Science of the Total Environment 821 (2022) 153093

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Monitoring of environmental DNA from nonindigenous species of algae, dinoflagellates and animals in the North East Atlantic

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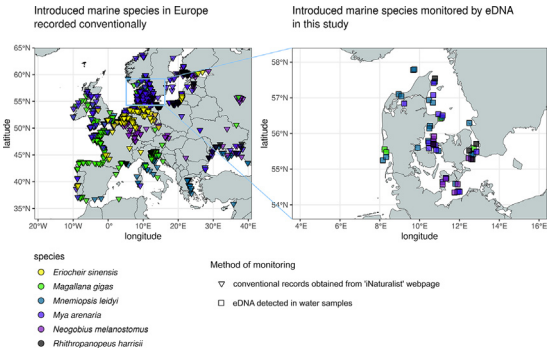
HIGHLIGHTS

- We monitored 18 non-indigenous marine species, using 12 specific eDNA assays we developed and six already published assays.
- Two sampling events in 16 harbours per year show seasonal variation.
- A positive relationship between the number of species detected by conventional monitoring and eDNA was significant.
- Rigorous tests of each assay ensures specific monitoring of eDNA for these species can be applied in seas around Europe.
- This study provides a proof of concept for how continuous monitoring of eDNA from marine introduced species can be achieved.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 August 2021
Received in revised form 4 December 2021
Accepted 9 January 2022
Available online 14 January 2022

Editor: Julian Blasco

Keywords:
Non-indigenous species
eDNA
Species-specific assays
North Eastern Atlantic Ocean
North Sea-Baltic Sea transition zone
Introduced species

ABSTRACT

Monitoring the distribution of marine nonindigenous species is a challenging task. To support this monitoring, we developed and validated the specificity of 12 primer-probe assays for detection of environmental DNA (eDNA) from marine species, all nonindigenous to Europe. The species include sturgeons, a Pacific red algae, oyster thief, a freshwater hydroid from the Black Sea, Chinese mitten crab, Pacific oyster, warty comb jelly, sand gaper, round goby, pink salmon, rainbow trout and North American mud crab. We tested all assays in the laboratory, on DNA extracted from both the target and non-target species to ensure that they only amplified DNA from the intended species. Subsequently, all assays were used to analyse water samples collected at 16 different harbours across two different seasons during 2017. We also included six previously published assays targeting eDNA from goldfish, European carp, two species of dinoflagellates of the genera *Karenia* and *Prorocentrum*, two species of the heterokont flagellate genus *Pseudochattonella*. Conventional monitoring was carried out alongside eDNA sampling but with only one sampling event over the one year. Because eDNA was relatively fast and easy to collect compared to conventional sampling, we sampled eDNA twice during 2017, which showed seasonal changes in the distribution of nonindigenous species. Comparing eDNA levels with salinity gradients did not show any correlation. A significant correlation was observed

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http://dx.doi.org/10.1016/j.scitotenv.2022.153093

Monitoring and assessment
Real-time polymerase chain reaction
Quantitative PCR

between number of species detected with conventional monitoring methods and number of species found using eDNA at each location. This supports the use of eDNA for surveillance of the distribution of marine nonindigenous species, where the speed and relative easy sampling in the field combined with fast molecular analysis may provide advantages compared to conventional monitoring methods. Prior validation of assays increases taxonomic precision, and laboratorial setup facilitates analysis of multiple samples simultaneously. The specific eDNA assays presented here can be implemented directly in monitoring programmes across Europe and potentially worldwide to infer a more precise picture of the dynamics in the distribution of marine nonindigenous species.

Abbreviations

Cq	Cycle of quantification
DNA	Deoxyribonucleic acid
dsPCR	Double stranded PCR
eDNA	Environmental DNA
HELCOM	Helsinki Commission
LOD	Level of detection
LOQ	Level of quantification
MDS	Multidimensional scaling
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
NIS	Nonindigenous species
NR	Not recorded
NTS	Non-target species
OSPAR	Oslo and Paris Commission
PCR	Polymerase chain reaction
PR	Previously recorded
qPCR	Quantitative PCR
SR	Recorded in this survey

1. Introduction

Nonindigenous species (NIS) can pose a threat to native species (Bax et al., 2003) by competing for food and space, which potentially can lead to eradication of the native species from their natural habitat (Blackburn et al., 2019; Karlson et al., 2007), but can also act as predators or parasites on native species, be vectors of parasites, result in critical modifications of the habitat, hybridize with native species, change inherent biodiversity in the area, or be of economic consequences to human agriculture, industries and disrupt wildlife conservation plans (Behrens et al., 2017; Simberloff, 2003; Sherpa and Després, 2021). The costs related to impacts of introduced species has been estimated to be several billion Euros annually in Europe alone, and the costs appear to increase exponentially across years (Haubrock et al., 2021). If native marine species and habitats are to be conserved by removing threats posed by NIS, a first step is to monitor the extent and dynamics of the distribution of the NIS (Ojaveer et al., 2017). This is especially challenging in cases including multiple and taxonomic different species, as monitoring efforts often will involve various types of field gear and sampling techniques. Such efforts will thus increase field expenses but also potential response time, due to limited availability of needed taxonomic experts to identify the specimens collected. An alternative approach to conventional monitoring is to analyse environmental DNA (eDNA) shed from the organisms living in the water column (Beng and Corlett, 2020; Kelly et al., 2014a; Valentini et al., 2016). Monitoring of marine species by eDNA is to a large extent done using metabarcoding (e.g. Sigsgaard et al., 2017; Stat et al., 2017, 2019; Thomsen et al., 2016), but Tsuji et al. (2019) reported that a higher proportion of studies have used species-specific primers and probes commonly in quantitative polymerase chain reaction (qPCR) setups (e.g. Jensen et al., 2018; Salter et al., 2019), though this may have changed today with the advancement of eDNA metabarcoding studies. Comparing qPCR with metabarcoding there are at least four major differences:

(1) When single pre-selected species from many different phyla and kingdoms are to be monitored continuous running costs are relatively low and data analysis is fast for already validated qPCR assays (Harper et al., 2018). Costs are low compared to those of Next Generation Sequencing (NGS) used in metabarcoding studies that can yield far more information than immediately needed, when only specific selected species are in focus. Provided the target sequence of the NIS is represented in genetic databases, metabarcoding of eDNA can be helpful for monitoring newly introduced NIS.
(2) Insufficient coverage of diversity in genetic databases (Yang et al., 2017) or a lack of genetic difference among congeners can lead to incorrect species identification and conclusions in metabarcoding studies (Weigand et al., 2019), but can be avoided with precautionary filtering and well-curated databases, and by starting out with *de novo* sequencing of the uncovered genetic diversity. In a species-specific qPCR approach, *de novo* sequencing of congeners is often a prerequisite before design and validation can be initiated, but can be less compared to what is required for metabarcoding, depending on the diversity of the group.
(3) Metabarcoding studies using generic primers typically target single classes or phyla (Kelly et al., 2014b, 2019; Harper et al., 2018), and will require multiple primer sets for different genetic regions when monitoring NIS from various phyla and kingdoms. A species-specific approach also require multiple primer sets for monitoring different species, but will not return the excessive and costly diversity data that a metabarcoding approach will, which would be irrelevant for monitoring of just a few pre-selected species.
(4) Specific detection gives a better quantitative measure of eDNA in water samples (Klymus et al., 2020) than a metabarcoding approach (Thomas et al., 2020; Tsuji et al., 2019; Salter et al., 2019). Although NGS in a metabarcoding approach returns read counts, primer affinity towards specific taxonomic groups might skew the observed number of reads (Kelly et al., 2014b).

Comparing conventional monitoring with eDNA monitoring point to two major differences: (1) Determining species identity in conventionally collected samples can be complicated, as it requires insight in how to differentiate between various distinct morphological characters for many different phyla. Even with such taxonomic experience identification can be difficult or even impossible if the organisms are damaged by collection. When monitoring eDNA in water samples, the dependency of intact morphological characters becomes irrelevant, as DNA from the sought organism is all that is required for making the identification - albeit with the shortcomings inherent in monitoring by eDNA as listed above. (2) Field work during conventional monitoring often requires several hours of labour in the field collecting different kinds of samples, and several hours of sorting collected material afterwards and determining the species identity by consulting identification guidebooks. Collection of a water sample is relatively easy and fast, and laboratorial work can be faster and more streamlined compared to manual sorting of conventional collected samples. Subsequent data analysis is most likely equally demanding for both eDNA monitoring and conventional monitoring, and probably mainly depends on the research questions asked. A strength of conventional monitoring is the precision in the locality of where the species was detected, as opposed to monitoring of eDNA that could stem from an individual that has shed

the eDNA further away from the sampling site. A diluted eDNA signal can make it hard to attempt an estimate of abundance, as infrequent eDNA detections just as well could be caused by sampling sites being situated too far away from the main occurrence of the species.

It is difficult to quantify biomass directly from levels of eDNA in seawater samples (e.g. Günther et al., 2018; Knudsen et al., 2019), probably because production and decay rates of eDNA vary between life stages, organisms and habitats, and is influenced by numerous abiotic and biotic factors (Andruszkiewicz et al., 2017; Wood et al., 2020). Nevertheless, evaluation of the qualitative composition of species from the diversity of eDNA in water samples has been shown to provide a more accurate approximation to what species can be found by conventional monitoring (Thomsen et al., 2012a, 2012b, 2016; Valentini et al., 2016). However, in these studies (Thomsen et al., 2012b, 2016) such a correlation has been based on metabarcoding, covering the biodiversity within a defined taxonomic group.

Species-specific detection of eDNA from water samples offers an additional tool for monitoring the occurrence, range and distribution of marine NIS (Simmons et al., 2015), in addition to more traditional catch and sightings, and to what a generalized eDNA metabarcoding approach can accomplish. Previous studies on NIS have focused on crayfish (e.g. Agersnap et al., 2017; Robinson et al., 2018; Strand et al., 2019), molluscs (Clusa et al., 2017; Thomas et al., 2020), ctenophore (Créach et al., 2021) and fish (e.g. Gargan et al., 2021; Adrian-Kalchhauser and Burkhardt-Holm, 2016; Robinson et al., 2019). Surveillance of the distribution of marine NIS in northern Europe is increasingly needed, as the numbers of introduced species is growing (Keller et al., 2011; Ojaveer et al., 2017; Tsiamis et al., 2018), and the consequences of these introductions on the native ecosystems are not necessarily easy to infer (Boltovskoy et al., 2021; Katsanevakis et al., 2014; Russel and Blackburn, 2017). For NIS where the abundance and distribution is still poorly known, a first step towards managing the potential threat of marine NIS on ecosystems is to obtain accurate information of their distribution. This is even more important if the NIS have negative economic consequences (Lovell et al., 2006). One way to get a better knowledge of the distribution of NIS is to analyse eDNA to supplement conventional monitoring. Here, multiple qPCR assays can be applied for surveying large ecosystems and several taxa representing different kingdoms and phyla (e.g. Hernandez et al., 2020), to make it possible to analyse multiple sample sites rapidly and reduce the time needed for evaluating whether a NIS is present.

Marine NIS are considered problematic worldwide and comprises very different group of organisms. In the Caribbean Sea nonindigenous seagrass (*Halophila stipulacea*) and lionfish (*Pterois volitans*) are a threat to the native species (Albins and Hixon, 2013; Guzmán-Méndez et al., 2020). In temperate seas such as the North Atlantic, algae, flagellates, arthropods, molluscs, bony fish and comb jellies have been introduced (Table 1) and are a threat not only to European seas, but have by their introduction to north and south western Pacific Ocean regions also become a threat to marine areas around Japan, New Zealand and eastern Australia. Fish, like the round goby (*Neogobius melanostomus*) that is native to the Black Sea has not only been introduced to the Baltic Sea and the western coast of Europe, but also been introduced in the Great Lakes in North America (Azour et al., 2015; Nurkse et al., 2018). Monitoring of NIS in European seas is – because of this worldwide nonindigenous introduction – an issue that is of major concern to many marine regions and freshwater areas around the world.

