DNA-BASED MONITORING OF Non-Indigenous Species (NIS)

GEANS PILOT 2.

Interreg North Sea Region GEANS
European Regional Development Fund

EUROPEAN UNION

Genetic tools for Ecosystem health Assessment in the North Sea region

DNA-BASED MONITORING OF Non-Indigenous Species (NIS)

Version: 3.0

Project:

GEANS– Genetic tools for Ecosystem health Assessment in the North Sea region

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Date:

30 June 2023

Lead Partner:

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Funding:

Funding for this research was received through the North Sea Program of the European Regional Development Fund of the European Union. Cofinancing was provided by the Sand Fund of the Federal Public Service Economy (Belgium), Senckenberg Research Institute (Germany), and from the Danish EPA.

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How to cite this report?

Hablützel P., Martínez P. A., Bilsen A., Christodoulou M., Delacauw S., Deneudt K., Khodami S., Lagaisse R., Heynderickx H., Obst M., Sapkota R., Sundberg P., Uhlir C., Stæhr P. A. U., Winding A. (2023).
DNA-based monitoring of Non-Indigenous Species.

Content

1. Summary

The introduction of non-indigenous species (NIS) has a significant human-driven impact on aquatic environments, causing the loss of native species, ecosystem integrity, ecosystem services, and economic benefits. However, monitoring efforts for new NIS introductions in European waters have been limited and inconsistent. Traditional monitoring methods for NIS are labor-intensive, taxonomically biased, and often ineffective for early detection. To address these limitations, DNA-based methods have emerged as promising tools for NIS monitoring. Two main types of DNA-based monitoring for marine NIS species are currently used: metabarcoding, which detects a wide range of taxa, including cryptic species, and quantitative polymerase chain reaction (qPCR), which uses specific assays for targeted NIS detection. This report presents results from four pilot studies from harbors in Belgium, Germany, Denmark and Sweden that combine metabarcoding, morphological analysis, and qPCR techniques to test the applicability of DNA-based methods for NIS detection in harbors in comparison to traditional monitoring following OSPAR-HELCOM protocols. We aimed to evaluate both methods in terms of cost- and time effectiveness as well as accuracy and detection power for NIS.

From the result of the sub-pilot studies, we conclude that DNA-based methods proved to have several strengths, including the ability to detect more species than traditional methods and identify cryptic species. However, it was also noted that for some pilot studies, NIS species detected from DNA-based and traditional methods were complementary, with an overlap ranging from 0 to 58 %. The time and cost-effectiveness may widely range due to numerous factors, including the available expertise and the specific methods that were used. We found that DNA-based methods were (on a per sample basis) 20-93 % less time consuming and either 65 % less costly or 28 % more expensive. Additionally, DNA-based methods require less training, and enable rapid screening of bulk samples. However, some weaknesses of DNA-based methods were also experienced. DNA-based methods offer no real quantitative information, and although qPCR gives some inside through copy numbers, the method needs further ground truthing to assess comparability with traditional abundance estimates in monitoring. Quantitative PCR (qPCR) based essays however, did prove to have higher detection power of NIS in the Danish subpilot compared to traditional metabarcoding. False positive detections resulted from incomplete reference databases, insufficient taxonomic resolution of the DNA-markers used and contamination in the field or in the lab. Expert consultation is often needed to reveal such false positives. The choice of the bioinformatic pipeline used to process the raw sequencing data and obtain taxon matches showed to influence the number of NIS detected in Danish harbors. Despite these weaknesses, DNA-based methods offer opportunities for early detection of NIS, rapid assessments, and standardization across countries. Automation and integration with biodiversity informatics initiatives are potential advancements. However, challenges include limited trust in the results, biases in monitoring methods, and difficulties in ground truthing. DNA-based methods can be applied to various sample types and provide real-time data processing, facilitating timely decision-making. Integrating DNA-based methods with traditional approaches is recommended for a comprehensive understanding of NIS presence and abundance. Overall, DNA-based methods offer valuable tools for monitoring and identifying NIS, providing accurate, cost-effective, and real-time detection and contributing to our understanding of their genetic diversity and population structure.

2. Introduction

Introductions of non-indigenous species (NIS) are one of the most detrimental anthropogenic impacts on global aquatic environments, leading to loss of native species, 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 and HELCOM conventions indicate that new NIS continue to arrive in most countries at alarming rates. The assessments of trends are highlighted as very uncertain as lack of standardized monitoring provides NIS records which likely do not accurately reflect the time and location of the introduction.

Early and cost-efficient detection of new NIS introductions and secondary spread 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 uncertainties associated with the patchy distribution of low population sizes of invaders typical of the early stages of the invasion process. As a result, conventional techniques for NIS monitoring limits 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-efficient and sensitive methods to detect NIS. The use of bulk metabarcoding has gained attention as a promising tool to complement traditional methods for monitoring aquatic species for standardized biodiversity assessments. Sampling community DNA is a rapid and efficient way to capture the majority of organisms within a given area. By avoiding visual species observation, capture and direct sampling metabarcoding has the potential to greatly reduce cost and time, while aiding ecosystem conservation and management through improved detection of species (Knudsen et al., 2022; Staehr et al., 2022; Thomsen & Willerslev, 2015).

Figure 1: Sampling NIS in the harbor of Ostend (VLIZ).

Two main types of DNA-based monitoring of marine NIS species are currently being applied. Monitoring of marine species by DNA is to a large extent done by using metabarcoding of bulk or eDNA samples for the whole community or by targeting selected taxa using qPCR where specific primer-probes assays are developed for detection of a number of marine NIS species (Knudsen et al., 2022).

This report provides results from four NIS pilot studies conducted within the GEANS project. All of these have used metabarcoding in combination with conventional morphological analysis following OSPAR-HELCOM protocols, and one has furthermore included the use of the qPCR technique.

3. Pilot studies

For this pilot study, different sub-pilots acted autonomously while keeping pre-defined minimal standards in their methodology. This approach allowed us to accommodate requests by local stakeholders and to implement newly available technologies (e.g. nanopore sequencing). This implied that methodological comparisons among sub-pilots are difficult. Different sub-pilots used different sample types and cost-calculations were only possible for 2 sub-pilots. Four sub-pilot sites were selected: the harbor of Ostend (Belgium, VLIZ), the harbor of Rostock (Germany, Senckenberg), the Danish coast (Aarhus University), and the western coast of Sweden (SeAnalytics).

Table 1: Summary of sub-pilot locations, sample types, DNA technology and target regions.

Country	**Belgium**	**Germany**	**Denmark**	**Sweden**
Site(s)	Harbor of Ostend	Harbors of Rostock	Harbor of Esbjerg, Hirtshals, Frederikshavn, Aarhus, Fredericia, and Copenhagen	Harbor of Wallhamn
Sample types for morphological analysis	Settlement plates	Van Veen grab, scrape samples	Settlement plates, Van Veen grab, scrape samples	Settlement plates, scrape samples
Sample types for DNA-based analysis	Settlement plates, plankton samples	Van Veen grab,scrape samples	Settlement plates, Van Veen grab, scrape samples, water samples (eDNA)	ARMS, settlement plates, artificial habitat, scrape samples, plankton, water samples (eDNA)
DNA platform and technology	Oxford Nanopore Metabarcoding	Illumina MiSeq Metabarcoding	Illumina MiSeq Metabarcoding, qPCR	Illumina MiSeq Metabarcoding
Markers for DNA barcoding	COI, 18S	COI	COI, 18S, 12S	COI, 18S

1. Sub-pilot Belgium (harbor of Ostend)

a. *Design and monitoring objective*

We conducted a rapid survey of the fauna in the harbor of Ostend (Belgium) using traditional microscopic examination of the organisms collected from settlement plates. In parallel, we metabarcoded samples (settlement plates and plankton samples) at two genetic markers (the mitochondrial COI and nuclear 18S rRNA).

b. *Collection of samples*

The sampling methodology follows the OSPAR-HELCOM recommendation whenever possible (OSPAR, 2019). Settlement plates were deployed to sample benthic communities at three locations in the harbor of Ostend on the 16th of June 2020: Vuurtorendok (51.237634°N, 2.931726°E), Ponton Overzet (51.234216°N, 2.927157°E), and Marina Mercator (51.228220°N, 2.923981°E) to cover different microhabitats.

Figure 2. Sampling locations for NIS pilot in the harbor of Ostend (VLIZ).

Settlement plates were made of sanded gray PVC (15 x 15 cm) and suspended in the water column on a pier. One or two settlement plates were deployed per location, with the uppermost plate being suspended at 1 m depth and the lower plate at 7 m (at Marina Mercator, the shallow depth only allowed the deployment of the upper plate). We retrieved all settlement plates after two months. A preliminary screening of live organisms visible on both sides of each settlement plate was carried out to morphologically identify species, especially tunicates, whose morphology may be largely destroyed after preservation.
Next, the benthic community attached to each side of each settlement were scraped off with a steel blade and separately preserved in DESS. Note that in a number of samples, tunicates were extremely abundant, and only a subset of the individuals were preserved for later analyses.
In June of 2020, zooplankton samples were collected at each of the three locations using a vertical free-fall plankton drop net (Apstein net) with a mesh size of 10 µm. The net was dipped three times at each location, and the plankton retrieved at each dip was first poured over a 300 µm mesh and then over a 100 µm mesh, yielding two size categories per replicate per location. Plankton samples were immediately preserved in 30-40 ml of DESS upon collection. This sampling design was repeated at the same three locations in August of 2020 at the time that settlement plates were being retrieved. This resulted in 36 zooplankton samples in total.

c. *Lab processing*

i. Morphological analysis

Following preservation, each scrape sample (corresponding to one side of a settlement plate) was investigated under a stereomicroscope to identify as many taxa as possible to as detailed taxonomic level as possible. Morphological identification of the preserved organisms was based on Hayward & Ryland (2017). Afterwards, samples were stored at room temperature until DNA extraction for metabarcoding. At least one specimen of each species was kept as a voucher specimen.

