AARHUS UNIVERSITY

Roskilde Universitet

Master thesis

„Non-indigenious species in the Danish Wadden Sea - are environmental DNA analyses the future tool for monitoring?"




Kathrina Zimmer

Study number 74356

M. Sc. Environmental Biology and Molecular Biology

Supervised by Benni Winding Hansen[1]

Anne Winding[2]

Rumakanta Sapkota[2]

1 Department of Science and Environment, Roskilde University

2 Department of Environmental Science, Aarhus University

Table of Contents

Preface

In my thesis, the presence or absence of non-indigenous species (NIS) in the Danish Wadden Sea was investigated. To do so, different detection methods were used, including metabarcoding and qPCR analyses as eDNA tools as well as conventional methods. This project can be seen as a monitoring of the health status of the Wadden Sea as non-indigenous species could pose a threat. Therefore, early detection of new NIS could help to prevent further spreading and damage.

My thesis is part of the Advisory Report from DCE – Danish Centre for Environment and Energy No. 547 with the title "Identification, dispersal, and possible mitigation responses for non-indigenous species in the Danish Wadden Sea area" (Stæhr et al., 2023). The Ministry of Environment was financing the project and it was developed in agreement with the Danish Environmental Protection Agency, which also reviewed the project.

I went on a field trip to the Danish Wadden Sea together with intern M.Sc. Karolina R. Andersen and special consultant Dr. Nikolaj R. Andersen (Department of Ecoscience, Aarhus University) from 12.09. to 14.09.2023. Samples were taken at the three harbors Esbjerg, Fanø and Rømø along with four different sampling stations in local tidal channels. I took the water samples for the following eDNA analyses and contributed sampling of the settlement plates and soft sediment probes for conventional analyses. I performed the laboratory work to analyze the water samples and settlement plates with metabarcoding and qPCR, which included extracting and cleaning DNA, performing PCRs, preparing the standards for qPCR, and conducting metabarcoding and species-specific qPCR. Afterwards, the eDNA data was processed and analyzed by me. The frame samples for conventional analyses were taken by academic assistant M.Sc. Helle Buur (Department of Ecoscience, Aarhus University), Karolina R. Andersen and Nikolaj R. Andersen. Helle Buur identified the taxa in the conventional samples and shared the data with me.

31.05.2023 KZimmer

Kathrina Zimmer

Summary

In the present project, non-indigenous species (NIS) in the Danish part of the Wadden Sea were detected. For this purpose, molecular and conventional methods were used to monitor the present NIS. In my thesis, I focused on environmental DNA (eDNA) techniques and performed species-specific qPCR and metabarcoding analyses.

NIS are species which immigrated into an area either caused by human activities like shipping or actions related to aquacultures, or in a natural way by e.g., brought with the currents. Some NIS can damage the ecosystem, for example by eliminating native species, bringing parasites, or transferring diseases to their new habitat, and are therefore categorized as invasive species. Other NIS are harmless or can even be beneficial for the receiving habitat.

The research area was the Danish Wadden Sea, which is a unique habitat formed out of intertidal sand and mudflat with a high biodiversity. As it is a very dynamic ecosystem, many different ecological niches are provided, which lowers the risk of a species extinction. Nevertheless, NIS could pose a threat for this ecosystem and a continuous monitoring of the Wadden Sea is important to recognize new NIS in an early stage and if considered necessary be able to act.

For the detection, traditional methods can be used, which include identifying organisms from environmental samples with a dissecting microscope and a species key. An alternative approach is the usage of molecular techniques like eDNA analyses, which turned out to be very efficient in the past. For those molecular analyses, water samples were collected from four tidal channels and three different harbors, as many NIS are introduced through shipping traffic and could further distribute through transportation in the tidal channels. Furthermore, settlement plates were placed in the three harbors and were collected again after 3.5 months. Species-specific qPCR analyses were performed to determine the presence or absence of 23 NIS. Moreover, metabarcoding analyses with the primers 12S rDNA, 18S rDNA and COI were conducted to detect further NIS.

With this study the efficiency of eDNA analyses for monitoring NIS in an aquatic ecosystem was confirmed as 40 NIS could be detected with eDNA tools, compared to 22 NIS identified with conventional methods. Combining the results from all three detection tools, 50 NIS could be identified in total. With metabarcoding analyses, the presence of 31 NIS could be confirmed, and the use of qPCR assays revealed 16 NIS in the Danish Wadden Sea. To analyze the qPCR data, two different approaches for setting the detection limit were tested and compared. It turned out that the approach mainly used in the present project and in previous reports for the Danish EPA is more sensitive than the one suggested by Klymus et al. (2020a).

On one tidal channel station, additional samples at various time points were taken to examine the effects of low and high tide on species diversity detected by molecular detection tools. The qPCR results and metabarcoding data revealed only minor differences between the different samples.

In conclusion, 50 NIS could be detected in the Danish Wadden Sea. Comparing the different methods for detection, metabarcoding identified with 31 species the most NIS and can be seen as the most efficient tool for monitoring an aquatic ecosystem. The species-specific detection with qPCR analyses turned out to be more sensitive than metabarcoding, but the development of the detection systems is time-consuming and expensive, and only a limited number of species can be detected. Further studies are required to conclude if eDNA analyses can completely replace conventional tools for species detection.

Introduction

Non-indigenous species

Non-indigenous species (NIS), also known as alien species, are defined by the European Commission as “a species or subspecies of an aquatic organism occurring outside its known natural range and the area of its natural dispersal potential” (EC, 2007). In the Oslo and Paris Conventions (OSPAR) Intermediate Assessment 2017, they are further described as organisms moved outside their natural range by human activity, contrary to species which expanded their habitat naturally and are therefore not considered as NIS (OSPAR, 2017).

Most NIS have a high reproduction rate and ecological opportunism along with the ability to spread widely. However, it is not possible to define further the typical characteristics of NIS as they occur in a wide range of taxa and biological features (Cardeccia et al., 2018).

Not all NIS have a negative impact on the ecosystem, but some can be a threat to an ecosystem as they could be at least partly responsible for endangerment or extinction of native species, which was shown several times in the past (Shrader-Frechette, 2001). To describe those NIS, the term invasive alien species (IAS) is used. The European Commission defined IAS as species which damage nature and economy by e.g., outcompeting native species or spreading infections with threats for wildlife or humanity. Nowadays, 354 native species are endangered by IAS in Europe, with increasing numbers in the past years (EC, 2020). Interestingly, those IAS do not show a different pattern of biological attributes than the harmless NIS (Cardeccia et al., 2018).

Looking at the Wadden Sea, no introduced species lead to the extinction of a native one on a larger scale and some of them can also be beneficial for the ecosystem. For example, the empty shells of introduced bivalves provide hiding possibilities and functions as a habitat for epibionts. The reefs created by Pacific oysters deflect and intercept the tidal currents. Another NIS stabilizing the sediment is the gras *Spartina anglica*, which has longer roots and higher growth than the native equivalent and can therefore better counter erosion of the sediment. Overall, most of the introduced species in the Wadden Sea increased the habitat and biodiversity and may help the ecosystem to deal with upcoming environmental changes (Reise et al., 2023).

There are many different ways species can get introduced to non-indigenous habitats, but the most common vectors are by vessels e.g., in the ballast water or as biofouling on the outside of the ships, and with activities related to aquacultures like intercontinental stock movement or escapes of the cultured organisms. Once a NIS is established, they can spread further by natural processes, like carried by the currents or by organisms from another species (Galil et al., 2014). Focusing on the Wadden Sea, twelve percent of NIS got directly introduced from oversea locations, but the majority of NIS got established first in neighboring regions southwards. From there, the species either spread by natural dispersal towards the Wadden Sea or were secondarily introduced to it. Interestingly, the rate of introduced species is the highest in the estuarine regions of the Wadden Sea with low salinity (Reise et al., 2023). The first arrival of a non-indigenous species in the Wadden Sea happened during the Viking age with the introduction of *Mya arenaria*, which originates from North America (Petersen et al., 1992). More species found their way to Northern Europe and today over 100 NIS are established in the Wadden Sea (Buettger et al., 2022; Reise et al., 2023).

Due to climate change, the average temperature and the number of extreme weather conditions is increasing in most places (Rius et al., 2014). Consequently, species with a greater thermal resilience will profit from it, while it could threaten native species, which are already close to their tolerance threshold (Laubier, 2001; Rius et al., 2014). This additional stress could cause death of many benthic organisms, resulting in free niches for non-indigenous species and could subsequently lead to an even stronger increase of NIS in the future (Cerrano et al., 2000; Garrabou et al., 2001; Occhipinti-Ambrogi, 2007).

Wadden Sea as an ecosystem

Since 2009 the Wadden Sea is one of the United Nations Educational, Scientific and Cultural Organization (UNESCO) World Heritage Sites as the world's largest contiguous intertidal sand and mudflat system with an area of 1,143,403 ha (*UNESCO World Heritage*). It is a unique ecosystem, which has a high diversity of terrestrial and aquatic species with approximately 10,000 taxa (Reise et al., 2010).

Due to the tides, the sand and mud flats are exposed to the air twice a day. Additionally, the Wadden Sea is partitioned by five major estuaries and over 30 branching tidal inflows (Reise et al., 2010). The Wadden Sea has in general a lower salinity than the open oceans, but a higher one than in estuaries, although there are large variations in salinity over the day and a year due to the tides and seasonal changes (Reise et al., 2010; van Aken, 2008). The difference in the sea level caused by the tides ranges between 1.5 and 4 m, but strong onshore winds can increase the sea level further (Reise et al., 2010; Weisse & Plüβ, 2005).

The Wadden Sea contains multiple different habitats like tidal areas, an offshore belt and mussel beds. They differ in their environmental gradients like salinity, wind exposure or depth, but are functionally connected to each other (Reise et al., 2010). For example, the phytoplankton bloom takes mainly place in the offshore belt due to a high nutrient content and lower turbidity, which ensures enough light for the algae (van Beusekom & de Jonge, 2002; Wolff & Zijlstra, 1980). The tidal channels and further inlets transport the phytoplankton as well as fish- and other larvae to the inshore regions. Some animals like fish, seals or shrimps oscillate between offshore and inshore regions, whereas most of the benthic fauna can be found in the tidal flats (Wolff & Zijlstra, 1980). The high number of different and very dynamic habitats is a major prerequisite for the high biodiversity in the Wadden Sea as it lowers the hazards of species extinction by providing many different niches (Reise et al., 2010).

In general, the availability of algal food is a major factor for the abundance of zoobenthos in the Wadden Sea. In regions of the Wadden Sea with high intertidal levels, a higher primary production of benthic microalgae and low zoobenthic biomass can be found, because the environmental conditions like strong currents and high waves are limiting the zoobenthic biomass. In parts of the Wadden Sea with more moderate environmental conditions, the food supply is the limiting factor for zoobenthos. Furthermore, cold winters can cause a decline in abundance of more thermosensitive species. A local reduction of the benthic biomass can be observed as consequences of intensive fishing activities for benthic organisms like mussels (Beukema et al., 2002).

In the present report, the northern part of the Wadden Sea, which belongs to Denmark, was analyzed. This part is protected against high waves and westerly winds by an offshore barrier consisting of eight islands and elevated sand bars (Reise et al., 2010).

eDNA analyses compared to conventional species identification methods

The term “environmental DNA” (eDNA) describes a genomic DNA mixture from different organisms obtained from an environmental sample. Examples for environmental samples are soil, water, or sediment samples, but feces and bulk samples are also included. eDNA incorporates both intracellular and extracellular DNA. Intracellular DNA can only be found in living cells or organisms, whereas extracellular DNA originates from dead and subsequently degraded cells plus secretes and excretes (Taberlet et al., 2018).

eDNA analyses are non-invasive, cost-effective, and comparably fast. No classical taxonomic knowledge is required for eDNA analyses, and every life stage and organism of every size can be detected. Additionally, they can detect low abundant species with a high sensitivity, which makes them a good tool for monitoring and detection of species with small population sizes like endangered or non-indigenous species (Beng & Corlett, 2020). This method can be used for different purposes: it is possible to determine the presence or absence of the target species and can indicate spawning activities due to a usually higher release of DNA in the water. Furthermore, eDNA analyses can show the genetic diversity of a population and monitor the biodiversity of an aquatic ecosystem as well its microbiota, which delivers an insight of the ecosystem’s health status (Huang et al., 2022).

There are two common methods to analyze eDNA samples: with metabarcoding and real-time quantitative PCR (qPCR).

For a metabarcoding analysis, universal primers are used, which can detect DNA fragments from individuals belonging to different species and trophic levels. The aim is to get a broad overview of the present species in the environmental sample, which enables analyses of the biodiversity and trophic interactions, gives an insight of the trophic food web and helps to monitor the ecosystem’s health (Beng & Corlett, 2020). A metabarcode is a DNA sequence, which has a short, variable part suitable for taxonomic identification and is bordered by conserved regions serving as primer regions (Taberlet et al., 2018). Those barcode regions are amplified and optionally get a unique tag with PCR, before the products are sequenced with next-generation sequencing (NGS) (Huang et al., 2022). This high-throughput technique enables identifying many different species at the same time (Pawlowski et al., 2022). Finally, the resulting reads need to be processed with bioinformatic tools to assign the taxonomy (Deiner et al., 2017; Huang et al., 2022).

The method of qPCR can either be used for non-specific or specific detection. In the first case, a dye is used, which binds to all double-stranded DNA leading to an increased fluorescence of the dye. When more DNA products are produced in every PCR cycle, the intensity of the fluorescence increases. However, as the dye is unspecific, it binds also to primer dimers. This does not happen with the TaqMan assay, which is based on specific detection. In that case, the target species can be detected and the amount of DNA in the original sample can be roughly estimated by using species-specific primers and probes. This technique is very sensitive as it can detect a single copy of a sequence in a sample. The principle of TaqMan qPCR is based on the method of a conventional PCR with an added fluorescent indicator, which makes it possible to quantify the amplified DNA after every cycle. This reporter fluorescent dye is located at the 5' end of an oligonucleotide probe, which can hybridize with a specific sequence and allows species-specific analyses. On the 3' end of the probe there is a quencher dye, which absorbs and therefore strongly decreases the emitted fluorescence from the reporter dye if the probe is intact. The thermostable Taq polymerase is used to amplify the target sequences, starting from the primer region, and adding nucleotides in the 5' to 3' direction. As soon as the polymerase reaches the probe, the probe is cleaved by an exonuclease activity from the Taq polymerase and consequently the quencher and the reporter dye are disconnected. This leads to a strong increase of fluorescence from the reporter dye (see Figure *1*). Furthermore, the rest of the probe is removed from the target strand, which enables the further synthesis of the new strand (Arya et al., 2005; Heid et al., 1996). Afterwards, a new qPCR cycle starts, and the procedure begins again. The intensity of the fluorescence increases proportional to the amplified DNA as more and more probes are cleaved and more DNA is synthesized with every cycle. In the present study, the BioRAD Real-time PCR system (Life Technologies) was used, which measures the fluorescence and tracks it over the complete qPCR analyses to creates amplification plots with the software CFX Manager (Version 3.1.1517.0823). These plots show, if the target DNA was present in the sample and makes a quantification of it possible (Arya et al., 2005). With species-specific qPCR analyses, only one species per run can be detected, whereas metabarcoding can detect multiple species simultaneously.

