDNA from either *H. americanus* or *H. gammarus* (Table 3).

**Table 3.** Detection of lobster eDNA in field samples. Positive controls had succeccful amplification, and no amplification in negative controls.

| Sample | Location | Detection of *H. americanus* (copies/µL) | Detection of *H. gammarus* (copies/µL) | Amount of filtrated water |
|---|---|---|---|---|
| 1-9 | Vinnes (April) | 0 | 0 | 4.2-5 |
| Neg. field control 1-3 | | | | 1.8-2 |
| Neg. extraction control. | | | | - |
| Neg. PCR control | | | | |
| Positive control | | 182 | 247 | |
| 10-18 | Ramsholmane (April) | 0 | 0 | 3-3.4 |
| Neg. field control 4-6 | | | | 2.3-2.75 |
| Neg. extraction control. | | | | - |
| Neg. PCR control | | | | |
| Positive control | | 182 | 247 | |
| 19-23 | Vinnes (August) | 0 | 0 | 2-2.1 |
| Neg. field control 7-8 | | | | 5 |
| Neg. extraction control. | | | | - |
| Neg. PCR control | | | | |
| Positive control | | 7 | 224 | |
| 19-23 | Vinnes (August) with increased extract volume | - | 0 | 2-2.1 |
| Neg. field control 7-8 | | | | 5 |
| Neg. extraction control. | | | | - |
| Neg. PCR control | | | | |
| Positive control | | - | 223 | |

## 3.4 Results from reanalysis of field samples collected in August

Calculations (from Wilcox et al. (2018), Appendix D) indicate that the effective quantity of potential DNA analysed from laboratory experiment samples was 1.1%, and between 2.6% and 6.6% in the field samples. This indicates that if there was 100 copies/L present in the laboratory samples, only an average of 1.1 copies would be analysed. For the field samples between 2.6 to 6.6 copies would actually be analysed. In the new extractions the rest of the sample volume (1.4 mL) was used in the extraction and analysed for *H. gammarus* eDNA. The effective quantity increased to 21% compared to the 2.6-6.6% from the previous analysis. The increase in effective quantity did however not result in any detection of *H. gammarus* eDNA (Table 5).

# 4 Discussion

Traditional methods used for biomonitoring, is typically dependent on catch or visual observation. Such approaches are often hampered by high cost and low detection precision, particularly if the population density of the target species is low. Molecular methods may in such cases be better suited if they can provide both better cost efficiency and higher detection probability.

The main aim of the present study was to investigate if eDNA from *Homarus americanus* can be used for detecting this introduced species in coastal waters. Laboratory experiment confirmed that *Homarus* lobsters release detectable concentrations of eDNA in tanks, however the levels were low. This is under the assumption that *H. americanus* and *H. gammarus* shed same levels of eDNA as *H. americanus* x *H. gammarus* hybrids. No *H. americanus* eDNA was detected in any of the water samples collected in the field. Field samples from a location with a known *H. gammarus* population did not contain detectable levels of eDNA from that species either, even when extraction volume was increased to increase detectability. This may suggest that these two lobster species release too low quantity of eDNA for detection in the wild. That indicate, like previous studies, that crustaceans release low levels of eDNA compared to species without exoskeleton e.g., fish (Allan et al., 2021; Crane et al., 2021; Forsström et al., 2016).

Results from the laboratory experiment show that the lobsters shed enough eDNA to be detected in a system with low dilution. The difference in eDNA concentrations among the tanks 1 hour after removal was 36 copies/µL at the most. The individuals used were all males with a similar size, and no moulting was observed. Hence, the difference in shedding rates could be due to different stress levels or difference in other physiological activities. Although no moulting activity was recorded, moulting could have started for lobsters in tank 1 and 3. The moulting process can last for more than 24 hours (Agnalt A-L, pers. comm.), and since the lobsters were only present in the tank for 24 hours, moulting could have started without being noticed. Difference in shedding rates between individuals of the same sex and similar size has also been observed in the study of Forsström et al. (2016) and Crane et al. (2021).