Zooplankton samples were not identified morphologically.

ii. DNA extraction

The DNA extraction protocol for the settlement plate communities was largely based on that of the Global ARMS (Autonomous Reef Monitoring Structures) Project of the Smithsonian Institution (https://www.oceanarms.org). DNA extraction was carried out with the DNeasy® PowerSoil® Pro kit of Qiagen, with the added step of incubating each subsample in 50 µL of a 10 mg mL-1 proteinase K solution overnight at 56 °C after the addition of solution CD1 from the kit. The CTAB protocol by Cullings (1992) was used for DNA extraction of plankton samples (with slight modifications). Prior to extraction, the samples were bead-beaten using 0.1 mm and 0.5 mm glass beads. For more detailed information on the DNA extraction, we refer to Bilsen (2021).

iii. PCR amplification, library preparation and sequencing

All samples from the zooplankton and the settlement plates were metabarcoded twice, once with the F-566/R-1200 primer pair (which targets the V4-V5 region of the 18S rRNA; Hadziavdic et al., 2014) and another time with the dgLCO1492/dgHCO2198 pair (targeting the Folmer region of the COI gene, Meyer et al., 2005). We opted for these two regions because the COI gene is generally considered a universal genetic marker for animal species (Valentini et al., 2009; Taberlet et al., 2012) due to its comparatively high interspecific and low intraspecific genetic distances (Bucklin et al., 2011), while the 18S region is more conserved allowing its application for a broader range of taxa than the COI gene. For each set of primers, two consecutive PCRs usingPhire Hot Start II polymerase (2X) (ThermoFisher Scientific) were carried out on the samples before attachment of sequencing adapters. In the first reaction, the region of interest was amplified with the aforementioned primer pairs. Primers had a 5'-tail to which primers of the Oxford Nanopore Technologies PCR Barcoding Kit (SQK-PBK004) would bind in the second PCR reaction in order to label individual samples with unique barcodes for the purpose of multiplexing. Because not more than 12 unique multiplexing barcodes from the SQK-PBK004 could be used at once, the samples analysed in this study had to be split into two runs. In the first run, all samples from the settlement plates plus an extra replicate from Ponton Overzet (one of the two most speciose samples) were sequenced alongside a negative control that had likewise been subjected to the same PCR amplification protocol. In the second run, all plankton samples (both 18S and COI) were sequenced. Note that due to constraints in time and resources, not all extracted samples could be sequenced. The barcoded libraries were sequenced on the MinION Mk1B sequencer (Oxford Nanopore Technologies, UK, 2021). In the first run, 680,020 reads were generated on a FLO-MIN111 flow cell. In the second run, 7.53 million reads were generated on a FLO-MIN106 flow cell (Oxford Nanopore Technologies).

d. Bioinformatic processing

For the harbor of Ostend samples, basecalling was done in Guppy (v4.3.4, Oxford Nanopore Technologies Ltd., UK), followed by read demultiplexing with qcat (v1.0.1, , Oxford Nanopore Technologies Ltd., UK) and filtering of the reads with NanoFilt (v2.8.0; De Coster et al., 2018). Next, a density based clustering approach implemented in ASHURE (V1.0.0; Baloğlu & Chen, 2021) was used for error correction (by consensus of clusters) and potential chimeras were removed with VSEARCH (Rognes et al., 2016). Taxonomy annotation was performed with BLASTn using the SILVA 138 and the PR2 databases for 18S and the MIDORI2 database for COI.

e. Data-analyses

Taxonomic annotations at species level were filtered for alignment length > 600 base pairs and alignment match of > 98 % for both 18S and COI. Non-indigenous species were identified by comparing the obtained species list with a curated list of species known to occur in Belgium (BeRMS, VLIZ Belgian Marine Species Consortium (2010 onwards)), a list of known marine NIS in Belgium (Verleye et al., 2020) and the world register of introduced marine species (WRIMS, Rius et al., 2023).

f. Results

i. Morphological identification of settlement plate communities

Morphological examination of the communities on the settlement plates resulted in the discovery of 4 NIS . In addition, a fifth species, *Magallana gigas*, was observed in large numbers at the harbor wall during sampling (Table 2). *Diplosoma listerianum, Amphibalanus improvisus,* and *Monocorophium sextonae* were also identified. But these are cryptogenic species (Hayward & Ryland, 2017; Verleye et al., 2020) and not included in the OSPAR-HELCOM list. Several taxa could not be identified at species levels because either the choice of preservative was not suited for that particular taxon, or because no adequate identification literature was available, or because only juvenile stages were found.

Table 2: NIS found via morphological examination.

Phylum	**Class**	**Order**	**Species**
Arthropoda	Malacostraca	Amphipoda	*Caprella mutica*
Arthropoda	Thecostraca	Balanomorpha	*Austrominius modestus*
Chordata	Ascidiacea	Phlebobranchia	*Ciona intestinalis*
Chordata	Ascidiacea	Stolidobranchia	*Molgula manhattensis*
Mollusca	Bivalvia	Ostreida	*Magallana gigas*

ii. Metabarcoding of settlement plate communities

Metabarcoding of the 18S rRNA gene region from settlement plate samples recovered 7 NIS (Table 3). All of the NIS have been previously reported for Belgium (Verleye et al. 2020).

Table 3: NIS detected on the settlement plates via metabarcoding (18S and COI). Max. match is the highest percentage match of the query sequence with the reference database for that species.

Phylum	**Class**	**Order**	**Family**	***Species***	**Barcode**	**Sample type**	**max. match**
Annelida	Polychaeta	Sabellida	Serpulidae	*Ficopomatus enigmaticus*	18S	plankton, plates	99.848
Arthropoda	Copepoda	Calanoida	Acartiidae	*Acartia tonsa*	18S	plankton	99.836
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus marinus*	18S, COI	plankton	99.035

Arthropoda	Copepoda	Calanoida	Temoridae	*Eurytemora carolleeae*	18S	plankton	98.873
Arthropoda	Copepoda	Cyclopoida	Oithonidae	*Oithona davisae*	18S	plankton	98.548
Arthropoda	Thecostraca	Balanomorpha	Balanidae	*Amphibalanus amphitrite*	18S, COI	plankton, plates	99.848
Arthropoda	Thecostraca	Balanomorpha	Elminidae	*Austrominius modestus*	18S	plankton, plates	100
Bacillariophyta	Bacillariophyceae	Thalassiosirales	Thalassiosiraceae	*Thalassiosira punctigera*	18S	plankton	98.86
Bacillariophyta	Bacillariophyceae	Triceratiales	Triceratiaceae	*Odontella sinensis*	18S	plankton	98.867
Chordata	Ascidiaceae	Phlebobranchia	Cionidae	*Ciona intestinalis*	18S	plankton, plates	99.838
Chordata	Ascidiaceae	Stolidobranchia	Molgulidae	*Molgula manhattensis*	18S	plankton, plates	100
Chordata	Ascidiaceae	Stolidobranchia	Styelidae	*Botrylloides violaceus*	18S	plankton, plates	100
Chordata	Ascidiaceae	Stolidobranchia	Styelidae	*Styela clava*	18S	plankton, plates	99.355
Ctenophora	Tentaculata	Lobata	Belinosidae	*Mnemiopsis leidyi*	18S	plankton	99.18
Mollusca	Bivalvia	Ostreida	Ostreidae	*Magallana gigas*	18S, COI	plankton	99.359
Mollusca	Bivalvia	Venerida	Mactridae	*Mulinia lateralis*	18S	plankton	99.197
Mollusca	Bivalvia	Venerida	Veneridae	*Petricolaria pholadiformis*	COI	plankton	99.088

iii. Metabarcoding of plankton communities

Metabarcoding of the 18S rRNA and COI gene region of plankton samples recovered 17 NIS (Table 3). 16 of those were identified with 18S, one with COI and three with both barcoding regions (Table 3). Among the taxa detected, two are reported for the first time for the Belgian part of the North Sea: *Eurythemora carolleeae* and *Oithona davisae*.

iv. Overlap among different monitoring methods

Comparing the results from the morphological examination with those of metabarcoding is challenging because of the varying levels of taxonomic resolution achieved across the samples. However, when comparing organisms identified to species level, it becomes clear that the two methods are highly divergent, with four species (*Austrominius modestus*, *Ciona intestinalis*, *Molgula manhattensis* and *Magallana gigas*) being recorded by both methods, and one species (*Caprella mutica*) only registered by morphological examination (Figure 5).

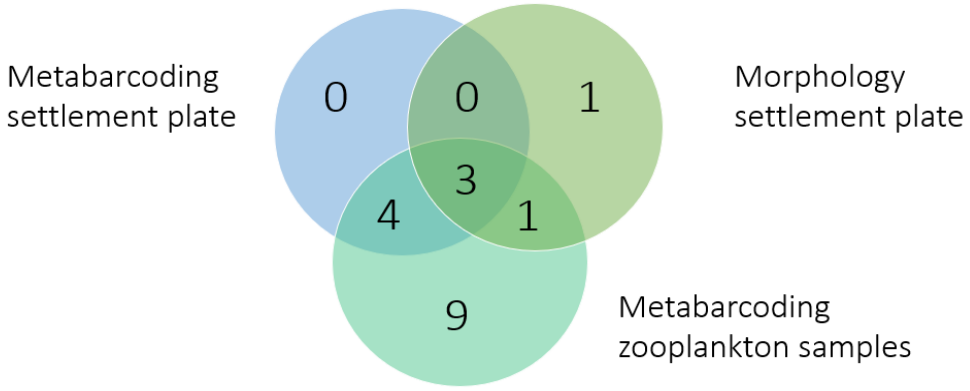

Figure 3. Comparison of the number of species detected using either identification method.

v. time and cost comparison

For time and cost comparison we took into account both staff cost and purchase costs of consumables. For morphological analysis time spent on analyzing 10 samples was recorded and for genetic analysis time spent on extraction, PCR and sequencing of 22 samples was recorded. A junior expert performed both analyses, therefore the hourly rate of staff costs for both analyses was equal. For morphological analysis purchase cost of ethanol and vials to store sample material was calculated for 22 samples. For genetic analysis extraction kits, cost of both PCRs and clean-ups and library preparation kit and flowcells purchase costs were calculated for 22 samples. In a next step, purchase and staff costs were summed for each method (=total cost), and divided by the number of samples analyzed for each method (=cost/sample). Gain in time and cost was calculated as (morphological-genetic)/morphological. Metabarcoding proved to take 93 % less time while it was 65 % cheaper (Table 4).

*Table 4.Summary of time and cost calculation for NIS samples in the harbor of Ostend. Total cost includes both staff cost, calculated as hours spend on all samples * hourly rate, and purchase costs.*

	Number of samples	Level of experience	Total time (h)	Time/sample (h)	Total cost (€)	Cost/sample (€)
Morphology based	10	Junior expert	135	13,5	4429	443
DNA-based	22	Junior expert	22	1	3452	157
DNA-based versus morphology				**93 % less time**		**65 % cheaper**

g. Conclusions

A wide range of NIS were recovered through the combined application of metabarcoding zooplankton and settlement plates and morphological examination of the settlement plate communities. The DNA-based method resulted in the discovery of 17 NIS. For the settlement plate samples, metabarcoding was able to detect seven NIS compared to five NIS detected using morphological analysis, with both methods overlapping in detection of three NIS species, and uniquely discovering 4 (metabarcoding) and 2 NIS (morphological analysis) not detected by the other method. Metabarcoding of plankton samples led to an additional detection of 9 NIS, not detected with metabarcoding or morphological analysis of the plates. Metabarcoding of zooplankton samples was also able to detect all NIS detected by metabarcoding of settlement plates, and four out of five NIS detected by morphological analysis of settlement plates. The discrepancy with morphology-based analysis can be attributed to the fact that many species were difficult to identify (e.g. annelids or small tunicates) and that many species were present as juvenile stages (e.g. molluscs). No plankton samples were analysed morphologically due to the lack of expertise and resources. However, the plankton samples were especially a rich source of NIS species. Several non-indigenous copepod and diatom species were detected in these samples along with a number of benthic species. The latter were putatively collected in their planktonic larval stage.Morphological analysis of plankton samples possibly could have detected more NIS that metabarcoding of the plankton samples might have missed.

Metabarcoding enabled the identification of difficult species to lower taxonomic resolution than was possible by morphological analysis. Two examples are; i. the *Botrylloides* species recognized on the settlement plates during morphological analysis which was subsequently identified to the invasive *B. violaceus* via metabarcoding, and ii. the serpulid worms challenging identification experienced during morphological examination, which was later assigned to *Ficopomatus enigmaticus* using metabarcoding.