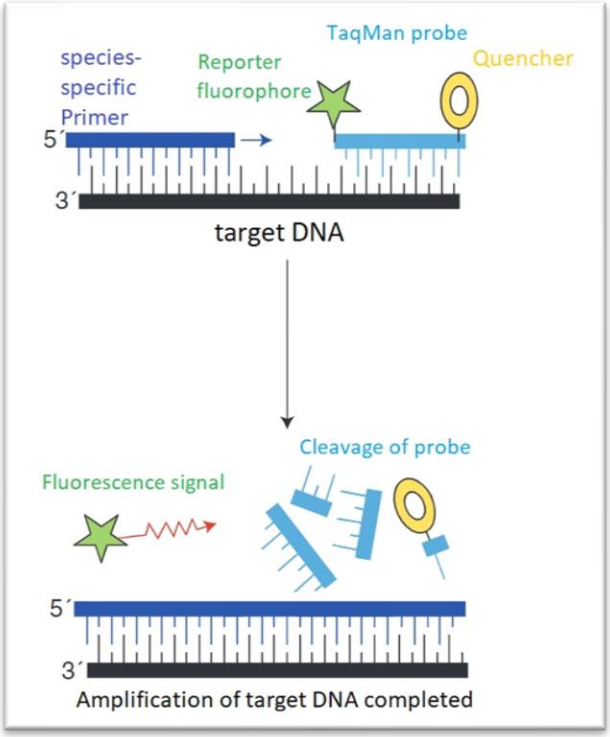

Figure 1: Molecular processes during qPCR. The amplification of target DNA causes a fluorescence signal because the probe is cleaved, which separates the quencher of the reporter fluorophore. Figure edited after Arya et al. (2005).

Before these molecular methods were invented, conventional tools were used for species detection and identification. The sample types for this method can be very diverse, ranging from sediment samples to scraping samples and fishing harvest. The organisms in the samples are usually identified with a species key from a taxonomist, partly by using a (dissecting) microscope. Therefore, the results and correctness of the species identification depends on a high level of the skills from the taxonomist, which is not the case for eDNA analyses. For some taxonomic groups a differentiation

between related species can be very difficult with conventional methods, for example the juveniles of the two NIS *Hemigrapsus sanguineus* and *Hemigrapsus takanoi* are very hard to distinguish, which could lead to a false species determination (Jensen et al., 2023). For species-specific qPCR analyses, the differentiation between closely related species can pose a challenge as well, because a detection system with primers and probes needs to be developed, which only amplifies DNA sequences from the target species. This is contrary to metabarcoding, which works with universal primers and sequencing of the PCR products. The species are later identified from the sequenced reads, which are usually long enough DNA sequences to make a clear differentiation between species possible. Another disadvantage of conventional methods is that some of the physical sample methods could damage the ecosystem, which is not suitable for conservation projects. Furthermore, some species are difficult to detect with conventional methods, if they are e.g., very small or evasive organisms, which are hard to catch or observe (Deiner et al., 2017). When using eDNA analyses, a water sample can be sufficient to detect most of the present species. Furthermore, the size of an organism is not a criterion for detection as long as enough DNA is present in the sample.

Objectives and hypothesis

The objective of the project is to detect non-indigenous species of various phyla from the benthic and pelagic zone in the Danish Wadden Sea. For this purpose, eDNA studies consisting of species-specific qPCRs and metabarcoding analyses as well as conventional methods were conducted. Therefore, settlement plates and water samples from tidal channels and harbors were collected, because a high concentration of NIS was expected in those areas as NIS often spread through ship traffic. For the qPCR analyses, species-specific primers, and probes for 23 non-indigenous species were used. With the method of metabarcoding, the presence of further non-indigenous species was examined. Additionally, it was investigated if sampling during different time points in the tidal cycle will influence the species composition resulting from eDNA analyses. This study provides an updated overview about the currently existing NIS in the Danish Wadden Sea and examines the accuracy and efficiency of eDNA analyses compared to conventional ones. Hence, is designed to answer the question “are environmental DNA analyses the future tool for monitoring?”.

My hypotheses are:

- All used methods will detect NIS in the Danish Wadden Sea
- eDNA analyses will detect certain species, which are hard to identify with the here used conventional methods (e.g., fish or single cell algae) and will result in more detected NIS
- NIS will be detected, which were not identified by previous studies in the Danish Wadden Sea and will result in a growth of the NIS list
- Tidal effects will have an impact on species composition detected by eDNA analyses

Material and Methods

Sampling in the Wadden Sea

Water samples

Water samples for the following eDNA analysis were taken at four tidal channels and three harbors to identify NIS from different phyla living in the benthic or pelagic zone (see Table 1, Figure 2). Many NIS are introduced by ships and therefore first appear in harbors before spreading to other regions by e.g., tidal channels. In every tidal channel three water samples were taken at the same location but with one minute waiting time between the samples or if possible, with 20 m between each other to ensure a water exchange to increase the chance of detecting further NIS in the biological replicates. The latter one was only feasible at the tidal channel Juvre Dyb for sample 1 and 2, but sample 3 was taken at the same location as sample 2. The water samples from the tidal channels were taken one to two hours after start of the rising tide. In the tidal channel Grådyb, two additional samples were taken at the same location one hour before and at the peak of the high tide to investigate if there are tidal effects on eDNA analyses. The water samples were taken with a two liter Van Dorn water sampler (KC Denmark A/S) one meter below the surface, as far as this was doable. To avoid additional sand/particles in the samples, the water samples were always taken before the sediment samples.

Every water sample was filtered twice to create technical replicates and filtering took place max. 1.5 h after sampling. A modified 8 l pressure sprayer (Tryksprøjte 8 l, Art. 45-577, BILTEMA) with an attached Millipore® Sterivex™ filter unit (0.22 µm pore size) was used for this purpose and the volume of the filtered water was measured and noted (see Table 1). The pressure in the sprayer should be always as high as possible without leaking water at the connection between pressure sprayer and filter. The water samples were filtered until the water was very slowly dripping through the filter although the pressure in the sprayer was high. After removing the filter from the pressure sprayer, water inside the filter was taken out with a 10 ml syringe and both ends of the filter were closed with sterile caps. This process was repeated with a second filter for every sample. The pressure sprayer and the water sampler were washed with the new sample water twice, if the sample originated from the same location as the last one, and six times if it was a new location. The filters were frozen in liquid nitrogen and partly stored on ice for a few hours before they were taken to a -20°C freezer (after Knudsen et al. (2020a)).

Table 1: Sampling sites for water samples with GPS coordinates, date, and time of sampling and amount of filtered water in ml.

Location	Sample name	GPS coordinates	Date and time of sampling	Filtered water [ml]
Esbjerg	Esbjerg St. 1a	55.475929°N 8.414073°E	13.09.2022 08:30 am	600
	Esbjerg St. 2a	55.464722°N 8.432887°E	13.09.2022 10:42 am	600
	Esbjerg St. 3a	55.477502°N 8.419556°E	13.09.2022 09:30 am	600
	Esbjerg extra	55.477144°N 8.419481°E	13.09.2022 09:45 am	500
Fanø	Fanø	55.443422°N 8.407235°E	13.09.2022 02:00 pm	600
Rømø	Rømø	55.087974°N 8.566713°E	12.09.2022 01:00 pm	500
Lister Dyb	Lis 1a	55.085521°N 8.57177°E	12.09.2022 12:41 – 12:43 pm	300
	Lis 2a			250
	Lis 3a			250
Juvre Dyb	Juv 1a	55.17224°N 8.57246°E	12.09.2022 11:42 – 11:45 am	400
	Juv 2b	55.172430°N 8.572552°E		350
	Juv 3a			390
Grådyb	Grå 1a	55.471691°N 8.422195°E	13.09.2022 12:25 – 12:27 pm	600
	Grå 2a			450
	Grå 3a			440
	Grå 16a		13.09.2022 04:30 pm	450
	Grå 17a		13.09.2022 05:30 pm	400
Knudedyb	Knu 1a	55.293562°N 8.580369°E	14.09.2022 01:21 – 01:23 pm	190
	Knu 2a			200
	Knu 3a			190

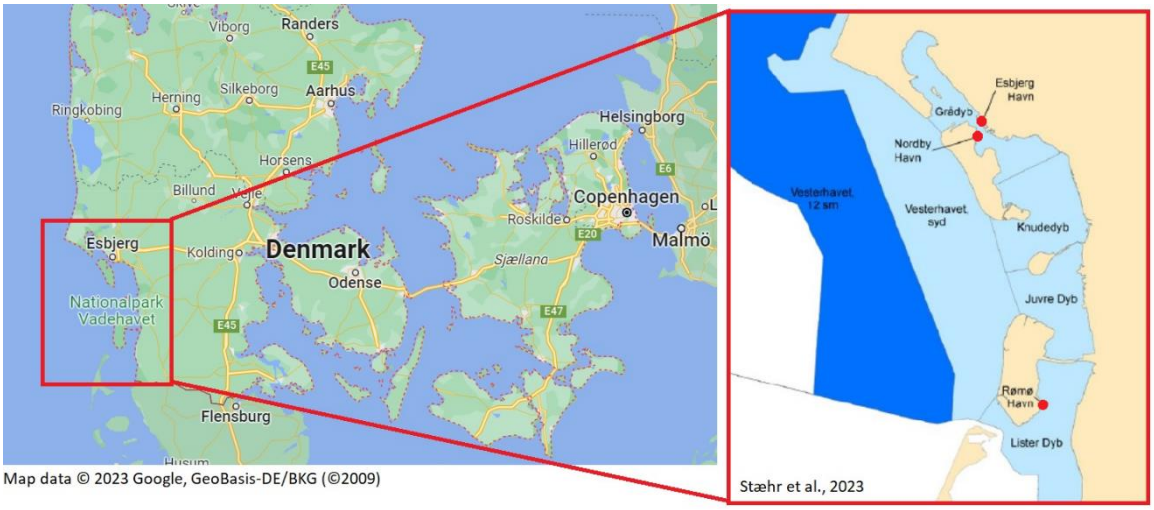

Figure 2: Map of Denmark and the Danish part of the Wadden Sea. The red dots mark the harbors, in which the samples were taken. Nordby Havn is another name for Fanø Harbor.

Settlement plates samples

The use of settlement plates can help to detect benthic organisms attaching to surfaces like algae, mussels, or snails as well as pelagic organisms like fish, if their spawn is attached to the plates. The used settlement plates consisted of 150 x 150 x 5 mm hard PVC plates, which had a hole in the middle to be able to attach them on a rope. The plates were strung on the rope in a way that one plate was 1 m above the sea floor, one was 1 m under the surface and the third plate was placed in between the others. This construction was weighted with an attached brick, marked with a small floating buoy, and affixed to the pier. One settlement plate construction was placed in each of the harbors Fanø and Rømø and in the harbor of Esbjerg three of them were placed on three different spots in May 2022 (21st to 23rd). After removing them from the water in September (12th to 14th), the settlement plates were stored separately in boxes with ethanol by room temperature to fixate the biological material (see Figure 3).

Figure 3: *Left:* Three settlement plates attached to a rope, which were launched in Esbjerg harbor for over 3 months. A brick is attached at the bottom end and a buoy on the top end of the rope. *Right:* Close-up of one settlement plate from Esbjerg harbor, which were stored in a box with ethanol after removing from the harbor until further analyses.

Conventional samples

Multiple conventional analyses were also conducted to detect NIS in the Danish Wadden Sea. For this purpose, the ethanol fixated settlement plates were observed with a dissecting microscope and the species were identified with a taxonomic key to the lowest taxonomic level possible.

Furthermore, soft sediment samples were taken at the harbors and tidal channel stations with a hand-held Van Veen grab (0.025 m^2) and sieved with a 1 mm mesh. The resulting organic material was conserved in ca. 70 % ethanol and brought to a laboratory, where the species were identified as described above.

Besides the sediment samples, scraping samples were collected at all stations with a 10 cm wide scraping device and an attached 1 mm mesh. For this purpose, hard structures like pier walls or big stones were scraped. The obtained material was conserved, and the organisms identified in the same way as the soft sediment samples.

Another conventional technique to detect NIS was the so-called frame-based sampling. Here, a frame with the dimensions 50 x 50 cm was randomly placed on mussel reefs in the four different tidal channel areas. The organisms inside the frame were mapped and collected, which was repeated three times at each station. Additionally, one sample at the border of each reef was collected. The collected species were later identified by a taxonomist, who provided me with the list of detected species. For detailed description of the material and methods from the conventional samples see Stæhr et al. (2023).

DNA extraction, metabarcoding and qPCR

DNA extraction – water samples

To extract the DNA from the Sterivex filters, the DNeasy® Blood & Tissue Kit (Qiagen®) was used based on the protocol of Spens et al. (2017). Before initiating the process, the flow bench was irradiated 1 h with UV-light and afterwards cleaned with a 0.05 % Sodium Hypochlorite solution and 96 % ethanol. In every filter 720 µl Buffer ATL and 80 µl proteinase K were injected by removing the cap at one end and inserting the solutions with a P1000 microliter pipette. After closing the filter again, both caps were covered with parafilm. The samples were mounted to a shaker (200/min) and incubated at 53 – 56 °C. After 02:40 h the filters were turned in a way that the other half of the filter was covered by the buffer ATL-proteinase K solution, and they incubated additional 17 h on the shaker.

On the next day, after cleaning the flow bench as described above, the filters were shaken by hand and the Parafilm and one cap on every filter was removed. The solution with lysate filtrate was taken out of the filters with a 5 ml syringe, the volume of the removed liquid was noted, and it was transferred to a 5 ml Eppendorf-tube. Then the AL-buffer was added to the solution and the samples were vortexed. The amount of added AL-buffer equals the volume of the lysate filtrate solution (ratio 1:1). Subsequently, the samples were incubated in 56 °C for 14 minutes. After incubation, cold 96% ethanol was added with the same amount as the AL-buffer before to have a ratio of 1:1:1

between the lysate filtrate, the AL-buffer and the 96 % ethanol. The samples were vortexed and 600 µl were transferred to a DNeasy Mini spin column placed in a 2 ml collection tube, which were centrifuged for 1 min at 6000 x g. The flow-through was discarded and the procedure was repeated until the whole volume of the sample was used. Next, the steps five to eight from the DNeasy® Blood & Tissue Kit protocol were executed and the extracted DNA was eluted in 350 µl Buffer. The DNA product from one water sample was divided into 7 Eppendorf tubes with 50 µl in each and stored in a -20 °C freezer.

DNA extraction – settlement plates

To extract DNA from the settlement plates, samples from at least five different spots of the top and bottom side from each plate were taken in a flow bench. If any other organisms were visible on the plate, which were not covered by the chosen five spots, they were included as well. The organisms attached to the settlement plate within the spots were carefully removed with a scalpel and tweezers and transferred to a TPP® 50 ml centrifuge tube. For every plate, a new sterile scalpel and tweezers were used. Every tube could contain up to 40 g wet weight of the samples. The tubes were centrifuged at 3000 rpm for 5 minutes and the supernatant was removed with a pipette. Afterwards, the samples were air-dried in a flow bench for 4 hours and stored in a -20 °C freezer afterwards. On the following day, the samples were transferred to a -80 °C freezer for 3 hours before putting them into the freeze drier Coolsafe 110-4 (LaboGene) for 3 days. To homogenize the freeze-dried samples, a bead mill homogenizer (Bead Ruptor Elite, Omni International) was used with 15 metal beads with a size of 2.4 mm. The samples F1.1a, F1.2a and F1.3a were shaken in 3 cycles with 4 m/s for 30 sec and a 4. cycle with 4 m/s for 45 sec as some parts of mussel shells were not pulverized enough after three rounds. The other samples were shaken with 3 cycles à 4 m/s for 30 sec. The samples were stored at -20 °C until the DNA extraction was processed with the DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen®). For the extraction, a maximum of 0.26 g dry weight of each sample was transferred to the provided PowerBead Tubes and stored at room temperature for 5 days. The DNA was extracted as described in the protocol of the kit with a few exceptions. At step three the tubes were inverted three times and afterwards vortexed for 3 seconds. The samples were shaken in the bead beater at 4000 RPM for 45 seconds and subsequently centrifuged at 10,000 x g for 3 minutes. The supernatants were pipetted to a 2 ml collection tube together with 250 µl of Solution C2 and after briefly vortexing they incubated for 10 minutes at 4 °C. The next steps were carried out as described in the kit's protocol and the extracted DNA from the settlement plates was eluted with a volume of 100 µl and stored at -20 °C.