We here focus on the warty comb jelly (*Mnemiopsis leydii*), the round goby, the North American mud crab (*Rhithropanopeus harrisii*), freshwater hydroid (*Corpdylophara caspia*), two heterokont flagellates (*Pseudochatonella* spp.) and a Pacific red algae (*Bonnemasionia hamifera*) as these species are considered NIS (Guiry, 2001; Hariot, 1891; van der Land, 2001; Projecto-Garcia et al., 2009) along many European coastal regions (Forsström and Vasemägi, 2016; Jaspers et al., 2018; Riisgård, 2017; Roche and Torchin, 2007), and some of them also considered NIS in North and South America as well as in the western Pacific (Table 1). We also focus on NIS that mainly are a concern for North European seas, such as the Chinese mitten crab (*Eriocheir sinensis*), the sand gaper (*Mya arenaria*) and oyster thief or bulb seaweed (*Colpomenia peregrina*) (Gofas et al., 2001; Green et al., 2012; Herborg et al., 2005; Køie and Kristiansen, 2000).

These species all represent very different organismal groups that will be very difficult to monitor using either conventional monitoring or with an eDNA metabarcoding approach. The latter would require large genetic databases with detailed coverage of species, and require multiple primer sets targeting different genomic regions, because the same genomic region is unlikely to provide an adequate resolution of species and diversity. Instead, eDNA from a limited selection of relevant NIS can be targeted with species-specific qPCR assays. Using species-specific assays in a qPCR approach allows for higher sensitivity towards gene regions from single species, and reduces laboratorial running costs compared to metabarcoding by NGS once assays have been developed and validated (Tsuji et al., 2020). Subsequent bioinformatic analysis is more straightforward for qPCR results, compared to the intricate analysis of NGS metabarcoding data.

Salinity levels has been suggested to be able to influence the distribution and spread of NIS, such as the round goby (Behrens et al., 2017), an introduced amphipod (Cuthbert and Briski, 2021) and an indigenous mussel (Knöbel et al., 2021). Seasonality might also be able to affect the distribution of NIS, like the warty comb jelly is (Jaspers et al., 2018). When species-specific qPCR assays are used for eDNA monitoring, the number of target molecules in the volume of filtered water can be estimated, which allows for determining a proxy of the prevalence of the species in the monitored area. The seas between the North Sea and the western Baltic Sea displays a gradient in salinity that drops from 34 ppt in the northwest to 10 ppt in the southeast (Maar et al., 2011; Momigliano et al., 2018). Estimating levels of eDNA, allows for comparison with seasons and general salinity levels, if the samples are collected from harbours that represents different salinity levels, and makes it possible to check whether the difference in salinity and seasons affects the distribution and prevalence of NIS in brackish and more saline marine environments.

We decided to monitor species from very different phyla. Which underlines the benefit of using qPCR for targeting NIS, compared to employing an eDNA metabarcoding NGS approach. Once assays have been developed and validated samples can also be analysed faster with qPCR compared to metabarcoding. A downside to developing multiple species-specific assays is the difficulty in obtaining reference samples from both the targeted NIS, and non-target species (NTS) for validation of the designed assays. When NTS representatives cannot be obtained, it can be difficult to verify if the assay is sufficiently specific for application on water samples. Optimization of primer and probe concentration and rigorous testing of the species-specific assays against NTS can help ensure that each eDNA assay is sensitive only towards eDNA from the intended targeted NIS.

In this study, we wanted to compare eDNA monitoring with conventional surveillance methods of NIS from major Danish harbours to investigate the potential for using species-specific eDNA analysis to target a wide range of diverse taxonomic species. We focused on harbours, as larger vessels with ballast tank water occasionally visit these harbours, which are considered one of the more common sources for introduction of marine NIS. Since there is a need for better monitoring of NIS, and a demand for being able to make early detections of NIS to limit the impact they may have, this study had three aims: (1) To evaluate which NIS would be relevant for monitoring by eDNA and for which it would be possible to develop species-specific eDNA assays, (2) to develop species-specific eDNA assays for a selected group of NIS known from north European marine waters that potentially could pose a threat on the ecosystems, and (3) to apply the new eDNA assays on water samples, to map the eDNA levels inferred and compare them with conventional monitoring, and across different seasons and gradients in salinity to see if these factors influence the prevalence of NIS. Additionally, we wanted to investigate if eDNA monitoring can help pinpoint focal harbours where NIS are present in higher numbers. The goal is that these species-specific assays easily can be implemented in future monitoring programmes to ensure cheap and accurate information of the distribution and spread of marine NIS in the northeastern Atlantic Ocean, but also be useful for monitoring of these NIS outside Europe in both North America and in the western Pacific Ocean.

Table 1
Nonindigenous species monitored in the present study, with the native distribution and their non-indigenous distribution listed. The references listed have more detailed information on the global spread and dispersal of these species. (A) Associated with relative low salinities.

Species name	Native distribution	Nonindigenous distribution	Reference
Bonnemaisonia hamifera	Northwestern Pacific Ocean	European coastal areas, North Sea, Skagerrak, Baltic seas	Guiry, 2001; Guiry, 2021; Hariot, 1891; Breeman et al., 1988; Harder and Koch, 1949
Carassius auratus (A)	East Asia	Introduced to freshwater systems in Japan and Europe, and Denmark	Carl, 2012c; Lusková et al., 2010
Colpomenia peregrina	Eastern Pacific Ocean	Western coast off Europe, North Sea, Skagerrak, Ireland	Abbott and Hollenberg, 1976; Guiry, 2001; Min Lee et al., 2014; Nielsen et al., 1995
Cordylophora caspia	Black Sea-Caspian Sea	Baltic Sea, North American Atlantic and Pacific coast, central America, Hawaii, New Zealand, southeastern Australia, Europe, Mediterranean	Occhipinti-Ambrogi et al., 2010; van der Land, 2001; Seyer et al., 2017, Cairns et al., 2009
Magallanas gigas	Northwestern Pacific Ocean Japan, Taiwan	Mediterranean, France, British Columbia, Portugal, Holland, Germany, Danish Wadden Sea, South Africa, New Zealand	Robinson et al., 2005; Smaal et al., 2009; Melo et al., 2010
Cyprinus carpio (A)	Eurasia, eastern central Europe	Scandinavia, western Europe southwestern Europe, North America	Carl, 2012b; Zhou et al., 2004; Nedoluzhko et al., 2020
Eriocheir sinensis	Pacific coast of China and Korea	Southern England and northeastern coast of Germany, Holland and Denmark, California, east coast of North America	Benson and Fuller, 2021; Herborg et al., 2005
Karenia mikimotoi	Northwestern Pacific Ocean	East coast of the United States of America and the west coast of Europe, New Zealand	Guiry, 2018; Li et al., 2019
Mnemiopsis leidyi	Northwestern Atlantic Ocean, northeast American coast	Denmark, Germany, Holland, France, English Channel, Mediterranean	Créach et al., 2021; Jaspers et al., 2018; Riisgård, 2017;
Mya arenaria (A)	Northeast American coast	Europe, Scandinavia, Ireland, Portugal, Baltic Sea, west coast off North America	Gofas et al., 2001; Powers et al., 2006; Strasser, 1999; Strasser and Barber, 2009
Neogobius melanostomus (A)	Caspian Sea, Black Sea	Baltic Sea, Netherlands, Freshwater areas in eastern Russia, central Europe, North America	Azour et al., 2015; Behrens et al., 2017; Phillips et al., 2003
Oncorhynchus mykiss (A)	West coast of North America, Alaska	Freshwater areas in North America, Europe, New Zealand, Australia	Crawford, 2001; Page and Burr, 1991; Carl and Møller, 2012
Oncorhynchus gorbuscha (A)	West coast of North America, Alaska	Northern Europe, White Sea, Barents Sea Russia, Norway, Britain	Armstrong et al., 2017; Carl and Møller, 2012; Gargan et al., 2021;
Prorocentrum minimum (A)	Caspian Sea	European seas, Japan, France, Netherlands, Norway, east coast of the North America.	Brandt, 2001; Heil et al., 2005; Velikova and Larsen, 1999
Pseudochattonella farcimen	Japan	North Sea, east coast of North America, New Zealand, Australia	Bowers et al., 2006; Edvardsen et al., 2007; Eikrem et al., 2009
Pseudochattonella serruculata	Japan	North Sea, east coast of North America, New Zealand, Australia	Bowers et al., 2006; Edvardsen et al., 2007; Eikrem et al., 2009
Rhithropanopeus harrisii (A)	East coast of North America	Baltic Sea, Scandinavia, California, Russia, Romania, Britain, Caspian Sea, Black Sea, Italy	Forsström and Vasemägi, 2016; Perry, 2021; Turoboyski, 1973
Acipenser baerii, Acipenser gueldenstaedtii, Acipenser ruthenus	Russia, Eurasia	Scandinavia, western Europe, South America	Demonte et al., 2017; Ludwig et al., 2009; Møller, 2012

2. Methods

2.1. Target species list

A list of invasive marine species was prepared from previous registrations of nonindigenous and/or invasive species in the Danish marine areas (Stæhr and Thomsen, 2012; Stæhr et al., 2016); the Oslo and Paris Conventions (OSPAR) and the Baltic Marine Environment Protection Commission (i.e. the Helsinki Commission, HELCOM). This list covered more than 150 species from various phyla and kingdoms (App. A. Table S1.01). Based on five selection criteria, we selected 12 target species for development of eDNA assays, and six species for which assays already were developed. The selection criteria were based on: (1) whether the species might colonise conservational valuable habitats, (2) their impact on native species, (3) their effect on ecosystem function, (4) economic as well as public health effects (Madsen et al., 2014), and (5) reference sequence data availability in the GenBank database provided by National Center for Biotechnology Information (NCBI). We prioritised the last selection criteria and started out by examining the sequence availability on NCBI for both the targeted species as well as for a number of closely related, non-target species known from European waters. The last criterion was critical for determining whether it would be possible to distinguish the different species in a qPCR setup.

Marine NIS in northern Europe comprise a wide range of taxonomic groups spanning Ctenophora, Decapoda, Hydrozoa, Rhodophyta and Teleostei. Among some of the widespread NIS is the warty comb jelly, which is native to the east coast of North America but has been introduced to western Eurasia (Bayha et al., 2015; Bologna et al., 2017; Jaspers et al., 2018). The round goby originating from the Black Sea is now widespread in northern Europe, the Baltic Sea and the great lakes in North America, and may be limited in dispersal by salinity (Azour et al., 2015; Nurkse et al., 2018). The ichthyotoxic microalgae *Pseudochattonella* spp. are originally known from Japan but have in northwestern Europe been linked with massive fish deaths, and algal blooms have severe consequences for the fish industries in these seas (Dittami et al., 2013; Riisberg and Edvardsen, 2008). The warty comb jelly is recorded from the Baltic Sea where it blooms in the summer (Riisgård, 2017). The North American mud crab has spread across the Baltic Sea, and been recorded in western and southern Europe, with a distribution that has been keyed with brackish waters (Roche and Torchin, 2007; Forsström and Vasemägi, 2016; Nurkse et al., 2018). The freshwater hydroid (*Cordylophora caspia*) stems from the Black Sea and inhabits brackish waters and have spread to the Baltic Sea, Australia and middle America (van der Land, 2001). The Pacific red algae (*Bonnemaisonia hamifera*) has spread across European seas (Guiry, 2001), but is native to the northwestern Pacific Ocean (Hariot, 1891).

