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Methods comparison

Scientific Report from DCE – Danish Centre for Environment and Energy no. 267 2023

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Data sheet

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Abstract: This report provides a comparison of non-indigenous species (NIS) in the marine environment detected using three different methods: conventional monitoring, and two eDNA molecular based methods: qPCR and metabarcoding. Conventional NIS detection is limited in early rapid detection of NIS, and eDNA are proposed as an alternative method, as eDNA is less invasive, is supposed to cover a larger area and potentially with a high level of detection. In the monitoring of NIS in six Danish harbors in 2021 by conventional methods and NIS specific qPCR detection systems for 23 species, eDNA was collected from three stations in each of the six harbors. This eDNA was used for metabarcoding with three different primer sets: 18S rDNA, cytochrome oxidase I (COI) and 12S rDNA, targeting eukaryotes, invertebrates and fish, respectively. The results show a higher number of NIS detected by metabarcoding followed by conventional and qPCR techniques. Only three NIS were found by all three methods, while metabarcoding found 23 unique species. While metabarcoding has advantages compared to conventional and qPCR analyses; metabarcoding results are sensitive to the applied bioinformatics pipelines which should be standardized and optimized along with the reference databases of the marine species in Danish and regional waters.

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Contents

Preface

This report provides a comparison of non-indigenous species (NIS) detected using three different approaches. The report is a supplement to a more comprehensive report on NIS detection in six Danish harbors. Also, this report is a result of a collaboration with Vlaams Instituut voor de Zee vzw, Belgium, established through the EU Interreg project GEANS.

Sammenfatning

Denne rapport præsenterer en sammenligning af tre forskellige tilgange til at detektere ikke-hjemmehørende arter (non-indigenous species: NIS) i det marine miljø: konventionel overvågning med flere forskellige indsamlingsmetoder og to miljø-DNA (eDNA) baserede metoder: qPCR artsspecifikke detektionssystemer og metabarcoding med tre forskellige primersæt analyseret med to forskellige bioinformatiske pipelines. Konventionel NIS-detektion er tidskrævende, hvilket begrænser en tidlig og hurtig detektion. eDNA baseret detektion er derfor foreslået som et alternativ, da eDNA er mindre invasivt, formodes at dække et større område og potentielt har et højere detektionsniveau. I 2021 blev NIS overvåget i seks danske havne med konventionelle metoder og NIS-specifikke qPCR detektionssystemer. Ved denne overvågning blev eDNA indsamlet fra vandprøver og begroningsplader fra tre stationer i hver af seks havne. I nærværende projekt blev dette eDNA anvendt til metabarcoding med tre primersæt: 18S rDNA, cytochrome oxidase I (COI) og 12S rDNA, målrettet mod henholdsvis eukaryoter, invertebrater og fisk. Resultaterne viser det højeste antal NIS detekteret ved metabarcoding og det laveste antal detekteret med qPCR. Sammenfald mellem påviste arter var begrænset: kun tre NIS blev fundet med alle tre metodiske tilgange, mens metabarcoding fandt mange arter, som ikke blev fundet ved qPCR og konventionel prøvetagning. Begge eDNA-metoder påviste fisk og planktonarter, hvilket ikke var muligt med de anvendte konventionelle metoder. Metabarcoding data blev analyseret ved hjælp af to forskellige bioinformatiske pipelines (hhv. DCE og VLIZ). Mens VLIZ påviste flest NIS (40 mod 30 med DCE) detekterede DCE-pipelinen NIS i flere prøver end VLIZ. Metabarcoding (DCE-pipeline) viste, at ca 1/3 af de påviste NIS findes i de fleste havne. En direkte sammenligning mellem qPCR og metabarcoding (DCE-pipelinen) viste, at sammenfaldet i artsdetektionen steg, jo flere prøver man sammenlignede. På trods af disse forskelle, vurderes metabarcoding at kunne berige NIS detektionen foretaget med konventionelle metoder og qPCR detektionssystemet. Metabarcoding er således den eneste metode, som både registrerer fastsiddende, mobile og planktoniske organismer, den er nem og billig at gennemføre, og gør det muligt at detektere helt nye NIS for danske farvande. Dog er der et behov for at standardisere og optimere bioinformatik pipelines, og ligeledes bør referencedatabaserne for marine arter i danske farvande optimeres.

Summary

This report provides a comparison of three different approaches to detect non-indigenous species (NIS) in the marine environment: conventional monitoring, and two environmental DNA (eDNA) molecular based methods: qPCR species specific detection systems and metabarcoding with three different primer sets analyzed using two different bioinformatics pipelines. Conventional NIS detection is time consuming and not efficient for early and rapid detection of NIS. eDNA based detection is proposed as an alternative, as eDNA are less invasive, are supposed to cover a larger area and potentially with a high level of detection. In 2021 NIS was monitored in six Danish harbors by conventional methods and NIS specific qPCR detection systems. During this monitoring eDNA was collected from water samples and settlement plates at three stations in each of the six harbors. In the present project, this eDNA was used for metabarcoding using three different primer sets: 18S rDNA, COI and 12S rDNA, targeting eukaryotes, invertebrates, and fish, respectively. The results show the highest number of NIS detected by metabarcoding and the lowest number by qPCR. The overlap between species detected was limited: only three NIS were found by all three methodological approaches, while metabarcoding found several species not detected with either qPCR or conventional sampling. Both eDNA methods detected fish and planktonic species, which was not possible with the conventional methods used. The metabarcoding data was analyzed using two different bioinformatics pipelines. Interestingly, more NIS (40 species) were detected by metabarcoding using the VLIZ pipeline compared to the DCE pipeline (30 species) using the Danish NIS gross list as reference. However, the VLIZ pipeline recorded NIS in much fewer samples, while the DCE pipeline as well as qPCR detected NIS at several sampling stations. Metabarcoding (DCE pipeline) detected ca 1/3 of NIS in all six harbors. A direct comparison between qPCR and metabarcoding (DCE-pipeline) showed that the level of agreement in NIS detection increased with the number of samples compared. Using a newly published European gross NIS list as reference, indicated that seven new NIS for Danish waters were detected by metabarcoding. Although metabarcoding currently appears less sensitive than qPCR for NIS detection, metabarcoding has several advantages which should be considered when implementing a monitoring strategy. Metabarcoding is the only method which enables detection of both sessile, mobile and planktonic species, it is comparatively cheaper, and it provides a fast approach to detect NIS new to a region. However, the bioinformatics pipelines should be standardized and optimized along with the reference databases of the marine species in Danish and regional waters.

1 Introduction

Introductions of non-indigenous species (NIS) are one of the most detrimental anthropogenic impacts on global aquatic environments, causing loss of native species and reductions in ecosystem integrity, ecosystem services (Rilov & Crooks, 2009; Simberloff et al., 2013), and economic losses (Williams et al., 2010). Despite global recognition of the threat from NIS, there has been limited coordinated and sustained monitoring of new NIS introductions in European waters. While some countries do monitor for NIS, others do not, and of those monitoring programs that do exist, none have been in place long enough to facilitate assessment of long-term temporal trends. Recent reports from both OSPAR (Stæhr et al. 2023) and European member states (Zenetos et al. 2022) show that new NIS continue to arrive in most countries at alarming rates. The assessments of trends are however highlighted as being very uncertain as lack of standardized monitoring provide NIS records which likely do not accurately reflect the time and location of the introduction.