Measuring DNA concentration

The DNA concentration in the samples was measured using a Qubit™ 4.0 fluorometer (Thermo Fisher Scientific Inc®). First, the samples were vortexed briefly and then centrifuged for 10 sec at 1000 x g. To prepare the two standards, 190 µl of the Qubit™ working solution was transferred to an assay tube each and 10 µl Standard (from kit) was added. For the samples, 198 µl of the working solution and 2 µl of the samples were pipetted into assay tubes. All tubes were vortexed for 3 sec and afterwards incubated for 2 min at room temperature. The Qubit™ Fluorometer was used with

the settings “dsDNA”, “1xdsDNA High Sensitivity” and “ng/µl” as the output unit along with “2 µl” accordingly to the actual sample volume. After reading both standards, all samples were measured.

Metabarcoding (COI, 12S and 18S)

Two step PCR

Before starting the first PCR for metabarcoding, the samples and needed reagents were briefly vortexed and centrifuged for 5 sec at 1000 x g. If the DNA concentration from the samples was above 10 ng/µl, they were diluted with PCR water. The flow bench was irradiated for 30 min with UV-light and subsequently cleaned with 70 % ethanol. A technical replicate (2 PCR-plates) was used to minimize variations and one negative control per PCR-plate was included as well (Staehr et al., 2022). For the master mix PCR water was pipetted in a 1.5 ml Eppendorf tube, followed by BSA (20 mg/ml), forward- and reverse-primer (10 µM, see Table 2), and KaPa Hifi- Master (Volumes see appendix Table 9). An aliquot of 23 µl Master Mix for each sample was transferred to a PCR-plates and afterwards 2 µl of sample DNA were added. After closing the two PCR-plates with lids, they were briefly centrifuged before starting the PCR (Protocol see appendix Table 10). Afterwards, 15 µl of each PCR product was pooled with 15 µl of its replicate. The pooled products were run on a 1.5% agarose gel (1 µl loading buffer and 4 µl PCR product, 5 µl of 100 bp ladder) at 140 V for 60 min. The gel was checked and photographed under UV light.

Table 2: Used primers targeting the different genes and communities for metabarcoding.

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

After checking the PCR products on a gel, they were cleaned by using magnetic beads from a HighPrep™ PCR Clean-up System (MagBio Genomics Inc. Gaithersburg, USA). PCR 1 products with 12S primers were not cleaned before PCR 2. To clean the products, the plate with the samples was centrifuged to collect condensation. The MagBio beads were vortexed for 30 seconds and afterwards 15 µl of them were added to each sample and mixed by using the pipette. Afterwards the official protocol by MagBio was followed, with the exception that 190 µl of 80 % ethanol was used for the two washing steps and 27 µl 1x TE buffer for the elution. An aliquot of 25 µl of the cleared supernatant with the cleaned product was transferred to a new plate.

The DNA concentration of these cleaned PCR products was measured with Qubit™ as described above and the products were used afterwards for a second PCR.

For the second PCR the cleaned PCR 1 products were diluted with PCR water according to their DNA concentration (see Table 11). The PCR was prepared as described above for PCR1, but a negative

control and technical replicates were not included for this procedure. For the ingredients and volumes see Table 12 and for the protocol see Table 13. The samples were labeled with a unique combination of two indexes, which made it possible to assign the sequenced reads to the samples later. The resulting PCR products with COI, 12S and 18S primers were cleaned with HighPrep™ magnetic beads as described above and checked afterwards on a 1.5% agarose gel (1 µl loading buffer and 4 µl PCR product, 5 µl of 100 bp ladder) at 140 V for 60 min. An uncleaned PCR1 product was included on the gel as well for comparison next to both ladders.

Pooling samples

The DNA concentrations of the cleaned PCR 2 products were quantified with Qubit™ as described above. With those values the needed volume from every sample for pooling was calculated, so that for COI 100 ng DNA per sample and for 12S and 18S 40 ng DNA per sample were added. Afterwards the DNA concentrations of the three pools were determined with Qubit™.

With TapeStation 4150 by Agilent, which is an automated electrophoresis tool, a D1000 ScreenTape Assay was performed to analyze the pools and control their quality before starting the sequencing process. To do so, the official protocol for D1000 ScreenTape Assay Operating Procedure from Agilent was followed for one ScreenTape device.

The results from the TapeStation indicate if the PCR product pools are contaminated or if there are, for example, primer dimers. Furthermore, the size of the DNA fragments can be measured. In this case the ladder was not recognized properly, which led to incorrectly measured DNA fragment sizes. The true length of DNA fragments could be checked on the agarose gels ran before.

The PCR product pools were stored at -20°C freezer until they were sequenced with the Illumina MiSeq platform at DCE, Aarhus University.

Bioinformatic analyses

The resulting DNA reads were analyzed with QIIME2 (Bolyen et al., 2019) and its plugin DADA2 (Callahan et al., 2016). The reads were trimmed to exclude the primer sequences and any bases after 230 bp. With QIIMES2 naive Bayes classifier, which was trained on 99% Operational Taxonomic Units (SILVA rRNA database v. 138, Quast et al. (2013)), the trimmed 18S rDNA amplicon sequence variants (ASVs) were classified. ASVs from the 12S dataset with less than ten reads were removed before the remaining 12S reads were blasted against the Mitofish database (Iwasaki et al., 2013). For the COI reads, the database MIDORI2 (Leray et al., 2022) was used and additionally the COI ASVs were blasted against BOLD database with the sequence-id tool (www.gbif.org). All used databases are to a large degree curated. The resulting taxa with a high similarity and coverage over 97 % were identified to species level.

With the software R (v. 4.2.2, R Core Team (2021)) and the package “phyloseq” (McMurdie & Holmes, 2013) the OTU/ASV tables and taxonomy files were statistically analyzed and visualized.

qPCR analyses

With species specific qPCR analyses, small amounts of DNA from a target species can be detected and quantified (Arya et al., 2005). This method was used to detect 23 non-indigenous species in the water samples and samples from the settlement plates.

Preparation of NIS standards

First, the standards needed to be prepared. For some organisms already extracted DNA or PCR products were available and in other cases the DNA still needed to be extracted. To do so, the DNeasy® Blood & Tissue Kit by QIAGEN was used and the official protocol by QIAGEN was followed. For the first step of the official protocol from the Kit, option 1d (for cultured cells) was used. At the second step, the incubation time was extended to 20 min and the optional step 9 was carried out. After the DNA was extracted, the concentration was measured with Qubit™ as described above. The DNA samples did not need to be diluted for the following PCR, if their concentration was lower than 2.1 ng/µl. The other samples were diluted in a way that their final DNA concentration was between 2 and 1 ng/µl. In the next step, a PCR was run with the extracted DNA and depending on whether the extracted DNA was diluted or not, different amounts of PCR water and DNA were used for the PCR (Master Mix composition see Table *14*, PCR protocol see Table *15*).

The DNA concentration of the resulting PCR products was measured with Qubit™ and afterwards the PCR products were cleaned with QIAquick® PCR Purification Kit. The reagents were prepared as described in the Quick-Start Protocol from QIAGEN. All centrifugations were performed with 8000 x g and a duration of 1 minute. An aliquot of 115 µl of Buffer PB were added to 23 µl of each sample to reach a 5:1 ratio. Furthermore, 10 µl 3M sodium acetate and 0.5 µl of pH indicator I were added and mixed with a pipette. The next steps were carried out as described in the protocol and as a last step the products were eluted with 50 µl Buffer EB.

After cleaning the standards, the DNA concentration was determined by using Qubit™ 4.0 fluorometer.

qPCR runs and data analyses

To run a qPCR, the master mix was prepared in a clean room to avoid contamination. This room is free from DNA samples, and it is deeply cleaned every week. Furthermore, a UV light was turned on 30 min before starting pipetting and plastic arm cover sleeves were used to minimize the risk of contamination. After making the master mix for qPCR (for composition see appendix Table *16*; primers and probes after Andersen et al. (2018); Knudsen et al. (2020b); Knudsen et al. (2022), see appendix Table *18*), it was transported to a different room, in which the master mix was distributed in a 96-wells qPCR plate in a flow bench. Triplicates were used to be able to spot false positive signals and enable a more precise determination of the detection limit and species detection. Different standard dilutions were prepared, which ranged from 10^{-5} to 10^{-11} ng/µl. An aliquot of 3 µl of standards and samples were added to each well as well as PCR H_2O as a negative control. During this process, the qPCR plate as well as the samples, standards and master mix were stored on ice. The wells were closed with qPCR lids and the plate shortly centrifuged, before starting the qPCR run in a BioRAD Real-time PCR system (Life Technologies) as described in Table *17*. The software CFX Manager Version 3.1.1517.0823 was used for running and editing the qPCRs as well as calculating

the standard curve. If the negative controls of a qPCR run had positive signals in the range of the standards, the run was repeated up to five times until this issue was solved. A standard signal was excluded from the analysis if its cycle of quantification (cq value) was too far away from the other standards with the same dilution and would lead to an R-squared value below 0.9. This was the case in seven runs with one or more triplicates from standard 11 or 10. Some samples were excluded in the run testing for *Oncorhyncus gorbuscha*, because their signal curve did not show the characteristic s-shape, but showed a nearly horizontal, slow rising curve instead. In some cases, the base line was adjusted to exclude noise, e.g., if a signal increases in one cycle, but decreases approximately in the same amount in the following cycle. By entering the amount of copy numbers of every standard dilution, the software could calculate those of the positive samples. To determine the limit of detection (LOD) and limit of quantification (LOQ) of each qPCR run, two different approaches were used. In the first approach, LOD equals the average Cq of the lowest standard dilution with at least one signal. For the limit of quantification, the average of the lowest standard concentration with signals in all three triplicates was computed. This definition was also used in Stæhr et al. (2023). The second approach after Klymus et al. (2020a) describes LOD as the average of the lowest standard concentration with 95% positive signals, which equals three positive replicates in this study. LOQ corresponds to the lowest standard concentration with a CV value ≤ 35. The CV value was calculated with the formula $CV_{ln} = \sqrt{(1+E)^{(SD(C_q))^2 * ln(1+E)} - 1}$ with E as the qPCR efficiency and SD (Cq) as the standard deviation of replicate Cq values.

Afterwards, the samples were categorized into four groups: no detection, detection with copy numbers below LOD, detection with copy numbers between LOD and LOQ, and detection with copy numbers above LOQ.

Results

NIS-detection methods

Metabarcoding

One of the methods used to detect NIS in the water samples and settlement plate samples was metabarcoding. After conducting PCRs with universal primers and unique tags for the different samples, the PCR products were pooled and sequenced with MiSeq Illumina. After sequencing, the quality of the resulting reads was checked. The reads from every primer set had an overall good quality with a quality score above 30 except for the first and last few base pairs, where the quality dropped to a score of 15.

The majority of reads from the COI region had a length of 313 bp. The shortest read was 204 bp long and the longest 396 bp, which leads to a standard deviation of 31. Before the quality check, a total of 2,300,176 reads of the COI region were obtained, which reduced to a number of 1,510,491 reads after the quality control.

The reads resulting from the 12S primer set had a median length of 255 bp. The minimum length of reads was 209 bp and the maximum 396 bp, which results in a standard deviation of 7. After sequencing, 1,836,114 reads were obtained, which decreased to 226,265 reads after the quality control and measures. It is noticeable that the settlement plates samples produced less reads than the water samples with this primer set. That is most likely the case, because the target organisms of 12S primers are fish and it is quite unlikely to find a large amount of fish DNA on settlement plates.

The median length of the reads targeting the 18S region was 362 bp, the minimum 222 and the maximum 408 bp. The calculated standard deviation of the read length was 18 and lays therefore in between the reads of 12S and COI region. The number of reads declined from 2,462,800 to 1,858,651 through the quality measures.

The quality of all sequenced samples was decent as the quality score was above 30 in most positions, so that the data could be further treated and analyzed.

Found non-indigenous species

After the quality control and measures, the taxonomy of the amplicon sequence variants was assigned by blasting them against a database to identify the species in the samples. Afterwards, a list of marine NIS in Europe was used to filter out the NIS from the identified species. The results were manually checked and corrected, which lead to a list of 31 NIS detected with metabarcoding (see Table 3). Most NIS were detected at Esbjerg with 16 species found in the water samples and settlement plates, respectively. The tidal channel with the most identified NIS is Grådyb with 14 and the least number of NIS was found in Knudedyb with 3 species. Comparing the different sample types, the settlement plates and water samples from the tidal channels both detected 19 NIS each and with the water samples from the harbors 18 NIS could be found (see Table 3). All three sample techniques detected the same eight NIS. The water samples from the harbors and tidal channels

uniquely identified 3 species each, whereas the analyses of the settlement plates resulted in eight species not detected by the other sample types (see Figure 4).

Table 3: Detected NIS at the different samples sites and types by using metabarcoding. An "x" indicates that the species was detected in at least one sample. In total, 31 NIS could be detected with this method. The species are in a taxonomic order and the bold horizontal lines indicate which species belong to the same taxonomic group.