2.2. Design of species-specific primers and probes

Using the software Geneious vR7.1.7 (Kearse et al., 2012) DNA sequences were aligned with MAFFT v6.822 (Katoh and Toh, 2010) while primers and probes were designed with Primer3 v0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012). Alignment and assay design was dependent on availability of sequences from NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/) and possibilities for Sanger sequencing (App. A. Table S01.2). Six assays were adopted from previously published studies (Table 2, App A. Table S01.3). Genomic DNA was extracted from tissue

samples (App. A setup 04.01), preferably from vouchered museum specimens (Table S01.02), and an initial polymerase chain reaction (PCR) (App A setup S04.02) served to obtain products for bidirectional *de novo* Sanger sequencing performed by Macrogen Sequencing services (Amsterdam). Chromatograms were manually inspected and assembled in Geneious vR7.1.7 and deposited on the NCBI GenBank database (accession numbers: OM320999-OM321004, OM368632, OM368662 in App. A. Table S01.2). Alignments comprised different mitochondrial (mtDNA) genes and different nuclear markers dependent on the marine NIS targeted (Table 2): mitochondrial cytochrome *b* (*cytb*), cytochrome oxidase 1 (*co1*), and nDNA regions of the internal transcribed spacer region 2 (*its2*), the ribosomal subunit *28s* and the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*). We aimed to include as many NTS representatives in our comparison of sequences, as missing NTS can make design of primers and probe unspecific towards NTS. Specificity of the 3′-end of the oligos can help ensure better specificity. If more than three base pair mismatches can be identified in the 3′-end of both primers, then the risk of obtaining false positive detection from co-occurring NTS is very low, as mismatches in 3′-end of primer influences primer specificity (Whiley and Sloots, 2005; Wilcox et al., 2013).

Multiple sequences from both the targeted NIS and other co-occurring species known from the northeastern Atlantic Ocean were downloaded from the NCBI GenBank database (App. A. Table S01.2). For the different target-species, potential co-occurring non-target species (NTS) were identified for Dinoflagellata, Heterokontophyta, Ochrophyta and Rhodophyta based on literature (Dittami et al., 2013; Eckford-Soper and Daugbjerg, 2016; Guiry, 2001, 2021; Scorzetti et al., 2009); for Arthropoda based on species lists for European known species (Türkay, 2001; WoRMS, 2020a, 2020b, 2020c); for Ctenophora and Hydrozoa based on species lists of invertebrates (van der Land, 2001); and for Mollusca based on literature listing known European species (Bouchet et al., 2017; Coan and Valentich-Scott, 2012). Sequences around 300–1200 bp from the target species were used in Primer3 v.0.4.0 to obtain primers and probes, which were tested with Primer-BLAST (Ye et al., 2012) to check their specificity towards the intended NIS. For six species (goldfish, European carp, the dinoflagellates *Karenia mikimotoi* and *Prorocentrum cordatum*, and the heterokont flagellates *Pseudochattonella farcimen* and *Pseudochattonella verruculosa*) we used already published assays (Nathan et al., 2014a, 2014b; Eckford-Soper and Daugbjerg, 2016; Smith et al., 2014; Takahara et al., 2012; Yuan et al., 2012). For the dinoflagellate *P. cordatum* we modified a previously published assay (Scorzetti et al., 2009) (Table 2). We did not use the assay developed for round goby (Adrian-Kalchhauser and Burkhardt-Holm, 2016), as it did not work consistently on DNA extracted from round goby.

2.3. Test and optimization of species-specific primers and probes

Test of specificity by PCR and qPCR followed previous published protocols (Agersnap et al., 2017; Knudsen et al., 2019). First, a PCR was prepared with the designed primers (App. A setup 04.03) and DNA extracted from tissue samples from both target species and other co-occurring NTS, and PCR results were checked on a 2% agarose gel. Primer pairs found to be specific were then tested again using qPCR with the specific probe added (App. A setup 04.04). The assay only amplifying DNA from the target species, returning the highest relative fluorescence and earliest amplification as found by the cycle of quantification threshold (Cq) (App. A setup 04.04) was selected for further analysis. For every specific assay, a second qPCR was performed to determine the optimal primer concentrations (App. A setup 04.05). The primer concentrations returning the lowest Cq in the second qPCR (App. A setup 04.05) were then used in a third qPCR setup (App. A setup 04.06) where the optimal concentration of the probe was tested. All assays used included a standard dilution series based on dsPCR amplicons, which were generated in a PCR using the DNA extracted from the target species as template. This PCR was performed with a polymerase with 3′-*exo*-nuclease activity (App. A setup 04.07). The resulting amplicon (App. A setup 04.07) was then purified using a QIAquick PCR Purification Kit (Qiagen, catalogue number 28104) and the concentration measured with a Qubit flourometer. The nucleotide sequence of the targeted fragment (Table 2) was then used to calculate the molecular weight using OligoCalc (Kibbe, 2007). The purified dsPCR fragment was then diluted to 10^7 copies per μL and stored at −20 °C until they were to be used in a final qPCR including the extractions from the water samples (App. A setup 04.08).

2.4. Filtration of water samples, storage and extraction from filters

Two water samples per harbour were collected from 16 harbours in Denmark during June and July 2017 (hereafter referred to as: 'summer samples'), and two more per harbour again between September to November 2017 (hereafter referred to as: 'autumn samples') (Table 3, Fig. 1). The water samples were after collection immediately placed in a disposable sterile plastic bag. Subsequently, samples were filtered through a Sterivex™-GP filter unit with a pore size of 0.22 μm, using either HSW Soft-Ject R 60 mL syringes or a pressure assisted filtration canister in the 'eDNA sampling kit' from NIRAS (www.niras.dk) that provides a pressure between 100 kPa and 300 kPa. Residual water was forced out, and samples were stored on dry ice within 1 hour of sample collection. Filters were transferred to a freezer (< −15 °C) within 24 hours. Extraction from filters were done using Qiagene Blood and Tissue DNeasy extraction kits and then followed previous published protocols on retrieval of eDNA from Sterivex™-GP filters (Sigsgaard et al., 2016, 2017; Spens et al., 2017). An extraction blank performed on only ddH_2O was included for each set of filters extracted per season. Extracted filters, and blanks, were analysed individually in subsequent qPCR analysis.

2.5. Detection of eDNA from nonindigenous species

For each of the 18 marine NIS (Table 2), two qPCR analyses (one for summer and one for autumn) were prepared (App. A setup S04.08) using the determined optimal concentrations of primers and probes (Table 2). All qPCR analysis, including standards, non-target controls (NTC) and samples, was performed in triplicates, using an MxPro 3005 qPCR machine (Agilent) at Eurofins Miljø A/S in Vejen, Denmark. The standard dilution series were based on a purified amplicon obtained in a PCR setup (App. A setup S04.07). Details on preparation of the standard dilution series are described in supporting information (App. A setup S04.07). For each species-specific qPCR, the corresponding primers and probes were added in constant volumes (App. A setup S04.08) but in different concentrations to ensure the final concentrations per reaction matched the optimal concentrations (App. A setup S04.05 and S04.06) (Table 2).

2.6. Conventional monitoring of nonindigenous species

In all 16 harbours conventional monitoring was performed using snorkelling and fishing gear. In Aarhus and Esbjerg additional conventional sampling techniques were applied. These extra sampling techniques focused on collecting phytoplankton above the pycnocline with a 10 μm mesh size net, and collecting zooplankton with a 200 μm and 500 μm mesh net, and subsequent identification performed with light microscopy. Mobile epifauna (i.e. decapods, echinoderms, molluscs and fish) were caught in a standard trap 60 cm by 40 cm by 20 cm with 2.5 cm mesh size being deployed for two days. Benthic infauna was collected with a Van Veen grab from the top 10 cm layer and a Kajak corer with a 5.5 diameter. Sediment infauna was collected with a 40 cm wide and 20 cm high handheld dredge dragged 50 cm along the bottom and the collected material sieved on 0.5 mm screens. Fouling organisms were scraped from 14 cm by 14 cm settlement plates fastened at 1 m, 3 m, 5 m and 7 m of depth collected after three months of deployment. For all harbours, including Aarhus and Esbjerg harbour, conventional fishing was carried out from August to September by setting three fyke nets and three gill nets with mesh size varying from 11 mm to 90 mm. Snorkelling was completed within 1 hour and carried out along a 500 m transect line at 1–5 m depth. Past conventional recordings of the NIS were included based on literature (Azour et al.,

Table 2
Primer and probe qPCR detection systems for 18 marine invasive species in the Northeastern Atlantic. Optimal concentrations for primers and probes are given as final concentrations in the individual qPCR reaction. Probes are 5′-end modified with a FAM-fluorescent dye, and equipped with a black hole quencher-1 (BHQ1)-modification at the 3′-end. The primers and probes used to detect eDNA from *Cyprinus carpio* was adopted from the study by Takahara et al. (2012). The assay for *Prorocentrum minimum* was modified from the study by Scorzetti et al. (2009). Two primer assays targeting *Pseudochatonella* were adopted from the literature (Eckford-Soper and Daugbjerg, 2016). For *Karenia mikimotoi* an assay was adopted from literature (Smith et al., 2014; Yuan et al., 2012). The assay for *Carassius auratus* was adopted from the study by Nathan et al. (2014a, 2014b).

Phyla, Class	Target species	Primer (F and R) and probe name (P)	Sequence, primer and probe 5′ → 3′ direction, with FAM and BHQ1 modifications	Optimal primer-/probe concentration (nM) per individual qPCR reaction	Molecular weight of dsDNA [Da]	Target fragment length (bp)
Rhodophyta, Florideophyceae	*Bonnemaisonia hamifera*	Bonham_rbcL_F02	CAATTACTAGATTACCTGGGCAAT	1200	96,873	157
		Bonham_rbcL_R02	CTTCTTTTACAAAGTCCCGACCT	200		
		Bonham_rbcL_P01	FAM-TCGTGCCATAACCATAGACTCTAAAG CC-BHQ1	300		
Myzozoa, Dinophyceae	*Prorocentrum minimum*	Promin_28S_F03	CTTGGCAAGATTGTCGGGT	1200	73,410	119
		Promin_28S_R03	TATTCACTCACCCATAGACGA	1200		
		Promin_28S_P03	FAM-ACACACAAGGCAAGAGACGATCAA GC-BHQ1	300		
Ochrophyta, Dictyochophyceae	*Pseudochattonella farcimen*	PsFa28SF	GGGAGAAATTCTTTGGAACAAGG	200	59,819	97
		PsFa28SR	GCAACTCGACTCCACTAGG	800		
		PsVeFa28SP1	FAM-TCAGAGAGGGTGACAATCCCGTCT-BHQ1	300		
Ochrophyta, Dictyochophyceae	*Pseudochattonella verruculosa*	PsVe28SF	GGGAGAAGTCCTTTGGAACAAGG	200	59,820	97
		PsVe28SR	GCAACTCGACTCCATTAGC	600		
		PsVeFa28SP1	FAM-TCAGAGAGGGTGACAATCCCGTCT-BHQ1	300		
Myzozoa, Dinophyceae	*Karenia mikimotoi*	KarmikF3	CCGAGTGACTGAATGTCCTC	200	54,251	88
		KarmikR3	GATCGCAGGCAAGCACATGA	200		
		KarmikP3	FAM-GCAGTGCTACCAGACACACAGAG-BHQ1	300		
Chordata, Actinopterygii	*Carassius auratus*	Caraur_COI_F01	TTCTTCCCCCATCATTCCTGT	200	47,456	77
		Caraur_COI_R01	GTATACTGTCCATCCGGAGG	600		
		Caraur_COI_P02	FAM-TAGCTTCCTCTGGTGTTGAAGCCG GAG-BHQ1	100		
Chordata, Actinopterygii	*Cyprinus carpio*	CpCyB_496_F	GGTGGGTTCTCAGTAGACAATGC	200	48,069	78
		CpCyB_550_P	GGCGGCAATAACAAATGGTAGT	400		
		CpCyB_573_R	FAM-CACTAACACGATTCTTCGCATTCCAC TTCC-BHQ1	200		
Ochrophyta, Phaeophyceae	*Colpomenia peregrina*	Colper_COX_3_F01	GCAAGCTTTTGAATATGCTAATG	400	75,242	122
		Colper_COX_3_R01	CAGCTAAAAATATTGTACCGATT	600		
		Colper_COX_3_P01	FAM-TTCAGTTTTTTACATGGCTACAGGCT TC-BHQ1	100		
Chordata, Actinopterygii	*Neogobius melanostomus*	Neomel_COI_F01	CTTCTRGCCTCCTCTGGWGTTG	200	90,719	147
		Neomel_COI_R01	CCCWAGAATTGASGARATKCCGG	600		
		Neomel_COI_P01	FAM-CAGGCAACTTRGCACATGCAG-BHQ1	100		
Chordata, Actinopterygii	*Oncorhynchus mykiss*	Oncmyk_Co1_F01	ACCTCCAGCCATCTCTCAGT	400	55,489	90
		Oncmyk_Co1_R01	AGGACGGGGAGGGAAAGTAA	600		
		Oncmyk_Co1_P01	FAM-TGAGCCGTGCTAGTTACTGCTGTC CTT-BHQ1	100		
Chordata, Actinopterygii	*Oncorhynchus gorbuscha*	Oncgor_CO1_F09	TCCTTCCTCCTCCTCTTTC	400	100,599	163
		Oncgor_CO1_R06	TGGCCCCTAAAATTGATGAG	1000		
		Oncgor_CO1_P06	FAM-CAGGGGCATCCGTCGACTTAA CTAT-BHQ1	300		
Mollusca, Bivalvia	*Magallanas gigas*	Cragig_CO1_F07	TTGAGTTTTGCCAGGGTCTC	200	95,018	154
		Cragig_CO1_R09	ACCAGCAAGGTGAAGGCTTA	1200		
		Cragig_CO1_P06	FAM-AACATTGTAGAAAACGGAGTTGGG GC-BHQ1	200		
Mollusca, Bivalvia	*Mya arenaria*	Myaare_CO1_F01	CCCTCCGTTGTCGAGAAATA	200	82,044	133
		Myaare_CO1_R02	ACGCATGTTACCCCAAGTTC	1200		
		Myaare_CO1_P06	FAM-TATCCCTTCATATTGGAGGGGCTT CAT-BHQ1	200		
Arthropoda, Malacostraca	*Rhithropanopeus harrisii*	Rhihar_co1_F03	GTCAACCTGGTACTCTCATTGGT	200	101,188	164
		Rhihar_co1_R03	ACGAGGAAATGCTATATCAGGGG	1200		
		Rhihar_co1_P03	FAM-TGTTGTAGTAACAGCTCACGCCTT TGT-BHQ1	150		
Arthropoda, Malacostraca	*Eriocheir sinensis*	Erisin_cytb_F02	ACCCCTCCTCATATCCAACCA	200	70,305	114
		Erisin_cytb_R02	AAGAATGGCCACTGAAGCGG	1200		
		Erisin_cytb_P02	FAM-TTTGCTTACGCTATTTTACGATCAAT TCCT-BHQ1	200		
Cnidaria, Hydrozoa	*Cordylophora caspia*	Corcas_COI_F01	TCATCTGTACAAGCACATTCTGG	200	46,831	76
		Corcas_COI_R01	TTGAAGAAGCTCCTGCACAGT	200		
		Corcas_COI_P01	FAM-CCTTCTGTAGACATGGCTATATTTAG TC-BHQ1	100		
Ctenophora, Tentaculata	*Mnemiopsis leidyi*	Mnelei_its2_F04	ACGGTCCCTTGAAGTAGAGC	400	77,127	125
		Mnelei_its2_R06	TCTGAGAAGGCTTCGGACAT	1000		
		Mnelei_its2_P06	FAM-GTGCCTCTCGGTGTGGTAGCAATA TCT-BHQ1	300		
Chordata, Actinopterygii	*Acipenser baerii*	Acibae_CR_F02	CAGTTGTATCCCCATAATCAGCC	800	122,207	198
		Acibae_CR_R03	TTATTCATTATCTCTGAGCAGTCGTGA	1200		
		Acibae_CR_P01	FAM-ATGCCGAGAACCCCATCAACATTT GGT-BHQ1	250		