The use of metabarcoding of plankton samples has the advantage that it can both detect planktonic larval stages (meroplankton) as well as holoplankton that spends its entire life cycle as plankton. These planktonic groups organisms are notoriously difficult to identify visually due to their small size and lack of discernible adult diagnostic characters displayed by meroplankton larvae. In this study we were able to detect plankton stages of benthic fauna like *M. gigas* larvae using both COI and 18S rRNA mariners, and 18S metabarcoding of plankton samples was also able to detect the shell-boring *Ficopomatus enigmaticus.* Morphological observations of plankton samples were not possible due to lack of in-house expertise, metabarcoding of these samples was able to detect non-indigenous zooplankton species *Acatia tonsa, Eurythemora coralleae, Oithona davisae* and *Pseudodiaptomus marina* (Verleye e al., 2020) and two non-indigenous phytoplankton species for Belgium (Verleye et al., 2020).

Further, metabarcoding of plankton samples and bulk material from settlement plates was able to detect a native endoparasite copepod, *Lichomolgus canui,* and an apicomplexan parasite *Lankesteria sp.,* both specific to tunicates (not shown in tables). This highlights another advantage of DNA-based methods over morphological analysis as endoparasitic species are often overlooked in traditional analysis and can have devastating effects on host populations.

However, we also experience a number of weaknesses of metabarcoding. Multiple closely related species may share a (nearly) identical COI or 18S rRNA marker region, making their distinction challenging. This problem is especially important in the detection of NIS, which may be closely related to native species (Duarte et al., 2021). For example, we detected the tunicate endoparasite *Lankesteria halocynthiae* using settlement plate 18S metabarcoding, which was not previously recorded for the North Sea region according to WoRMS. However, it is unlikely that the corresponding reads represent an introduced organism. Lecudinid apicomplexans such as *L. halocynthiae* are highly host-specific (Rueckert et al., 2015), and together with the low database match of the corresponding reads, this fact suggests that the species detected from the plates may well have been a native organism. A similar example is *Styela plicata* which showed up in the raw results. This detection was likely a misidentification with the congeneric *Styela clava*, which has been recorded from the Belgian part of the North Sea (Verleye et al., 2020) because of the low read match of the generated sequences with those in the SILVA database. The difficulties of the 18S rRNA gene region in discriminating some species stems from the fact that it has a slower rate of mutation leading to smaller differences between closely related species (Bucklin et al., 2011) and a tendency to underestimate biodiversity (van der Loos & Nijland, 2020). For this reason, COI metabarcoding is preferred for macrobenthos communities (van der Loos & Nijland, 2020).

This study demonstrates the capacity of metabarcoding to survey communities for non-indigenous species in a cost-effective way producing a large array of species. However, because of the disparity between morphological and metabarcoding species lists and the potential inaccuracies using metabarcoding (false positives, false negatives) and lack of abundance information, we propose to monitor NIS by combining both methods. Metabarcoding can help taxonomists in identifying small, (semi-) cryptic and or poorly-know species. However, since every step of the metabarcoding process from sampling, to lab protocols, primer choice and bioinformatics processing and reference database choice and completeness and correctness can bias results, morphological observations can be important for ground-truthing and supplementing metabarcoding data.

2. Sub-pilot Germany (harbors of Rostock)

a. *Design and monitoring objective*

We conducted a rapid survey of the macrobenthic fauna in the harbor of Rostock area, located in the estuary of the Warnow river in Germany using Van Veen grabs and sheet pile wall scraping. The collected specimens were identified morphologically and, in parallel, we metabarcoded samples at the mitochondrial DNA barcode region COI.

b. *Collection of samples*

The sampling methodology followed the OSPAR-HELCOM recommendation whenever possible (OSPAR, 2019). Van Veen grab and sheet pile wall scrape samples were taken in triplicates (A-C) at three different locations within the greater harbor area: Station HRO-1 (54°05'40.3"N 12°07'00.8"E; marina in city harbor, southernmost location, strongest influence of the river Warnow, lowest salinity), HRO-2 (54°08'39.3"N 12°05'49.9"E; international port), HRO-3 (54°10'50.7"N 12°05'27.7"E; marina in Warnemünde, in immediate proximity of the Baltic Sea, highest salinity). In 2020, a total of nine grab samples and nine scrape samples were taken. For quantitative comparison between years, a total of six replicates per station were taken in 2021, resulting in a total of 18 sheet pile wall scrape samples. No grab samples were taken in 2021. All samples were immediately sieved after sampling with a 0.5 mm mesh size sieve.

Figure 4. Sampling locations for the NIS pilot in the harbor of Rostock (Germany).

c. *Lab processing*

Traditional morphological examination of collected samples was conducted by the Institute of Applied Ecosystem Research (IfAÖ, Neu Broderstorf, Germany). Collected biological specimens were identified at species level when possible and kept in 98 % ethanol. For metabarcoding, the previous morphologically examined sample was divided into 2 x 300 mL to retain an unprocessed subsample as a backup. Subsequently the nine scrape and nine grab samples were homogenized with a commercial high-performance kitchen mixer. DNA was subsequently extracted from the homogenizate using the PowerSoil Pro Kit (Qiagen) following the manufacturers recommendations. The following settings were used for the PCR cycle: Initial denaturation at 98°C for 3 min, x 25 cycles of denaturation at 98°C for 30 s, primer hybridization at 50°C for 30 s, elongation at 72°C for 30 s, and a final elongation step at 72°C for 5 min. From each successful amplification, 1 µL of product and a unique combination of dual Nextera-compatible IDT for Illumina 10bp was used for the second PCR with identical settings, but only 13 cycles in total. Successful amplifications were purified and normalized using the SequalPrep Normalization Plate 96 kit (Invitrogen), and 2 µL of each product was added to a pooled library. The concentration of the final library was measured using the Qubit 3.0 Fluorometer (Invitrogen), and the molarity was measured using the Collibri Library Quantification Kit (Invitrogen). The pooled library was denatured and 20 % PhiX genomic control DNA was added before a test sequencing run with a MiSeq Reagent

Nano Kit v2 (250 cycles paired-end) and a final run with a MiSeq Reagent Kit v3 (300 cycles paired-end) on a high-throughput Illumina MiSeq sequencing platform.

d. Bioinformatic processing

For harbor of Rostock samples, the de-multiplexed NGS reads were trimmed by primer's sequences using BBmap (sourceforge.net/projects/bbmap/). Further the Illumina reads were de-noised, truncated to make contigs, filtered by length and quality scores, chimera detected and de-replicated to high resolution ASVs using DADA2 pipeline (Callahan et al., 2016). A costume script (SGN Metabarcoding pipeline) was used to blast the ASVs against the NCBI database incorporating BLASTn pipeline. Total of ten best blast hits were retrieved and pooled with GEANS reference library of macrofauna and this merged dataset was used as final costume BLAST database (db) to assign the best and closest taxonomic assignment to each ASV including the percentage identity, query coverage, length of the fragment, GenBank/reference library accession number and number of reads per library. All ASVs were checked against WoRMS (WoRMS Editorial Board, 2023) to ensure accurate taxonomic assignment. Taxonomic annotations were assumed to be correct at species level after a two-step-quality-control of the database match by filtering ASVs with >97 % percent identity (*pident*) and >90 % query coverage (*qcovs*).

e. Data-analyses

The community analyses were performed at species (aggregated ASVs), order and phylum levels using different R packages: vegan (Okansen et al. 2023), limma (Ritchie ME, 2015), dada2 (Callahan 2016), dada2pp (Martinez Arbizu, 2020), pairwiseAdonis (Martinez Arbizu, 2020), ggplot2 (Wickham H., 2016), UpSetR (Conway, 2014) and DECIPHER (ES Wright, 2016). The results of metabarcoding and morphology have been compared for the present/absence of species to evaluate shared and unique species in each method.

f. Results

i. Method comparison

Morphological identification conducted by the IfAÖ yielded 74 macrozoobenthic taxa, 57 identified to species level. Sequencing of metabarcoding samples generated ~10.7 million reads, of which ~2.8 million reads were removed as chimeras from the dataset, resulting in ~7.9 Million reads of interest. After bioinformatic processing, 910 unique macrofauna amplicon sequence variants (ASVs) were categorized as target taxa, which yielded 102 single taxa, 79 on species level. Most of the ASVs were 313 bp (base pairs) in length. ASV outliers were identified according to the frequency distribution of the following parameters: sequence length (read length), percent identity (*pident*) and query coverage (*qcovs*) of the sequence length compared to the best matching GenBank sequence. Outliers are defined as values that fall outside 1.5 times the quartile distance. The lower limit for length was 226 bp, for *pident* 71.7 % and for *qcovs* 75 %. All ASVs that were below the threshold for at least one parameter were removed.

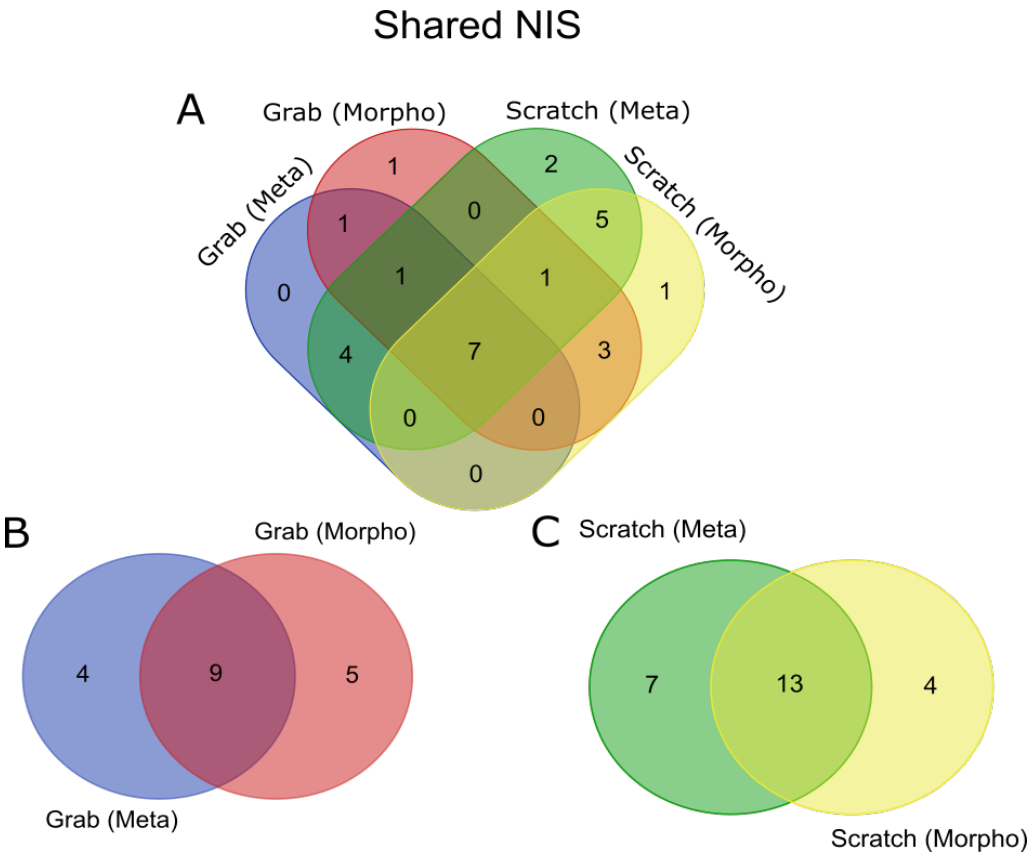

Figure 5. Venn diagrams show the number of common macrozoobenthic NIS identified by a combination of methods (A), in Grab- (Grab, B) or Scrape (Scratch, C) samples, and by morphological determination (Morpho), or detected using the Metabarcoding method (Meta).