Another notable result from the laboratory experiment was the drop in eDNA concentration 6 hours after removal of lobsters. Due to organic material clogging the filters used for sampling 1 hour after removal, the circulation pump was stopped. The drop in concentration could be

caused by eDNA settling and resuspension in the water column, increasing the availability for the next sampling. In another similar study by Kutti et al. (2020) eDNA concentrations was also higher after the initial sampling. It was assumed that removal process flushed of DNA from the animals' surface, temporarily increasing the DNA concentrations in the water.

Degradation of *Homarus* eDNA followed an exponential decay curve as observed in other aquatic species (Forsström et al., 2016; Kutti et al., 2020; Thomsen et al., 2012). The half-life of target eDNA was estimated to 27 hours. In contrast, Forsström et al. (2016) indicated a significant degradation rate (55%) of white fingered mud-crab (*Rhithropanopeus harrisii)* eDNA between 72 and 120 hours. Collins et al. (2018) indicated that the half-life of European green crab (*Carcinus maenas*) eDNA was between 32 to 35 hours depending on temperature. A study of the degradation rate of *Lophelia* coral eDNA was conducted in the same laboratory as the current study, using the same water source, rooms, pumps and tanks, and suggested an eDNA half-life of 41 hours (Kutti et al. 2020). Due to similar environmental conditions like the current study, it is likely that this comparison is valid suggesting that the exploited lobster eDNA degrades at a significantly higher rate than *Lophelia* eDNA. Knowledge about degradation rate of a species eDNA can help understanding how recent the species was present in that environment (Barnes et al., 2016).

The results from the field samples analyses are more challenging to interpret. Whether no detection of eDNA in any of the field samples signifies that there are no lobsters present in the local environment or that shedding from lobsters actually present is below the detection limit, is uncertain. Even if there are lobsters present in an area, several factors may reduce the possibility of detection. eDNA can be transported away from the sampling site, it can be degraded before sampling, PCR inhibitors can be present in samples or not enough eDNA is released for successful detection (Barnes et al., 2016).

## 4.1 False negative results

It is important to consider the probability of detecting a species eDNA when analysing results from seawater samples (Furlan et al., 2016). The lower the probability is, the higher the chance is that no detection of target eDNA is a false negative result. Different physical factors in the marine environment may affect the fate of eDNA. The complex current patterns and possible density stratification, typical for the coastal marine environment, may distribute eDNA in an unpredictable way and away from the source population (Barnes et al., 2016; Thomsen et al.,

2015). Modelling of ocean currents indicate that eDNA can be transported tens of kilometres in only a few days (Andruszkiewicz et al., 2019). eDNA in the marine environment will at the same time be significantly diluted. In addition, the degradation rate is found to be faster in marine than freshwater systems (Thomsen et al., 2012), presumably due to higher microbial activity (Strickler et al., 2015). Transport away from the sampling site, the dilution, and degradation decreases the chance of detection. Mapping local current pattern and distribution of eDNA in seawater is therefore relevant. The latter can be done by sampling along a horizontal or vertical gradient. Such a design was planned in the present study by using caged lobster hybrids, however approval from the environmental authorities was not given and the study could not be carried out.

Wilcox et al. (2018) emphasize the importance of assessing PCR inhibitors as the presence of such components may contribute to false negative results. PCR inhibitors prevents amplification of potential target amplicon in the sample and can be found in several of substances in environmental samples. Non-target DNA can inhibit PCR amplification and is affected by template amount (Harper et al., 2018). However, the present study used a relatively low template amount (4 µL in a total PCR reaction volume of 22 µL) reducing the risk of such inhibition. Relative to the alternative technique qPCR, ddPCR used in the present study has been shown to be less vulnerable to PCR inhibition (Doi et al., 2015; Whale et al., 2012), but inhibition can still occur. Droplet rain in the *QuantaSoft™ Software* plots observed in the present study (Figure 5) may indicate inhibition (Kokkoris et al., 2021). Further studies could focus on the presence and possible removal of inhibitors, to reduce the probability of false negative results.