Location	**Esbjerg**		**Fanø**		**Rømø**		**Grå-dyb**	**Juvre Dyb**	**Knude-dyb**	**Lister Dyb**	**Total**		
Species	settlement plates	water samples	settlement plates	water samples	settlement plates	water samples	water samples	water samples	water samples	water samples	**settlement plates harbors**	**water samples harbors**	**water samples tidal channels**
Pseudochattonella verruculosa		x		x			x			x		x	x
Fibrocapsa japonica		x				x	x			x		x	x
Chattonella marina	x		x		x			x	x		x		x
Prorocentrum cordatum							x			x			x
Prorocentrum triestinum		x										x	
Saccharina japonica						x				x		x	x
Fucus distichus		x					x			x		x	x
Agarophyton vermiculophyllum							x	x					x
Dasysiphonia japonica	x	x								x	x	x	x
Tricellaria inopinata	x	x									x	x	
Magallana gigas	x	x	x	x		x	x	x		x	x	x	x
Ruditapes philippinarum					x						x		
Mya arenaria	x	x	x			x	x	x	x	x	x	x	x
Haliclystus tenuis	x		x		x						x		
Mnemiopsis leidyi		x					x					x	x
Polydora websteri	x	x			x					x	x	x	x
Polydora cornuta	x	x	x		x	x	x	x			x	x	x
Streblospio benedicti		x					x	x		x		x	x
Mytilicola intestinalis					x						x		
Acartia tonsa	x	x		x			x	x	x	x	x	x	x

Location	Esbjerg		Fanø		Rømø		Grå-dyb	Juvre Dyb	Knude-dyb	Lister Dyb	Total		
Species	settlement plates	water samples	settlement plates	water samples	settlement plates	water samples	water samples	water samples	water samples	water samples	**settlement plates harbors**	**water samples harbors**	**water samples tidal channels**
Austrominius modestus	x	x	x		x	x	x				x	x	x
Amphibalanus improvisus	x	x	x		x		x	x			x	x	x
Balanus glandula	x				x						x		
Hemigrapsus takanoi	x										x		
Jassa marmorata					x						x		
Caprella mutica	x										x		
Diplosoma listerianum	x						x				x		x
Molgula manhattensis	x				x						x		
Chromis multilineata								x					x
Rastrelliger kanagurta						x						x	
Cyprinus carpio		x										x	
Total number detected species	16	16	7	3	11	7	14	9	3	11	**19**	**18**	**19**

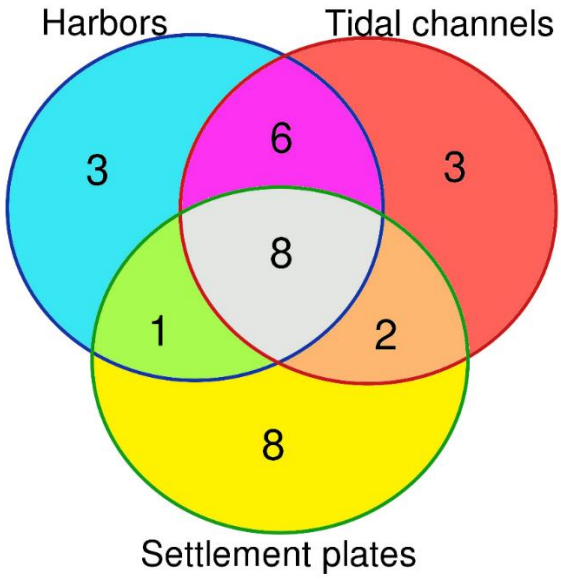

Figure 4: Venn diagram of the number of detected NIS with metabarcoding differentiate by the three used sample types. The category “Harbors” only includes the water samples taken from the harbors.

qPCR

Besides metabarcoding, species-specific qPCR analyses were conducted to detect NIS. For this method, detection systems for 23 non-indigenous species were available. By using qPCR, 16 out of 23 non-indigenous species could be detected with approach 1 to determine the limit of detection (LOD) and limit of quantification (LOQ). A low cycle of quantification (Cq value) stands for a high DNA concentration of the target sequence in the sample. A species is considered as detected, if at least one sample of the triplet had a Cq value below LOD and is marked in the tables with an orange, red or black background. In the result tables for qPCR analyses, a white background means that no signal was received in any of the sample triplicates and therefore the species could not be detected. Yellow highlighted samples have a signal in at least one of the triplicates, which is still above the limit of detection and consequently the species is not seen as detected here either. An orange background indicates that at least one sample of the triplicates had a Cq value below LOD, but above the limit of quantification. Nevertheless, the species is considered as detected in that case. Samples, which are highlighted in red, had minimum one sample of the triplicate with a Cq value below the limit of quantification and the species is therefore classified as detected. Samples with a black background had signals from all triplicates below the limit of quantification and the species is subsequently seen as detected.

The location with the most detected species was Rømø harbor for the water samples and Esbjerg station 3 for the settlement plates with 10 detected species each. If the water samples from all four stations in Esbjerg harbor were taken together, 11 NIS could be identified in that harbor (see appendix Table 19, 20 and 21).

By comparing the two different approaches used for determining LOD and LOQ, it gets clear that approach 1 is more sensitive in detecting non-indigenous species than approach 2. With approach 1, 13 species in the water samples from the harbors and tidal channels, as well as 13 species from the settlement plate samples could be detected (see appendix Table 19, 20 and 21). Thus, the detected species variate by using the different methods (see Table 5).

With approach 2, 12 species in the water samples from the harbors and 11 in the ones from the tidal channels could be detected (see appendix Table 22 and 24). Only 9 species were classified as detected from the settlement plates with this approach (see appendix Table 23).

Comparing the two approaches directly for the harbor samples, approach 2 classified the qPCR results for species *Pseudochattonella verruculosa* at all stations one or two categories below the results from approach 1. In case of the species *Mya arenaria*, all three replicates from Esbjerg harbor station 3 had a Cq value below LOQ with approach 1, but a Cq value between LOD and LOQ when applying approach 2. At Fanø harbor the species was detected with two of the triplicates by analyzing with approach 1, but not detected with approach 2 as the Cq values were above LOD. By using the first approach, the species *Hemigrapsus takanoi* was categories as detected in any of the harbors, while the use of approach 2 led to a detection only in Esbjerg harbor station 3 (see Table 4). The same trend is shown when comparing the total results from all stations and sample types with the two different approaches for determine LOD and LOQ. Here, big differences can be seen for the species *P. cordatum*, *P. farcimen, M. gigas* and *M. leidyi* as well as for the three species mentioned before (see Figure *5*).

Table 4: Comparison of the two different approaches to determine LOD and LOQ (defined as described above) shown on qPCR results from water samples taken from the harbors. The triplicates from every sample were categorized in “No Cq” / “Signal with Cq above than LOD” / “Signal with Cq between LOD and LOQ” / “Signal with Cq lower than LOQ”. Yellow highlighted samples have at least one positive signal, which has a Cq above LOD, but are not considered as detected. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD, which is the case for orange, red and black highlighted samples.

Species	Approach	Esbjerg harbor 1	Esbjerg harbor 2	Esbjerg harbor 3	Esbjerg harbor extra	Fanø harbor	Rømø harbor
Pseudochattonella verruculosa	1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/1/2
	2	0/0/3/0	0/0/1/2	0/0/3/0	0/0/1/2	0/0/3/0	0/1/2/0
Mya arenaria	1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/1/2/0	0/0/0/3
	2	0/0/0/3	0/0/0/3	0/0/3/0	0/0/0/3	0/3/0/0	0/0/0/3
Hemigrapsus takanoi	1	2/0/1/0	2/0/1/0	1/0/1/1	1/1/1/0	0/2/1/0	1/0/2/0
	2	2/1/0/0	2/1/0/0	1/1/1/0	1/2/0/0	0/3/0/0	1/2/0/0

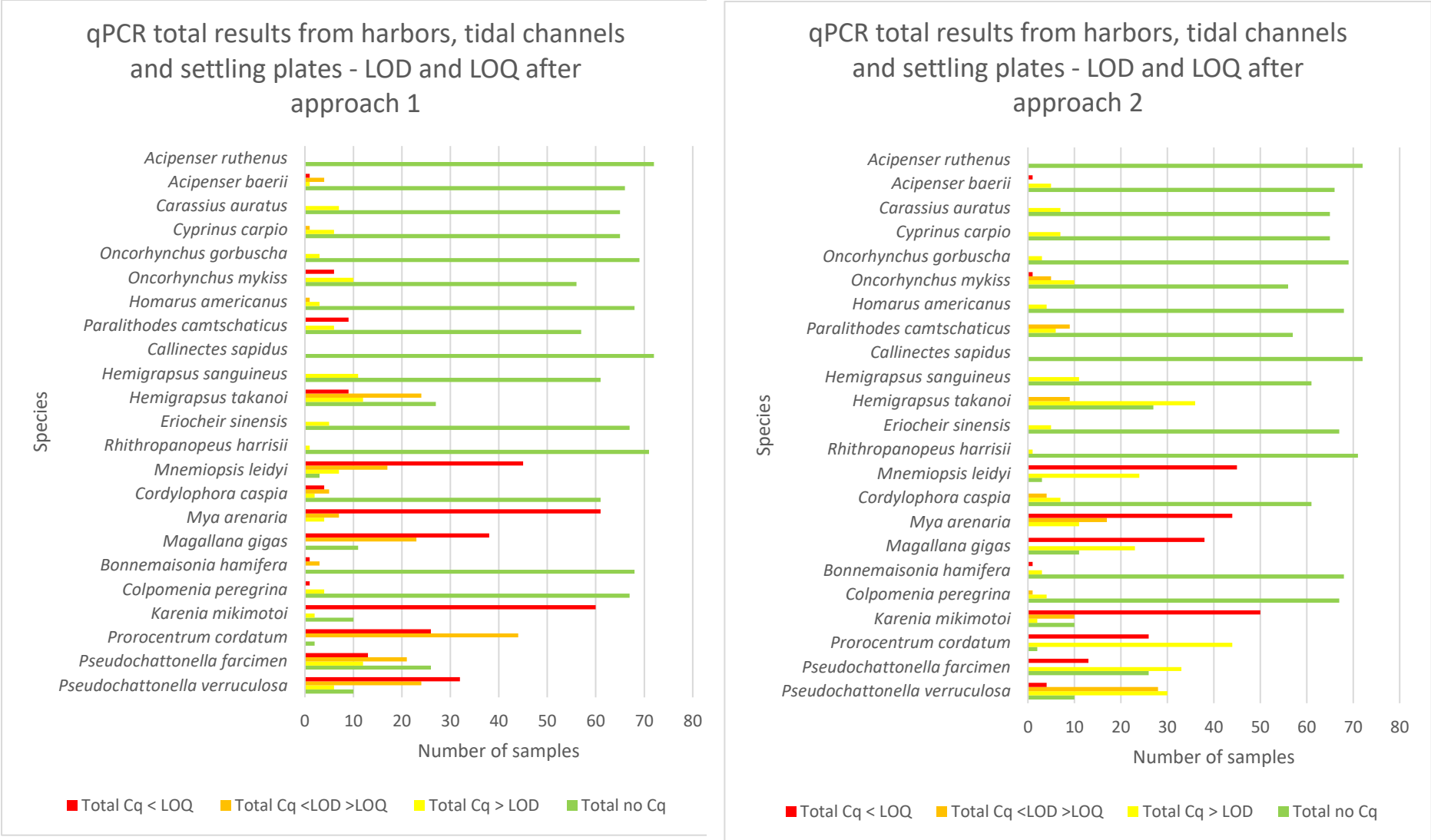

Figure 5: Summary of qPCR analyses results from all samples and locations. LOD and LOQ were determined after approach 1 (left graph) and approach 2 (right graph) as explained above.

In the next section, the detected species (at least one triplicate had a Cq < LOD (using approach 1)) from the different sample origins (water samples taken at the harbors and tidal channels, and settlement plates) were compared. With each sample type, 13 NIS could be detected and moreover an intersection between all three sample types of 10 species is revealed (see figure Figure 6). The species *Homarus americanus* is only detected from the settlement plate samples and the two species *Colpomenia peregrina* and *Cyprinus carpio* were only found in the water samples from the harbors (see Table *5*).

Table 5: Intersection of detected species with different sample types and locations for qPCR analyses. A species is considered as detected, if at least one Cq values of the triplets is lower than LOD. LOD was determined with approach 1 here.

Detected by	**Number species**	**Species name**
All three sample types	10	*Pseudochattonella verruculosa* *Prorocentrum cordatum* *Karenia mikimotoi* *Bonnemaisonia hamifera* *Magallana gigas* *Mya arenaria* *Cordylophora caspia* *Mnemiopsis leidyi* *Hemigrapsus takanoi* *Oncorhynchus mykiss*
Water samples from harbors and tidal channels	1	*Pseudochattonella farcimen*
Settlement plates and water samples from tidal channels	2	*Paralithodes camtschaticus* *Acipenser baerii*
Water samples from harbors	2	*Colpomenia peregrina* *Cyprinus carpio*
Settlement plates	1	*Homarus americanus*

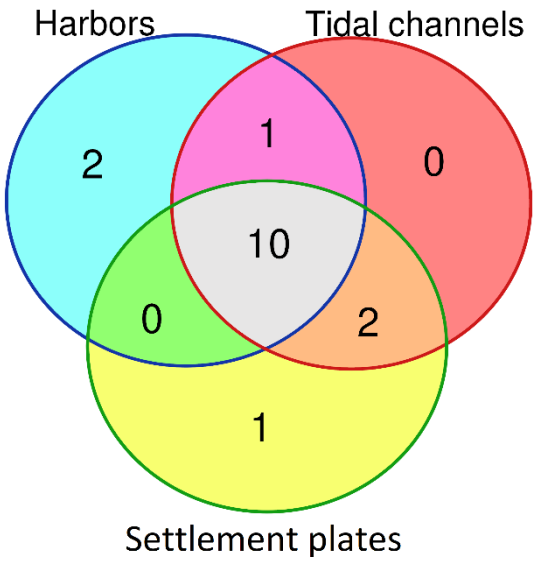

Figure 6: Venn diagram of the intersection from the detected species within the different methods and sampling locations. A species is considered as detected if at least one Cq values of the triplets is lower than LOD. LOD was determined with approach 1 here. The category "Harbors" includes only the water samples taken from the harbors.

Tidal effects on eDNA analyses

One research question of the present study was if the sampling time point in the tidal cycle affects the detected biodiversity with eDNA analyses. The effect of the tides was examined by sampling at three different time points at the tidal channel Grådyb. The first three samples are technical replicates (a – c) and were collected one hour after the lowest water level. The next sample was taken at 16:30, which was one hour before the peak of the flood. The last sample at 17:30 was taken during the peak of the flood and subsequently the highest water level.
The metabarcoding analyses only showed minor differences between the samples from different time points. Two species were only detected in the samples around the high tide (Grådyb 16:30 and 17:30), but with a very low copy number of 2. Interestingly, *P. cordatum* was not detected in the 16:30 and 17:30 samples as well as *P. verruculosa* could not be identified in the 17:30 sample by metabarcoding, which differs from the qPCR results. The comb jelly *Mnemiopsis leidyi* was only found in the triplicates taken at 12:30, which also varies from the qPCR results, where the species was detected in all samples. Furthermore, the huge range of copy numbers from *Magallana gigas* is remarkable. The samples collected an hour after low tide (12:30) showed copy numbers between 1583 and 3019, which stand in contrast to the other samples with 562 and 63 copies respectively (see Table 6).
A similar trend is shown by the qPCR results from the same samples. A species is seen as detected if the cycle of quantification is below the limit of detection, which is marked with the colors orange, red and black. The two species *Paralithodes camtschaticus* and *Oncorhynchus mykiss* were only detected at the 16:30 sample. Apart from this, no pattern of detected species between the samples is cognizable, except for a slightly higher detection rate of the phytoplankton species *Pseudochattonella farcimen* and *Prorocentrum cordatum* in the sample taken at 16:30. Also the other phytoplankton species *Pseudochattonella verruculosa* and *Karenia mikimotoi* were detected with a high concentration in the two later samples (see Table 7).

Table 6: Detected NIS from metabarcoding analyses and their copy numbers of the targeted genes are displayed. The analyses were conducted with water samples taken at the tidal channel Grådyb. To analyze the tidal effects, samples were taken at different time points. The samples at 12:30 (a – c) are technical triplicates. Red marked numbers show that those species were only detected in samples from one time point.