Among the 102 taxa a total of 26 NIS (Figure 5A, Table 5) were detected, 18 NIS within the grab samples (Figure 5B) and 24 NIS within the scratch samples (Figure 5C). Here, only taxa that could be unambiguously identified morphologically and/or had trustful sequence matches to species level and are known NIS for the region are counted. NIS taxa identified exclusively morphologically include *Nippoleucon hinumensis, Cordylophora caspia, Arachnidium lacourti, Streblospio benedicti,* and *Amathia imbricata*. The exclusively genetically discovered NIS taxa are *Balanus trigonus, Euplana gracilis, Sinelobus vanhaareni, Austrominius modestus, Marenzelleria viridis* and *Mulinia lateralis*.

Table 5. NIS detected in grab- & scrape samples by metabarcoding (Meta,) and/or identified by taxonomic identification (Morpho).

NIS	GRAB SAMPLES		SCRAPE SAMPLES	
	Meta	Morpho	Meta	Morpho
Alitta succinea	X	X	X	X
Amathia gracilis	X	X	X	X
Amathia imbricata				X
Amphibalanus improvisus	X	X	X	X
Arachnidium lacourti		X		X

Austrominius modestus	X		X	
Balanus trigonus	X		X	
Cordylophora caspia		X		X
Euplana gracilis	X		X	
Ficopomatus enigmaticus			X	X
Gammarus tigrinus		X	X	X
Grandidierella japonica	X	X		
Hemigrapsus takanoi			X	X
Marenzelleria viridis			X	
Melita nitida	X	X	X	X
Mulinia lateralis			X	
Mya arenaria	X	X	X	X
Mytilopsis leucophaeata	X	X	X	X
Nippoleucon hinumensis		X		X
Palaemon elegans			X	X
Potamopyrgus antipodarum			X	X
Rhithropanopeus harrisii	X	X	X	X
Sinelobus vanhaareni	X		X	
Streblospio benedicti		X		
Telmatogeton japonicus			X	X
Tubificoides heterochaetus	X	X	X	

Due to being part of a larger study, calculation of cost and time between traditional morphological and DNA-based methods was not possible.

g. Conclusions

DNA-based and traditional monitoring methods detected about the same number of species (21 vs. 20). While 15 NIS were detected by both methods, six were only detected genetically (*Balanus trigonus, Euplana gracilis, Sinelobus vanhaareni, Austrominius modestus, Marenzelleria viridis* and *Mulinia lateralis*) and five were only detected morphologically (*Nippoleucon hinumensis, Cordylophora caspia, Arachnidium lacourti, Streblospio benedicti,* and *Amathia imbricata*). DNA-based monitoring was able to identify NIS that are difficult to detect or identify morphologically. It also has high potential for automation and can therefore easily be scaled up when sample size increases for large surveys. However, DNA-based methods are still limited by incomplete reference libraries and the inability to deliver accurate quantitative results. In addition, false positive detection may occur more often in DNA-based monitoring. Our study also showed that some species are difficult or impossible to detect with the DNA-based methods that we used. For these reasons, we recommend to use DNA-based methods as a tool for rapid screening and use it in combination with traditional methods when the NIS monitoring should be more exhaustive or when morphological ground truthing for specific NIS is required.

6. Subpilot Denmark

a. *Design and monitoring objective*

This report provides a comparison of non-indigenous marine species (NIS) detected using three different methods: 1. Conventional methods with microscopic identification of species, 2. NIS specific qPCR based detection systems for 23 species based on qPCR of DNA samples (Staehr et al. 2022b), 3. DNA metabarcoding with three different primer sets: 18S, cytochrome oxidase I (COI) and 12S, targeting eukaryotes, invertebrates and fish respectively.

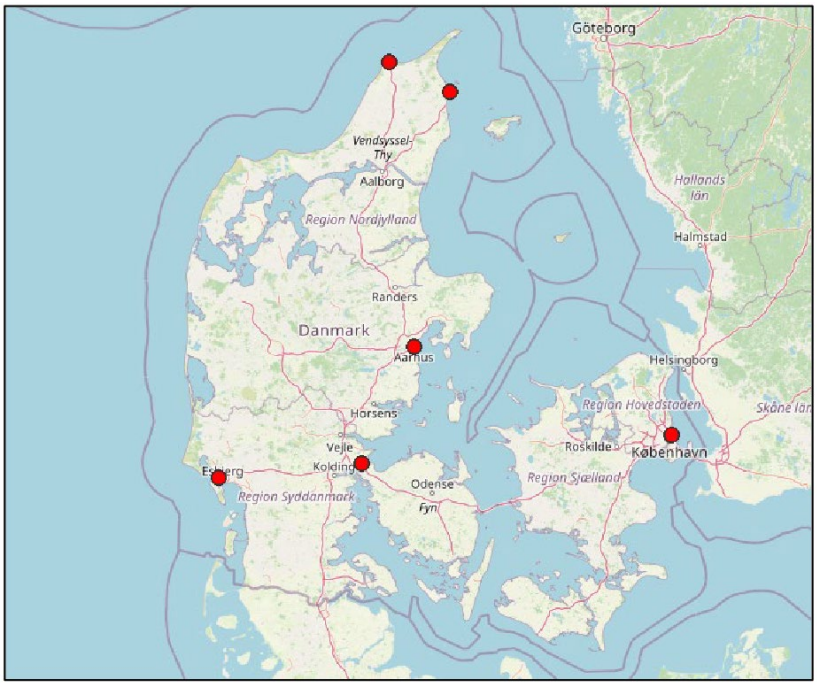

Figure 6. Sampling for NIS detection was performed in six major harbors. In each harbor three stations were sampled.

b. *Collection of samples*

Sampling was done at three stations in each of six selected major Danish harbors (Esbjerg, Hirtshals, Frederikshavn, Aarhus, Fredericia, and Copenhagen) providing a total of 18 stations. Sampling was only conducted in the industrial sections of the harbors. Details of the sampling program are described in Staehr et al. (2022b).

At each station, a vertical series of settlement plates 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, central and 1 m below surface). Upon retrieval, plates were preserved in ethanol at a final concentration of 70 %. In the laboratory, photos of each plate and species were identified. Later, biological material was scraped off for DNA extraction.

One sediment sample per station was taken with a Van Veen grab and immediately sorted through a 1 mm sieve. Remaining material was preserved in 96 % ethanol for final concentration of 70 % for later species identification.

Material associated with hard structures in harbors was collected by scraping using a 10 cm wide handheld scraping device. Three scrapings (app. ½ m) were made in each harbor. Collected material was sorted through

a 1 mm sieve and preserved in ethanol at a final concentration of 70 % for later species identification.
Water sampling for eDNA analyses generally followed the technical guideline TA 30 (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 Sterivex filters with 2 replicates per station. The filters were flash frozen in liquid nitrogen and upon arrival to the lab stored at -80 °C until DNA extraction.

c. Lab processing

i. Morphological analysis

All collected material from settlement plates, sediment samples and scrapings was identified in the laboratory by a trained taxonomist to the lowest possible taxonomic level using a stereomicroscope.

ii. eDNA positive controls

For positive control samples and ability to create standard curves for qPCR, DNA from the 23 individual NIS were needed. This DNA was extracted from tissue DNA. Unicellular and microscopic NIS were cultured in the lab followed by centrifugation (7000 rpm for 10 min) to concentrate cells and tissue prior to DNA extraction. Larger NIS specimen tissue obtained either from our sampling campaigns or from national and international colleagues and the species identification varied by a trained taxonomist. DNA was extracted from the animal parts avoiding gut, mouth, or skin parts, and subsequently subjected to grinding using mortar, pestle and liquid nitrogen. Macroalgal material was treated similarly. The collected tissue was used for DNA extraction using the DNeasy Blood & tissue kit (QiAGEN) following the manufacturer 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 stored at −20°C prior to downstream processing.

iii. DNA extraction from Sterivex filters

DNA extraction from the filters was carried out using the DNeasy Blood & tissue kit (Qiagen) with 'spin-columns', 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 filter with ATL and proteinase K was incubated on a rotor in a heating cabinet at 55°C (± 1°C) for 4 -24 hours until lysis was complete. Further steps in the extraction followed the manufacturer's protocol. Extracted DNA was split into several replicates and stored at -20 °C until used for quantitative PCR.

iv. DNA extraction from settlement plates

DNA was extracted from the upper and lower plate surface separately. Settlement plates were gently removed from the box with ethanol to a flow hood at sterile conditions to avoid DNA contamination. Samples were collected from 5 different spots of individual settlement plates into a 50 ml tube. Collected samples were centrifuged at 3000 rpm for 5 min to remove supernatant ethanol. Further, samples were air dried to remove traces of ethanol for 1-2 hours at room temperature, and subsequently stored at -20 °C until further processed for DNA extraction. Samples were lyophilized for 24 h and then grounded using a bead beater. In total 10-15 metal beads of 2.4 mm diameter were used to ground three cycles of 30 s at 4 m/s speed using a bead mill homogenizer (Bead 301 Ruptor Elite, Omni International). Once ground, 250 mg of each sample was used for DNA extraction using the DNeasy PowerLyser PowerSoil kit (QIAGEN), following the manufacturer's protocol. DNA concentration was quantified using a Qubit 4.0 fluorometer. DNA of settlement plates was pooled for each station prior to qPCR and metabarcoding detection.

v. qPCR

TaqMan qPCR was used for the detection and quantification of NIS in eDNA from water and bulk DNA from settlement plates. Amplification was performed in a BioRAD Real-time PCR system (Life Technologies) using 96-well plates. The primers and probes developed and further described by Knudsen et al. (2022) were used for detection and quantification. 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 of NIS tissue DNA were used, respectively. Thermal cycles in the qPCR consisted of an initial denaturation 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. A standard curve was obtained by plotting the log quantification of cycle (Cq) values of the amount of NIS PCR product DNA added in a 10-fold serial dilution (10^-4 to 10^−11).
The 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/µl), 0.5 µl each of forward and reverse primers (10 µM stock), 14.25 µl of water, and 5 µl of PcrBio HiFi buffer, PCRBIO HiFi Polymerase (2U/µl) (PCR Biosystems). PCR thermal cycles consisted of an initial denaturation at 95°C for 1 minute followed by 35 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 60 s, and final elongation 72 °C for 5 minutes. The PCR products obtained were purified using a QIAquick PCR Purification Kit (Qiagen, catalog number 28104). For NIS with amplicon size less than 100 bp, we used Gel and PCR clean-up columns (Ma- cherey-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) using DNA concentrations of the serial dilutions.

vi. Metabarcoding

In total 36 samples from water filter samples, and 14 samples from settlement plates 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 (SSU F04, SSU R22, Fonseca et al. 2010), 12S rDNA (MiFish-F, MiFish-R, Miya et al. 2015) and COI region of mitochondrial DNA (mlCOIintF, jgHCO2198, Leray et al. 2013) to study eukaryote, fish, and invertebrate communities respectively. Sequencing was carried out using the Illumina MiSeq platform at DCE, Aarhus University.