Early and cost-effective detection of new NIS introductions and secondary spreads are needed to efficiently mitigate the impacts of NIS by enabling eradication or control efforts to be quickly implemented (Harvey et al., 2009). Conventional NIS sampling methods (e.g., traps, grabs, settlement plates) are however often labor intensive (Muirhead et al., 2008), associated with observer bias (Fitzpatrick et al., 2009) and associated with uncertainties due to the patchy distribution and small population sizes, which are typical of the early stages of the invasion process. As a result, conventional techniques for NIS monitoring limit our ability for early and rapid detection (Harvey et al., 2009).

In view of these limitations of conventional NIS monitoring, efforts are being made to develop and implement cost-effective and sensitive methods to detect NIS. In recent years, the use of environmental DNA (eDNA) has gained attention as a promising tool to complement more conventional methods in the monitoring of aquatic species and in standardized biodiversity assessments. Sampling DNA directly from the environment or bulk DNA from a specific community of organisms, referred to collectively as eDNA, are rapid and efficient methods of capturing the majority of organisms within a given area (Taberlet et al., 2012). By avoiding the need for visual species observation, capture and direct sampling (Goldberg et al., 2016), eDNA has the potential to greatly reduce cost and labor time, while aiding ecosystem conservation and management through improved detection of species (Knudsen et al., 2022; Staehr et al., 2022; Thomsen & Willerslev, 2015).

Currently, two main types of eDNA monitoring of marine NIS are being applied: metabarcoding and quantitative PCR (qPCR). Metabarcoding is to a large extent used in the monitoring of marine species (Sigsgaard et al., 2017; Staehr et al., 2022; Thomsen et al., 2016). Metabarcoding has been shown in studies to detect taxa that are not easily detectable, such as non-indigenous or endangered species (Dejean et al., 2012; Piaggio et al., 2014), as well as to document local scale patterns in benthic communities in marine habitats connected by water movement (Jeunen et al., 2019). The qPCR technique, in which specific primer-probes assays are developed for individual species has been used for detection of a number of marine NIS in Danish waters (Knudsen et al., 2022) and elsewhere (Hernandez et al., 2020).

This report provides results from a comparison of NIS detections using metabarcoding, qPCR and conventional morphological analysis. Samples were collected as part of a NIS monitoring program for six major Danish harbors (Andersen et al., 2023). The overall aim of the comparison in this report is to evaluate the usefulness of metabarcoding for detection of NIS in Danish harbors compared to conventional morphological analysis and detection with qPCR systems.

2 Methods

NIS identification was performed as part of a NIS monitoring project aimed at identifying the number of NIS in six major Danish harbors using replicated sampling with both conventional morphological approaches and a recently developed eDNA technique, which was developed to detect 23 selected NIS using a qPCR detection system (Andersen et al., 2023). In addition to these, we performed metabarcoding using three primer sets (18S rDNA, COI and 12S rDNA) on the same DNA that had been extracted for the qPCR analysis.

2.1 Sampling program

Sampling was done at three stations in six selected major Danish harbors (Esbjerg, Hirtshals, Frederikshavn, Aarhus, Fredericia, and Copenhagen) providing a total of 18 stations in 2021 (Figure 2.1). Only the industrial harbors were sampled. Details of the sampling program are described in Andersen et al., (2023).

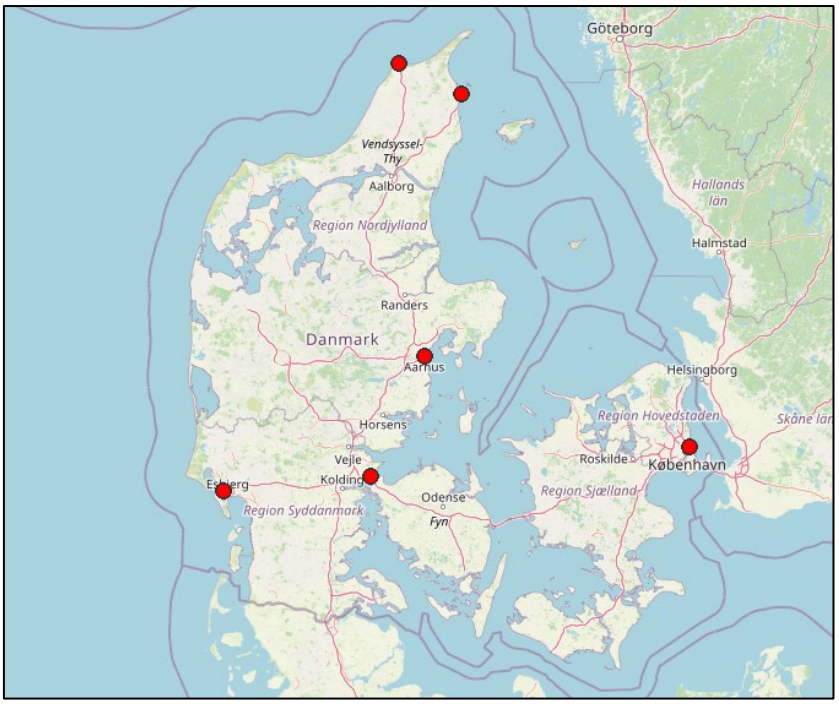

Figure 2.1. Sampling for NIS detection was performed in six major harbors. In each harbor three stations were sampled

2.2 Morphological detection

Three conventional morphological based techniques for sampling biological material were applied: settlement plates, sediment samples and scrapings. All collected material was identified in the laboratory by a trained taxonomist to the lowest possible taxonomical level using a stereo magnifier and microscope.

Settlement plates

At each station, a vertical series of settlement plates (Figure 2.2) were deployed in June 2021 and left for ca. three months until retrieval in September/October 2021. Plates were distributed evenly through the water column (1 m above sediment, centrally and 1 m below surface).

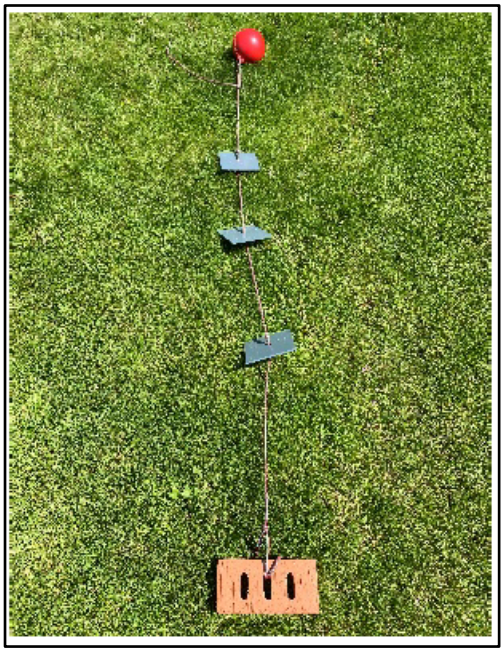

Figure 2.2. Settlement plates applied in each harbor.

Upon retrieval, plates were preserved in 96% ethanol and diluted to a final concentration of 70%. In the laboratory, photos of each plate and species were identified. Later, biological material was scarped in preparation for DNA extraction (see 2.3.2).

Sediment samples

Sediment samples were taken using a Van Veen grab (Figure 2.3).

Figure 2.3. Van Veen grab and sieve used for sediment sampling

One sediment sample was collected from each station and immediately sorted through a 1 mm sieve. The remaining material was preserved in 96% ethanol and diluted to a final concentration of 70% for later species identification.

Scrapings

Organisms associated with hard structures in the harbors were collected by scraping using a 10 cm wide handheld scraper (Figure 2.4).