Species	Grådyb 12:30 a	Grådyb 12:30 b	Grådyb 12:30 c	Grådyb 16:30	Grådyb 17:30
Pseudochattonella verruculosa	20	23	22	22	0
Fibrocapsa japonica	77	60	47	64	36
Prorocentrum cordatum	15	14	0	0	0
Fucus distichus	27	52	34	66	10
Agarophyton vermiculophyllum	0	0	0	0	2
Diplosoma listerianum	0	0	2	0	0
Magallana gigas	2509	3019	1583	562	63
Mya arenaria	10	12	20	32	11
Mnemiopsis leidyi	20	44	35	0	0
Polydora cornuta	0	8	28	14	0
Streblospio benedicti	0	0	0	15	0
Acartia tonsa	63	0	58	0	694
Austrominius modestus	0	0	23	0	0
Amphibalanus improvisus	0	0	5	72	0

Table 7: Results of qPCR analyses from water samples taken at the tidal channel Grådyb. To analyze the tidal effects, samples were taken at different time points. The samples at 12:30 (a – c) are technical triplicates. Every sample was analyzed with species-specific qPCRs, which were conducted with triplicates. The triplicates from every sample were categorized in "No Cq" / "Signal with Cq above than LOD" / "Signal with Cq between LOD and LOQ" / "Signal with Cq lower than LOQ". Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD and are highlighted in orange, red or black in this table. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	Taxonomic group	Grådyb 12:30 a	Grådyb 12:30 b	Grådyb 12:30 c	Grådyb 16:30	Grådyb 17:30
Pseudochattonella verruculosa	Dictyochophyceae	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	1/0/0/2
Pseudochattonella farcimen		0/0/2/1	0/0/3/0	0/0/2/1	0/0/0/3	0/0/2/1
Prorocentrum cordatum	Dinophyceae	0/0/1/2	0/0/0/3	0/0/3/0	0/0/0/3	0/0/3/0
Karenia mikimotoi		0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Colpomenia peregrina	Phaeophyceae	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Bonnemaisonia hamifera	Rhodophyta	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Magallana gigas	Bivalvia	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	1/0/0/2
Mya arenaria		0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Cordylophora caspia	Cnidaria	3/0/0/0	2/0/0/1	3/0/0/0	3/0/0/0	3/0/0/0
Mnemiopsis leidyi	Ctenophora	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/1/2/0
Rhithropanopeus harrisii	Decapoda	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0
Eriocheir sinensis		3/0/0/0	2/1/0/0	2/1/0/0	3/0/0/0	3/0/0/0
Hemigrapsus takanoi		2/0/1/0	0/0/1/2	0/0/3/0	1/2/0/0	1/0/1/1
Hemigrapsus sanguineus		3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	1/2/0/0
Callinectes sapidus		3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Paralithodes camtschaticus		3/0/0/0	2/1/0/0	3/0/0/0	1/0/0/2	3/0/0/0
Homarus americanus		3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	Actinopteri	3/0/0/0	2/1/0/0	3/0/0/0	2/0/0/1	3/0/0/0
Oncorhynchus gorbuscha		3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	0/3/0/0
Cyprinus carpio		3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0
Carassius auratus		2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser baerii		3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0	2/0/1/0
Acipenser spp.		3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Conventional methods and total results

Besides eDNA analyses, conventional methods were used to identify NIS in the Danish Wadden Sea. To do so, frame-, soft sediment- and scraping samples were taken and the settlement plates were examined with a dissecting microscope and a taxonomic key was used for species identification. With those methods, a total of 22 NIS could be detected, from which ten species could not be detected with eDNA analyses (see Table 8). Of those ten species, *Botrylloides violaceus* and *Bugulina stononifera* are here found in the Danish Wadden Sea for the first time. Nine species were identified with metabarcoding and conventional methods and the presence of three species could be confirmed with all three detection tools. Combining the results from all used methods, 50 NIS could be identified in the Danish Wadden Sea, of which 40 NIS were detected with eDNA analyses (see Figure 7, Table 8).

Table 8: List of detected NIS with metabarcoding, qPCR and conventional analyses. For further information about the detected species with conventional methods see Stæhr et al. (2023).

Detected with	**Total**	**Species**
Conventional, metabarcoding and qPCR analyses	3	*Mya arenaria*
		Magallana gigas
		Hemigrapsus takanoi
Metabarcoding and qPCR analyses	4	*Pseudochattonella verruculosa*
		Prorocentrum cordatum
		Mnemiopsis leidyi
		Cyprinus carpio
Conventional and metabarcoding analyses	9	*Amphibalanus improvisus*
		Agarophyton vermiculophyllum
		Polydora cornuta
		Austrominius modestus
		Caprella mutica
		Dasysiphonia japonica
		Jassa marmorata
		Streblospio benedicti
		Molgula manhattensis
Only with metabarcoding analyses	15	*Prorocentrum triestinum*
		Mytilicola intestinalis
		Haliclystus tenuis
		Fucus distichus
		Chattonella marina
		Polydora websteri
		Tricellaria inopinata
		Ruditapes philippinarum
		Saccharina japonica
		Chromis multilineata
		Fibrocapsa japonica

Detected with	Total	Species
Only with metabarcoding analyses	15	*Balanus glandula*
		Diplosoma listerianum
		Acartia tonsa
		Rastrelliger kanagurta
Only with qPCR analyses	9	*Colpomenia peregrina*
		Bonnemaisonia hamifera
		Homarus americanus
		Karenia mikimotoi
		Pseudochattonella farcimen
		Oncorhynchus mykiss
		Cordylophora caspia
		Acipenser baerii
		Paralithodes camtschaticus
Only with conventional analyses	10	*Botrylloides violaceus*
		Bugulina stononifera
		Melanothamnus harveyi
		Styela clava
		Alitta succinea
		Antithamnionella spirographidis
		Dasya baillouviana
		Aphelochaeta marioni
		Crepidula fornicata
		Hemigrapsus sanguineus

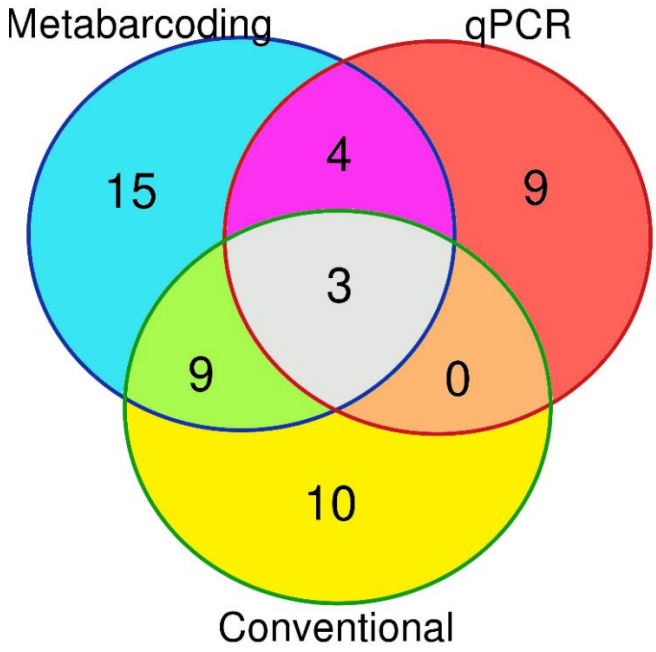

Figure 7: Venn diagram of the number of detected NIS with metabarcoding, qPCR and conventional analyses. A total of 50 NIS could be identified with all three tools. Metabarcoding revealed 31 NIS, qPCR analyses resulted in 16 NIS and with conventional methods 22 NIS were detected.

Discussion

In the present project, three different methods were used to detect non-indigenous species in the Danish Wadden Sea. Besides conventional methods, the DNA based tools metabarcoding and species-specific qPCR were utilized on water samples and samples from settlement plates. Both sample types were taken at the three harbors Esbjerg, Fanø and Rømø and water samples were additionally collected at the tidal channels Lister Dyb, Juvre Dyb, Grådyb and Knudedyb. With the present project, NIS in the Danish Wadden Sea were identified. It was investigated if the used eDNA methods can detect more NIS than by conventional tools and therefore may replace them in the future. Furthermore, the influence of sampling during different time points in the tidal cycle on the detected biodiversity with eDNA methods was an object of this study.

Detection of NIS with metabarcoding

For this study, metabarcoding was one of the techniques used to detect NIS in the Danish Wadden Sea. It was conducted with three different universal primers targeting the genes 12S rDNA, 18S rDNA and COI to cover different taxonomic groups. Different environmental samples were taken, which were either water samples or settlement plates. The water samples should mainly capture the DNA from organisms living in the pelagic zone, while the settlement plates should detect benthic species.

Metabarcoding analyses could detect 31 NIS in total, including 15 species, which could not be found with the other methods. Taking a closer look to the found species, it appears that *Acartia tonsa* and *Mya arenaria* were detected in all tidal channels. Both species were furthermore detected in samples from the harbors Esbjerg and Fanø, and *M. arenaria* was additionally identified in Rømø harbor (see Table 3). This indicates that those two species are very widespread in the Danish Wadden Sea. The mussel species *M. arenaria* can tolerate a wide range of environmental conditions, which is why it can settle in different habitats. Furthermore, it has a high fertility, and the juveniles can stick to hard substrate, which might lead to unintended dispersal by e.g., ship traffic and are the bases of its high spreading. The mussel species is known as very common in the Wadden Sea for over hundreds of years and contribute highly to the benthic biomass in this habitat (Beukema, 1982; Strasser, 1998).

Interestingly, the copepod *A. tonsa* was also detected on settlement plates samples, although it is not a benthic species, but lives in the pelagic zone instead. The positive settlement plate sample could be explained with either eggs from this species placed on the plate or feces from another species digesting this copepod species encountering the plate. The species was found in Denmark for the first time at Ringkøbing Fjord in the year 1921 (Jespersen, 1933). This copepod is a good example of an opportunistic species as it can tolerate a broad range of environmental conditions. It can be found in waters with a salinity between 5 and 36 psu, and temperatures ranging from -1°C to 32°C (Cervetto et al., 1999; Chaalali et al., 2013; Hansen et al., 2012). Nowadays, the species is one of the most abundant copepod species in the Wadden Sea, which is why the detection of it in this study was expected (Jensen, 2010; Peck et al., 2015).

Looking at the different sample types, it is noticeable that eight species, including five crustaceans, were only detected in the settlement plate samples (see Table 3). It could be that the crustaceans crawled up to the plates on the rope and left DNA traces e.g., in the form of feces, on them. A possible explanation, why their DNA was not captured in the water samples, could be that those were taken 1 m below the surface and therefore too far away from these benthic organisms as metabarcoding is not a very sensitive method compared to qPCR detection systems (Wilcox et al., 2013). From those five crustacean species, a species-specific qPCR detection system was only available for the crab *Hemigrapsus takanoi*, which made a detection possible in most of the water samples as from the harbors and tidal channels (see appendix Table 19 and Table 21). To conclude, the use of settlement plates for metabarcoding analyses can be recommended as it targets benthic species, which may not be detected in water samples.

With metabarcoding analyses, the presence of three fish species (*Chromis multilineata, Rastrelliger kanagurta* and *Cyprinus carpio*) could be only uncovered in the water samples. This matches the expectations as that kind of sample should target species living in the pelagic zone as these fish species do. The only way to detect fish DNA on settlement plates would be by fish spawning on the plates or leaving feces on them. This seems to be quite unlikely as the settlement plates are relatively small with dimensions of 15 cm x 15 cm.

On the other side, DNA from many benthic species like *M. arenaria*, *Magallana gigas* or *Fucus distichus* could be found in the water samples. In the case of benthic animals like the mentioned bivalves above, larvae or juveniles of the species could be identified in the water samples as they are partly moving in the pelagic zone. For benthic algae like *F. distichus*, eggs or sperm or even small parts of the algae thallus could have been cut off from the main organism and may float free in the pelagic zone. Another option could be that some organisms like *M. gigas* were very abundant in the research area. Consequently, a higher amount of their DNA was released to the water, which made detection in the water samples possible.
Interestingly, eight species could only be identified in the settlement plates samples and not in the water ones.

Detection of NIS with qPCR

Another detection method based on eDNA samples was qPCR analyses, for which species-specific primers and probes were used. For the present project, primers, and probes for 23 potential non-indigenous species were available (after Andersen et al. (2018); Knudsen et al. (2020b); Knudsen et al. (2022)) and the qPCRs were conducted with the collected water samples from tidal channels and harbors, and the settlement plate samples. The following results are based on approach 1 for defining the limit of detection (LOD) and limit of quantification (LOQ), which was used by Stæhr et al. (2023). In total, the presence of 16 from the tested 23 NIS could be shown in the samples by using approach 1. Ten NIS could be detected with a water sample in Rømø harbor, compared to nine species in Fanø and eleven species in Esbjerg harbor, when combining all four sample locations from that harbor (see appendix Table 19). Those results fit with the fact that Esbjerg harbor is the largest one out of the three and has the highest number of arriving ships with 5367 ship calls in 2021 (Gade et al., 2021). As ship traffic is one of the most abundant causes for the spreading of NIS (Galil et al., 2014), the number of detected NIS in Esbjerg was expected to be higher than in the other harbors. On the other hand, there is a ferry going between Esbjerg and Fanø harbor multiple times a day,

which could potentially bring newly introduced species from Esbjerg to Fanø. Furthermore, looking at the results from the sample sites at Esbjerg one by one, a maximum of nine NIS was detected. For the other two harbors, samples were taken at only one location and maybe more NIS could have been detected there, if multiple water samples were collected.

This hypothesis is supported from the data of the tidal channel results (see appendix Table 21). Here, triplicates and in the case of the location Grådyb five water samples were collected. In six cases, a non-indigenous species could be identified in only one of the samples from the same location. This could be explained by a patchy distribution of organisms and their DNA in the water, especially if they are rare in the research site.

Besides the water samples, samples of settlement plates were collected, which were placed in the three harbors for over three months. The qPCR analyses were conducted the same way as with the water samples. The idea behind the settlement plates was to target mainly benthic species, which could be hard to detect with water samples taken 1 m below the surface. Interestingly, only the species *Homarus americanus* could be exclusively detected with the settlement plate samples (see Table 5 and appendix Table 20). Other benthic species like *Hemigrapsus takanoi* or *Magallana gigas* could also be traced in the water samples. This could either be explained with the high sensitivity of qPCR analyses, which were for example described in Wilcox et al. (2013). This may make it possible to detect extracellular DNA from degenerated cells originating from benthic organisms in the water column. The other possibility is that larvae or juveniles of some benthic organisms can be found in the pelagic zone, so that their DNA got caught in the water samples.

Comparison of results with different LOD/LOQ

A crucial part of qPCR analyses and data treatment is to define the limit of detection (LOD) and limit of quantification (LOQ) as those parameters determine if a species can be seen as detected or not. Apparently, it is quite difficult to treat qPCR data, as they are often not normally distributed and differ greatly from analytical chemistry datasets, for which LOD and LOQ were defined in the first place (Currie, 1999; Klymus et al., 2020a). In past studies with qPCR analyses, different approaches were used to define LOD and LOQ due to the lack of a clear guideline (e.g. Armbruster and Pry (2008); Burns and Valdivia (2008); Klymus et al. (2020a)). In the present study, two different definitions of LOD and LOQ were applied to this dataset and the results were compared.

Approach 1 to determine LOD and LOQ was already used in the Wadden Sea Report by Stæhr et al. (2023). LOD is there defined as the average Cq-value of the lowest standard concentration with at least one positive signal. LOQ is calculated by taking the Cq average of the lowest standard concentration with positive signals in all triplicates. With this definition of LOD and LOQ the main results were produced and are discussed in the section above.

Besides this, a second approach used by Klymus et al. (2020a) was tested. LOD is defined in that study as the average Cq-value of the lowest standard concentration with a 95% detection rate. As only triplicates were used in this project, LOD equals here the average Cq-value of the lowest standard concentration with positive signals in every triplicate. Comparing the two approaches, the definition of LOD would equal the definition of LOQ from approach 1. However, LOQ is calculated in approach 2 as the lowest standard concentration with a CV value ≤ 35. The calculation of the CV

value includes multiple factors like the qPCR efficiency or the standard deviation from the Cq values of a triplicate.