Table 6.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	

d. Bioinformatic processing

The DNA reads obtained from the Illumina MiSeq runs were analyzed using a custom-made “VLIZ pipeline” and a “DCE pipeline”. In the “VLIZ pipeline” the initial quality control and filtering of pair-end reads was done

using FastQC (Andrews, 2010) and Trimmomatic (Bolger et al., 2014). Forward and reverse primers were trimmed and reads less than 200 bp were excluded. Paired- end sequences were merged using PANDAseq (Masella et al., 2012). VSEARCH was used for removing 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 dataset, MIDORI2 database for COI and Mifish/12S (Iwasaki et al., 2013; Machida et al., 2017; Quast et al., 2013). Taxonomic assignments were ranked by e-value and the first hit was accepted above thresholds of 2 % (18S and 12S) or 3 % (for COI) for alignment match and 180 bp for alignment length.
To compare the results, a second bioinformatics "DCE pipeline" using QIIME2 (Bolyen et al., 2019) was used. The DADA2 (Callahan et al., 2016) plugin in QIIME2 was used with default parameters except reads trimmed for primer sequence, and reads truncated after 230 bp. For 18S, the resulting amplicon sequence variants were classified using the QIIME2 naïve Bayesian classifier trained on 99% Operational Taxonomic Units from the SILVA rRNA database (v. 138) after trimming to the primer region (Quast et al., 2013). However, COI amplicons were blasted against the BOLD database using sequence ID tool (www.gbif.org) and blasted against Mitofish database (Iwasaki et al., 2013). Less abundant ASVs (with less than 10 reads) were filtered out before blasting for COI and Mifish. Blast taxa with high similarity and coverage (> 97%) at species were used for downstream processing.

e. Data-analysis

Statistical analyses and data visualizations were performed in R v.4.2.1 (R Core Team, 2022). Diversity based analysis (non-metric multidimensional scaling and rarefaction analyses) was carried out using the vegan (Oksanen et al., 2019) and phyloseq (McMurdie et al., 2013) packages.

f. Results

i. Comparison of methods

The eDNA and bulk DNA 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 and bulk DNA methods (metabarcoding DCE pipeline, metabarcoding VLIZ pipeline 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 referred to as "Danish NIS list" (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 include 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 23 species with qPCR detection systems. Using metabarcoding we identified 30 and 40 NIS with the DCE and VLIZ pipeline, respectively (Figure 7).

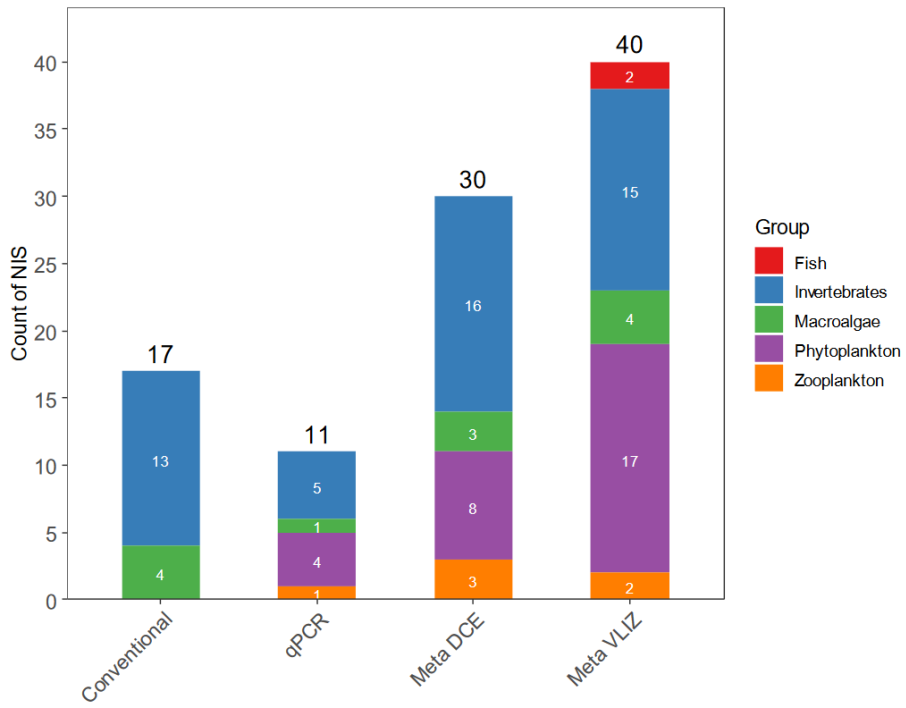

Figure 7. Number of NIS within five major groups detected in six Danish harbors during sampling in June and September 2021, using different methods. Results from metabarcoding (Meta) are shown for the two bioinformatic pipelines used.

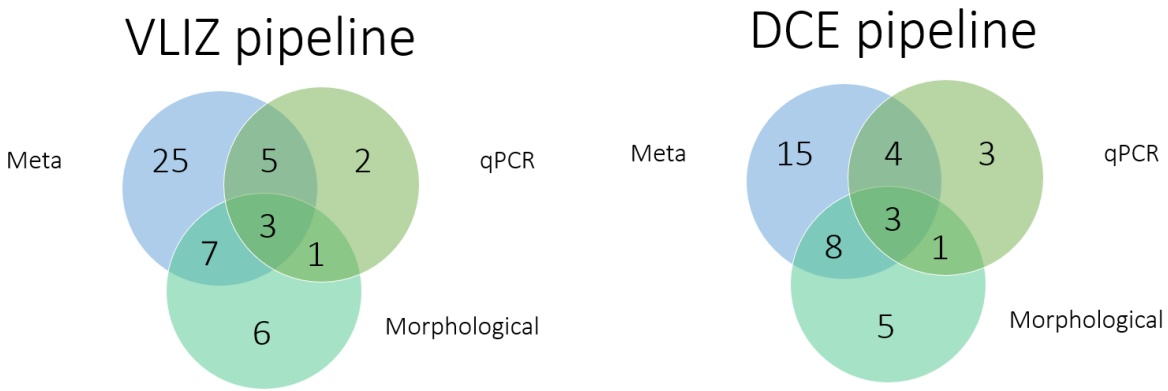

Figure 8.Venn diagram comparing the NIS in common between the three methods applied separated into comparing metabarcoding using VLIZ and the DCE bioinformatics pipelines.

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. More species were detected using the VLIZ bioinformatics pipeline (Figure 8).

With qPCR specifically, we detected 11 NIS species out of the 23 qPCR species with detection systems, of which two, *Pseudochatonella farcimen* (phytoplankton) and *Prorocentrum cordatum* (dinoflagellate), were uniquely detected with the qPCR assay. In addition to the NIS detected 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 Cq values below LOD can only be considered weak traces of the target DNA. In this report we report Cq values below LOQ as the species being detected, while we consider values between LOD and LOQ as the species being identified but not detected. When the Cq value is below LOQ, the species detection is considered certain. 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 7).

Table 7. 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 morphological analysis, 11 were also identified with the VLIZ pipeline, and 12 with the DCE pipeline. The seven NIS uniquely detected with morphological analysis were *Rhithropanopeus harrisii*, *Sargassum muticum*, *Schizoporella japonica*, *Sinelobus vanhaareni*, *Streblospio benedicti*, *Tharyx killariensis* and *Dasya* sp. Of these only *Rhithropanopeus harrisii* was 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 cryptic or new NIS in the monitoring area and the identification challenges with identifying these. 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 eight and seven, respectively) (Figure 8). 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 combine information on LOD, LOQ and number of technical replicates to justify NIS detection. In addition, very few NIS were common for all three methods suggesting the most comprehensive assessment of monitoring is a combination of methods, but also different sample types.

The overall higher number of NIS detected with metabarcoding than both conventional and qPCR (Figure 7 and 8), was evident in most harbors, especially with the DCE pipeline (Figure 9).

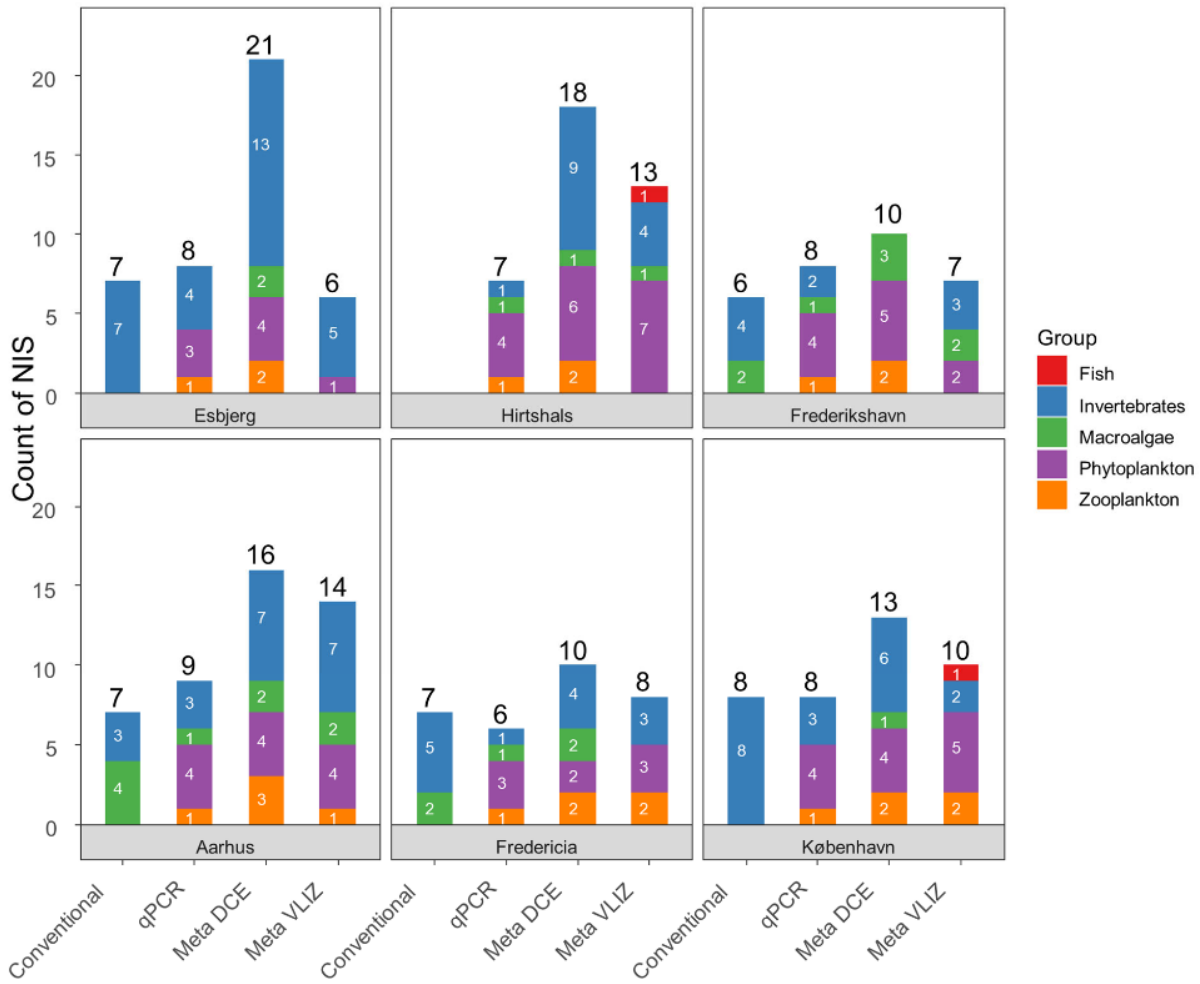

Figure 9. Number of NIS within five major groups. NIS were detected in each of the six monitored harbors during sampling in both June and September 2021. Color codes identify major taxonomic groups. Results from metabarcoding (Meta) are shown for the two bioinfo

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 rarer (maximum of eight stations). For metabarcoding, 11 out of 30 NIS detected with the DCE pipeline were recorded in more than half of the stations while the VLIZ pipeline recorded fewer NIS (Figure 10).