Figure 2.4. Scraper used to collect biological material on hard substrates in the harbors.

In each harbor, three scrapings (app. ½ m) were made covering hard surfaces in the upper meter below the water surface. Collected material were sorted through a 1 mm sieve and preserved in 96% ethanol and diluted to a final concentration of 70% for later species identification.

2.3 Molecular (eDNA) detection

Water samples

Sampling of water for eDNA analyses generally followed the technical guideline by Knudsen et al. (2020). Water was collected 1 m below the surface using a 2 L Van Dorn water sampler (KC Denmark A/S). From each water sample, 550-1500 ml of water was filtered through a Sterivex filter with two replicates per station. The filters were snap frozen in liquid nitrogen and upon arrival to the lab stored at -80 °C until DNA extraction.

DNA extraction

Tissue DNA extractions: Unicellular and microscopic NIS in growth cultures were centrifuged (7000 rpm for 10 m), to concentrate cells and tissues, prior to DNA extraction. Larger NIS were dissected avoiding the gut, mouth, or skin parts, and extracted tissues were then ground using mortar and pestle and liquid nitrogen. Algae material was treated in the same way. DNA extraction from the collected tissue using DNeasy Blood & tissue kit (QIAGEN) following the manufacturer's protocol except the samples were treated with 10 µl Proteinase K (600 U/ml) (QIAGEN) and incubated for at least three hours at

56 °C and 1000 rpm before the bead-based homogenization. All the DNA extracts were split into several Eppendorf tubes and stored at −20 °C before being used in qPCR.

DNA extraction from Sterivex filters: DNA extraction from the filters was carried out using DNeasy Blood & tissue kit (QIAGEN) with 'spin-columns' using the protocol as described by the manufacturer except proteinase K treatment was added. The filters were opened and processed at sterile conditions in a flow hood. A mixture of 720 µL ATL buffer and 80 µL proteinase K (600 U/ml) was used instead of 720 µL ATL buffer. The filters were then incubated on a rotor in a heating cabinet at 55 °C (± 1 °C) for 4 to 24 hours so the filtrates were completely lysed. Further steps in the extraction followed the manufacturer's protocol. Extracted DNA was split into several Eppendorf tubes and stored at -20 °C until used for quantitative PCR and/or metabarcoding.

DNA extractions from settlement plates: From the settlement plates fixed in ethanol, DNA was extracted separately from the upper and lower plate surface. Settlement plates were gently removed from the box with ethanol in a flow hood at sterile conditions to avoid DNA contamination. Samples were collected from five different spots of individual settlement plates into a 50 ml tube. Collected samples were centrifuged (3000 rpm for 5 minutes) to remove supernatant ethanol. Samples were then air dried for one to two hours at room temperature to remove traces of ethanol and subsequently stored at -20 °C until DNA extraction. Samples were lyophilized for 24 hours and then ground using a bead beater. In total 10-15 metal beads of 2.4 mm diameter were used to grind three times for 30 s at 4 m s-1 speed in a bead mill homogenizer (Bead Ruptor Elite, Omni International). Once ground, 250 mg of each sample was used for DNA extraction using the DNeasy PowerLyser PowerSoil kit (QIAGEN) and following the protocol described in the kit. DNA concentrations were quantified using a Qubit 4.0 fluorometer. The DNA from settlement plates were split into several Eppendorf tubes and stored at -20 °C until use. Prior to qPCR and metabarcoding analyses DNA was pooled for each station.

Quantitive PCR

TaqMan qPCR was used for the detection and quantification of NIS eDNA from water and settlement plates. Amplification reactions were performed in a BioRAD Real-time PCR system (Life Technologies) using 96-well plates. Primers and probes, developed and described earlier, were used for detection and quantification (Knudsen et al., 2022). A total reaction mixture of 25 µl was used, containing 3 µl of the DNA template (1-5 ng/ul), 1 µl each of forward and reverse primers (10 µM stock), 0.5 µl of probe (5 µM stock), 7 µl of water, and 12.5 µl of qPCRBio Probe Mix Lo Rox-Cobio (PCR Biosystems). As negative and standard curves, 3 µl of sterile water and serial dilutions of PCR products from NIS tissue DNA were used, respectively. Thermal cycles in the qPCR consisted of an initial denaturation phase at 95 °C for 10 min, followed by 50 cycles of 95 °C for 30 s and 60 °C for 45 s. Three technical replicates were prepared for each sample. Standard curves were obtained by plotting the quantification of cycle (Cq) values against the log10 of a 10-fold serial dilutions (10^{-4} to 10^{-11}) of NIS PCR product DNA.

For positive controls and standard curves, PCR products of the individual NIS DNA were obtained via PCR reaction mixture of 25 µl containing 4 µl of the tissue DNA template (1-10 ng/ul), 0.5 µl each of forward and reverse primers (10 µM stock), 0.5 µl of bovine serum albumin (20 mg/µL), 14.25 µl of water,

and 5 µl of PcrBio HiFi buffer, 0.25 µl of PCRBIO HiFi Polymerase (2U/µl) (PCR Biosystems). PCR thermal cycles consisted of an initial denaturation phase at 95 °C for 1 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 60 s, and final elongation at 72 °C for 5 min. The PCR products obtained were purified using a QIAquick PCR Purification Kit (Qiagen, catalogue number 28104). For NIS DNA with amplicon sizes less than 100 bp, we used a Gel and PCR clean-up column (Macherey-Nagel).

Standard curves were obtained using plots of critical threshold (Ct) versus the logarithm of a 10-fold serial dilution of DNA products. The NIS gene copy numbers were calculated from the standard curve by Bio-Rad CFX manager 3.1 (Bio-Rad, Hercules, USA).

Metabarcoding

In total 36 water filter samples, and 14 settlement plate samples were used for DNA metabarcoding. Invertebrates, eukaryotes, and fish sequencing libraries were generated by a two-step dual indexing strategy for Illumina MiSeq sequencing. We used three different primers targeting 18S rDNA, 12S rDNA and COI region of mitochondrial DNA to study eukaryote, fish, and invertebrate communities, respectively (Table 1). PCR amplicons were performed in a 25 µl reaction mixture consisting of 12.5 µl KaPa HiFi HotStart ReadyMix 2x (Roche), 1 µl of 10 mM forward and reverse primers, 0.5 µl of bovine serum albumin (20 mg/µL), 8 µl of water and 2 µL (10–20 ng) of DNA template. For 18S rDNA, the PCR cycle program included initial denaturation at 98 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final elongation at 72 °C for 10 min. Thermal cycles were performed similarly for invertebrates and fish, with the exception that the annealing temperatures were 48 °C for COI and 65 °C for 12S rDNA. This was followed by a 15-cycle indexing PCR, known as second PCR (PCR2), during which unique index combinations (i7 and i5) and adaptors were added. For PCR2, thermocycler conditions were 95 °C for 5 min, 13 cycles of 95 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min, and a final elongation at 68 °C for 10 min. The amplicon size of PCR products was confirmed by visualization in a 1.5% agarose gel using SYBR staining. Subsequently, the amplicon products were cleaned using HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, USA), according to the manufacturer's instructions. The amplicon concentrations were quantified using a Qubit 4.0 fluorometer. Finally, amplicons were equimolarly pooled for equal representation in the sequencing library, and sequencing was carried out using the Illumina MiSeq platform at DCE, Aarhus University.