In total, 16 NIS were classified as detected with approach 1 and only 14 with approach 2. Looking at the different sample types, the biggest difference can be seen in the settlement plates samples. With approach 1, 13 NIS could be identified, whereas approach 2 only recognized 9 species. The four species not considered as detected by approach 2 are *Bonnemaisonia hamifera*, *Pseudochattonella verruculosa, Magallana gigas* and *Homarus americanus*. Apart from *P. verruculosa*, all those species are living in the benthic zone and therefore were targeted with the settlement plates. In praxis, it is more likely that the pacific oyster and the lobster would only attach to the settlement plates during a larvae or juvenile stage as the adult organisms are very big and the settlement plates did not provide much space. In the water samples from the tidal channels, *P. verruculosa* and *M. gigas* were seen as detected with the Klymus-approach, and in the water samples originated from the harbors *B. hamifera* was additionally considered as detected (see appendix Table 22, Table 23 and Table 24).

The crustacea *H. americanus* is indeed a special case as it was only seen as detected in the settlement plate samples with approach 1 and traces could be found in the tidal channels (see appendix Table 21). In Denmark, only one individual of this species was caught in the Øresund in 2006. In Norway, over 23 lobsters were found, and single individuals were captured in Sweden. Some of them had rubber bands around their claws, which signaled that they escaped, presumably from live import or an auction hall. So far, no juveniles could be captured which is why this species is not seen as established in the Nordic countries at the moment (van der Meeren et al., 2010). Furthermore, no traces of this species could be observed in the study by Knudsen et al. (2022), in which species-specific qPCR analyses were conducted on water samples from 16 harbors in Denmark, including Esbjerg harbor. Taking this into account, the positive signal in the settlement plate sample from Esbjerg Harbor could originate from a dead individual sold by a restaurant or shop. Otherwise, a lobster could have escaped from a transport of live individuals or alternatively as organic trash thrown overboard from a larger vessel serving seafood to their guests. As this NIS was only detected in one sample, an established population of *H. americanus* seems to be very unlikely.

In general, it became clear that approach 1 is more sensitive and detected more NIS in the present study than the Klymus-approach. The use of approach 2 seems to be more reasonable for studies with a high number of replicates as it requires e.g., 95% positive replicates of standards to determine LOD. The study from Klymus et al. (2020a) used 20 to 96 replicants per standard concentration, which is much higher than the triplicates used in the present study. The reason why no more standards could be used in the present study is that more standards could not be financed with the given funding for this project. The use of additional standards would allow a more precise calculation of the detection limit and limit of quantification, which would make a species detection more certain. Alternatively, a qPCR run could be repeated if there are doubts about the certainty of a detected species. Another difference between the two approaches is that approach 2 takes more factors like the qPCR efficiency or standard deviation into account to determine LOD and LOQ, which makes their calculation more precise and adaptable to the data.

All in all, it depends on the purpose of the project and the numbers of replicates, which approach for calculation of LOD and LOQ should be chosen. If the project is focused on detecting new NIS in an early succession stage, a more sensitive approach like the first one would suit better, which was also the case for the present study. If a project has the purpose to monitor the spreading of a certain species and to decide if action to prevent further dispersal should be taken, a more conservative approach might be the better option. Otherwise, there would be a risk of false positive samples and taken measures against a species which does not really exist in the research area.

Comparison between qPCR and metabarcoding results

In this study, species-specific qPCR assays and metabarcoding analyses were conducted on eDNA samples. With qPCR analyses 16 NIS and with metabarcoding analyses 31 NIS could be detected in total. Both methods have advantages and disadvantages. For metabarcoding, different universal primers were used targeting the genes COI, 12S rDNA and 18S rDNA, which made it possible to detect a wide range of taxa. Therefore, this method is a good tool to get an overview of the biodiversity in the research side, but can also help to identify new NIS, which were maybe not expected in the area. That is underlined by the results from this study as 24 species could be found with metabarcoding, but not with qPCR analyses (see Figure 7).

In the present study, three different sample types consisting of water samples from harbors, settlement plate samples from harbors and water samples from tidal channels, were collected for both eDNA analyses. Comparing the results of the different sample types, it becomes clear that settlement plate samples were very efficient for metabarcoding, but not for qPCR. In the first case, eight NIS were only detected with settlement plates and most of them were benthic species (see Table 3). The results from qPCR analyses showed that only one species, namely *Homarus americanus*, was detected on the settlement plate samples, but not in the water samples (see Table 5). This can be explained by the limited amount of detection systems for qPCR analyses, because out of the eight detected species with metabarcoding, only the detection system for *H. takanoi* existed.

For the present study, 23 species-specific primers and probes of NIS were available. Consequently, only the presence or absence of those 23 NIS could be tested, but no other NIS could be detected with qPCR analyses. The reason for the small number of available detection systems is the time and money consuming development of species-specific primers and probes, as they must not target other species. To start the process, a list of species including the target species, closely related species, and sympatric species of it must be generated. Afterwards, the availability of suitable sequences from those species in a local or public database must be checked and potentially an extraction of DNA and followed sequencing must be conducted. For this case, a tissue of the target species must be available for DNA extraction, which can pose a challenge as well. Next, the sequences should be aligned and checked for suitable regions to design the primers and probes specific for the target species. Furthermore, a testing for PCR inhibition is recommended and assay tests including optimizations need to be conducted (Klymus et al., 2020b). An example for a detection system, which cannot differentiate between the target species and closely related species, is the one for *Acipenser baerii* used in Knudsen et al. (2022) and also in the present study. It became clear that it cannot distinguish the NIS *A. gueldenstaedtii* and *A. ruthenus,* which is why they are listed as *Acipenser* spp*.* in the present study. Knudsen et al. (2022) state furthermore, that those two species cannot be distinguished from *A. baerii* as well*.* This hypothesis seems to be debatable as *A. baerii* could be detected in multiple samples in the present study, but the detection system for *A. gueldenstaedtii* and *A. ruthenus* did not indicated DNA from the target species in any sample (see appendix Table 19, Table 20 and Table *21*). These problems cannot occur with metabarcoding, as it can detect a broad range of taxa with very few primer sets and as the PCR products are sequenced afterwards, related species can usually be distinguished easily. Another disadvantage of species-specific detection with qPCR is that it cannot be conducted successfully if the target species is interbreeding with another species. However, the detection of hybrids may also pose a problem for metabarcoding analyses as they will either detect one or two species, depending on the used DNA marker.

Of course, metabarcoding also has its downsides. The resulting detected species with metabarcoding are based on the used genetic database. It is very important to pick a well-curated database, which has a high coverage of species diversity and intraspecific genetic diversity. Otherwise, species could be identified incorrect or falsely not detected in the analysis. In the present study, all used databases are to a large degree curated, which reduces the risk of false identifications. This could usually not happen with qPCR analyses as the species-specific detection systems are often based on de novo sequences from the target species and closely related ones (Knudsen et al., 2022; Weigand et al., 2019; Yang et al., 2017).

Another disadvantage of metabarcoding compared to qPCR analyses is the poor sensitivity. In a study by Wood et al. (2019), the detection probabilities for qPCR were found to be 0.93 and for metabarcoding 0.27 to 0.57, depending on the targeted region of the primers. This is underlined by the fact that qPCR analyses have a very low detection limit as they can detect target DNA with a concentration down to 0.5 copies/µl (Wilcox et al., 2013). The results from the present study display those findings for multiple species. For example, *M. leydi* could only be detected in the water samples of Esbjerg harbor and Grådyb with metabarcoding (see Table *3*), while qPCR analyses could identify the species in every water sample from all harbors and tidal channels (see appendix Table 19, Table 20 and Table 21).

The question which eDNA method may be the best for a certain project, cannot be generally answered as it strongly depends on the purpose of the study. If the goal is to determine the presence or absence of only a few species, species-specific analyses might be the better option as it appears to be more sensitive and enables a rough quantification. For projects, which should determine the general biodiversity of an ecosystem or should check the presence of many species, metabarcoding analyses seems to be the preferred option as a broad range of taxa can be detected with very few primer sets. This leads to a lower time and effort for laboratory work compared to qPCR analyses in the case of a high number of species, although the treatment of the sequencing data needs additional time.

Nevertheless, a combination of both techniques would be optimal, if the budget allows it. Metabarcoding could detect NIS, for which a qPCR system is not available or working and the presence of e.g., invasive species could be determined with qPCR analyses, because they can detect a very low concentration of target DNA and make a rough quantification possible. This could help to spot new NIS at an early stage. In the present study, qPCR analyses could detect nine species and metabarcoding 15 species, which were not identified by other methods. Taking both eDNA methods together, they found 40 out of the 50 NIS in total (see Figure *7*).

Total results and comparison between eDNA and conventional methods

In the present project, three different detection methods were used. Besides the molecular methods based on eDNA samples, metabarcoding and species-specific qPCR analyses, conventional detection was used as well. The organisms in the conventional samples were identified by a taxonomist, who used a taxonomic key and a dissecting microscope to determine the species.

My hypothesis was that all used methods will detect NIS, which I can confirm now. In this study, 50 non-indigenous species could be detected in total. Most species could be found with metabarcoding

(31 NIS), followed by conventional methods (22 NIS) and qPCR analyses detected the lowest number of NIS with 16 species.

Only the species *H. takanoi*, *M. gigas* and *M. arenaria* could be identified with all three techniques (see Table *8*). Those three species are widespread in the Wadden Sea (Jensen et al., 2023) and are multicellular organisms, which leads to a relatively high concentration of DNA in the water. Thus, the chances are high that eDNA analyses can detect the species in water samples and they are also easy to identify with conventional methods.

The low number of commonly found NIS could be explained with the different target organism groups of each method: the conventional methods used in this study could only detect benthic, multicellular species. Besides settlement plate samples, water samples were taken for the eDNA analyses, which mainly target pelagic organisms, including plankton. Although the settlement plates were used for all three detection methods, eDNA analyses could target unicellular organisms, which are not detectable with the conventional methods used in this project. The species *Prorocentrum cordatum, Cyprinus carpio, Pseudochattonella verruculosa* and *Mnemiopsis leidyi* could only be detected with the two eDNA methods, but not with conventional ones. Taking a closer look at these species it becomes clear that they could not be identified with the conventional techniques used in the present study as they are single cell organisms (*P. cordatum* and *P. verruculosa*), fish (*C. carpio*) and comb jelly (*M. leidyi*), living in the pelagic zone. This confirms my hypothesis that eDNA analyses will detect certain species, which are hard to identify with the here used conventional methods (e.g., fish or single cell algae) and will result in more detected NIS. Taking the results from qPCR and metabarcoding analyses together, 40 species could be detected with eDNA analyses, which is far more than the 22 detected NIS with conventional methods.

A bit surprising is that ten species were detected with conventional methods, but not with any of the eDNA techniques (see Table 8). There are multiple explanations for this high number of species: First, additional frame samples as a conventional method were taken on different mussel reefs, but no eDNA samples were taken at these locations. A mussel reef usually provides a large surface for benthic organisms and provides different niches. These aspects lead to a high biodiversity and make mussel reefs to a very species rich area, especially compared to the sandy sediment in the tidal channels, in which water samples for eDNA analyses were collected. Another reason could be that the list of NIS in the EU, which was used to filter out the NIS from the metabarcoding results, does not include all newly introduced NIS. The detection systems for species-specific qPCR analyses were only developed for a limited number of species, which were known as NIS before. In the present study, the two species *Botrylloides violaceus* and *Bugulina stolonifera* were identified as new NIS in the Danish Wadden Sea, and they could only be found with conventional methods (Stæhr et al., 2023). A further cause for the high number of species uniquely detected with conventional methods is that the used primers for the metabarcoding analyses may not amplify every species. Furthermore, metabarcoding is not a very sensitive method, which make a detection of very rare species difficult (Wilcox et al., 2013; Wood et al., 2019).

A clear advantage of conventional detection methods is that it can be observed if the detected organism is alive or dead. This distinction is not possible with eDNA methods as they also amplify extracellular DNA originating from dead cells (Taberlet et al., 2018). Furthermore, species detection with conventional methods ensures that the species is really existing in the research area and false positive results can only appear if the taxonomist determines the species wrong. In general, species identification with conventional methods is highly dependent on the skills of the taxonomist. For the two eDNA technics, a taxonomist is not necessarily needed as the detected species are determined with molecular methods and the help of databases. Nevertheless, it would be good if a taxonomist

checked the results from eDNA analyses as false positive samples can occur, which can partly be detected by a taxonomist e.g., if it is very unlikely that the species is living in the area of study.

Using conventional methods for species detection can be quite expensive due to the laborious sampling as often multiple sample types are required with several gears. Furthermore, the identification of species with a taxonomic key is usually time consuming and subsequently expensive. Moreover, in some cases it can be very hard to differentiate between closely related species (Knudsen et al., 2022). This all together makes results depending on the personal skills of the observer and therefore introduces a certain element of subjectivity. Regarding this problem, metabarcoding might pose the better option as it can objectively tell apart close related species in most cases.

Another hypothesis was that NIS will be detected, which were not identified by previous studies in the Danish Wadden Sea, so that the list of NIS will grow. Matching this hypothesis, 14 new NIS could be detected with this project and therefore the list of NIS in the Danish Wadden Sea is growing (see Stæhr et al. (2023) for detailed information). Most of them were already detected in other European countries or even in other parts of the Wadden Sea like *Haliclystus tenuis*, which was detected in Helgoland (Germany) in 2010 (Stæhr et al., 2023). Those results underline that NIS are spreading further and the arrival of more NIS in the Danish Wadden Sea can be expected in the future.

In the present study 40 out of 50 NIS could be detected with the two eDNA methods metabarcoding and species-specific qPCR analyses. This result shows how effective eDNA methods are for monitoring purposes, especially compared to conventional monitoring techniques and that a combination of metabarcoding and qPCR analyses can be recommended. In some studies, the newer technique of droplet digital PCR (ddPCR) was compared with those two eDNA tools and turned out to have an even better detection rate than the two methods used in the present study (Doi et al., 2015; Wood et al., 2019). This could be the future tool for monitoring studies, but at the moment it is cost intensive and complex to operate (Xia et al., 2018).

As ten NIS could only be detected with conventional methods, the used eDNA tools cannot fully replace conventional methods yet. Further optimization of the eDNA methods is necessary to improve the sensitivity of metabarcoding detection and to develop further qPCR detection systems.

Which NIS are established in the Wadden Sea and how is NIS defined?

In this study, 50 NIS could be detected in the Danish Wadden Sea by using different detection methods. Nevertheless, this list of NIS is most likely not complete and maybe some of the detected species are false positive samples, which means that the identified species is not established in the research area. In the following paragraphs, this problem is described in detail as well as the definition of non-indigenous species is discussed.

Although 31 non-indigenous species could be detected with metabarcoding, it is very likely that more NIS were detected by this tool but were excluded in the results as they are not part of the NIS list from the EU. This list was used in the present study to filter out the NIS from all detected species with metabarcoding. For example, the fish species *Limanda aspera* could be detected on a settlement plate sample from Esbjerg. This fish is native in the North-Pacific and therefore must be considered as a NIS in the Danish Wadden Sea, if a population of the species is established in the

Wadden Sea. Nevertheless, *L. aspera* is not included in the results of this project, because it is not part of the list with NIS in the EU and was not filtered out as NIS from all found species with metabarcoding. As this fish species is a common food fish (https://www.fishbase.se/summary/Limanda-aspera.html), it is not unlikely that the detected DNA sequences originate from a dead individual, which was transported to Esbjerg for consumption. Since eDNA methods cannot distinguish between DNA originating from a living or dead organism, it cannot be determined if this species is living and maybe even established in the Wadden Sea or not.