2015; Carl, 2012b, 2012c; Forsström and Vasemägi, 2016; Guiry, 2001; Guiry, 2021; Heil et al., 2005; Herborg et al., 2005; Nielsen et al., 1995; Rasmussen, 2012; Riisgård, 2017; Strasser, 1999; van der Land, 2001). The results from the conventional monitoring were used to assign a status to each species as one of three possible categories: Not recorded in the past nor during the present study (NR), recorded in previous surveys but not during this study (PR), or recorded in the present study by conventional monitoring (SR). Confer with Andersen et al. (2017) for more information on the conventional sampling. Conventional monitoring can be difficult in cold waters, and requires more field work than monitoring of eDNA with collection of filters. We only performed one conventional monitoring event per harbour over the year. This is opposed to the sampling of filters that were carried out twice (i.e. in June–July and September–November) (Table 3). Preferably the conventional monitoring should be carried out at different seasons, like the sampling of filters for eDNA monitoring, to allow for evaluation of the effect of difference in seasons, but that was not possible in this study. For salinity levels for the sampled harbours we used rough estimates adopted from previous literature (Maar et al., 2011; Momigliano et al., 2018). The decision to try and compare eDNA levels with variation in salinity at sampling sites was made after all samples had been collected, and we were forced to adopt generalized salinity levels from the different harbours. On the other hand generalized salinity levels might be a better parameter for comparing eDNA levels, than stochastic sampled salinity levels can provide.

2.7. Analysis of levels of eDNA

The eDNA levels in the filtered water samples were estimated using a standard dilution series. The data was analysed in R v4.0.2 (R Core Team, 2020) (App. A. S04.09). The eDNA levels in the triplicate qPCR wells were scored in five categories from their relation to the limit of detection (LOD) and limit of quantification (LOQ) (Klymus et al., 2020). The categories were: (1) no amplification at any Cq threshold (NC), (2) below LOD (bL), (3) above LOD but below LOQ (aLbL), (4) minimum one replicate out of three above the LOQ (1aL) and (5) all three replicates above the LOQ (3aL). The five categories were colour coded white, yellow, orange, red and black, respectively, with an increase in colour being equal to an increase in eDNA detected. The added template for each qPCR result above LOQ was evaluated as a fraction of the volume of filtered seawater per filter, allowing for an estimation of the molecular copy number per L of water for each sample site for eDNA detections. An interpolation of $\log_{10}$ transformed eDNA levels, for data with at least one replicate above LOQ, was inferred between sampling locations with the 'ipdw' package (Stachelek, 2018), that makes use of inverse path distance weighting between sampled locations and using the coastline as barrier, preventing the interpolation going across land areas. The interpolation was set to have a mean neighbouring and resolution adjusted to allow for predicted areas around points to overlap. All samples and eDNA levels were compared in multidimensional scaling (MDS) and linear discriminant analysis (LDA) plots categorised for summer and autumn sampling and categorised with generalized salinity levels. For the MDS and LDA comparing eDNA for species with salinity, we excluded species with broad tolerance in salinity – i.e. Pacific oyster, warty comb jelly, sand gaper and oyster thief.

3. Results

3.1. Specificity of the primers and probes designed

Ten of the 12 primer-probe assays designed in this study were found to be specific and only returned amplification on DNA from the intended

Table 3
Filtered water samples collected during 2017 with volume of filtered water and approximate latitude longitude position for sampling. Harbours are assigned three letter abbreviation codes.

Harbour	Harbour abbrev.	Catch position	Spring-2017			Autumn-2017		
			Collection date Samples 1 and 2	Volume water filtered (mL) Sample 1	Volume water filtered (mL) Sample 2	Collection date Samples 1 and 2	Volume water filtered (mL) Sample 1	Volume water filtered (mL) Sample 2
Aalborg	ALH	57°03′N; 10°03′E	Jun.29	1000	1000	Oct.11	1000	1000
Aalborg Portland	ALP	57°03′N; 09°53′E	Jun.30	1000	1000	Oct.11	2500	2500
Aarhus	ARH	56°08′N; 10°19′E	Jul.05	2400	3100	Sep.19	1000	1000
Esbjerg	ESB	55°24′N; 08°10′E	Jun.27	1000	1000	Oct.17	200	200
Fredericia	FRC	55°33′N; 09°47′E	Jul.05	1600	2000	Oct.11	1000	1000
Frederikshavn	FRH	57°25′N; 10°34′E	Jun.28	1000	1000	Oct.06	3000	450
Gedser	GED	54°32′N; 11°56′E	Jul.04	700	700	Sep.23	1000	1000
Grenaa	GRE	56°24′N; 10°57′E	Jul.05	1100	1500	Sep.19	1000	1000
Helsingoer	HEL	56°03′N; 12°38′E	Jul.11	1000	1000	Sep.13	1000	1000
Hirtshals	HIR	57°36′N; 09°56′E	Jun.28	1000	1000	Nov.08	600	600
Kalundborg	KAB	55°41′N; 11°01′E	Jul.03	1500	1500	Sep.22	1000	1000
Kalundborg Statiol	KSH	55°40′N; 11°03′E	Jul.02	1500	1500	Sep.22	1000	1000
Koebenhavn	KBH	55°41′N; 12°42′E	Jun.09	1800	1800	Sep.12	1650	1000
Koege	KGE	55°27′N; 12°13′E	Jul.04	850	850	Sep.12	450	600
Odense	ODE	55°27′N; 10°27′E	Jul.19	800	800	Sep.15	800	800
Roedby	ROD	54°38′N; 11°20′E	Jul.04	500	500	Sep.23	200	200

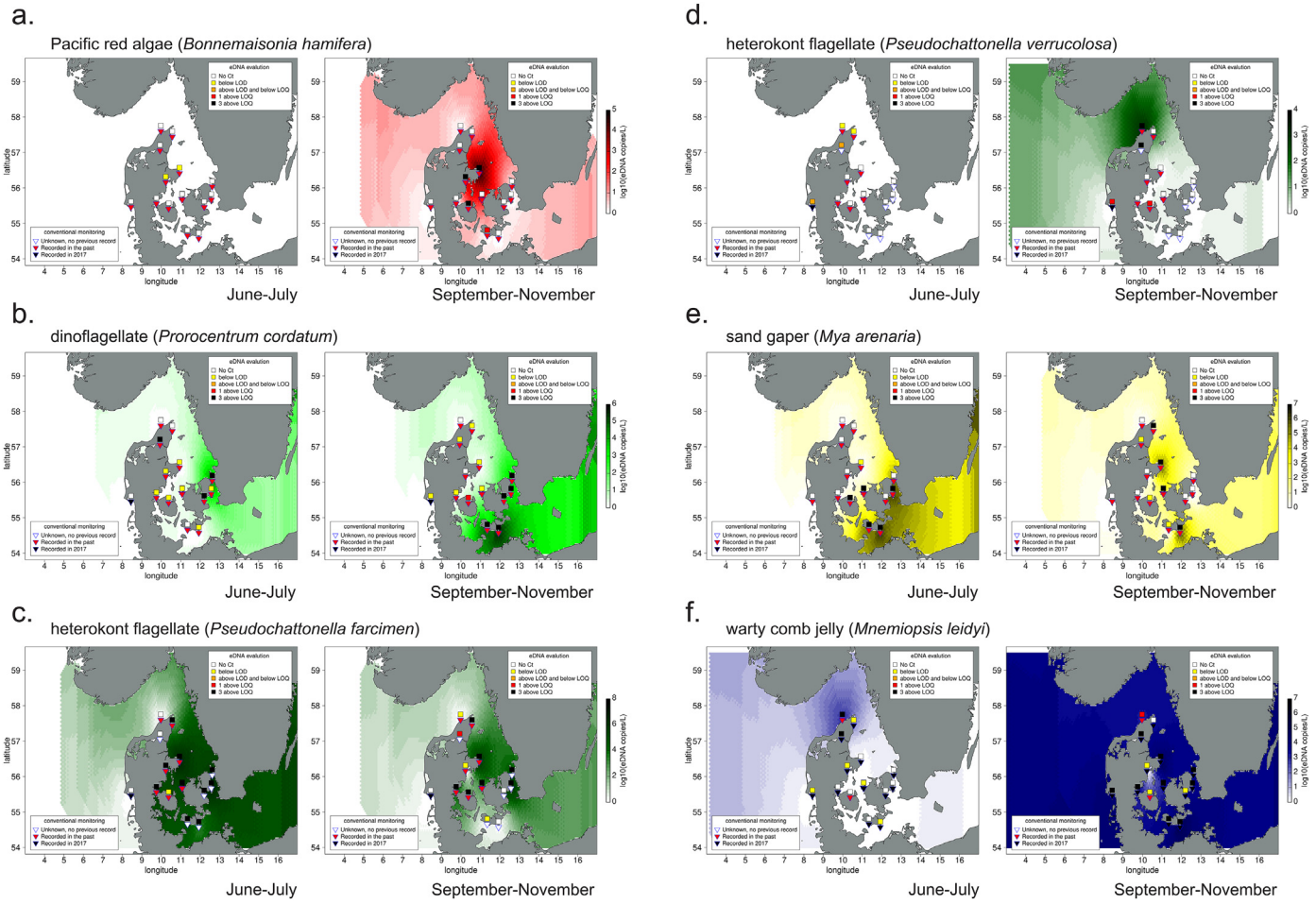

Fig. 1. Geographic representation of levels of eDNA recorded on a logarithmic scale with interpolation between sampled locations for June–July and September–November 2017 in the Inner Danish seas. The coastline was defined to act as barrier for the interpolation by inverse path distance weighting, and does not reflect distribution, dispersal or abundance because of the limited number of samples. Instead these representations are useful for visualization of the change across the two sampling periods. Higher eDNA levels across the entire sampling season is coloured with a darker colour. The squares indicate harbours sampled for eDNA. The squares are coloured to match the categories of eDNA in Table 4. The coloured triangles reflect the conventional monitoring results. The eDNA detected stems from (a) the Pacific red algae *Bonnemaisonia hamifera* (red), (b) the dinoflagellate *Prorocentrum cordatum* (green), (c) the heterokont flagellate *Pseudochattonella farcimen* (dark green), (d) the heterokont flagellate *Pseudochattonella verruculosa* (dark green), (e) the sand gaper *Mya arenaria* (yellow), and (f) the warty comb jelly *Mnemiopsis leidyi* (blue).