Table 2.1. Target genomic region, primer sets and their references used in this study

Locus/Target community	Primers	Sequence	References
12S rDNA /Fish	MiFish-F	GTCGGTAAAACTCGTGCCAGC	Miya et al. 2015
	MiFish-R	CATAGTGGGGTATCTAATCCCAGTTTG	
18S rDNA /Eukaryote	SSU F04	GCTTGTCTCAAAGATTAAGCC	Fonseca et al. 2010
	SSU R22	GCCTGCTGCCTTCCTTGGA	
COI / Invertebrates	mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al. 2013
	jgHCO2198	TANACYTCNGGRTGNCCRAARAAYCA	

Bioinformatics and data analysis

The DNA reads obtained from the Illumina MiSeq runs were analyzed using a custom-made "VLIZ pipeline" and a "DCE pipeline". By the "VLIZ pipeline" the initial quality control and filtering of pair-end reads was done using fastqc and Trimmomatic. Forward and reverse primers were trimmed and reads less than 200 base pairs were excluded. Paired-end sequences were merged using PANDAseq (Masella et al., 2012). VSEARCH was used to remove chimera and de-replication of the reads (Rognes et al., 2016). For COI, singletons were removed. Clean and de-replicated reads were subjected to clustering using the swarm algorithm (Mahé et al., 2015) to cluster amplicon sequence variants into operational taxonomic units (OTU). Taxonomy assignments for representative OTU sequence were done using nucleotide BLAST (blastn) against the SILVA v. 138 reference database for the 18S rDNA dataset, MIDORI database for COI and Mifish/12S rDNA (Iwasaki et al., 2013; Machida et al., 2017; Quast et al., 2013). Taxonomic assignments were ranked by e-value and the first hit above thresholds of 2 % (18S rDNA and 12S rDNA) or 3 % (for COI) for alignment match and 180 bp for alignment length was accepted.

In the "DCE pipeline" QIIME2 (Bolyen et al., 2019) was used. The DADA2 (Callahan et al., 2016) plugin in QIIME2 was used with default parameters, except the reads were trimmed for primer sequence and reads truncated after 230 bp. For 18S rDNA, the resulting amplicon sequence variants were classified using the QIIME2 naive Bayes classifier trained on 99% Operational Taxonomic Units from the SILVA rRNA database (v. 138) after trimming to the primer region (Quast et al., 2013). COI amplicons were blasted against the BOLD database using sequence-id tool (www.gbif.org) and 12S rDNA were blasted against Mitofish database (Iwasaki et al., 2013). Less abundant ASVs (with less than ten reads) were filtered out before blasting for COI and Mifish. Blast taxa with high similarity and coverage (>97%) were assigned at species level.

The OTU/ASV tables and taxonomy files were imported into the statistical software R and statistical analyses and data visualizations were performed in v.4.2.1 (R Core Team, 2022) using 'phyloseq' package (McMurdie et al., 2013).

3 Results and discussion

3.1 Comparison of methods

The eDNA based detection of NIS at three stations in each of the six harbors was carried out using qPCR species specific detection system and metabarcoding using three primer sets and two different bioinformatic pipelines (DCE and VLIZ). This resulted in three different lists of NIS identified using eDNA methods (DCE, VLIZ and qPCR), which were compared to the list of NIS detected using conventional monitoring of NIS.

In addition, we explored the importance of comparing different NIS reference lists to the NIS identified with metabarcoding. Thus, we matched the species list obtained with metabarcoding with A) the updated official list of NIS known to occur in Danish waters (Miljøstyrelsen, 2022) and B) a list of NIS recently published for all European waters (Zenetos et al. 2022) referred to as "EU NIS list". Both the Danish and the EU NIS lists includes cryptogenic species of unknown origin. The Danish NIS list was updated in January 2023 and includes 123 species. The EU list covers a total of 934 species, including the 123 NIS known to occur in Danish seas.

Overall, we detected 17 NIS using conventional methods, and 11 NIS with qPCR out of the 24 species with qPCR detection systems. Using metabarcoding we identified 26 and 39 NIS with the DCE and VLIZ pipeline, respectively (Figure 3.1).

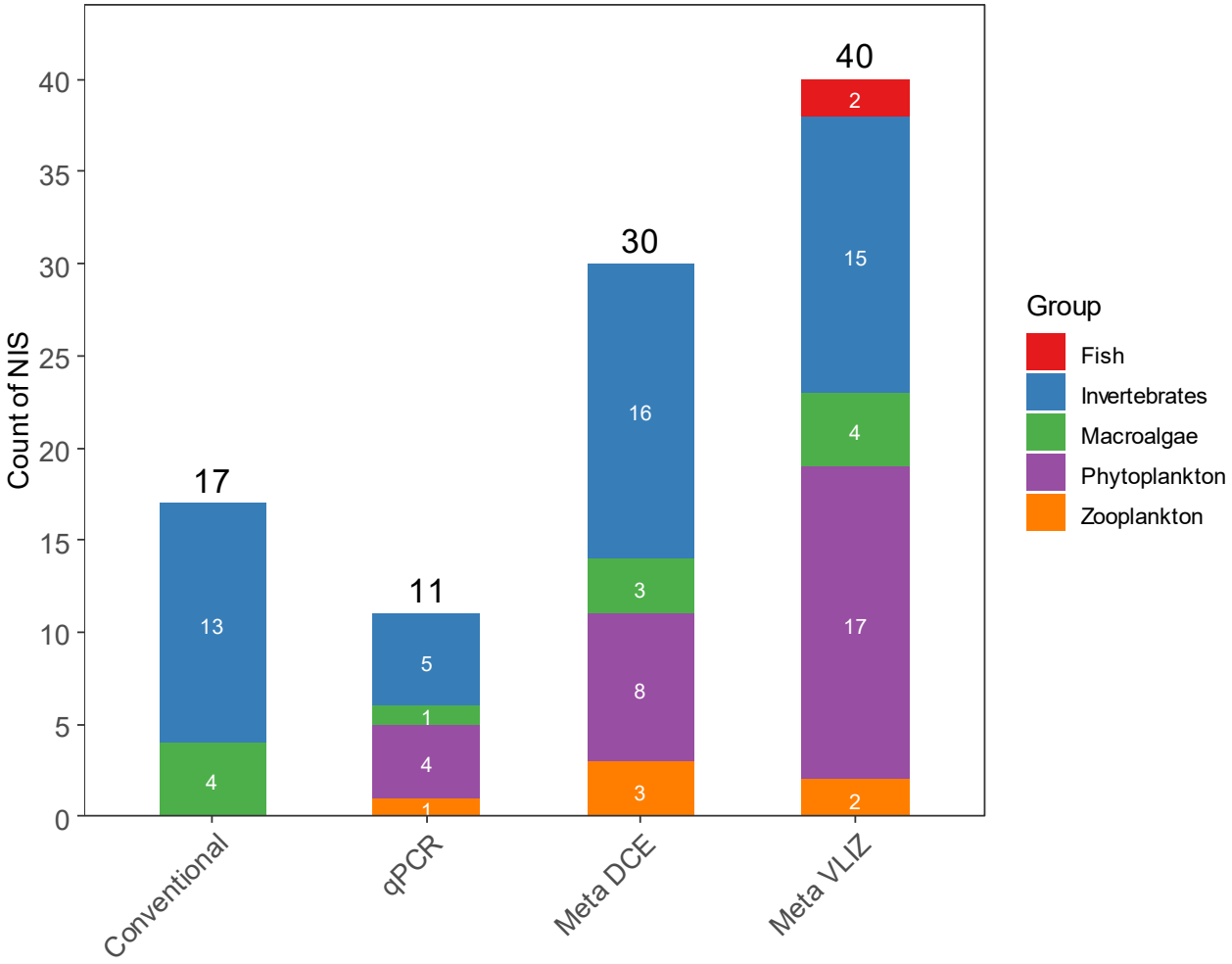

Figure 3.1. Number of NIS within five major groups detected in six Danish harbors using different methods in June and September 2021. Results from metabarcoding (Meta) are shown for the two bioinformatic pipelines used.