Table 8. 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

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 8). 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. Although metabarcoding overall (Figure 7 and 9) provides many more NIS species, this was only seen in three harbors

(Aarhus, Hirtshals and Fredericia harbor). This suggests that metabarcoding, compared to qPCR, has lower sensitivity in terms of detection. When looking more closely at how frequently the individual NIS has been observed with each method, the qPCR method generally registers five out of 12 qPCR detected species at almost all visited stations, which indicates great sensitivity to these species (Figure 10). In comparison, metabarcoding recorded each NIS at fewer stations using the VLIZ pipeline, suggesting a lower detection limit/sensitivity for the individual species. However, for the NIS detected using the DCE pipeline, these were detected with higher frequency (more stations), although for fewer species (Figure 10). These results highlight that the metabarcoding results are quite dependent on the chosen bioinformatic pipeline. Several available bioinformatics pipelines are developed for molecular ecology-based research, while this NIS study is based on detection of specific species in different locations. This indicates that the NIS data analysis bioinformatics pipeline needs to be customized.

In comparison, the conventional methods applied in our study registered the individual NIS at relatively few stations, suggesting that the observed NIS are generally rare, occurring in low abundances (Figure 10). For example, *Magallana gigas* was detected in Esbjerg and Copenhagen harbor by all methods tested.

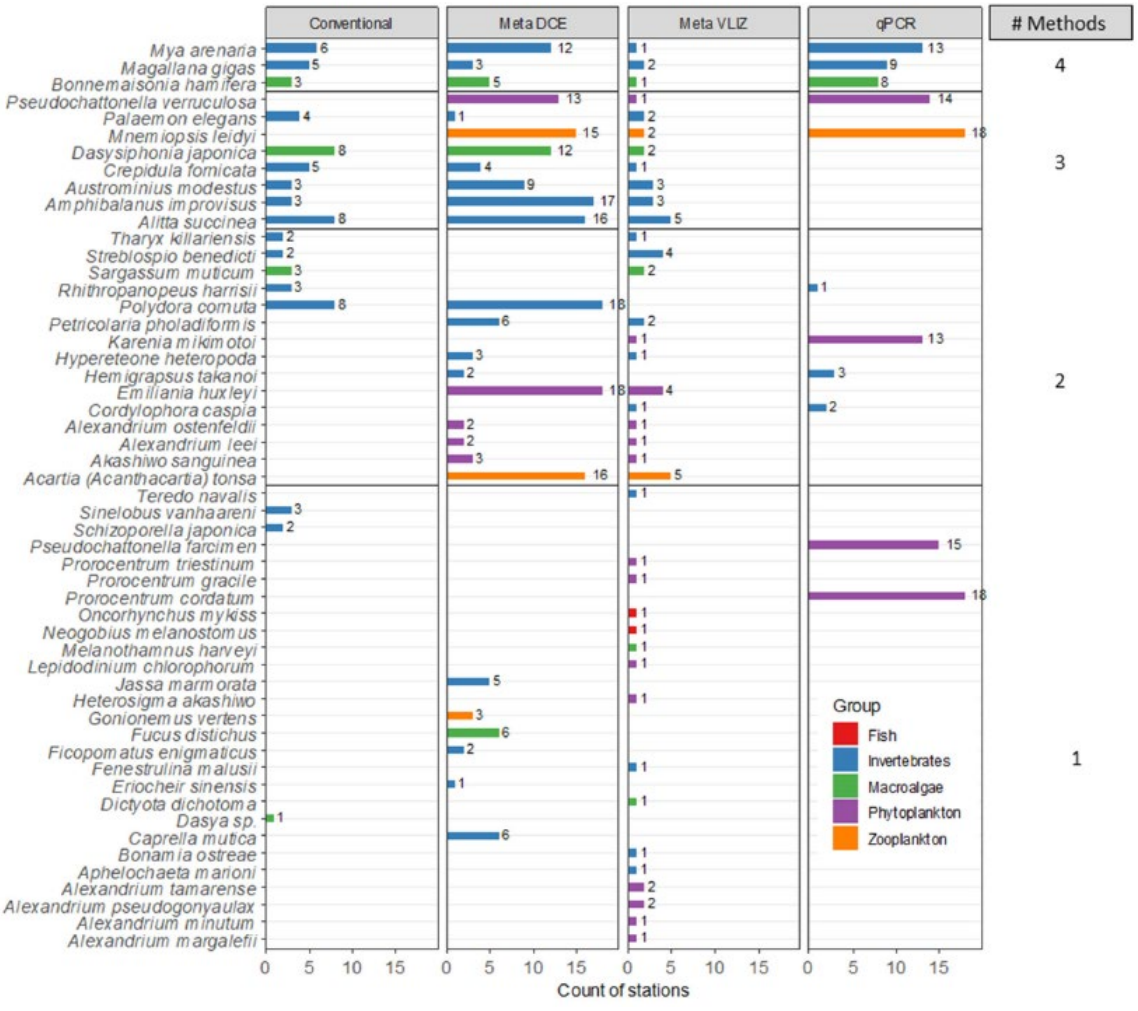

Figure 10. NIS species detected by conventional analysis, metabarcoding (Meta) and qPCR techniques in six Danish harbors. Results from metabarcoding are shown for the two bioinformatic pipelines used. Color codes identify major taxonomic groups. Species are sorted according to method of detection.

Of the three NIS in common for all three methods, qPCR detected these at far more stations compared to conventional and metabarcoding methods (Figure 10). In total, 27 NIS were detected by one method, 16 NIS were detected by two methods, seven NIS by three methods and only three NIS: *Mya arenaria*, *Magallana gigas* and *Bonnemaisonia hamifera*, were detected by all four methods (Figure 10). Considering these three NIS, it is noteworthy that these were detected at much fewer stations using conventional sampling techniques, but appeared to be very common with the qPCR and metabarcoding (DCE pipeline) (Figure 10). The metabarcoding analyses were based on three primer sets targeting 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 targeting 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 highlights 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 uses 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 resolution, are reusable across studies, and are independent of clustering algorithm and similarity percentages (Bolyen et al., 2019). 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.

ii. Time and cost comparison

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 14 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 time resources spent in the laboratory. For qPCR, the Danish protocol was followed, no multiplexing took place, and two plates of PCR were run separately per species, leading to a very high time spent per sample, also reflected in cost by scientist wages. This could be reduced following other laboratory protocols.

Table 9. 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 costs by eDNA or bulk DNA; yellow cells show a break-even situation, and green cells show that the introduction of eDNA or bulk DNA methods has led to a saving in either time and/or costs

Time and costs - quantifications								
Monitoring method	**Sampling type**	**# Samples**	**# Stations**	**Total time (h)**	**Time per sample (h)**	**Time per station (h)**	**Price per sample (kr)**	**Price per station (kr)**
Conventional	Settlement plates	54	18	135	2.5	7,5	2.560 kr.	7.679 kr.
qPCR (bulk DNA)	Settlement plates	54	18	270	5	15	12.816 kr.	38.448 kr.
Metabarcoding (bulk DNA)	Settlement plates	54	18	108	2	6	3.274 kr.	9.821 kr.
Conventional	Core + scraping + settlement plates	42	18	531	13	29,5	13.026 kr.	30.394 kr.
qPCR (eDNA)	Water	108	18	540	5	30	6.408 kr.	38.448 kr.
Metabarcoding (eDNA)	Water	108	18	216	2	12	2.674 kr.	16.042 kr.

Time and costs - %reductions						
Monitoring method	**Sampling type**	**Total time (h)**	**Time per sample (h)**	**Time per station (h)**	**Price per sample (kr)**	**Price per station (kr)**
qPCR (bulk DNA) vs conventional	Settlement plates	2X expensive	2X expensive	2X expensive	5X expensive	5X expensive
Metabarcoding (bulk DNA) vs conventional	Settlement plates	20% cheaper	20% cheaper	20% cheaper	30% more expensive	30% more expensive
qPCR (eDNA) vs conventional	Core + scraping + settlement plates	break-even	60% cheaper	break-even	50% cheaper	30% more expensive
Metabarcoding (eDNA) vs conventional	Core + scraping + settlement plates	60% cheaper	95% cheaper	60% cheaper	80% cheaper	50% cheaper
Metabarcoding (eDNA) vs qPCR (eDNA)	Water	60% cheaper	60% cheaper	60% cheaper	60% cheaper	60% cheaper

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 than eDNA and bulk DNA techniques.

g. Conclusions

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 groups 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; I. 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. II. 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. III. Bioinformatic pipeline used. Agreement should be sought among EU countries/countries around the North Sea on a single optimized pipeline. IV. 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 of bulk DNA had higher costs than conventional monitoring and qPCR of eDNA had lower costs than conventional monitoring. The latter might be due to combining several settlement plates for qPCR which was not done for the conventional measurements. 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 26 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 and the optimization of bioinformatic pipelines.

The eDNA technique of metabarcoding has the potential to supplement 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) taxonomic 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.

7. Sub-pilot Sweden (port of Wallhamn)

a. Design and monitoring objective

There is an ongoing yearly survey of invasive and non-indigenous species in 20+ commercial ports and marinas along the Swedish coast. These surveys have been based on the "Extended Rapid Assessment Survey" (eRAS) technique and methodology as outlined in Granhag (2016) and Bergqvist et al. (2021). The Swedish Water and Marine Management Agency (SWAM) is in charge of this monitoring program and from 2022 SeAnalytics is commissioned to carry out the surveys in a subset of the 20+ ports per year. There have been previous comparisons between using eRAS for monitoring non-indigenous species and molecular methods (Sundberg et al. 2018) but the SWAM agency has expressed an interest in additional comparisons for making a final decision which approach to be used in the future.
In view of this, a more detailed comparison was carried out during the 2022 monitoring schedule using the commercial port of Wallhamn on the eastern side of the island Tjörn just north of Gothenburg as study/pilot area (Figure 11). Wallhamn is one of the leading vehicle landing ports in Northern Europe. The port was established in 1962 and has a long history of shipping, hence making it a potential hotspot for NIS introductions.

b. Collection of samples

The eRAS assessment includes: settling panels (four sets), two artificial habitats (Figure 12), visual observations from the surface of invasive species as described by the RAS protocol, and scrapings from hard structures in the port/marina. Species are identified from morphology and habitus. Panels and scraping are intended to catch settling organisms, and those living among those. The artificial habitat is intended to catch moving animals like fish and crustaceans.
At each station, a vertical series of settlement plates (for details, see Bergqvist et al. (2021)) was left for app. 3 months (Table 10). Organisms associated with hard structures were collected with scraping using a 10 cm wide handheld scraping device.
For the DNA-based species identification we used three approaches to collect DNA: plankton samples, filtered water samples, and settling panels in the ARMS layout (Obst et al. 2020).
Opposite the port of Wallhamn there is a marina with floating jetties. For practical reasons, we used this marina as the main base for our sampling. We considered it likely that in view of the closeness to the port that we would find the same fauna at this location. Figure 13 shows the location of the sampling/panels.
Plankton samples were taken as vertical hauls from the bottom (5-10 m) up to the surface with a 90 µm plankton net of the same type as described in Sundberg et al. (2022). The samples were fixed in 95% ethanol directly in place with a final concentration of at least 70% ethanol. Upon returning home, the plankton samples were decanted and fresh alcohol was poured in to ensure that the ethanol content was sufficiently high. The samples were then stored in a freezer (-20 °C) until DNA extraction.