targeted species. The assay developed for sturgeons (Table 2) was unable to distinguish between the Siberian sturgeon (*Acipenser baerii*), diamond sturgeon (*Acipenser gueldenstaedtii*) and sterlet (*Acipenser ruthenus*), however, all three species are considered NIS in northern Europe. The assays developed for the heterokont flagellates *Pseudochattonella farcimen* and *Pseudochattonella verruculosa* targets the same gene region and use the same forward and reverse primers, but distinguishes the species using different internal probes (Table 2).

3.2. Estimated eDNA copies in water samples

Five out of the 18 species monitored by eDNA were not detected above LOD at any location across both seasons sampled (sturgeons, goldfish, freshwater hydroid, Chinese mitten crab, and pink salmon) (Table 4, App A. Figs. S21b, S24a, S26a, S26b, S27b). The inclusion of triplicates of each standard dilution series step and three NTCs per qPCR plate allowed discrimination between false and positive detection by monitoring NTCs.

For seven species (the Pacific red alga *B. hamifera*, oyster thief, the dinoflagellate *K. mikimotoi*, warty comb jelly, sand gaper, the dinoflagellate *P. cordatum* and the two heterokont flagellates *P. farcimen* and *P. verruculosa*) the levels of estimated eDNA copies per L of filtered water were higher than the estimated LOQ for at least one of the sampled localities across the two sampling periods. In these cases, the results could be analysed quantitatively, and plotted on maps to allow for interpolation between sampling sites (Fig. 1). The $\log_{10}$ levels of eDNA on maps shows a seasonal difference, with generally low levels of eDNA in the harbours from the targeted NIS in June–July, compared with the higher levels of eDNA recorded for August–November (Figs. 1–2). Five species (North American mud crab, Pacific oyster, rainbow trout, round goby and European carp) were detected by eDNA below LOQ (App. A. Figs. 24b, S25b, S23a–b, S22a). Two species (the dinoflagellate *K. mikimotoi* and oyster thief) were detected with eDNA above LOQ for less than three sampling locations per season (App. A. Figs. S21a, S22b). For six species the eDNA levels were above LOQ for three sampling locations per season (Fig. 1) allowing for a presumptuous interpolation between harbours.

If the organism was detected in past surveys with conventional monitoring, the eDNA monitoring could in most cases detect eDNA, although often below LOD (Table 4). We could not confirm the presence of European carp, dinoflagellate *K. mikimotoi*, warty comb jelly, round goby, the dinoflagellate *Prorocentrum cordatum*, the heterokont flagellate *P. farcimen* from eDNA in harbours where these species previously have been recorded by conventional monitoring (Fig. 1, Tables 5–6).

Comparison of the $\log_{10}$ transformed levels of eDNA copies per L of filtered water between the different species in the generalized linear model

(GLM) with one or two extra descriptors did not show any correlation between the eDNA levels for the different species (App. A. Figs. S29–S37).

3.3. Conventional monitoring

Eight NIS targeted in this study (the Pacific red alga *Bonnemaisonia hamifera*, Pacific oyster *Magallanas gigas*, the dinoflagellate *Karenia mikimotoi*, warty comb jelly, sand gaper *Mya arenaria*, round goby, the dinoflagellate *Prorocentrum cordatum* and the heterokont flagellate *P. verruculosa*) were found in the various harbours around the coast off Denmark using traps and gill nets. Sturgeons, goldfish, the freshwater hydroid (*C. caspia*), Chinese mitten crab, and pink salmon were not recorded in any harbours. During the conventional monitoring carried out in 2017, a maximum of eight NIS was recorded in Kalundborg Harbour. Inclusion of past recordings raises this to 11 NIS in Kalundborg Harbour (Table 6). Round goby and North American mud crab were recorded in the harbours in the south-eastern part of the Danish waters, in agreement with past records. Warty comb jelly was recorded in samples from all Danish ports, except for two harbours where the warty comb jelly is known from past records (Table 5). The Pacific red algae *B. hamifera*, sand gaper and the dinoflagellate *P. cordatum* were not recorded but have been recorded in the past in almost all the 16 harbours monitored in this study. The dinoflagellate *K. mikimotoi* has previously been recorded from Danish harbours but was only found in Esbjerg. Pacific oyster was found in the northern part of the Limfjord and in the southern North Sea west off Esbjerg, and in the central eastern part of the inner Danish seas. The heterokont flagellates *P. farcimen* and *P. verruculosa* were recorded in Esbjerg harbour and have previously been recorded from various harbours in the Danish seas (Table 5).

3.4. Comparison between conventional and eDNA monitoring approaches

Comparisons between the conventional and the eDNA monitoring were in agreement for many of the harbours sampled (Table 6). The two separate sampling events showed minor differences in respect to the regions of Danish waters where the highest number of species was detected by eDNA (Fig. 3). The highest number of species was found in the same region, near Kalundborg harbour, when conventional monitoring and eDNA based monitoring was compared (Fig. 4). For individual species there was seasonal differences in the levels of eDNA in the filtered water (Figs. 1a–d, 2a, e–f). When all the eDNA monitored species were evaluated across both summer and autumn samples (Fig. 2) fewer species were detected in the summer samples (Fig. 2a) when compared with the autumn samples (Fig. 2b), and for both summer and autumn, samples in the southern part of the Kattegat had the highest number of NIS. Interpolation between the number of NIS per harbour detected by eDNA and by conventional monitoring both indicate that the central parts of the Inner Danish waters have the highest number of nonindigenous species when the maximum number of species detected across the year is compared (Fig. 3). Comparing the number of species detected per harbour per season and plotting the squared residuals (App. A. Fig. S28) indicate that the two approaches (eDNA and conventional monitoring) had a significant (p-value of 0.04 on F-statistics and 30 degrees of freedom), although poorly explained, correlation (R^2 of 0.106) (Fig. 4).

A multidimensional scaling (MDS) plot of all eDNA levels recorded for the different species in the different harbours across the two sampling seasons grouped the technical qPCR replicates together for harbours, providing a strong indication of reproducibility across samples analysed by qPCR (Fig. 5a). However, it did not indicate that the grouping could be associated with the time period of sampling (Fig. 5b) or with salinity (Fig. 5c). The qPCR triplicates for each harbour per season sampled had identical eDNA diversity levels (Fig. 5), but the eDNA diversity profiles in each of the samples did not reflect the season sampled (Fig. 5a and c) nor did they group as a reflection of the different salinity levels (Fig. 5b and d). The close grouping of qPCR triplicates for each sampling location (Fig. 5) indicated that the technical qPCR replicates had very similar eDNA levels and diversity, which was expected as they ideally should return similar amplification for the same species. Triplicate sets varied across the two sampling seasons for the same locality sampled (Fig. 5).

4. Discussion

The 18 assays tested and used here for monitoring NIS in the North East Atlantic hold the potential for being applicable for monitoring of eDNA from these species worldwide. The NIS targeted here are considered a threat to ecosystems and native biodiversity in coastal areas in Europe, North and South America, as well as in Australia and New Zealand and northwestern Pacific seas. Once the assays were applied on water samples, the eDNA monitoring turned out to be capable of supporting the conventional monitoring (Figs. 2–3, Table 6). Comparison of the eDNA method

Table 4
Highest levels of environmental DNA (eDNA) recorded across the two sampling events in 2017 for the 18 marine nonindigenous species. The eDNA levels recorded are scored as one of five categories and coloured accordingly: No amplification at a cycle of quantification threshold (NC) (white), below limit of detection (bL) (yellow), above limit of detection but below limit of quantification (aLbL) (orange), at least one replicate above limit of quantification (1aL) (red), all three replicates above limit of quantification (3aL) (black). Sampling codes for the 16 harbours are as in Table 3.

species	ALH	ALP	AAR	ESB	FRC	FRH	GED	GRE	HEL	HIR	KAB	KAS	KOB	KOG	ODE	ROE
Acipenser spp.	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
B. hamifera	NC	NC	3aL	NC	NC	NC	NC	3aL	NC	NC	NC	NC	NC	NC	3aL	1aL
C. auratus	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
C. carpio	NC	bL	bL	NC	NC	NC	NC	NC	NC	NC	bL	bL	NC	NC	NC	NC
C. caspia	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
C. peregrina	NC	NC	bL	NC	bL	bL	NC	NC	bL	NC	bL	3aL	bL	NC	NC	NC
E. sinensis	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
H. americanus	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
K. mikimotoi	3aL	3aL	NC	bL	NC	bL	NC	bL	NC	NC	NC	NC	NC	NC	3aL	NC
M. arenaria	bL	3aL	NC	NC	NC	3aL	3aL	3aL	NC	NC	3aL	3aL	3aL	NC	3aL	3aL
M. gigas	NC	NC	NC	bL	NC	NC	NC	bL	NC	NC	NC	NC	aLbL	NC	NC	NC
M. leidyi	3aL	3aL	bL	3aL	3aL	bL	3aL	3aL	3aL	1aL	3aL	3aL	3aL	bL	bL	3aL
N. melanostomus	NC	NC	NC	NC	NC	NC	bL	NC	NC	NC	bL	bL	NC	bL	NC	bL
O. gorbuscha	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
O. mykiss	bL	bL	NC	NC	NC	bL	NC	NC	NC	bL	bL	bL	NC	NC	NC	NC
P. camtschaticus	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
P. farcimen	1aL	3aL	3aL	NC	3aL	3aL	3aL	3aL	3aL	bL	3aL	3aL	3aL	3aL	3aL	3aL
P. cordatum	3aL	3aL	bL	bL	bL	bL	3aL	bL	3aL	NC	3aL	bL	3aL	3aL	1aL	3aL
P. verruculosa	3aL	3aL	NC	1aL	NC	bL	NC	NC	NC	3aL	NC	NC	NC	NC	1aL	NC
R. harrisii	NC	NC	NC	NC	NC	bL	NC	NC	NC	NC	NC	NC	bL	bL	aLbL	NC
Total detections	7	8	6	5	4	9	5	7	4	4	8	8	7	5	8	6