When we compared the number of NIS detected by the different methods, we found that combining qPCR and morphological sampling resulted in a total of 24 NIS, two of which were new to Danish waters (Andersen et al. 2023). By adding the metabarcoding based NIS detection, additional NIS were detected, with 15 NIS for the DCE pipeline and 25 NIS for the VLIZ pipeline (Figure 3.2).

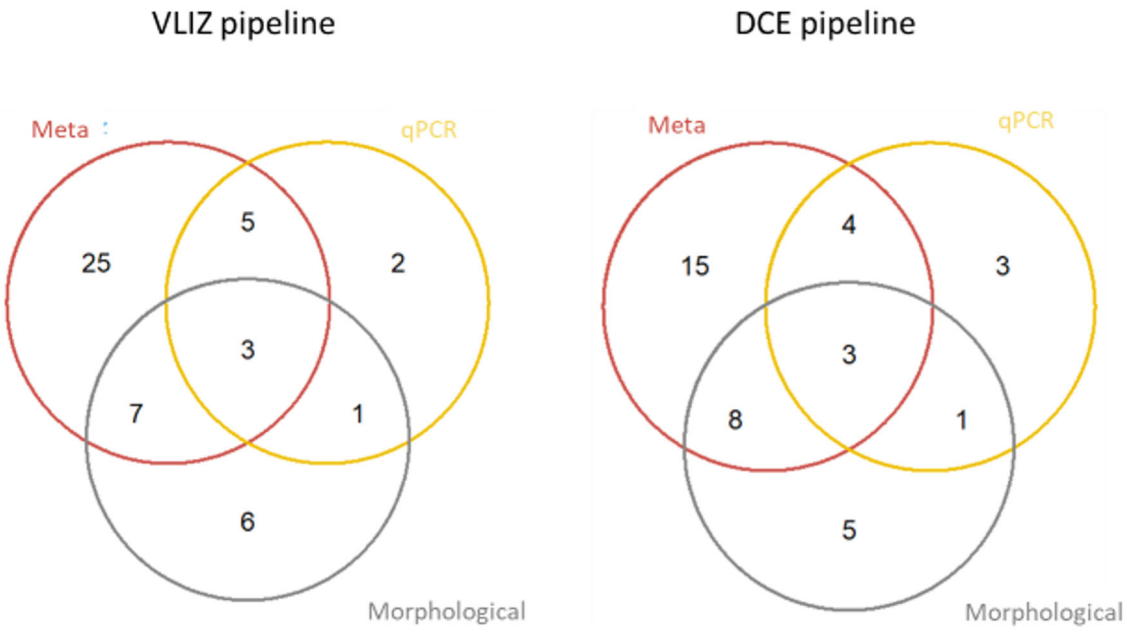

Figure 3.2. Venn diagram comparing the NIS shared by the three methods. The VLIZ and DCE bioinformatics pipelines were used separately to compare metabarcoding with morphological and qPCR methods. .

The total number of identified NIS was thus 49 and 39 when combining the VLIZ and DCE pipelines, respectively, with NIS identified with qPCR and morphological methods. Hence, combining both conventional and different eDNA methods for monitoring of NIS provides the most comprehensive detection of NIS. More species were however, detected using the VLIZ bioinformatics pipeline.

With qPCR specifically, we detected 11 NIS out of the 23 qPCR species with detection systems of which two, *Pseudochatonella farcimen* (phytoplankton) and *Prorocentrum cordatum* (invertebrate), were uniquely detected with the qPCR assay. In addition to the NIS detected reported above Limit of Detection, we also found traces (below Limit of Detection) of *Acipenser gueldenstaedtii, Hemigrapsus sanguineus, Oncorhynchus mykiss,* and *Paralithodes camtschaticus.* According to Knudsen et al. (2020), for a qPCR result to be considered as a detection of the NIS, the assay should include a standard series from which Limit of Detection (LOD) and Limit of Quantification (LOQ) are defined. Hence, qPCR results with Ct values below LOD can only be considered weak traces of the target DNA. In this report we report values above LOD as the species being detected, while we consider values below LOD as the species being identified but not detected. Such weak signals in the qPCR assay have the risk of also being due to technical errors during the qPCR or DNA traces in the water from other environments.

An advantage of the metabarcoding approach is that the extensive species provided, makes it possible to identify new NIS at the monitored sites. Matching the results from our metabarcoding (DCE pipeline) against the extended "EU NIS" list provided a total of 39 NIS compared to 30 when matching against the Danish NIS list. Among the extra NIS identified using the extended EU NIS list for matching, we identified seven species, which potentially could be considered as new NIS for Danish seas (Table 3.1).

Table 3.1. List of potential new NIS detected with metabarcoding using the DCE pipeline when matching against an extended EU NIS list.

Species	Group
Balanus glandula	Barnacle
Botrylloides violaceus	Ascidian
Crisularia plumosa	Bryozoan
Fibrocapsa japonica	Phytoplankton
Haliclystus tenuis	Cnidaria
Tenellia adspersa	Gastropod
Thalassiosira hendeyi	Phytoplankton

Of the 17 NIS detected by conventional sampling, 10 were also identified with the VLIZ pipeline, and 11 with the DCE pipeline. The seven NIS uniquely detected with morphological sampling were *Rhithropanopeus harrisii, Sargassum muticum, Schizoporella japonica, Sinelobus vanhaareni, Streblospio benedicti, Tharyx killariensis* and *Dasya* sp. Of these only *Rhithropanopeus harrisii* were among the 23 NIS searched for with the qPCR detection system. By conventional morphological detection, the certainty of the detection is high, and only limited by rare cyrptic or new NIS in the monitoring area and the identification challenges with identifying these.

Since the detected NIS covers a broad range of taxonomic clades and is not limited to a single phylogenetic clade, different sets of universal primers were used to cover invertebrates, eukaryotes, and fish communities by metabarcoding. Further, the data were analyzed by two contrasting bioinformatics pipelines. Interestingly, 25 and 15 of NIS uniquely identified with metabarcoding using the VLIZ and DCE pipelines, respectively, were not detected either by morphological or qPCR techniques. The majority of these species were planktonic and hence not looked for by the conventional technique. Interestingly, many of the species identified using the qPCR detection system were also detected using metabarcoding (VLIZ and DCE detected nine and eight, respectively) (Figure 3.2). Some of the qPCR detected NIS were not detected using metabarcoding (three NIS for VLIZ and four for the DCE pipelines), suggesting that metabarcoding is a less sensitive technique. NIS detection using metabarcoding is currently not assessed against quantitative information on species abundance, and should therefore be considered as having lower certainty compared to the qPCR detection systems which combines information on LOD, LOQ and number of technical replicates to justify NIS detection.

The overall higher number of NIS detected with metabarcoding than both conventional and qPCR (Figure 3.1 and 3.2), was evident in most harbors, especially with the DCE pipeline (Figure 3.3).