Even if a species is present in an environment, the amount of eDNA released may be too low for successful detection (Furlan et al., 2016). In an early phase, introduced species are typically low in numbers, and before recruitment is established, levels of eDNA in the environment may stay below the detection limit. Detection of the invasive crayfish *Procambarus clarkii* in freshwater ponds by eDNA was problematic at low abundances (Tréguier et al., 2014). The same may be the case for *H. americanus* (or hybrid) populations. The density of the potential population at the studied locations is unknown and could be very low for many years after introduction. A well-established population of *H. gammarus* is although documented in the sampling area (Vinnes) but the abundance needed for a positive amplification of eDNA is currently unknown.

Capability for eDNA based detection of hybrids is important to consider when evaluating whether results are false negative. As mtDNA is only inherited from the female, the present assay cannot distinguish between pure *H. americanus* and hybrids of *H. gammarus* male x *H. americanus* female. In addition, hybrids between *H. gammarus* females x *H. americanus* males will not be detected. In an early phase of an introduction event, most of the introduced individuals will have low probability of finding a mating partner of the same species. In this phase inter species mating may be more likely.

Models can be used to evaluate the suitability of eDNA for detection of organisms (Chambert et al., 2015; Furlan et al., 2016; Guillera-Arroita et al., 2017). To predict the probability of detection such models include input parameters like number of sampling sites, sample size at each site, sample volume, amount and volume of PCR replicates per sample. Further studies of lobster distribution could take advantage of models to estimate the theoretical probability of eDNA detection success.

## 4.2 Alternative sampling approaches

Despite eDNA being used in detection of species for over a decade, no standardized eDNA survey method suitable for different taxa has yet been established. Testing alternative sampling, extraction and analysis approaches could increase the likelihood of detecting target eDNA.

Water temperature is known to influence the activity of lobsters (Aiken et al., 1989; McLeese et al., 2011) and could consequently affect eDNA shedding rate. Biological events such as lobster moulting and spawning typically taking place during the summer months (Herrick, 1911) may also affect the eDNA release rate. Studies of the invasive European green crab (*Carcinus maenas*) show that the species shed varying levels of eDNA throughout its life cycle, with high levels being released from ovigerous females but otherwise low release (Crane et al., 2021). That study highlights the importance of considering life stage when designing sampling program for monitoring of marine crustaceans. Another study of an invasive freshwater crayfish (*Pacifastacus leniusculus*) in European waters indicate that the eDNA concentration is higher during spawning (Dunn et al., 2017).

The present study only tested eDNA levels at one specific water temperature in the lab experiment (10°C) and used only one life stages. To gain more information about the eDNA

release of *H. americanus,* laboratory studies including several environmental conditions and various life cycle stages is necessary. Low eDNA release may be a factor limiting detection success from field samples, particularly at low temperatures. However, samples were collected at two different seasons, with different water temperatures (~8°C vs. ~16°C). In addition, if lobsters shed sufficient eDNA for detection, the presence of both adults and juveniles would increase the likelihood of detection. Another factor to consider is that sampling has only been conducted during daytime. Lobsters are nocturnal animals primarily active at night (Moland et al., 2011). As they may release more eDNA while interacting with the environment, sampling at night may also increase chance of detection.

In the present study, samples were only collected from the water column. Other studies that compared eDNA presence in water vs. sediments, concluded that more eDNA was available in sediments (Crane et al., 2021; Holman et al., 2019; Turner et al., 2015). As lobsters are benthic species, eDNA concentrations may be higher in sediment. A study by Buxton et al. (2018) did however get a higher detection rate of eDNA in water sample than sediment samples. Crane et al. (2021) suggests that lower detection in sediment samples might be a result of the sample size being smaller as there is a limit to how much sediment that can be processed in extraction methods, compared to water samples. The study also recommends collecting water slurry samples as they may contain more DNA than water samples and are more effective than sediment samples. Further studies on eDNA on marine crustaceans could include sampling of alternative ecological compartments (e.g., sediment or water slurry).