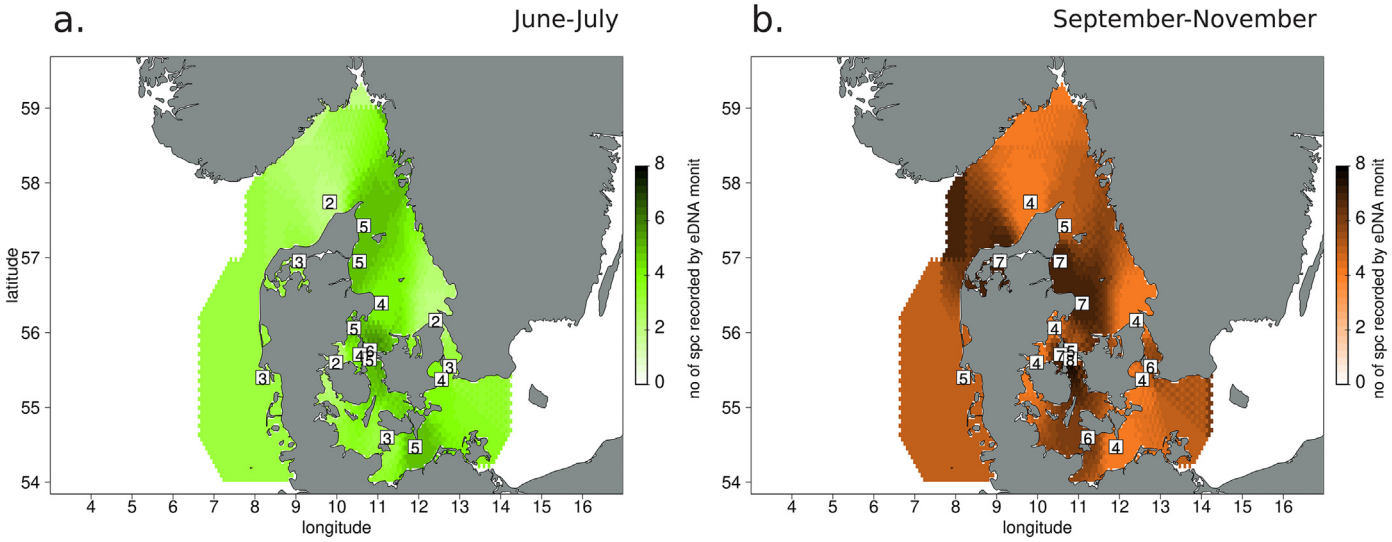

Fig. 2. Geographic representation with colour intensity interpolated between sampled harbours with the highest number of nonindigenous species recorded out of the 18 species monitored from eDNA in 2017 for (a) June–July (green colour) and (b) September–November (brown colour). Sampled harbours are marked with squares for the eDNA monitoring, with the number of species detected per harbour.

with conventional method demonstrated that for a large proportion of the species and sample sites both methods found matching results (Table 6).

4.1. Difference in eDNA levels for two sampling periods for the different species

The variation in distribution of NIS across the two sampling periods is especially evident for the warty comb jelly, being absent in the eDNA monitoring tests from the summer period (Figs. 2 and S27a, Tables S01.4, S01.6 and S1.08), but present in all parts of the Inner Danish seas in the autumn (Figs. 2 and S30a, Tables S01.5, S01.7 and S01.9). This match previous monitoring of warty comb jelly in the Inner Danish seas (Riisgård, 2017), where the warty comb jelly dies during the winter period, and then blooms again in the late summer. This may result in potential discrepancies between eDNA monitoring and conventional monitoring if the sampling does not take such temporal variation into consideration. Even though a widely distributed NIS such as the warty comb jelly is hard to overlook in a visual monitoring when it is abundant in the later part of the summer (Jaspers et al., 2018), it is far simpler to evaluate its abundance and distribution by collecting and analysing a few eDNA water samples over the year. A conclusion Créach et al. (2021) also reached when monitoring eDNA from warty comb jelly. Conventional monitoring is for some of the minor dinoflagellate and algal species dependent on whether the sampling is performed in a period where life stages, important for species identification, can be accurately identified. Whereas monitoring of eDNA depends on the presence of enough eDNA material at the sampling sites to allow for detection of the species at the time of sampling. The Pacific red algae *Bonnemaisonia hamifera* has previously been recorded in Danish waters (Køie and Kristiansen, 2000) and was found again with eDNA monitoring in September–November 2017 (Table 6, Figs. 1, S19a). The absence of the Pacific red algae (*B. hamifera*) and the heterokont flagellate (*P. verruculosa*) in the first season sampled (June–July) could be a reflection of these algae not yet having gained a sufficient population size this early in the year, and that these species wax and wane in their occurrence during the year, but it could also just as well be caused by the stochastic sampling

Table 5
Conventional monitoring of non indeginous species in 16 Danish harbours. Abbreviations for harbours follow Table 3. Numbers represent when each species has been recorded. For past records only findings in a 100 m radius from the center of the harbours is considered. Never found (0), found in the past before this study (1) and found during the conventional monitoring carried out in 2017 (2). Letters represents footnotes: (a) found in Frederikshavn in 1981, (b) Phytoplankton was collected with a net (10 μm mesh size), and identification carried out in light microscopy, (c) caught in 2010, (d) recorded in survey carried out by the Natural History Museum of Denmark in 2010, (e) recorded previously by NIRAS.

Species name	ALH	ALP	ARH	ESB	FRC	FRH	GED	GRE	HEL	HIR	KAB	KSH	KBH	KGE	ODE	ROD
Bonnemaisonia hamifera	0	0	0	0	0	1a	0	0	0	0	0	0	0	0	0	0
Carassius auratus	0	0	0	0	0	0	0	0	0	0	0	0	1d	0	0	0
Colpomenia peregrina	1d	1d	0	0	0	1d	0	0	0	0	0	0	0	0	0	0
Cordylophora caspia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Magallanas gigas	2	2	0	2	0	0	0	0	2	0	1d	0	2	0	0	0
Cyprinus carpio	0	0	0	0	0	0	0	0	0	0	1c	1c	0	0	0	0
Eriocheir sinensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Karenia mikimotoi	1e	1e	1e	2b	1e	1e	0	1e	1e	1e	1e	1e	1e	0	1e	0
Mnemiopsis leidyi	2	2	2	2	2	2	2	2	2	1	2	2	2	2	1	2
Mya arenaria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neogobius melanostomus	0	0	0	0	0	0	2	0	0	0	2	2	2	2	0	2
Oncorhynchus mykiss	1d	1d	1d	0	0	0	0	1d	1d	0	1d	1d	1d	1d	1d	0
Oncorhynchus gorbuscha	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Prorocentrum minimum	1e	1e	1e	2b	1e	1e	1e	1e	1e	1e	1e	1e	1e	1e	1e	1e
Pseudochattonella farcimen	0	0	1e	2b	1e	1e	0	1e	0	1e	1e	1e	0	0	1e	0
Pseudochattonella serruculata	0	0	1e	2b	1e	1e	0	1e	0	1e	1e	1e	0	0	1e	0
Rhithropanopeus harrisii	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	1
Acipenser baerii, Acipenser gueldenstaedtii, Acipenser ruthenus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6

Match between conventional monitoring and environmental DNA (eDNA) in water samples for 18 marine nonindigenous species in 16 Danish harbours. Evaluated from the highest levels of eDNA recorded on the two sampling events in 2017 compared with a single conventional monitoring the same year. The letter code combination in each cell reflects the results from the eDNA results (left of the underscore) and from conventional monitoring (right side of the underscore). The eDNA levels recorded are scored in one of five categories: No amplification at a cycle threshold (NC), below limit of detection (bL), above limit of detection but below limit of quantification (aLbL), at least one replicate above limit of quantification (1aL), all three replicates above limit of quantification (3aL). The conventional monitoring was categorised as: Never previously recorded (NR), recorded in past surveys (PR) and recorded in the survey in 2017 (SR). Correspondence between conventional monitoring and eDNA monitoring is coloured as to whether to both agrees the species is present (yellow) or both agrees the species is absent (white). Disagreements are in different hues of brown and blue. Disagreements where the eDNA approach detected the species but the conventional monitoring did not are brown, and disagreements where the conventional monitoring recorded the species, but the eDNA approach did not are coloured blue. Darker brown (1aL_NR, 3aL_NR) and darker blue (NC_SR) colouring indicates major disagreements between the methods, and minor disagreements between the two approaches are coloured in a lighter shade of brown (1aL_PR, 3aL_PR, aLbL_NR, bL_NR) and blue (aLbL_SR, bL_PR, NC_PR). Abbreviations for harbours are as in Table 3. Last row sums up the total number of agreements per harbour.

species	ALH	ALP	AAR	ESB	FRC	FRH	GED	GRE	HEL	HIR	KAB	KAS	KOB	KOG	ODE	ROE
A. baerii	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR
B. hamifera	NC_PR	NC_PR	3aL_PR	NC_PR	NC_PR	NC_PR	NC_PR	3aL_PR	NC_PR	NC_PR	NC_PR	NC_PR	NC_PR	NC_PR	3aL_PR	1aL_PR
C. auratus	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_PR	NC_NR	NC_NR	NC_NR
C. carpio	NC_NR	bL_NR	bL_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	bL_PR	bL_PR	NC_NR	NC_NR	NC_NR	NC_NR
C. caspia	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR
C. peregrina	NC_PR	NC_PR	bL_NR	NC_NR	bL_NR	bL_PR	NC_NR	NC_NR	bL_NR	NC_NR	bL_NR	3aL_NR	bL_NR	NC_NR	NC_NR	NC_NR
E. sinensis	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR
H. americanus	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR
K. mikimotoi	3aL_PR	3aL_PR	NC_PR	bL_SR	NC_PR	bL_PR	NC_NR	bL_PR	NC_PR	NC_PR	NC_PR	NC_PR	NC_PR	NC_NR	3aL_PR	NC_NR
M. arenaria	bL_PR	3aL_PR	NC_PR	NC_PR	NC_PR	3aL_PR	3aL_PR	3aL_PR	NC_PR	NC_PR	3aL_PR	3aL_PR	3aL_PR	NC_PR	3aL_PR	3aL_PR
M. gigas	NC_SR	NC_SR	NC_NR	bL_SR	NC_NR	NC_NR	NC_NR	bL_NR	NC_SR	NC_NR	NC_PR	NC_NR	aLbL_SR	NC_NR	NC_NR	NC_NR
M. leidyi	3aL_SR	3aL_SR	bL_SR	3aL_SR	3aL_SR	bL_SR	3aL_SR	3aL_SR	3aL_SR	1aL_PR	3aL_SR	3aL_SR	3aL_SR	bL_SR	bL_PR	3aL_SR
N. melanostomus	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	bL_SR	NC_NR	NC_NR	NC_NR	bL_SR	bL_SR	NC_SR	bL_SR	NC_NR	bL_SR
O. gorbuscha	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR
O. mykiss	bL_PR	bL_PR	NC_PR	NC_NR	NC_NR	bL_NR	NC_NR	NC_PR	NC_PR	bL_NR	bL_PR	bL_PR	NC_PR	NC_PR	NC_PR	NC_NR
P. camtschaticus	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR
P. farcimen	1aL_NR	3aL_NR	3aL_PR	NC_SR	3aL_PR	3aL_PR	3aL_NR	3aL_PR	3aL_NR	bL_PR	3aL_PR	3aL_PR	3aL_NR	3aL_NR	3aL_PR	3aL_NR
P. minimum	3aL_PR	3aL_PR	bL_PR	bL_SR	bL_PR	bL_PR	3aL_PR	bL_PR	3aL_PR	NC_PR	3aL_PR	bL_PR	3aL_PR	3aL_PR	1aL_PR	3aL_PR
P. verruculosa	3aL_NR	3aL_NR	NC_PR	1aL_SR	NC_PR	bL_PR	NC_NR	NC_PR	NC_NR	3aL_PR	NC_PR	NC_PR	NC_NR	NC_NR	1aL_PR	NC_NR
R. harrisii	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	bL_NR	NC_PR	NC_NR	NC_NR	NC_NR	NC_NR	NC_NR	bL_PR	bL_PR	aLbL_NR	NC_PR
Total agreements	11	10	10	14	13	10	14	12	12	12	9	10	9	12	11	14

and distribution of eDNA at the sampling events. Continuous and frequent sampling would help to show whether expansions and disappearance of species can be monitored in this way. Conventional monitoring could perhaps just as well have succeeded in finding a difference in occurrence and distribution across the year, but would have been more cumbersome and more costly compared to what the monitoring of eDNA would have required.