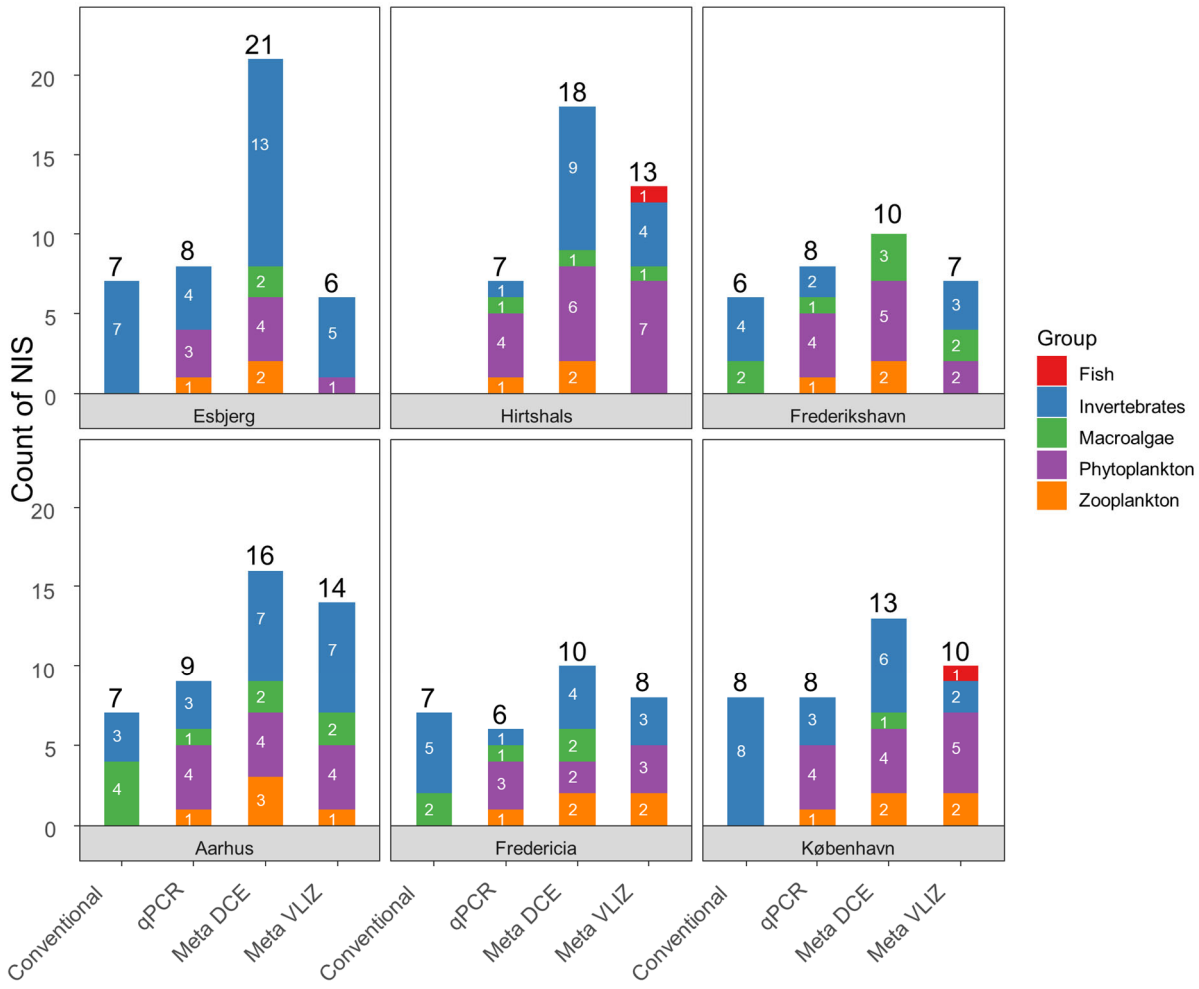

Figure 3.3. Number of NIS within five major groups. NIS were identified in each of the six monitored harbors during sampling in both June and September 2021. Color codes indentify major taxonomic groups. Results from metabarcoding (Meta) are shown for the two bioinformatic pipelines used.

Looking at the number of NIS records by the different detection methods, showed that eight of the 11 qPCR-detected species were found at half of the 18 stations sampled. In comparison NIS identified using conventional sampling was more rare (maximum of 8 stations). For metabarcoding, 11 out of 30 NIS detected with the DCE pipeline were recorded in more than half of the stations (Figure 3.4).

To further compare the sensitivity of NIS detected only using the qPCR system and metabarcoding (DCE pipeline only), we compared the number of NIS recorded with metabarcoding out of the 11 NIS detected with the qPCR system (Table 3.2).

Tabel 3.2. Level of agreement between qPCR and metabarcoding (DCE pipeline). Seasons were spring and autumn, methods refer to settlement plates and water samples.

Comparison level	Agreement (%)
6 Harbors x 3 station x 2 season x 2 methods	21
6 Harbors x 2 season x 2 methods	25
6 Harbors x 2 seasons	28
6 Harbors	32
All samples	64

From this simple analysis, it is clear that the level of agreement (ability of metabarcoding to detect a NIS found by qPCR) increases with the number of samples compared.