Geerts et al. (2018) concluded that detection of eDNA from the invasive crayfish *Procambarus clarkii* was affected by sampling strategy, type of extraction method and choice of primers. Other studies comparing different sampling and extraction methods also indicate that the detection potential may be affected by techniques used (Eichmiller et al., 2016; Kumar et al., 2020). The DNeasy Blood and Tissue kit that was used in the present study, successfully extracted DNA from the tissue samples used for positive controls with the chosen primers and probe for both lobster species. This confirms that the primers and PCR conditions used, provides species-specific identification. Lack of signal from water samples collected in the field, may therefore be caused using sub optimal filter type. eDNA-bearing particles may vary in size, larger pore size could cause the smallest particles to escape through the filter. Too small pore size on the other hand, could lead to clogging meaning that less water can be filtered, resulting in lower chance of detection (Turner et al., 2014). Spens et al. (2017) compared multiple filters and suggested using Sterivex-GP capsule filter instead of nitrocellulose

membrane filters (used in the present study) for detecting macrobial eDNA. Another study compared different filters (using the same extraction kit as in the present study), where nitrocellulose filters had the highest yield of eDNA concentration (Djurhuus et al., 2017). That study did however not test Sterivex filters. The nitrocellulose filters used in the present study captured lobster eDNA in the laboratory experiment, but alternative filters may increase yield.

## 4.3 Importance of results for conservation management

Monitoring the distribution of introduced species to understand and prepare for potential negative ecological impacts are important, as they can become invasive and be a significant threat to native biodiversity (Clavero et al., 2005). The use of eDNA in conservation has, relative to traditional monitoring methods, sometimes proven to be more cost efficient and less invasive (Lugg et al., 2018; Rees et al., 2014). Effort is therefor put into studying the use of eDNA for biomonitoring (Dunshea et al., 2021). eDNA is used to sample for metabarcoding, a technique that can provide information about the composition of communities, in addition to detecting introduced species. In metabarcoding environmental samples are analysed for multiple species' eDNA at the same time, by sequencing an unique DNA marker in their genome as a "barcode" for identification (Valentini et al., 2016). The present confidence in eDNA approaches for monitoring is generally high but it is important to take into consideration that methods are still under development. Different species release various quantities of eDNA into the environment, depending on local population density, how they interact with the environment and their physiology (Barnes et al., 2016). The suitability of eDNA approaches may therefore vary, and in some cases provide less precise data. Very low frequency of decapod reads in sediment metabarcoding suggest that eDNA from this taxon is generally missing from the environment (Anders Lanzén pers. comm.). Even though the field sampling conducted in the present master project was limited in scope, the results indicate that monitoring of low-density populations of crustaceans by eDNA in a coastal environment where dilution typically is significant may not be feasible. Further method development needs to be implemented before eDNA can be completely trusted for use in bio monitoring.

# 5 Conclusion

In the present study species-specific primers and probes were developed and PCR assays were optimised to detect eDNA from *H. americanus* and *H. gammarus*. Observations from the laboratory experiments indicates that both species release low amounts of eDNA. Low eDNA shedding rates has also been observed in other studies of decapods and could, relative to other taxa, be a general challenge for all species with exoskeleton. No eDNA of either *H. americanus* or *H. gammarus* was detected in any of the field samples analysed, even though presence of the latter species has been documented in the local environment. The results therefore indicate that the monitoring approach in its current form, does not seem to be suitable for detection of lobster species in coastal waters. It is although important to investigate factors that can cause false negative results, preventing detection. Further studies should focus on oceanographic conditions (dilution and transport) that may decrease the detection probability, and inhibitors that may prevent amplification of target DNA. In addition, alternative sampling from different ecological compartments (e.g., sediment or water slurry) and sampling at different times, together with optimisation of extraction and analysis approaches, may increase the detection potential and should hence be tested in future studies.