The dinoflagellate *Prorocentrum cordatum* was originally identified from the Caspian Sea (Velikova and Larsen, 1999) and algae blooms of this dinoflagellate have been reported from Japan, France, Netherlands, Norway, and the eastern coast of the United States of America (Brandt, 2001; Heil et al., 2005), and generally under relative high temperatures and low salinities (Heil et al., 2005). We detected eDNA from the dinoflagellate *P. cordatum* in September–November 2017 where the sea surface temperature is higher in the southeastern part of the Danish seas where the salinity is lower. This eDNA detection match previous recordings of the dinoflagellate *P. cordatum* in the Danish seas (Figs. 1, S21b, Table 6). For these different algal species, monitoring of eDNA provides an easier and likely also a cheaper alternative, compared to conventional monitoring that is forced to deal with difficult discernible life cycle stages, microscopic morphology, and difficult taxonomy. Surveillance of marine algal NIS by eDNA provides the

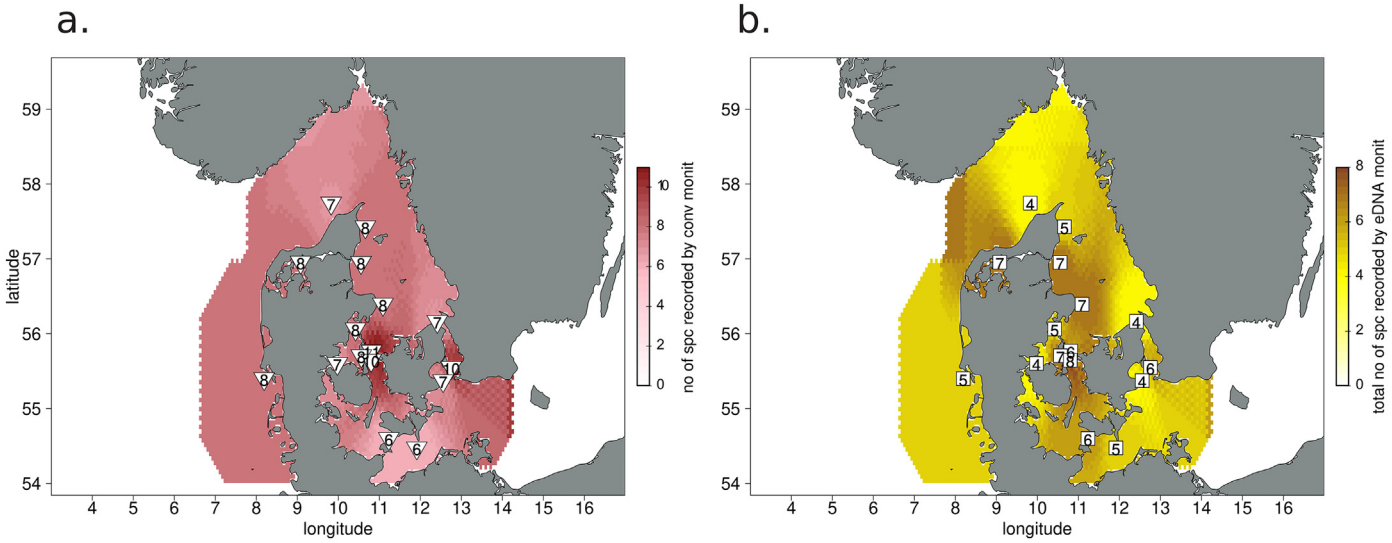

Fig. 3. Geographic representation with colour intensity for the highest number of nonindigenous species recorded for both sampling seasons with (a) conventional monitoring and historical records and (b) recorded with the species-specific detection of eDNA. Sampled harbours are marked with triangles and squares for the conventional monitoring and the eDNA monitoring, respectively, with number of species detected per harbour.

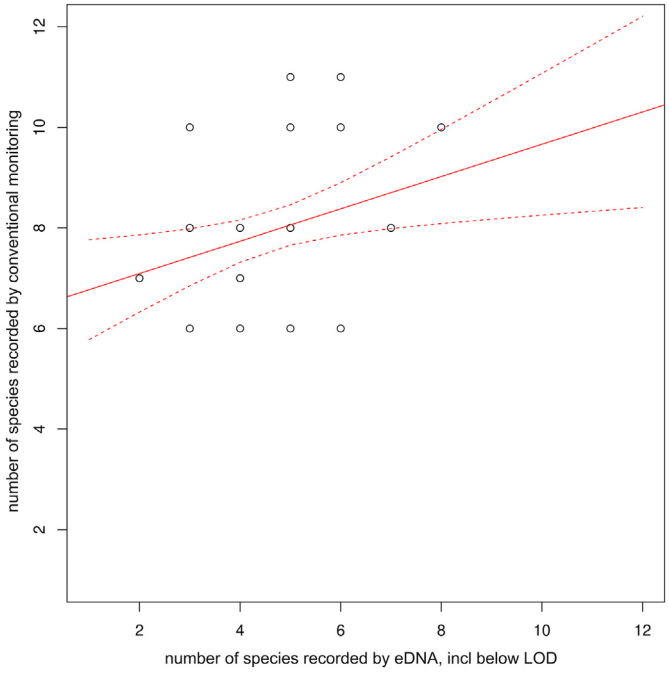

Fig. 4. Linear regression for the 18 nonindigenous species between number of recorded species from conventional monitoring and eDNA monitoring. Each point represents a harbour. R2: 0.106, *p*-value: 0.04, with F-statistics, and 30 degrees of freedom. The number of species recorded by conventional monitoring is inferred by adding up the results from all non-molecular surveillance methods performed in 2017.

possibility for monitoring harmful or unwanted algal blooms (Jacobs-Palmer et al., 2021), and with frequent sampling it also provides a chance of identifying onsets of blooms and make early warnings for when undesired spread of algae is on the verge of having a harmful influence on other co-occurring species. This is especially relevant for monitoring the spread of ichthyotoxic algae such as the heterokont flagellates of *Pseudochattonella* (Dittami et al., 2013; Riisberg and Edvardsen, 2008).

The eDNA distribution inferred for round goby (Fig. S23a) and North American mud crab (Fig. S25a) in the southeastern part of the Inner Danish seas, reflects the current known distribution of these NIS (Azour et al., 2015; Carl, 2012a; Nurkse et al., 2018; Forsström and Vasemägi, 2016), where these two species have been associated with lower salinity levels. Both are benthic species, and the eDNA detection below LOD (Tables 4 and 6, Fig. S25a) might reflect low eDNA levels in the surface water due to water stratification (Jeunen et al., 2020; Yamamoto et al., 2016).

4.2. Limitations in detection of eDNA

If there happens to be high levels of suspended solids and/or algae in the water, the filter may easily become clogged, reducing the obtainable sampling volume (e.g. Esbjerg harbour – Table 3). This then hampers the ability to detect low concentrations of eDNA (Table 4). Pre-filtration can increase the detection of species (Takasaki et al., 2021) and could be a solution to help obtain better sensitivity for eDNA in turbid waters.

Environmental DNA from the Pacific oyster was detected from the western coast of Denmark and the Øresund region near Copenhagen and supports previous conventional monitoring (Table 6, Fig. S24b). The Pacific oyster is common in the Wadden Sea (Wrange et al., 2010) and has spread from the coasts of the Netherlands, Germany, Denmark, Norway and Sweden (Anglès d'Auriac et al., 2017), and as anticipated, we found relative high levels of eDNA from Pacific oyster in this area. Suspended material in the water collected in the autumn in Esbjerg (Table 3) can have influenced the volume of water it was possible to filter, and thereby reduced the probability of detecting Pacific oyster by eDNA.

The goldfish and European carp are common in all freshwater systems in Denmark (Carl, 2012c, 2012b), and eDNA may get transported via freshwater streams and reach the shores around Denmark. However, low eDNA levels in Aarhus and Aalborg for European carp (Fig. S22a) and no eDNA detected for goldfish (Fig. S21b) suggests that detection of these fishes are rare in brackish waters, or that a more extensive sampling effort is needed to detect these fish in the harbour areas.

The oyster thief is native to the eastern Pacific Ocean (Abbott and Hollenberg, 1976; Green et al., 2012) but has been recorded along the western coast of Europe (Min Lee et al., 2014), Ireland (Minchin, 1991; Guiry, 2001), and at Hirsholmene in Kattegat, the North Sea and Limfjorden (Køie and Kristiansen, 2000; Nielsen et al., 1995), and was detected by eDNA in Kalundborg harbour in September–November-2017 (Fig. S22b). The absence of eDNA from oyster thief in the water samples can perhaps be a reflection of scarce occurrence, insufficient sampling or sampling outside prominent abundance levels associated with life stages.

4.3. Comparison of eDNA diversity and salinity

Higher precision and better grouping of similar samples might be obtained with additional replicates (Ficetola et al., 2015; Klymus et al., 2020), and might also allow for inferring a better correlation between conventional and eDNA monitoring (Schmelzle and Kinziger, 2016), or show whether eDNA monitoring can outperform conventional monitoring (Fediajevaite et al., 2021). Monitoring of biodiversity by conventional and eDNA approaches are both encumbered by uncertainties, and accurate species indices might not necessarily be obtainable.

Salinity levels are around 16–18 ppt in the southeastern part of the Inner Danish seas, 28 ppt in the northwestern part of Skagerrak and 32 ppt on the most western coast of Denmark (Maar et al., 2011; Momigliano et al., 2018), and we suspected this would impact the composition of NIS and thereby also the eDNA levels monitored. Especially as the round goby and the North American mud crab over the past decade have displayed a spread from the low saline seas in the Baltic Sea towards the more saline North Sea (Forsström and Vasemägi, 2016), and salinity was presumed to be a hindrance for further dispersal towards more saline areas (Azour et al., 2015). The freshwater hydroid (*Cordylophora caspia*) that inhabits brackish waters is also a good NIS candidate for evaluating whether higher salinity levels hinders further dispersal. Two species (i.e. round goby and North American mud crab) monitored by eDNA exhibited a more local distribution fixed at a smaller regional level (App. A. Figs. S23a and S25a) in the southeastern part of the Danish waters where salinity is low, when compared with the more widespread NIS (e.g. warty comb jelly and sand gaper) which are more evenly distributed across the salinity gradient in the Danish waters (Fig. 1). Other NIS exhibited a more central distribution, coinciding with some of the harbours in Denmark having highest levels of ship traffic (i.e. Kalundborg and Copenhagen) (Fig. 3). Studying levels of eDNA from NIS in a saline transition zone like the Baltic Sea can be helpful for determining whether salinity levels can act as a barrier on the NIS. This may in turn be helpful for evaluating if the spread of the NIS monitored in this study, that also are considered NIS in other areas around the world, may be mitigated by higher levels of salinity. This is especially relevant for the round goby, that in Baltic Sea is found at low salinity levels, but have been assumed to be hindered in further dispersal towards the saline areas in the North Sea (Azour et al., 2015; Nurkse et al., 2018). The limited number of NIS monitored in our study could, however, not indicate if there was a relationship between high prevalence of eDNA from NIS and low levels of salinity. The non metric MDS plot (Fig. 5) and the linear discriminant analysis did not indicate any groupings of species by salinity in the harbours, even though species that are considered to have a broad tolerance to salinity levels were excluded from the MDS and LD analysis. It is possible that the limited number of species included in the analysis is insufficient to detect dependency of salinity levels.