A somewhat similar case is the identification of the red king crab (*Paralithodes camtschaticus*) in Esbjerg harbor and the tidal channels Grådyb and Knudedyb, with qPCR analyses (see appendix Table *20* and Table *21*). The native habitat of the crab is the Okhotsk and Japan Sea, Bering Sea, and Northern Pacific Ocean. Between 1961-1969, 1.5 million larvae, 10.000 juveniles and over 2.500 adults of this species were intentionally released by scientists in the east Barents Sea (Russia), because they wanted to establish this species as a fishing resource. The species spread further to the east and west, and the first individual in a Norwegian water body was found in Varangerfjord in the year 1992. Over the following years, the crab was found further west along the Norwegian cost (Jørgensen, 2013). In 2008, a 4 kg heavy male crab was found in the Mediterranean Sea, which age was estimated to 10 years. The pathway of introduction is not clear, but an unintentional transport of it in a larvae stage e.g., in the ballast water of a ship seems to be the most likely scenario (Faccia et al., 2009; Jørgensen, 2013). This finding indicates that this NIS is capable of surviving and growing in way warmer water than in its native habitat, which would be the requirement for its further spread in southern seas. Considering this, it seems possible that the found DNA in the present project originates from living individuals of *P. camtschaticus*. Especially, because it was detected in two tidal channels and Esbjerg harbor, which would not be possible if the DNA originated from one dead organism. However, it would be very surprising as the Danish Wadden Sea is over 1.600 km further south than the locations at the Norwegian cost, where individuals were spotted in the past. One explanation for the finding could be that the red king crab is seen as a delicacy and was maybe shipped to Esbjerg and sold in local restaurants, from where at least parts of it ended in the harbor. Another possibility could be that the qPCR detection system perhaps also amplifies DNA from related species and those findings are subsequently false positive ones. This example shows that only because a species was detected with eDNA methods, it is not necessarily an established species in the research area and further studies are needed to confirm or decline the presence of it.

The term non-indigenous species in an ecological context was first used in papers from the late 90's (e.g., Grizel (1996); Reusch and Williams (1998)). One of the first official definition of NIS was stated by the Office of Technology Assessment (OTA), United States in 1993. They defined non-indigenous as "The condition of a species being beyond its natural range or natural zone of potential dispersal; includes all domesticated and feral species and all hybrids except for naturally occurring crosses between indigenous species." (OTA et al., 1993). In the next years, other institutions and researchers defined NIS, which is why multiple definitions exist nowadays. While the definition used by the European Commission is very similar to the one from OTA (EC, 2007), the Oslo and Paris Conventions (OSPAR) emphasized that non-indigenous species were introduced by human activities (OSPAR, 2017). A standardized definition of NIS would be very useful and would help to decide if a species is considered as NIS or not. Moreover, it should be defined if cryptogenic species, which origin, and subsequently native habitat is unknown, should be considered as NIS or not. Furthermore, I think that it should be discussed how a species can be removed again from the list of NIS. Now, no regulation has been made for this aspect, which means that the species will always be considered as NIS, regardless of how long the species has been established in the immigrated area. One example is the mussel *Mya arenaria*, which is considered as a NIS in the Danish Wadden Sea. In 1992,

researchers took shell samples from *M. arenaria* in Skagen, Denmark and used conventional radiocarbon dating to determine the age of the samples. One of the samples were dated to the 13th century, which differs from the previous assumption that the species was introduced to Europe in the 16th century after Columbus journey to America. The researchers assume that the mussel was introduced to Europe by the Vikings, which established small settlements in North America (Petersen et al., 1992). Consequently, *M. arenaria* was probably introduced to Danish Waters over 800 years ago. It seems a bit strange to consider this species as NIS after it has been present in Denmark for so many years. A removal of *M. arenaria* from the list of NIS in the Danish Wadden Sea would be reasonable, especially as all NIS species need to be monitored. As monitoring is quite expensive, money could be saved by excluding species which has been established in the Danish Wadden Sea for e.g., over 100 years as no big change in their status, like abundance or effects on other species, is expected.

Effect of sampling at different time points in the tidal cycle

One of my research questions was if sampling at different time points in the tidal cycle would affect the outcome of eDNA analyses. To investigate this matter, water samples were taken at three different time points in the tidal channel Grådyb. The first three samples were taken at 12:30, one hour after the start of the rising tide. The other two samples were collected one hour before and during the peak of high tide (at 16:30 and 17:30, respectively). For all five samples, metabarcoding and species-specific qPCR analyses were performed.

The results from metabarcoding analyses showed only a few differences between the different sample time points. The two species *Mnemiopsis leidyi* and *Prorocentrum cordatum* could only be detected in the samples taken at 12:30, but not in the later ones (see Table 6). Interestingly, both species were detected at all time points with qPCR analyses, which may be caused by the higher sensitivity of species-specific qPCR analyses compared to metabarcoding (see Table 7). This could indicate a lower concentration of DNA from those two species in the later samples. Nevertheless, this might not be caused through the tidal cycle as only one sample was taken at 16:30 and 17:30, while three samples were collected at 12:30. As both species are mainly occurring in the pelagic zone, they might be distributed patchy, and it could be only a coincidence that not enough DNA for metabarcoding detection were captured in the later water samples.

Another difference between the samples is shown for the pacific oyster *M. gigas*. Looking at the copy number, the samples collected at 12:30 had way higher ones between 1583 and 3019 compared to the later samples (562 for 16:30, 63 for 17:30, see Table 6). This could be explained by the different water levels and therefore the distance of the sampling points to the sediment as the water sampler was always placed 1 m below surface. *M. gigas* are benthic organisms and therefore the most DNA fragments from them can be found in the benthic zone. The water level was rising during sampling and consequently the water sampler was closest to the sediment at the samples taken at 12:30, which also showed the most copy numbers of DNA from *M. gigas*.

Looking at the results of the qPCR analyses, it is noticeable that the species *Paralithodes camtschaticus* and *Oncorhynchus mykiss* were only detected in the sample taken at 16:30, one hour prior to the peak of the flood. As the crab *P. camtschaticus* is a benthic species and the rainbow trout *O. mykiss* lives in the pelagic zone, no clear trend of a stronger represented group can be

observed. Furthermore, a slightly higher detection for the phytoplankton species *Pseudochattonella farcimen* and *Prorocentrum cordatum* is shown for the sample taken at 16:30 compared to the earlier ones (see Table 7).

All in all, no clear pattern could be observed regarding the effects of sampling at different time points in the tidal cycle. This result is confirmed by the study from Kelly et al. (2018), which took samples at four different time points: two water samples were collected during the incoming and two during the outgoing tide, respectively. The samples were used for a metabarcoding analysis with primers targeting the COI region. They concluded that the metabarcoding results were largely consistent over the different sample times, so that the sampling point in the tidal cycle does not affect the metabarcoding results to a large extend (Kelly et al., 2018).

Another interesting factor to investigate is seasonal changes, which could affect the outcome of eDNA studies. A study by Andersen et al. (2023) used eDNA analyses to monitor NIS in different Danish harbors, including Esbjerg harbor. They took water samples in June and September/October to later perform species-specific qPCR analyses, besides other analyses. In that study, more NIS were detected in the September/October samples than in the ones from June, but on the other hand a few species could only be detected in June with qPCR analyses. A similar result can be found in Knudsen et al. (2022), where eDNA samples were taken during two sample periods, one in June/July and the other in August – November in different Danish harbors. Knudsen et al. also used species-specific qPCR assays for NIS detection. They recorded a higher level of eDNA and could detect more NIS in the samples originating from the later period. The possible reasons for these seasonal differences variate from species to species. For example, the detection of the comb jelly *M. leidyi* is more likely in the late summer and fall as some of the jellies die during the Danish winter, are starving during summer and bloom again in the late summer (Knudsen et al., 2022; Riisgård, 2017). The red algae *Bonnemaisonia hamifera* could only be detected in the later sampling period by Knudsen et al. (2022) as well. This could be caused by a too small population size in June/July, which probably made it undetectable in the samples from June/July. All in all, a sampling for eDNA analyses seems to be most promising in the late summer or early fall to detect the highest amount of NIS.

Considerations

While performing eDNA analyses for NIS detection, there are a few things which need to be considered.

First, not every NIS will probably be detected with eDNA analyses for various reasons. One issue for metabarcoding analyses is that some species have multiple names or got a new name recently. Some databases will possibly list those species under a different name than the NIS list used for filtering out NIS from all the detected species. It could also occur that some databases will list the species multiple times under its different names. This poses a problem as those NIS may not be filtered out correctly and consequently would be seen as not detected in the samples but are in fact only listed under a different name. To avoid those false negative samples, all variations of species names from the tested NIS were used to filter them out of the species detected with metabarcoding.

Another reason why a species could falsely not be detected with eDNA analyses, could be a too low DNA concentration in the sample. The eDNA concentration in aquatic ecosystems is mainly

determined by the eDNA production from the organisms, which is dependent on biological factors like life stage, body size or reproductive status of the organisms as well as the size and structure of the community. Besides the production, the eDNA elimination including transportation and degradation of eDNA is the other variables influencing the eDNA concentration in a sample to a great extent (Huang et al., 2022). The persistence of eDNA in water can be affected by abiotic factors like temperature, salinity, pH, and UV radiation as well as currents and turbulences (Huang et al., 2022; Stoeckle et al., 2021). Therefore, DNA from some organisms, which are present in the analyzed habitat, could already be eliminated at the time of sampling and those species could consequently not be detected. So, the risk is high that more NIS reside in the Wadden Sea than the ones we detected in this study.

Picking fitting primers for the target groups is crucial. Primers targeting longer DNA fragments could be problematic as longer DNA fragments tend to degrade quickly in water and are consequently only present in low concentrations, which makes the detection of a species more difficult. On the other hand, eDNA analyses with primers targeting longer DNA sequences will represent a very recent picture of the biodiversity. Contrary, shorter fragments are in general more stable in the aquatic environment, which leads to a higher concentration in the water compared to longer DNA sequences (Beng & Corlett, 2020). Furthermore, primers targeting mitochondrial DNA (mtDNA) leads usually to a more sensitive detection as up to 10^5 more copy number per cell exist from mtDNA compared to nuclear DNA (Beng & Corlett, 2020; Chinnery & Hudson, 2013). In the present study, two out of three universal primers used for metabarcoding, namely 12S rDNA and COI, targeted different regions from the mitochondrial genome. Furthermore, the primers were relatively short, which should ensure a sensitive detection of target DNA.

Possible ecological effects of found NIS

The introduction of non-indigenous species can have a huge effect on the local ecosystem. NIS with a negative impact is called invasive species and could for example outcompete native species, which can lead to their extinction. Furthermore, NIS could bring parasites or diseases with them, which would pose a new threat to the ecosystem (Shrader-Frechette, 2001). In some cases, the introduction of alien species for aquacultures can bring economic profits, but carries the risk of unintended introduction of non-target species (Savini et al., 2010). In the following paragraphs, I will describe the ecological effects of a selection of NIS from different branches of the taxonomic system, Mollusca: the Pacific oyster *M. gigas*, Ctenophora: the comb jelly *M. leidyi* and Arthropoda: the brush-clawed shore crab *H. takanoi* in the Danish Wadden Sea, which could be detected in multiple samples in the present study (see Table *8*).

The pacific oyster *M. gigas* is a very interesting case, as it can bring economic benefits to the region, but due to its fast spreading and high abundance could outcompete native species. The European Union classified the pacific oyster as an economical valuable species as it is an important resource for the aquaculture industry (EC, 2007; Hansen et al., 2023).

The bivalve was introduced in 66 countries outside its native habitat and in at least 17 of them the oyster is established with self-sustaining populations. It was first introduced in Europe about 50 years ago for aquaculture purposes, but through escapes the species could establish itself in big

biogenic reefs (Gillies et al., 2015; Hansen et al., 2023). The first individual in the Danish Wadden Sea was found in the mid-90s, but nowadays the population size of pacific oysters in the Danish Wadden Sea is approximately over 70,000 t (Nielsen et al., 2019; Wrange et al., 2010). The risk assessment of this species in Scandinavia was conducted for different habitants. A moderate impact of the oyster was stated for littoral biogenic reefs in areas with little or no tides, currents, and low wave exposure, which are summarized as low energy areas. Contrary, a high ecological impact was found in high energy areas, which are defined by large tidal fluctuations, strong currents and a high wave exposure (Herbert et al., 2016). The Danish Wadden Sea is one of those high energy areas and consequently, the ecological impact of the pacific oyster is considered as high. In general, pacific oysters do not pose a threat to the ecosystem if they only appear sporadically. However, the species has a high reproduction rate and somatic growth rate, which enables the oyster to form dense reefs in short time (Hansen et al., 2023).

The introduction of the pacific oyster brought certain positive effects in some areas: it partly increased the biodiversity and biomass of to the oyster reef associated macro fauna and could replace and keep up the ecosystem function of the nearly extinct European oyster *Ostrea edulis*. Another positive effect caused by the pacific oyster reefs is the interception and deflection of the tidal currents, which stabilize the sediment (Reise et al., 2023). Furthermore, the pacific oyster can be used as foodstuff, for pearl production and as a bivalve it filters the water from contaminants (Hansen et al., 2023). Nevertheless, this NIS could also affect the ecosystem in a negative way. After its introduction, many mussel beds formed by native species were invaded by *M. gigas* and became mixed bivalve beds or transformed completely to oyster reefs. As a mussel bed and an oyster reef differ in their provided resources for other species e.g., nesting sites or hiding places for prey, the shift from one to the other can also lead to a shift of the associate community and food web structures. For example, the bird species *Haematopus ostralegus, Larus canus* and *Calidris canutus* are negatively affected by the pacific oyster reefs in the Dutch Wadden Sea (Waser et al., 2016).

Another prominent NIS in the Wadden Sea is the comb jelly *Mnemiopsis leidyi*, which native habitat is the east coast of America. The species was first sighted in different locations in North Europe including the Nissum Fjord in Denmark in 2005 and could be found in all Danish waters two years later (Jaspers et al., 2018; Jensen et al., 2023; Tendal et al., 2007). There are indices that the North Sea serves as a refuge for *M. leidyi* if it disappeared from other waters due to unfavorable conditions. If the conditions changed to more favorable ones again, the comb jelly probably uses the North Sea as a starting point to spread to all Danish waters once more (Jaspers et al., 2018; Jensen et al., 2023). *M. leidyi* is feeding on zooplankton and in region, where the NIS is very abundant a strong predation control on zooplankton could be observed with cascading effects on copepods and diatoms (Jensen et al., 2023; Tiselius & Møller, 2017). Moreover, the comb jelly is preying on fish eggs and larvae, which could have a negative impact on the fish population (Riisgård, 2017).

The crab *Hemigrapsus takanoi* is native to the northwest pacific coast. It has a wide thermal tolerance range and has a high fertility with up to 50,000 eggs per female. Besides this, the fact that the planktonic larval stage last circa one month is another factor fostering the spreading of the species (Epifanio, 2013). This NIS was registered in Europe for the first time in France in 1994 and reached the Danish Wadden Sea in 2011, when the species could be identified on the island Rømø (Jensen et al., 2023; Landschoff et al., 2013). The crab probably has an impact on the mussel stocks as it eats smaller mussels. Interestingly, it managed to inhabit a Pacific Oyster reef in Denmark (Vogensen et al., 2020). Furthermore, it is competing with the European shore crab, *Carcinus maenas*, in the Wadden Sea and even predates on the juveniles from *C. maenas*. Nevertheless, it

seems unlikely that *H. takanoi* has a big negative impact on the population of the native crab (Jensen et al., 2023; Landschoff et al., 2013).