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# Appendix A - ddPCR assays used in study for optimisation of detection of targeted amplicons

**Table A1. Reaction components for the ddPCR assays – *H. americanus***

| | Volume per reaction (µL) | | |
|---|---|---|---|
| **Components** | **Test assay no. 1** | **Test assay no. 2** | **Test assay no. 3** |
| Bio-Rad ddPCR Supermix for Probes (no dUTP) | 11 | - | 11 |
| Bio-Rad EvaGreen | - | 11 | - |
| Forward primer (10 µM) | 0.88 | 1 | 0.88 |
| Reverse primer (10 µM) | 0.88 | 1 | 0.88 |
| Probe (2,5 µM) | 0.88 | - | 0.88 |
| Water | 4.36 | 5 | 4.36 |
| Template | 4 | 4 | 4 |
| Total volume | 22 | 22 | 22 |

**Table A2. PCR program for ddPCR test assays for *H. americanus*. Ramp rate was set to +2°C $s^{-1}$ for all steps.**

| | Test assay no. 1 | | | Test assay no. 2 | | | Test assay no. 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| **Step** | **Temp** | **Time** | **# of cycles** | **Temp** | **Time** | **# of cycles** | **Temp** | **Time** | **# of cycles** |
| Enzyme activation | 95°C | 10 min | 1 | 95°C | 5 min | 1 | 95°C | 10 min | 1 |
| Denaturation | 95°C | 30 sec | 40 | 95°C | 30 sec | 40 | 95°C | 30 sec | 50 |
| Annealing/Extension | 61°C | 1 min | | 61°C | 1 min | | 53-65°C | 30 sec | |
| Signal Stabilization | 98°C | 10 sec | 1 | 4°C | 5 min | 1 | 72°C | 10 sec | 1 |
| Hold | 4°C | hold | 1 | 90°C | 5 min | 1 | 4°C | ∞ | 1 |

| | Volume per reaction (µL) | |
|---|---|---|
| **Components** | **Test assay no. 5** | **Test assay no. 6** |
| Bio-Rad EvaGreen | 11 | 11 |
| Forward primer (10 µM) | 1 | 1 |
| Reverse primer (10 µM) | 1 | 1 |
| Water | 5 | 5 |
| Template | 4 | 4 |
| Total volume | 22 | 22 |

**Table A3. Reaction components for the ddPCR assays – *H. gammarus***

**Table A4. PCR program for ddPCR test assays for *H. gammarus*. Ramp rate was set to +2°C $s^{-1}$ for all steps.**

| | Test assay no. 5 | | | Test assay no. 6 | | |
|---|---|---|---|---|---|---|
| **Step** | **Temp** | **Time** | **# of cycles** | **Temp** | **Time** | **# of cycles** |
| Enzyme activation | 95°C | 5 min | 1 | 95°C | 5 min | 1 |
| Denaturation | 95°C | 30 sec | 40 | 95°C | 30 sec | 40 |
| Annealing/Extension | 52-63°C | 1 min | | 56,4°C | 1 min | |
| Signal Stabilization | 4°C | 5 min | 1 | 4°C | 5 min | 1 |
| | 90°C | 5 min | 1 | 90°C | 5 min | 1 |
| Hold | 4°C | hold | 1 | 4°C | hold | 1 |

# Appendix B - Lab experiment

**Table B1. Biometrics, *H. americanus* x *H. gammarus* hybrid lobsters used in lab experiments**

| Lobster ID | Tank no. | Sex | Weight (g) | Total length (cm) | Carapace length (mm) | Number of claws |
|---|---|---|---|---|---|---|
| F5 | 1 | Male | 653 | 25 | 87 | 2 |
| L4 | 2 | Male | 686 | 26 | 97 | 2 |
| M6 | 3 | Male | 703 | 26 | 97 | 2 |

**Table B2. Water sampling intervals**

| Tank no. | Tank content | Times sampled |
|---|---|---|
| 1 | Seawater + lobster F5 "Frank" | 1h, 6h, 25h, 49h, 121h |
| 2 | Seawater + lobster L4 "Lars" | 1h, 6h, 25h, 49h, 121h |
| 3 | Seawater + lobster M6 "Mikkel" | 1h, 6h, 25h, 49h, 121h |
| 4 | Distilled water | 6h, 24h, 48h, 121h |
| 5 | Seawater | 6h, 24h, 48h, 121h |
| Tap water | Tap water | 6h, 25h, 49h, 121h |