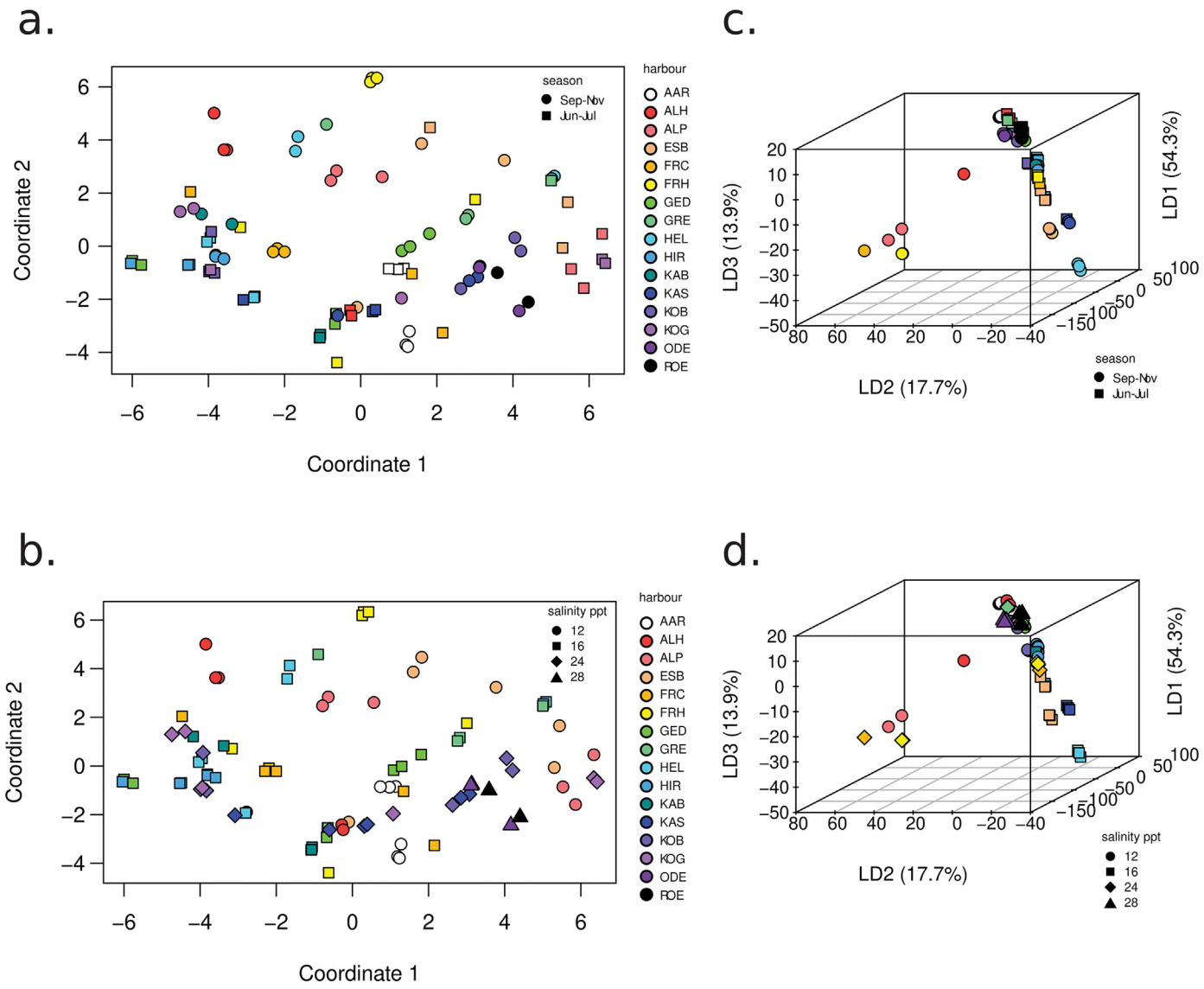

Fig. 5. Non metric multidimensional scaling plot (a, b) comparing eDNA levels recorded for the different harbours with seasonal sampling (a) in the summer (June–July) and autumn (September–November) of 2017, and (b) comparing eDNA with estimated salinity levels for each harbour. Linear discriminant analysis (c, d) of three most influential discriminators for eDNA levels in relation to (c) season sampled and (d) estimated salinity levels. Abbreviations for harbours sampled are explained in Table 3. Species with broad tolerance in salinity (i.e. Pacific oyster, warty comb jelly, sand gaper and oyster thief) were excluded from the salinity levels comparison plots (b, d).

Inclusion of other salinity-dependent species in eDNA surveillance might lead to a better correlation.

4.4. Benefits of eDNA monitoring of introduced marine species

Previous species-specific eDNA monitoring has reported incongruence between conventional monitoring and molecular based monitoring (e.g. Knudsen et al., 2019; Sigsgaard et al., 2015; Takahara et al., 2013). Conventional monitoring is likely to be more influenced by weather conditions than sampling of eDNA is, and conventional monitoring might only be possible to perform once or twice per year, due to the cost associated with labour, and because it requires various types of gear and sampling material.

For conventional monitoring it can be problematic to distinguish between closely related species. Past misidentifications or a low effort for finding these species might lead to conventional monitoring unintentionally overlooking the presence of species such as the Pacific red alga (*B. hamifera*), the dinoflagellates (*K. mikimotoi*) and (*P. cordatum*), and the heterokont flagellates (*P. farcimen* and *P. verruculosa*). Monitoring by eDNA is mainly plagued by eventual misidentification of species at the initial design and validation of oligos, and false positive detection if primers are unspecific. Continuous and frequent monitoring of eDNA alongside conventional monitoring can help evaluate how often conventional monitoring overlooks the presence of species because of incorrect identification.

The NIS monitored in the present study are also considered a threat to ecosystems at various other coastal areas in Europe (e.g. sand gaper, warty comb jelly, Chinese mitten crab, and the North American mud crab) in the great lakes in North America (e.g. round goby), and brackish waters in Australia and middle America (e.g. the freshwater hydroid *C. caspia*). This underlines that there is a scope for the applicability of these developed specific eDNA assays, and allows for more rapid and broad scale monitoring of these NIS globally. Monitoring of eDNA from NIS is not new (e.g. Clusa et al., 2017; Strand et al., 2019; Thomas et al., 2020), but has seen steady increase over the past decade with monitoring of zebra mussels (*Dreissena polymorpha*) (Gingera et al., 2017), swamp crayfish (*Procambarus clarkii*) (Agersnap et al., 2017), bluegill sunfish (*Lepomis macrochirus*) (Takahara et al., 2013), and the warty comb jelly (Créach et al., 2021) – all examples of NIS that are considered a problem worldwide. The eDNA monitoring attempted here shows that regular and parallel monitoring of several NIS is possible to carry out using several fractions of the same filtered water samples. Because the assays target very different

genetic markers, it is likely multiple assays can be combined in multiplex qPCR setups using different dyes, although this is dependent on initial tryouts to make sure primers do not interact and reduce sensitivity (Hulley et al., 2019). Multiplex qPCR detection of eDNA could in tropical seas be applied on nonindigenous seagrass (*Halophila stipulacea*) and lionfish (*Pterois volitans*) that have heavy impacts on marine tropical ecosystems globally (Albins and Hixon, 2013; Guzmán-Méndez et al., 2020; Christianen et al., 2019). In temperate marine coastal areas parallel multiplex qPCR monitoring of eDNA from NIS can be used for detection of American blue crab (*Callinectes sapidus*), Chinese mitten crab (*Eriocheir sinensis*), Japanese shore crab (*Hemigrapsus sanguineus*), Asian brush crab (*Hemigrapsus takanoi*) and American lobster (*Homarus americanus*) which all have been introduced to European coastal areas (Tendal and Jensen, 2017; Türkay, 2001; WoRMS, 2020c). Monitoring of eDNA from NIS has reached a level where there needs to be an increased focus on training of managers and stakeholders to allow for implementing continuous and regular surveys of the distribution of NIS (Sepulveda et al., 2020). With specific assays already developed and validated (Thalinger et al., 2021), the cost of frequent monitoring of eDNA from NIS by multiplex qPCR can be reduced compared to broad scale monitoring (Harper et al., 2018) and conventional monitoring (Valentini et al., 2016), and will allow for detection of very low levels of eDNA molecules from NIS presumed present in the vicinity of the sampling locality.

We observed no correlation between the eDNA levels on a logarithmic scale between the different species (Figs. S29–S37). A possible explanation might be that the NIS monitored in the present study have independent ecology and habitat preferences, despite of their common introduction, and to some degree establishment status, to northern European seas. It is possible that organisms sharing either ecological niches or associated life cycles will allow for inferring a correlation. Whether the presence of the NIS (Parker et al., 2013) have direct negative impacts on native species is difficult to assess (Boltovskoy et al., 2021; Davis and Chew, 2017; Russel and Blackburn, 2017). We recommend frequent continuous parallel monitoring of eDNA from both NIS and native species, to evaluate whether ratios between eDNA from these species are correlated. Since 2018 the Danish Environmental Agency have collected filter samples from all over the Danish Exclusive Economic Zone. With collection of filtered water samples twice each year, there is a possibility of evaluating whether NIS have any negative impacts on native fauna.

Our results show that eDNA monitoring has not reached a level where it can completely replace conventional monitoring. Here the two methodologies both infer the highest number of NIS to be in the central part of the Inner Danish seas (Fig. 3). The similarity between the NIS detected (Fig. 4) underlines that the eDNA assays presented here can be used as a supplementary tool. Ongoing research in eDNA monitoring shows there is a continuous development of additional species-specific eDNA assays, optimization and more frequent and strategically better water sampling, together with an increased understanding of the dispersal and production of eDNA. Our present study shows that there is an incentive for regularly monitoring marine waters by specific eDNA-assays to investigate the distribution and spread of introduced species.

5. Conclusions

Monitoring of marine nonindigenous species using eDNA-based assays in Danish marine waters shows that there is seasonal change in the occurrences and distribution of the different species from summer to autumn. This is something that is difficult to show with traditional monitoring, as it is costly, and sometimes logistically complicated, to undertake multiple traditional monitoring surveys each year. The eDNA monitoring found the same marine areas for the highest occurrence of NIS as the conventional monitoring. This supports the use of eDNA for monitoring NIS. Although the eDNA concentrations inferred here does not reflect abundance or biomass of NIS, the quantification of eDNA can still provide a rough image of seasonal changes in distribution of the NIS. The results obtained here show that species specific monitoring of eDNA by qPCR is an advantageous approach for continuous, fast and easy monitoring of the distribution of these 18 NIS in European seas. In this study, we have not only shown that surveillance of NIS by specific eDNA monitoring is an important supplemental tool for evaluating where the highest numbers of marine NIS are occurring, but also provided a proof of concept for the development, testing and use of eDNA-based methods in marine monitoring targeting nonindigenous species.

CRediT authorship contribution statement

SWK, PRM, JHA, MH conceived the ideas; JHA, PRM, DB, MH, SWK secured the funding; SWK, PRM, JHA, MH designed the study; SWK, JT, BKH, MWJ, SA, SKSJ collected and produced experimental data; SWK analysed the collected data and wrote the first draft of the manuscript; SWK, PRM, JHA finalized the manuscript. All authors contributed to the final manuscript and approved the manuscript for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the Danish Environmental Agency (Miljøstyrelsen; MONIS project, phase 1-4) and Innovation Fund Denmark (Grant J.nr. 104-2012-1). We thank Joseph Stachelek for help on the 'ipdw' package. We are grateful for help with sample material provided by Øresundsakvariet in Helsingør, and material from Florian Lüskow and Hans-Ulrik Risgård (Syddansk University), Henrik Carl, Marcus Krag, Christian Aakjaer Olesen, Jørgen Olesen, Tom Schiøtte, Gert Hansen and Danny Eibye Jacobsen (Natural History Museum of Denmark), Dennis Ulrik Andersen, Thomas Møller, Stine Kærulf Andersen, Britta Sønderskov Pedersen (DTU AQUA National Institute of Aquatic Resources), Marc Anglès d'Auriac, Vladyslava Hostyeva (Norwegian Institute for Water Research), Ulrik Berggreen (Danish EPA), Line Le Gall (NHMN, France), Ignacio M. Bárbara (UCD, Spain), Janne Kim Gitmark (NIVA), Sandra Lindstrom (UBC, Canada), Sherri Pucherelli (Bureau of Reclamation, Technical Service Center, Hydraulic Investigations and Lab Services, AZ, USA). Thanks to Philip F. Thomsen and Eva E. Sigsgaard at Aarhus University for initial advice on protocols for extraction from filtered samples. Finally, we thank Ciarán Murray (NIVA), for linguistic corrections.

Appendix A. Supplementary data

Supplementary data, information, and codes to this article can be found online at https://github.com/monis4567/MONIS3_4_v1.git. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153093.

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