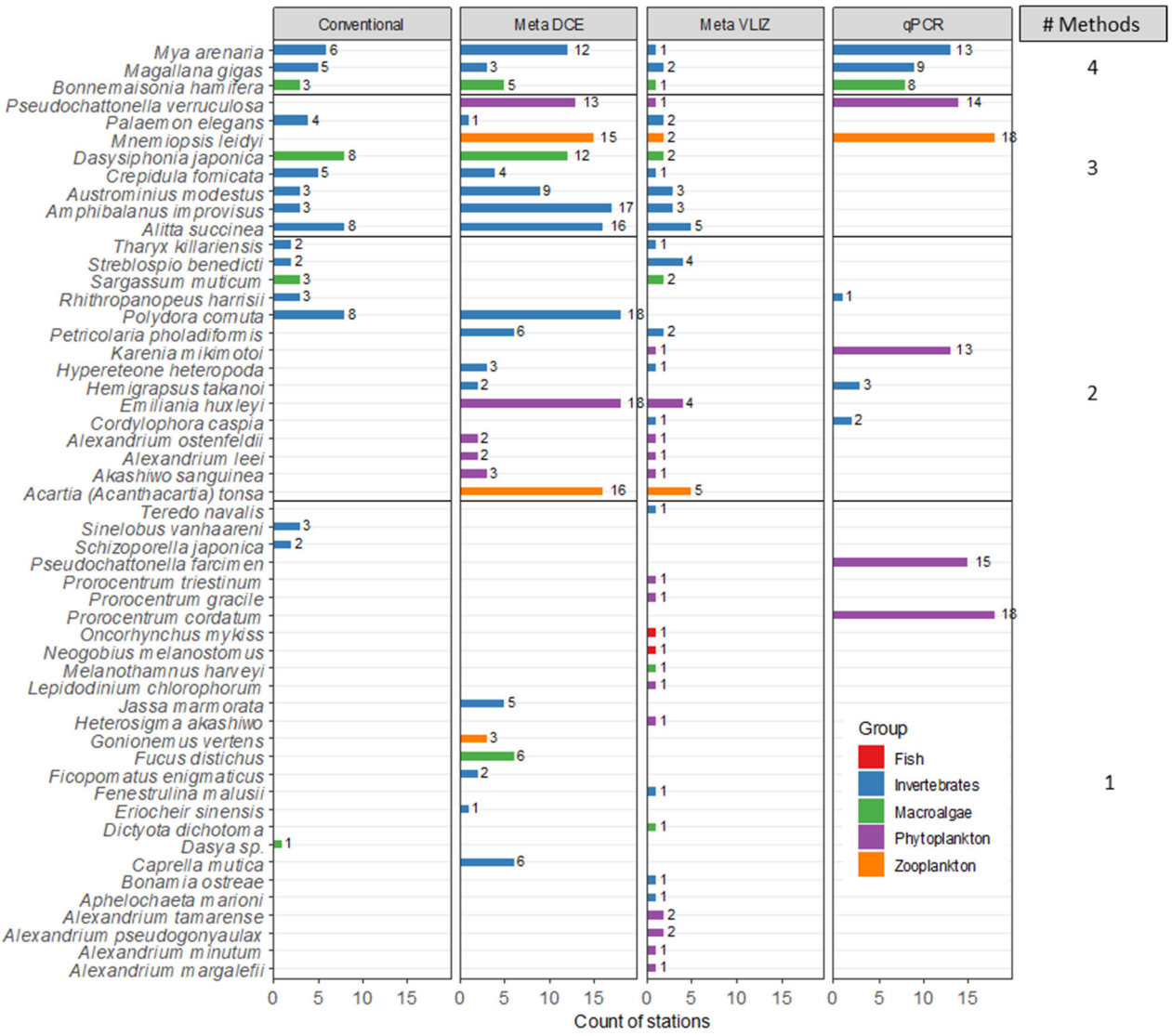

Figure 3.4. Total number of NIS records detected by conventional, metabarcoding (Meta) and qPCR techniques in six Danish harbors. Maximum is 18 records (6 harbors x 3 stations). Results from metabarcoding are shown for the two bioinformatic pipelines used. Color codes indentify major taxonomic groups. Species are sorted according to overlap between methods detection.

Of the three NIS in common for all three methods, qPCR detected these at far more stations compared to conventional and metabarcoding methods (Figure 3.4). In total 27 NIS were detected by one method, 16 NIS were detected by two methods, 7 NIS by three methods and only 3 NIS: *Mya arenaria, Magallana gigas* and *Bonnemaisonia hamifera,* were detected by all four methods (Figure 3.4). Considering these three NIS, its noteworthy that these were detected at much fewer stations using conventional sampling techiques, but appeared to be very common with the qPCR and metabarcoding (DCE pipeline) (Figure 3.4).

The metabarcoding analyses were based on three primer sets targetting invertebrates, eukaryotes, and fish. However, the design of primers can constantly be improved the more sequences are available in databases. Hence, we anticipate that more optimal primers targetting a larger fraction of the biome will be available for future monitoring.

Interestingly, metabarcoding recorded the individual NIS at much fewer stations using the VLIZ pipeline, suggesting a lower detection limit/sensitivity using this pipeline. This result highlight that the metabarcoding results are quite dependent on the chosen bioinformatic pipeline. Several available bioinformatics pipelines are currently being developed for molecular ecology-based research. Differences in bioinformatics pipelines concerns the workflow involving different quality control steps, clustering of the reads to amplicon sequence variant (ASV) or operational taxonomic units (OTUs), and taxonomy assignments against a reference database (Prodan et al., 2020). Many tools for each step and various workflow combinations have been developed and tested, however, each has its own pros and cons, and is dependent on the genomic region used for amplicons (Antich et al., 2021; Pauvert et al., 2019). For this study, we used widely used bioinformatics tools with two workflow pipelines. VLIZ is based on VSEARCH and swarm algorithm for OTU clustering, while DCE used the DADA2 plugin using QIIME2 environment for ASVs without clustering step. Use of OTU after denoising is recommended for markers such as COI (Antich et al., 2021), however, several studies propose ASVs as future replacement of OTUs arguing that ASV have higher genetic resulution, are reusable across studies, and are independent of clustering algorithm and similarity percentages (Bolyen et al., 2019). The ongoing GEANS project is also comparing different bioinformatics pipelines across different laboratories and is ongoing to address workflow based differences in North Sea species identification. Given the observed influence of detection pipeline, we recommend that these are further evaluated and if possible, customized to the monitoring area of interest.

Conventional methods such as those used here (scraping, bottom samples, settlement plates) can be considered time-consuming (Muirhead et al., 2008), with results highly dependent on taxonomic knowledge (Fitzpatrick et al., 2009) and high uncertainties for species with low population densities, as is typically the case for NIS. Hence, there is a desire to promote the use of methods that reduce these uncertainties and promote the rapid and safe detection of alien species (Harvey et al., 2009). Here it has been highlighted that eDNA techniques have great potential (Dejean et al., 2012), especially due to a greater certainty of species identification which makes it possible to distinguish between closely related species and assess whether a species is cryptogenic or non-indigenous.

In this study we did not use information on the abundances of the observed species, although the conventional methods produced quite extensive species lists, that indicate the quantity of each species in terms of either individual density or degree of coverage (%). If such true abundance data were needed, neither the metabarcoding nor the qPCR method would have provided the necessary data. However, relative measures of abundance can be obtained for qPCR (copy numbers) and metabarcoding (reads numbers). The application of this information needs investigation.

In our evaluation, we have compared some very different methodological approaches (conventional detection vs. qPCR and metabarcoding based detection), which in many ways do not allow comparing 1:1 but rather supplement each other.

3.2 Cost-efficiency of methods

In addition to the quality of the species lists obtained through the conventional and DNA based techniques, it is of interest to assess cost-efficiency of the different methods. Table 3.1 provides a simple assessment of the resources (time and costs) associated with the three types of NIS sampling applied in this study. Here we have excluded time and costs associated with the field-based sampling and only focus on resources spent in the laboratory and office.

Table 3.1. The top table gives an overview of the time and costs of processing samples collected for monitoring non-indigenous species (NIS). The bottom table lists any savings calculated as the percentage reduction / increase in time consumption and economy for the individual techniques. Red cells mark an increase in either time or economy by eDNA; yellow cells show a neutral change, and green cells show that the introduction of eDNA methods has led to a saving in either time and/or economy.

Time and costs - quantifications

Monitering method	Sampling type	# Samples	# Stations	Total time (h)	Time per sample (h)	Time per station (h)	Price per sample (kr)	Price per station (kr)
Conventionel	Settlement plates	54	18	135	3	7,5	2.560 kr.	7.679 kr.
qPCR (bulk)	Settlement plates	54	18	270	5	15	12.816 kr.	38.448 kr.
Metabarcoding (bulk)	Settlement plates	54	18	216	4	12	5.347 kr.	16.042 kr.
Conventionel	Core + scraping + settlement plates	42	18	531	13	29,5	13.026 kr.	30.394 kr.
qPCR (water)	Water	108	18	540	5	30	6.408 kr.	38.448 kr.
Metabarcoding (water)	Water	108	18	432	4	24	2.674 kr.	16.042 kr.

Time and costs - %reductions

Monitering method	Sampling type	Total time (h)	Time per sample (h)	Time per station (h)	Price per sample (kr)	Price per station (kr)
qPCR (bulk) vs conventionel	Settlement plates	200%	200%	200%	501%	501%
Metabarcoding (bulk) vs conventionel	Settlement plates	160%	160%	160%	209%	209%
qPCR (water) vs conventionel	Plates + scraping + settlement plates	102%	40%	102%	49%	126%
Metabarcoding (water) vs conventionel	Plates + scraping + settlement plates	81%	32%	81%	21%	53%
Metabarcoding (water) vs qPCR (water)	Water	80%	80%	80%	42%	42%

The assessment indicates that major savings are encountered when comparing metabarcoding of water samples with both conventional monitoring and qPCR detection. If only considering information obtained through settlement plates, such as being applied in the ARMS program (Obst et al. 2020), the conventional monitoring was less expensive that either of the eDNA techniques.