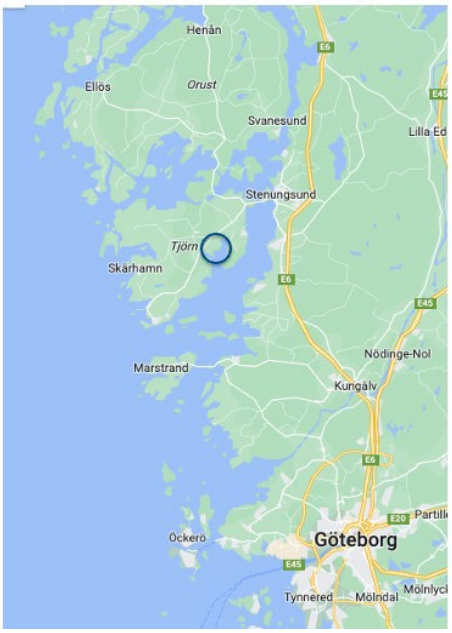

Figure 11. Location of Wallhamn Port (circle) on the east side of Tjörn

Water samples for eDNA were taken with a Ruttner sampler at slightly different depths on the premises and pooled in a 1 L vessel. Water was filtered (Sterivex 0.45 µm) in situ and fixed with 95% ethanol (see also Staehr et al. (2022)). The filters were stored in a freezer (-20 °C) until DNA extraction.

Figure 12. The kind of artificial habitats used in the eRAS protocol to catch mobile fauna.

The number of samples per category, and dates for the various actions are given in Table 10.

Table 10. Timing for each sampling activity, and number of samples/panels at Wallhamn in connection to the comparison of eRAS and DNA-based NIS monitoring.

	Activity					
Date	eRAS panel	artificial habitat	ARMS	plankton samples	eDNA (water)	scraping/RAS
2022-06-22 (date I)	deployed (1+3)	deployed (2)	deployed (2+1)	3 samples	3 samples	
2022-08-04 (date II)		retrieved		3 samples	3 samples	
2022-09-09 (date III)	retrieved		retrieved	3 samples	3 samples	3 sites

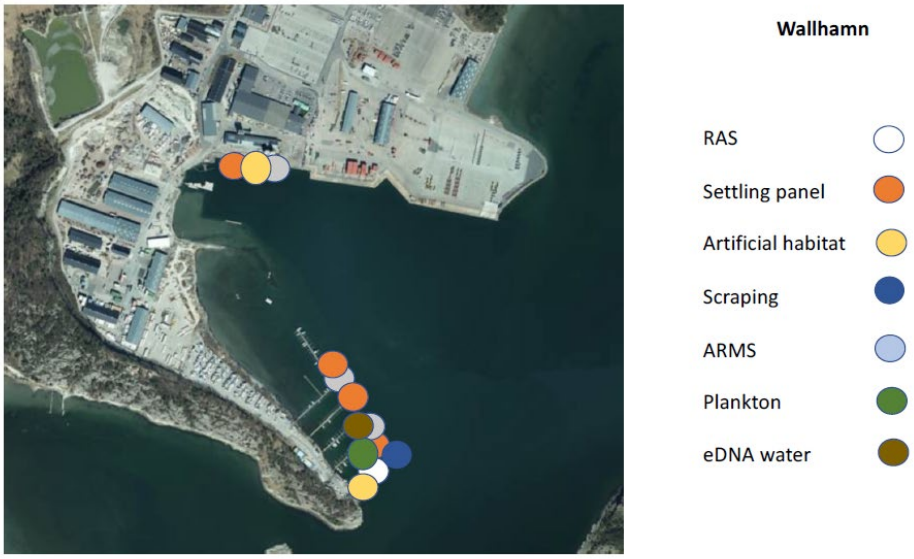

Figure 13. Sampling sites at the port of Wallhamn (northern location) and the Wallhamn Marina (south). RAS is visual observation of the presence of NIS.

c. Lab processing

Specimens for morphological identification (eRAS) were brought back to the laboratory and identified by Dr. Björn Källström, Göteborgs Marinbiologiska Laboratorium.

Sundberg et al. (2022) describes how DNA is extracted from plankton samples and the ARMS fouling panel. DNA was extracted from the filters with the Nucleospin eDNA water kit (Macherey-Nagel) using the technique and standard developed in the laboratory. Concentration of DNA in the extracts was measured with a Qubit®

fluorometer. Note that this measures the concentration of all DNA in the sample and not specific DNA from the target species. This is done to ensure that the extractions have worked.
PCR, library preparations, and sequencing followed the procedures described in Sundberg et al. (2022).

d. Bioinformatic processing and data analysis

The genetic analysis and comparison are based on two molecular markers (COI and 18S). All steps of the molecular biology and bioinformatics protocols used in this study are described by Sundberg et al (2022). The sequence data is published and available in the European sequence archive ENA under the following link https://www.ebi.ac.uk/ena/browser/view/PRJEB60147. Obtained sequences were matched against reference libraries in BOLD (for COI) and PR2 (for 18S). All genetic species observations are available through GBIF under the DOIs https://doi.org/10.15468/z8pm63 (18S) and https://doi.org/10.15468/cw5jrv (COI). The obtained species lists were then matched against two national lists for non-indigenous species, one from the Swedish Agency for Marine and Water Management and the other from the Swedish Species Information Center.

e. Results

All species recorded (from eRAS and DNA) within this multi-year monitoring programme are or will be reported in the HELCOM-OSPAR database:
https://portal.helcom.fi/workspaces/HELCOM-OSPAR%20Port%20survey%20data-165/default.aspx

i. Morphological analysis - eRAS

The surveys with settling panels, artificial habitats, RAS and scrapes identified a total of 14 species (of which 12 on the panels and six in the artificial habitats). No non-indigenous species were found in Wallhamn with eRAS.
The settling panels were covered to a very large extent by large ascidians (mainly *Ciona intestinalis*), which made it difficult to find other fouling organisms on the panels. As an example, the wet weight of a single fouling panel from the surveys in Wallhamn (which was covered to 100% by *C. intestinalis*) was 2340 g.
The problem with overgrown growth panels occurred in all the ports that were examined on the west coast in 2022 (Strömstad, Smögen, Kungshamn). The artificial habitats were relatively clean of growth and had been colonized by a smaller number of mobile organisms. RAS and scraping did not generate any findings of sessile organisms or NIS other than those found on the fouling panels.

ii. Analysis based on DNA

Number of species detected by the different markers and sample types is displayed in Figure 14. A total of 289 species could be identified. The marker COI identified 136 species (45 species in the water samples, 90 in the plankton samples, 46 on the ARMS plates), while the marker 18S could identify 170 species (130 species in the water samples, 113 in the plankton samples, 54 on the ARMS plates). There was little overlap in species identification between the markers (17 species). COI identified mostly metazoa while the 18S marker identified unicellular eukaryotes such as dinoflagellates. Species determination is more certain with COI than with 18S sequences that can be identical between closely related species. But in both cases, one can examine identified species more carefully by, for example, BLAST searching and discussions with taxonomic experts to confirm the genetic identifications.

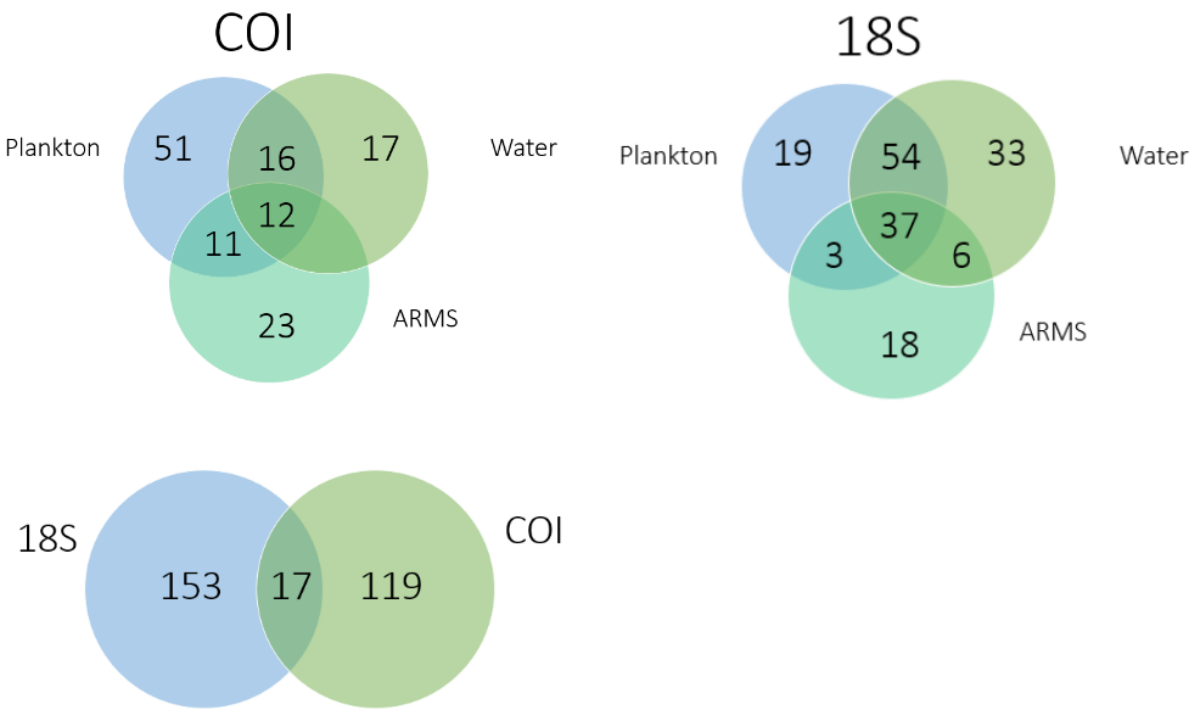

Figure 14. Species composition identified by the molecular markers (COI and 18S) respectively, and overlap between sampling methods. Light green = plankton samples, blue = filtered water samples (eDNA), red = ARMS panels.

Non-indigenous species identified with DNA metabarcoding are displayed in Table 11. We found ten species identified as NIS of which some have been risk assessed as invasive in Sweden. It should be noted that to be defined as non-indigenous in Sweden the species should have been introduced, by man, after the year 1800 (Strand et al. 2018). It should also be noted that it may be difficult to know whether a new record for Sweden is a case of lack of knowledge (the area not sampled), or if the species would be declared as a NIS. We have a few observations of new records/NIS but have decided to leave them out since it is beyond what we can clarify in the present study given time and economic constraints.