**Table B3. Sampling Information. * Reduced volume due to clogged filters.**

| Tank no. | Sample | Date (All 2019) | Time starts | Time stops | Volume filtered (L) | Time since removal (hours) |
|---|---|---|---|---|---|---|
| 1 | 1 | Sept. 18th | 09:15 | 12:45 | 1 | 1 |
| 2 | 2 | | 09:15 | 14:00 | 1 | |
| 3 | 3 | | 09:15 | 13:00 | 0.85 * | |
| 4 | 4 | | 14:00 | 16:00 | 1 | 6 |
| 5 | 5 | | 14:00 | 16:30 | 1 | |
| 2 | 6 | | 14:00 | 20:00 | 0.385 * | |
| 3 | 7 | | 14:00 | 20:00 | 0.645 * | |
| 1 | 8 | | 14:00 | 17:00 | 1 | |
| Tap water | 9 | | 14:00 | 16:30 | 1 | |
| Tap water | 10 | Sept. 19th | 08:30 | 09:00 | 1 | 24 |
| 4 | 11 | | 08:45 | 09:10 | 1 | |
| 5 | 12 | | 08:50 | 09:20 | 1 | |
| 1 | 13 | | 09:25 | 10:10 | 1 | 25 |
| 2 | 14 | | 09:25 | 10:10 | 1 | |
| 3 | 15 | | 09:25 | 10:10 | 1 | |
| 4 | 16 | Sept. 20th | 08:50 | 09:20 | 1 | 48 |
| 5 | 17 | | 08:50 | 09:20 | 1 | |
| Tap water | 18 | | 08:50 | 09:15 | 1 | |
| 1 | 19 | | 09:30 | 10:05 | 1 | 49 |
| 2 | 20 | | 09:30 | 10:05 | 1 | |
| 3 | 21 | | 09:30 | 10:05 | 1 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | 22 | Sept. 23rd | 09:20 | 09:25 | 1 | 121 |
| 5 | 23 | | 09:20 | 09:25 | 1 | |
| Tap water | 24 | | 09:20 | 09:25 | 1 | |
| 1 | 25 | | 09:50 | 10:10 | 1 | |
| 2 | 26 | | 09:50 | 10:15 | 1 | |
| 3 | 27 | | 09:50 | 10:10 | 1 | |

# Appendix C - Field sampling

**Table C1. Sampling information 16th and 17th of April**

| Sample | Location | Latitude | Longitude | Depth (m) | Depth sample taken (m) | Amount filtrated (L) | Temperature of water |
|---|---|---|---|---|---|---|---|
| 1 | Ramsholmane | 5930.6072 N | 00511.1772 E | 35.5 | 33.9 | 5 | Missing data |
| 2 | | 5930.5754 N | 00511.1138 E | 35.5 | 34.6 | 5 | |
| 3 | | 5930.5595 N | 00511.2061 E | 37.5 | 36 | 4.2 | |
| 4 | | 5930.5198 N | 00511.1543 E | 22.1 | 20.7 | 5 | |
| 5 | | 5930.5586 N | 00511.1288 E | 31.9 | 28.8 | 5 | |
| 6 | | 5930.5895 N | 00511.1824 E | 27.5 | 24.4 | 5 | |
| 7 | | 5930.6641 N | 00511.1866 E | Ca. 20 | 18.7 | 5 | |
| 8 | | 5930.6320 N | 00511.0912 E | 23 | 17,2 | 5 | |
| 9 | | 5930.6104 N | 00511.0425 E | 21.1 | 15.8 | 5 | |
| 10 | Vinnes | 6008.9472 N | 00535.1428 E | 8.5 | 7.9 | 3.4 | Around 8°C |
| 11 | | 6008.9138 N | 00535.1489 E | 12.8 | Ca 11 | 3.3 | |
| 12 | | 6008.8722 N | 00535.1770 E | 11.4 | 10 | 3 | |
| 13 | | 6009.1122 N | 00534.6280 E | 7.9 | Ca 6 | 3 | |
| 14 | | 6009.1437 N | 00534.5361 E | 84 | 6.1 | 3 | |
| 15 | | 6009.0790 N | 00534.7137 E | 9.3 | 7.9 | 3 | |
| 16 | | 6009.0116 N | 00534.9306 E | 8 | 6.4 | 3,5 | |
| 17 | | 6009.0355 N | 00534.8134 E | 12.4 | 10.3 | 3,1 | |
| 18 | | 6008.9789 N | 00534.9831 E | 9.35 | 7.9 | 3,15 | |