3.3 Pros and cons of metabarcoding for NIS monitoring

A summary of the pros and cons of implementing metabarcoding as a tool to monitor NIS in Danish harbors, is presented as a SWOT analysis (Table 3.1).

Table 3.1. Summary of the Strengths, Weaknesses, Opportunities and Threats (SWOT-analysis) of implementing metabarcoding for monitoring of NIS.

Strengths:	**Weaknesses:**
• eDNA based methods such as qPCR and metabarcoding are less invasive for the environment (Veilleux et al. 2021). • Metabarcoding detects more species than both conventional and qPCR sampling. • Metabarcoding detects rare species, including taxonomically challenging ones. • After initial investments, metabarcoding is more time- and cost efficient than both conventional methods and qPCR for NIS detection. • Use of metabarcoding for species detection does not require the specific taxonomical expertise necessary for morphological identification. • It is straight forward to replicate sampling and investigate changes in species composition along gradients (e.g., within and outside of harbors)	• Assessment of false positives and false negatives requires taxonomic expertise. This can however, also be seen as a weakness of conventional monitoring. • Ground-truthing with conventional methods of detected NIS is challenging for many species. • Reference libraries are still insufficient and for some species groups (e.g., macroalgae), still under development. • Barcoding regions can sometimes not distinguish between closely related species. • Currently not possible to conduct fast on-site species detection. • The origin (local vs remote) of DNA-material is associated with uncertainty. • DNA from shedding or accidental release, makes it difficult to know if a detected species is part of an established population. • Presence/Absence data → not quantitative as conventional methods. • To reduce the risk of false positive results, the risk of false negatives increases (i.e., removing species that were present, but in low concentrations).
Opportunities:	**Threats:**
• Fast and early detection of NIS is possible. • It is possible to standardize and reproduce data. • Data can easily be shared. • Data can be reanalyzed. • Metabarcoding provides semi-quantitative data → relative abundance and impact of NIS is possible to achieve. • Development of in situ / on-site NGS using Nanopore technology is ongoing. • The technological and data analytical development is very fast, giving promise of faster, cheaper, better, and more efficient metabarcoding based monitoring of NIS in the near future. • Increase sensitivity by optimizing and potentially increasing number of primer sets targeted towards relevant taxa. • Increase detection of NIS through higher sequencing depth and more replicates.	• Choice of pipelines affect the outcome. • Contamination during sampling and lab-analysis. • Lack of confidence with stakeholders to results when publishing doubtful results. • Lacking resources for development of reference libraries and for tests of protocols • Lack of taxonomic expertise hampers documentation of DNA-based results. • Data bases and reference libraries need curation and errors therein give false results. • Standard Operating Procedures are needed.

4 Conclusions

Based on our results and previous studies we find that:

- It is beneficial to continue the collection of eDNA material for reprocessing.
- qPCR based detection is a good supplement to the conventional methods of species detection. This is especially true regarding observations of planktonic and mobile NIS, such as crabs and fish, which are not detected by the conventional methods currently used in the Danish national monitoring programme. However, zoo- and phytoplankton, fish and crabs are only monitored to a limited extent because of the limited number of the qPCR detection systems, that have been developed and employed. If these species group are to be thoroughly monitored, the number of qPCR detection systems have to be significantly expanded either by national development or based on published detection systems (e.g. Hernandez et al. 2020), that should be evaluated and tested at Danish conditions prior to use.
- Metabarcoding has a good potential to monitor NIS in Danish waters, but the method applied in this study needs to be optimized to increase the level of certainty in NIS detection. The method is not limited to a predetermined number of NIS and additionally makes it possible to register other NIS, which are both common and new to Danish waters. However, compared to qPCR, the metabarcoding method appears to have a somewhat lower sensitivity to the detection of NIS, which promotes the risk of false negative results, associated with low abundance of NIS DNA and poor match / binding of the applied primers. Thus, there are some of the NIS registered by qPCR at many stations that metabarcoding only recorded a few times. Conversely, there is also a risk of false positive results.
- The observed uncertainties related to metabarcoding include:
    - Low abundance of a given NIS → low DNA signal from that NIS → underestimation of frequency of NIS (few stations). This can be partly overcome by increasing the sequencing depth, more replicates and higher filtration volume at each station.
    - Poor binding of applied primers. Customized primers towards specific taxa can be developed to raise the sensitivity for NIS detection. We performed metabarcoding with three primer sets. Agreement should be sought among EU countries/countries around the North Sea to optimize primer sets and use fewer primer sets.
    - Bioinformatic pipeline used. Agreement should be sought among EU countries/countries around the North Sea on a single optimized pipeline.
    - In Danish and regional waters, the existing reference libraries are incomplete for several taxonomic groups, especially arthropods.
- If these uncertainties are improved, data from metabarcoding have the potential to be used to assess how environmental conditions affect and define habitats. Further, it is of scientific interest to develop and test genetically based indices to be compared with existing biodiversity indices.
- The cost analyses showed major potential savings by replacing conventional monitoring with metabarcoding, while qPCR had similar costs to conventional monitoring. These figures should be taken with great caution as for all three methods, the cost and time provided are just estimates and unexpected issues, as e.g., unfamiliar species demanding longer time for

identification, redoing of DNA extraction, qPCR or the PCRs for metabarcoding may be needed, changing the input data to the comparisons considerably. The cost analysis should also consider the costs of developing and testing qPCR detection systems for additional NIS and compared to the costs of improving metabarcoding-based detection of NIS.

- The eDNA technique of metabarcoding have the potential to supplement the conventional monitoring.
- Using metabarcoding as a tool to detect rare species or NIS is indeed possible. However, in addition to the need for standardized bioinformatics pipelines, a second step is recommended to ensure that the detection is optimized with regards to 1) the approaches used for ASVs or clustering of OTUs; 2) taxonomical assignment method and threshold levels used and finally, 3) choice of the reference database.
- Finally, we identified that the level of agreement between metabarcoding and qPCR increases with the number of samples compared. This underlines the importance of replicated sampling.

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USE OF METABARCODING TO DETECT NON-INDIGENOUS SPECIES IN DANISH HARBORS

This report provides a comparison of non-indigenous species (NIS) in the marine environment detected using three different methods: conventional monitoring, and two eDNA molecular based methods: qPCR and metabarcoding. Conventional NIS detection is limited in early rapid detection of NIS, and eDNA are proposed as an alternative method, as eDNA is less invasive, is supposed to cover a larger area and potentially with a high level of detection. In the monitoring of NIS in six Danish harbors in 2021 by conventional methods and NIS specific qPCR detection systems for 23 species, eDNA was collected from three stations in each of the six harbors. This eDNA was used for metabarcoding with three different primer sets: 18S rDNA, cytochrome oxidase I (COI) and 12S rDNA, targeting eukaryotes, invertebrates and fish, respectively. The results show a higher number of NIS detected by metabarcoding followed by conventional and qPCR techniques. Only three NIS were found by all three methods, while metabarcoding found 23 unique species. While metabarcoding has advantages compared to conventional and qPCR analyses; metabarcoding results are sensitive to the applied bioinformatics pipelines which should be standardized and optimized along with the reference databases of the marine species in Danish and regional waters.

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