Table 11. Non-indigenous species identified by DNA from the water eDNA sample (W), plankton (P), and ARMS panels (A). The table also lists from which sample period the species was found in addition to the sampling approach. Dates: 2022-06-22 = I; 2022-08-04 = I

Phylum	Family	Species	Sample	Barcoding region
Pyrrophycophyta	Goniodomataceae	*Alexandrium minutum*	W:I	COI
Arthropoda	Balanidae	*Amphibalanus improvisus*	A:III	COI
Rhodophyta	Bonnemaisoniaceae	*Bonnemaisonia hamifera*	P:III	COI
Mollusca	Calyptraeidae	*Crepidula fornicata*	P:II	COI
Ochrophyta	Vacuolariaceae	*Fibrocapsa japonica*	W: I, III	COI
Ctenophora	Bolinopsidae	*Mnemiopsis leidyi*	P:III	COI
Chordata	Gobiidae	*Neogobius melanostomus*	W:III	COI
Rhodophyta	Rhodomelaceae	*Neosiphonia harveyi*	P:III	COI
Arthropoda	Sididae	*Penilia avirostris*	P:II	COI
Dinoflagellata	Goniodomataceae	*Alexandrium minutum*	P:III	18S
Ochrophyta	Polarcentric-Mediophyceae	*Chaetoceros seiracanthus*	W:II,III P:III	18S
Ochrophyta	Raphidophyceae	*Fibrocapsa japonica*	W:III	18S
Metazoa	Branchiopoda	*Penilia avirostris*	W:II,III P:I,II,III	18S

The results clearly show that DNA-based methods have a much higher probability of finding NIS species. We also see that the three different collection methods (ARMS, plankton, water) give slightly different results and in that way they complement each other. The finding of just one single NIS on the ARMS plates is somewhat surprising given previous results (Sundberg et al. (2022)). One explanation could be that this time all three fractions were pooled to keep costs down. A very dominant species with a lot of tissue on the plates then risks "overshadowing" species that were only present in low concentrations and thus also left small amounts of DNA. We recommend sequencing each fraction separately in the future like in Sundberg et al. (2022). In that study all organisms were scraped off from the plates and sieved into three different size fractions (< 40 µm, 100–500 µm, > 500 µm) and metabarcoded separately. Larger organisms (like tunicates and mussels) were removed before sieving.

The matching of 18S sequences against reference libraries is difficult, partly because these libraries are not as extensive as for COI, and because the gene does not always separate at the species level. We will not use that marker in our future comparisons in the Gulf of Bothnia and Stockholm area in 2023.

iii. Time cost comparison

Since this study was part of a larger monitoring program in 2022 it was not possible to separate individual costs from other expenses connected to the program. However, roughly the amount of time for field work for eRAS and DNA-based sampling was about equal. In addition to that there is lab work for the DNA and for morphological identification which was also about the same in time. There is an additional cost for sequencing and bioinformatic processing when it comes to the DNA-based identification, so overall this approach costs more. However, in view of the much higher number of NIS detected by the DNA approach, it will still be considered more efficient and cost-effective. Our recommendation is therefore to use some alternative DNA-based approach (in this case the eDNA and plankton samples were the most successful in finding NIS) in the future monitoring of NIS, and this move has already happened in some cases.

f. Conclusions

DNA-based approaches turned out to be more efficient in finding non-indigenous and invasive species compared to the eRAS approach hitherto been used in the Swedish monitoring program. We recommend a mixture of settling panels, plankton samples, and water eDNA sampling to cover a wider range of taxa. In the comparison reported here we could not see any advantage of including morphological species identifications but it may have added information if a more detailed morphological analysis had been employed, but that would also come with a greater cost.

8. SWOT analysis

DNA-based methods for monitoring NIS offer several strengths and weaknesses, as well as opportunities and threats. In terms of strengths, these methods typically detect more NIS species compared to traditional monitoring approaches, and they can identify species with cryptic morphology, like the meroplankton larvae detected in the harbor of Ostend, or unique lifestyles, like the endoparasites detected in the harbor of Ostend. Once initial investments are made, DNA-based methods become cost and time-effective. The time and cost-effectiveness may widely range due to numerous factors, including the available expertise and the specific methods that were used. We found that DNA-based methods were (on a per sample basis) 20-93 % less time consuming and either 65 % less costly or 28 % more expensive, depending on lab protocols, sequencing platform used and/or local staff and consumable costs. DNA-based methods may require less training because no specific training is needed per taxonomic group (e.g. copepods, bryozoans, etc.) as in morphological analysis. DNA-based methods also enable rapid and detailed screening of bulk samples, allowing for the detection of even rare species. However, there are several weaknesses associated with these methods. Expert consultation is necessary to exclude false positives, and contamination is a potential issue. In the different sub-pilot, species lists had to be curated manually in order to filter out obvious wrong assignments, such as those of some tropical species that have never been reported from temperate waters. Such errors stem from incomplete reference databases and the lack of species-level resolution in short DNA-barcoding regions. Absence of a species from a database can also lead to its reads being misidentified rather than unidentified, leading to false positives (Zaiko et al., 2016). Not only are missing entries a problem in databases, but also the fact that some reference sequences originate from misidentified organisms (Kress et al., 2015) or contain sequencing errors (Valentini et al., 2009). In this respect metabarcoding is also dependent to some extent on taxonomic expertise, with possible errors amplifying across studies rather than being limited to the study in which the misidentification occurred. The failure of metabarcoding to detect these species points to the fact that DNA-based detection methods are not immune to generating false negatives (Duarte et al., 2021) despite their potential for high sensitivity and accuracy (von Ammon et al., 2018). Notably, many of these species were small organisms represented by only a few individuals per sample. The low biomass of many of these organisms in the samples may have led to the exclusion of their genetic material from the small subsamples taken for DNA extraction, despite efforts to homogenize the samples as thoroughly as possible beforehand. In order to avoid such biomass-dependent biases, van der Loos & Nijland (2020) recommend sorting samples according to size in addition to carrying out a homogenization. Another possibility is primer mismatch with the DNA sequences of these organisms, as well as amplification bias in general (Trebitz et al., 2017; Duarte et al., 2021). Another weakness of DNA-based methods for NIS detection is the lack of ability to estimate relative abundances. Such estimates of abundance may be useful, for example, to determine if an eradication plan for an established NIS still is feasible. A morphological examination, on the other hand, might miss many organisms but can give a reasonably accurate estimate of the relative abundances of the most dominant species in a community.

Lastly, integration with biodiversity informatics initiatives is not yet seamless, and quick on-site identification is not possible. Additionally, accidental detection by non-experts is not feasible. Despite these weaknesses, DNA-based methods present opportunities for early detection of NIS, rapid assessments, and continuously improving methodologies. For example, stream-lined Nanopore sequencing workflows can produce results within a few days. Data can be easily shared and workflows generalized for different projects or samples. Automation and the use of robots for sample processing are also potential advancements. However, there are threats to consider, such as limited trust in the results by certain stakeholders. False positives due to contamination or incomplete reference databases are an important issue and DNA-based monitoring methods have their own intrinsic biases, systematically missing specific species. Ground truthing is sometimes necessary, but challenging for many species due to factors like cryptic morphology, and certain non-indigenous species may be missed altogether even when both DNA-based and morphological methods are applied.

Table 12. List of internal strengths and weaknesses and external opportunities and threats of DNA-based NIS monitoring.

Strengths

- Typically more species detected compared to traditional NIS monitoring methods
- Species with cryptic morphology or life styles can be detected
- After initial investments, DNA-based methods become time and cost-effective
- Less training needed compared to traditional NIS monitoring methods
- Rapid and detailed screening of bulk samples

Weaknesses

- Expert consultation needed to exclude false positives or false negatives
  - Contamination
  - Errors in databases -> Ostend
  - false negative -> Ostend, Denmark
  - false positive -> Ostend
- No seamless integration with biodiversity informatics initiatives (linking resulting data to OBIS, GBIF, WRIMS, taxonomic backbones etc. is not yet automated)
- No quick identification on site
- Accidental detection by non-experts not possible
- Barcoding regions not always discriminate at species level (recent split between species, short barcoding regions used, hybridization)
- Reference databases incomplete
- Quantification is difficult/unreliable
- No information on establishment/reproduction
- Excessively dominant taxa can overshadow others in DNA-based analysis

Opportunities

- Early detection of NIS
- Rapid assessments
- Rapidly improving methodology promising for future
- Standardization across countries feasible
- Data can be easily shared
- Reproducible
- Workflows can be generalized across different type of projects or samples
- Automation and use of robots for sampling processing
- Semi quantitative data possible through qPCR methods but needs further investigation
- Can optimize analysis to be more sensitive to certain taxa under investigation (primers)

Threats

- Possibly not sufficient trust in results by some stakeholders
- New monitoring methods that comes with its own systemic biases (i.e. systematically misses specific species due to primers not binding or individuals that do not shed DNA)
- Ground truthing is difficult for many species (e.g. due to cryptic morphology or lifestyle)
- Sample archiving
- Need for standardization of bioinformatic pipelines
- Need for better reference database curation

9. General conclusions and recommendations

Monitoring NIS is a critical step for understanding, preventing, and managing the impacts of biological invasions. Here, we demonstrated the usefulness of DNA-based approaches, such as (e)DNA metabarcoding or qPCR assays, for monitoring NIS in aquatic environments. These methods are highly complementary to traditional NIS monitoring techniques and can increase sensitivity, accuracy, and cost-effectiveness of detecting and identifying non-indigenous species.

DNA-based methods can be applied to various sample types, including water, plankton, scrape (biofouling), and sediment samples. They have the ability to detect even low-abundance, morphologically cryptic, or rare species, which may be challenging to identify using traditional methods. Another significant benefit of DNA-based methods is their rapid and efficient data processing and analysis capabilities. These methods can generate results in near real-time, allowing for timely decision-making in management and mitigation efforts. This real-time aspect is particularly beneficial when dealing with rapidly spreading or potentially harmful non-indigenous species. By employing DNA-based techniques, researchers can also gain insights into the genetic diversity and population structure of non-indigenous species. In this pilot study, we did not conduct analyses at the within-species level. But such information could be vital for understanding their ecology and potential impacts on the ecosystem.

Once initial investments are made, DNA-based methods become cost and time-effective. The time and cost-effectiveness may widely range due to numerous factors, including the available expertise and the specific methods that were used. We found that DNA-based methods were (on a per sample basis) 20-93 % less time consuming and either 65 % less costly or 28 % more expensive, depending on lab protocols (e.g. number of markers chosen, pooling PCR, ...), sequencing platform used and local costs for consumables and staff.

To obtain a comprehensive and robust assessment of non-indigenous species, we recommend to integrate DNA-based methods with other monitoring approaches. Visual surveys and physical sampling can complement the genetic analysis, providing a more holistic understanding of the species' presence and abundance. Considering the practical aspects of monitoring, it is advisable to analyze a subset of the samples morphologically. This approach allows for a cost-effective and time-efficient preliminary assessment. However, it is essential to periodically conduct a full assessment, including morphological analysis, to ensure accuracy and account for any changes in species composition or abundance. Conducting a full assessment at the beginning of a time series or for risk assessments is also recommended to establish a baseline and identify potential threats. By integrating DNA-based methods with other monitoring approaches, a comprehensive understanding of non-indigenous species and their impacts can be achieved.

qPCR assays are limited compared to DNA metabarcoding in their capability to detect only species for which the assay was designed. However, qPCR is a sensitive method and can be used to detect the targeted species at low abundance.

In summary, DNA-based methods offer valuable tools for monitoring non-indigenous species in marine ecosystems. They provide sensitive, accurate, and cost-effective means of detection and identification. Additionally, these methods offer insights into genetic diversity, population structure, and real-time data processing.

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Genetic tools for Ecosystem health Assessment in the North Sea region

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