## Appendix D – Effective quantity

**Volume extracted from cryogenic vials**

Lab: (0.180 mL subsample/1.98 mL buffer in cryogenic vial) *100= 9.09%

Field: (0.180 mL subsample/1.6 mL buffer) *100 = 11.25%

**Effective volume (L)**

Lab:

9*1/100=0.09

Field:

11*2/100= 0.22

11*5/100= 0.55

**Effective quantity (%)**

*Volume collected * Volume extracted * Aliquot volume (PCR template volume x three replicas)*

Lab:

1 * 0.09 * (0.04*3) = 0.0108 → Effective quantity= 1.1%

Field:

2 * 0.11 * (0.04*3) = 0.0264 → 2.6%

5 * 0.11 * (0.04*3) = 0.066 → 6.6%

## New extractions

### Volume extracted from cryogenic vials

Field: (1.4mL subsample/1.6 mL buffer) *100 = 87.5%

### Effective quantity (%)

*Volume collected * Volume extracted * Aliquot volume*

Field:

2*0.88*(0.04*3) = 0.21 → 21%

**Table D1. Overview of the quantity of different solutions in sampling, extraction and analysis used to calculate the effective quantity. Reasoning based on Wilcox et al. (2018).**

| Samples | Volume filtrated (L) | Volume extracted (%) | Effective volume (L) | Volume eluted (µL) | Template volume aliquot (µL) | Effective quantity (%) |
|---|---|---|---|---|---|---|
| Laboratory experiments | 1 | 9 | 0.09 | 100 | 12 | 1.1 |
| Field samples | 2-5 | 11 | 0.22-0.55 | 100 | 12 | 2.6-6.6 |
| Field samples from August – new extractions | 2 | 88 | 1.76 | 100 | 12 | 21 |

## Appendix E – Degradation constant and eDNA half-life

**Table E1. eDNA concentration and mean of all tanks, at each time sampled in lab experiment**

| Tank | Time (hrs) | Copies /µL | Mean (copies/µL) |
|---|---|---|---|
| 1 | | 39.9 | |
| 2 | 1 | 2.6 | 23 |
| 3 | | 26.5 | |
| 1 | | 9 | |
| 2 | 6 | 2.4 | 6 |
| 3 | | 6 | |
| 1 | | 2.7 | |
| 2 | 24 | 1.9 | 14 |
| 3 | | 15.4 | |
| 1 | | 11 | |
| 2 | 49 | 0.8 | 5 |
| 3 | | 2.7 | |
| 1 | | 2.8 | |
| 2 | 121 | 0.3 | 1 |
| 3 | | 0.8 | |

**Calculation of degradation constant and target eDNA half-life**

$C(t) = C_0 e^{-\alpha t}$

$C_0$ was the initial measurement of mean concentration from all the tanks: 23 copies/µl.

$6 = 23e^{-\alpha 6} \rightarrow \alpha = \frac{ln\frac{6}{23}}{-6} = -0.22$

$14 = 23e^{-\alpha 24} \rightarrow \alpha = \frac{ln\frac{14}{23}}{-24} = -0.021$

$5 = 23e^{-\alpha 48} \rightarrow \alpha = \frac{ln\frac{5}{23}}{-48} = -0.032$

$1 = 23e^{-\alpha 121} \rightarrow \alpha = \frac{ln\frac{1}{23}}{-121} = -0.025$

The mean of the degradation constant was α = 0.026. Using the decay constant from 6 hours after removal would have given a half time of 9 hours, this was thus excluded.

$11.5 = 23e^{-\alpha t} \rightarrow$ t= $\frac{ln\frac{11.5}{23}}{0.026} = 27$