Those three examples show that some NIS can have a negative impact on the ecosystem, but others could also bring e.g., economic benefits to the region, create new habitats for other species or stabilize the sediment. A detailed assessment of the effects from the different NIS is necessary to decide if action should be taken to prevent further spreading of the NIS.

Methods to prevent further NIS introductions or their spreading

Some NIS can have negative effects on an ecosystem, which may make it necessary to take action to prevent further harm. As it is very difficult to combat already established NIS, a focus on prevention of further NIS introductions could be a good strategy to reduce the number of new NIS.

First, it is important to identify the major introduction vectors of NIS to decide the focus of the management efforts. As most NIS are distributed unintentionally by ship, it could be effective to establish an exchange of the ballast water at sea for vessels coming from outside Europe. The ballast water could contain eggs, larvae, or small individuals of non-indigenous species, which would be released in a new habitat. The International Maritime Organization developed a convention to reduce the risk of species introductions through balance water, which demands a ballast water management system (BWMS) implemented in most international vessels by 2024 (IMO, 2004). Different kinds of BWMS have been developed and most of them include multiple steps of ballast water treatment. Many systems use a mechanical process like filtration to remove larger organisms. Afterwards, a chemical or physical disinfection step is incorporated, for example ultra-violet irradiation or electro chlorination (Bailey et al., 2022). Furthermore, a regulation to prevent or limit biofouling on ships would also contribute to limit the unintentional introduction of NIS (Galil et al., 2014).

Besides unintentional transportation by global shipping, escapes from aquacultures and associated organisms to the cultured species like parasites are further sources of NIS. To prevent the latter, a quarantine of the imported species is recommended by the European Commission (EC, 2007).

If a species is already established, it can spread through natural processes like transportation by currents or organisms from other species. Further studies of the dispersal potential of those natural processes are needed as they could hinder measures to prevent further spreading (Galil et al., 2014).

Another way is to develop species-specific management actions for NIS, which are seen as problematic or spread very fast. To give an example, different measures are proposed to stop further spreading of the pacific oyster in the Wadden Sea. They include hand-picking for human consumption, destroying or removal of oysters with mechanical tools, and commercial fishing of oysters. Moreover, biological measures are suggested like breeding sterile triploids, covering the reefs with e.g., sand to starve and choke the oysters, or place native blue mussels on the oyster reefs, so they could outcompete the oysters (Hansen et al., 2023).

In general, it should be secured that a NIS is established in a region before taking action to combat it. Especially by using eDNA analyses for species detection, there is a risk of false positive results, which means that a species is detected with the used methods, but in reality, no population exists in the

research site. If management measures were falsely applied, money would be wasted, and non-target species could be negatively affected by the measures. To conclude, enough research should be conducted before starting action to ensure that a species is really established, but it should be balanced with the advantages of starting regulations at an early point to stop a NIS from further spreading.

Future perspectives

In the future, more studies to detect NIS and investigate the health of an ecosystem need to be conducted. Due to climate change, the arrival of more NIS is expected, and the ecosystems need to be monitored on a regular basis. For future studies and monitoring projects, the following aspects should be discussed.

Besides the three different methods used in the present study, the collection of sediment samples for eDNA analyses could be useful to detect rare species. Through vertical transport, eDNA from organic matter and such directly adsorbed onto sediment particles, can be found in the sediment. Consequently, DNA from pelagic organisms can also be detected in the sediment samples. DNA attached to the surficial sediment is preserved for a longer time (29 to 93 days) than DNA dissolved in seawater with 6.5 to 25 hours, because the DNA is protected against UV radiation and free oxygen in the sediment, which prevent an oxidation and irradiation of the DNA (Torti et al., 2015). These conservation mechanisms of DNA in the sediment makes it possible that ancient DNA until the late Pleistocene, which was circa hundred thousand years ago, can be found in sediment samples (Boere et al., 2011; Torti et al., 2015). This leads to the disadvantage that eDNA analyses from sediment samples potentially also detect ancient species and species, which are already extinct in the research area, which makes it difficult to get an overview of the present species (Deiner et al., 2017).

In the present study, the use of settlement plates in combination with metabarcoding analyses was a success as it revealed eight species, which could not be found in the water samples with metabarcoding. The use of species-specific qPCR analyses can be recommended as well because this method seems to be more sensitive than metabarcoding. The development of qPCR detection systems would increase the biological spectrum covered by qPCR analyses, but the development of species-specific primers and probes is very time intense and costly.

Moreover, further studies are needed to compare conventional detection methods with eDNA analyses. For those studies, sampling for both methods should be conducted at the same sampling stations and time to enable a comprehensive comparison. This would help to answer the question, if eDNA analyses can completely replace conventional methods for species detection.

Concluding remarks

In this project, 50 non-indigenous species could be detected in total in the Danish Wadden Sea with various methods, which aligns my expectations. Out of the 50 NIS, 14 species were spotted for the first time in the Danish Wadden Sea with the present study. All three used techniques could identify NIS from the samples, but as expected the most efficient one was metabarcoding with 31 detected NIS. Taking both eDNA based methods together, they revealed the presence of 28 NIS, which were not detectable with the here used conventional methods. Surprisingly, the conventional methods made it possible to detect ten species, which were not discovered with the two eDNA methods. This could have many reasons, for example that some samples were taken at mussel reefs, which were close to but not the exact same sampling sites as for the eDNA analyses.

As already indicated in previous studies, species-specific qPCR analyses appear to be more sensitive than metabarcoding, as it can detect lower copy numbers of the targeted DNA sequence in each sample. Compared to the results of metabarcoding, qPCR analyses detected nine NIS, which could not be found with the other two techniques. A clear disadvantage of qPCR analyses compared to metabarcoding is the required time and the relatively high cost associated with intensely development of species-specific primers and probes, which leads to a limited number of available species detection systems. Therefore, qPCR analyses cannot detect all NIS in an ecosystem and need to be supplemented with another technique for a more complete picture in the monitoring.

Different sample types were collected, which targeted different taxa: water samples were taken to mainly detect species living in the pelagic zone, whereas settlement plates and the conventional samples mainly aimed for benthic organisms. For metabarcoding, the additional use of settlement plates can be recommended, because they revealed eight species which could not be identified in the water samples. Interestingly, this was not the case for qPCR analyses as only one species was exclusively detected in the settlement plate samples.

In the present project, the effect of the timepoint in the tidal cycle for taking water samples on eDNA analyses was investigated. Contrary to the expectation, negligible effect was observed on species diversity by the metabarcoding and qPCR analyses. Nevertheless, more studies in the future with additional sampling time points, especially during the outgoing water phase, could be conducted to find if there is a best timepoint for sampling.

Since 14 new NIS in the Danish Wadden Sea could be detected in the present study, further monitoring programs in the future are important to track the NIS status of this ecosystem and be able to act if a NIS pose a threat to it. To answer my question “are environmental DNA analyses the future tool for monitoring?”: The use of eDNA analyses for monitoring an aquatic ecosystem can indeed be recommended as 40 out of 50 NIS found in the present study could be identified with eDNA methods. However, we are not yet there where eDNA can stand alone and a combination with conventional analyses seems to be the best choice for now.

Acknowledgment

First of all, I would like to acknowledge my three supervisors Professor Benni Winding Hansen, Professor Anne Winding and Associate Professor Rumakanta Sapkota for their great help and guidance throughout the whole project. I am very grateful that you made me feel as a part of the research group and included me in many events!

I want to thank researcher Helle Buur for providing me with her data resulting from conventional methods. Furthermore, special thanks to M.Sc. Karolina R. Andersen and Dr. Nikolaj R. Andersen for taking me on the fieldtrip and made it an amazing experience.

A big thank you to our lab technicians Tanja Begovic and Tina Thane for their unlimited patience in answering all my questions and teaching me everything I needed to know in the lab.

I am also thankful for the help of Associate Professor Lea Ellegaard and Assistant Professor Thanassis Zervas for their help in the sequencing process and data treatment.

I am grateful for all advice and mental support from the PhD students of our working group and the interesting discussions during our journal club.

I am extremely grateful for having such an amazing family, which always supports my dreams and encouraged me throughout my years of study.

And finally, I want to thank my friends and “the office”, who became so much more than just fellow students. Thank you for making my time in Denmark and at RUC unforgettable!

References

Andersen, J. H., Kallenbach, E., Thaulow, J., Hesselsøe, M., Bekkevold, D., Hansen, B., Jacobsen, L., Olesen, C., Moller, P. R., & Knudsen, S. W. (2018). Development of species-specific eDNA-based test systems for monitoring of non-indigenous species in Danish marine waters. *NIVA, 2018. 77 p. (NIVA Report; No. 7204 - 2017)*.

Andersen, N. R., Stæhr, P. U., Andersen, K. R., Buur, H., Jakobsen, H. H., Winding, A., & Sapkota, R. (2023). Havneovervågning af ikke-hjemmehørende arter 2021. Havstrategiens deskriptor 2. *Aarhus Universitet, DCE – Nationalt Center for Miljø og Energi, 65 s. - Videnskabelig rapport nr. 534.* http://dce2.au.dk/pub/SR534.pdf

Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The Clinical biochemist*.

Arya, M., Shergill, I., Williamson, M., Gommersall, L., Arya, N., & Patel, H. (2005). Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn. , 5 (2)*, 209 - 219.

Bailey, S. A., Brydges, T., Casas-Monroy, O., Kydd, J., Linley, R. D., Rozon, R. M., & Darling, J. A. (2022). First evaluation of ballast water management systems on operational ships for minimizing introductions of nonindigenous zooplankton. *Marine Pollution Bulletin*, *182*, 113947. https://doi.org/https://doi.org/10.1016/j.marpolbul.2022.113947

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089-2121. https://doi.org/10.1007/s10531-020-01980-0

Beukema, J. J. (1982). Annual variation in reproductive success and biomass of the major macrozoobenthic species living in a tidal flat area of the Wadden Sea. *Netherlands Journal of Sea Research*, *16*, 37-45. https://doi.org/https://doi.org/10.1016/0077-7579(82)90015-1

Beukema, J. J., Cadée, G. C., & Dekker, R. (2002). Zoobenthic biomass limited by phytoplankton abundance: evidence from parallel changes in two long-term data series in the Wadden Sea. *Journal of Sea Research*, *48*(2), 111-125. https://doi.org/https://doi.org/10.1016/S1385-1101(02)00162-4

Boere, A. C., Damsté, J. S. S., Rijpstra, W. I. C., Volkman, J. K., & Coolen, M. J. (2011). Source-specific variability in post-depositional DNA preservation with potential implications for DNA based paleoecological records. *Organic Geochemistry*, *42*(10), 1216-1225.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodriguez, A. M., Chase, J., . . . Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*, *37*(8), 852-857. https://doi.org/10.1038/s41587-019-0209-9

Buettger, H., Christoph, S., Buschbaum, C., Gittenberger, A., Jensen, K., Kabuta, S., & Lackschewitz, D. (2022). Alien species. *Wadden Sea Quality Status Report . Eds.: Kloepper S. et al., Common Wadden Sea Secretariat, Wilhelmshaven, Germany.* qsr.waddensea-worldheritage.org/reports/alien-species

Burns, M., & Valdivia, H. (2008). Modelling the limit of detection in real-time quantitative PCR. *European Food Research and Technology*, *226*(6), 1513-1524. https://doi.org/10.1007/s00217-007-0683-z

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*, *13*(7), 581-583. https://doi.org/10.1038/nmeth.3869

Cardeccia, A., Marchini, A., Occhipinti-Ambrogi, A., Galil, B., Gollasch, S., Minchin, D., Narščius, A., Olenin, S., & Ojaveer, H. (2018). Assessing biological invasions in European Seas: Biological traits of the most widespread non-indigenous species. *Estuarine, Coastal and Shelf Science*, *201*, 17-28. https://doi.org/10.1016/j.ecss.2016.02.014

Cerrano, C., Bavestrello, G., Bianchi, C. N., Cattaneo-vietti, R., Bava, S., Morganti, C., Morri, C., Picco, P., Sara, G., Schiaparelli, S., Siccardi, A., & Sponga, F. (2000). A catastrophic mass-mortality episode of gorgonians and other organisms in the Ligurian Sea (North-western Mediterranean), summer 1999. *Ecology Letters*, *3*(4), 284-293. https://doi.org/https://doi.org/10.1046/j.1461-0248.2000.00152.x

Cervetto, G., Gaudy, R., & Pagano, M. (1999). Influence of salinity on the distribution of Acartia tonsa (Copepoda, Calanoida). *Journal of Experimental Marine Biology and Ecology*, *239*(1), 33-45.

Chaalali, A., Beaugrand, G., Raybaud, V., Goberville, E., David, V., Boët, P., & Sautour, B. (2013). Climatic facilitation of the colonization of an estuary by Acartia tonsa. *PLoS ONE*, *8*(9), e74531.

Chinnery, P. F., & Hudson, G. (2013). Mitochondrial genetics. *Br Med Bull*, *106*(1), 135-159. https://doi.org/10.1093/bmb/ldt017

Currie, L. A. (1999). Detection and quantification limits: origins and historical overview. *Analytica Chimica Acta*, *391*(2), 127-134. https://doi.org/https://doi.org/10.1016/S0003-2670(99)00105-1

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872-5895. https://doi.org/https://doi.org/10.1111/mec.14350

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K., & Yamanaka, H. (2015). Droplet digital polymerase chain reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. *Environmental science & technology vol. 49,9 (2015): 5601-8.*(1520-5851 (Electronic)).

EC. (2007). *Council Regulation (EC) No 708/2007 of 11 June 2007 concerning use of alien and locally absent species in aquaculture*. OJ L 168, (BG, ES, CS, DA, DE, ET, EL, EN, FR, IT, LV, LT, HU, MT, NL, PL, PT, RO, SK, SL, FI, SV) Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32007R0708

EC. (2020). *COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS. EU Biodiversity Strategy for 2030 - Bringing nature back into our lives. European Commission*. Retrieved from https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:52020DC0380

Epifanio, C. E. (2013). Invasion biology of the Asian shore crab Hemigrapsus sanguineus: A review. *Journal of Experimental Marine Biology and Ecology*, *441*, 33-49. https://doi.org/https://doi.org/10.1016/j.jembe.2013.01.010

Faccia, I., Alyakrinsky, A., & Bianchi, C. N. (2009). The crab that came in from the cold: first record of Paralithodes camtschaticus (Tilesius, 1815) in the Mediterranean Sea. *Aquatic Invasions*, *4*, 715-718.

Fonseca, V. G., Carvalho, G. R., Sung, W., Johnson, H. F., Power, D. M., Neill, S. P., Packer, M., Blaxter, M. L., Lambshead, P. J., Thomas, W. K., & Creer, S. (2010). Second-generation environmental sequencing unmasks marine metazoan biodiversity. *Nat Commun*, *1*(7), 98. https://doi.org/10.1038/ncomms1095

Gade, S., Rasmussen, J. F., Olsen, D. M., Sørensen, J., Kirkegaard, M., Thomsen, J., Hansen, F., & von Qualen, J. B. (2021). *Port Esbjerg - Annual report 2021*. https://portesbjerg.dk/en/about-us/annual-reports

Galil, B. S., Marchini, A., Occhipinti-Ambrogi, A., Minchin, D., Narščius, A., Ojaveer, H., & Olenin, S. (2014). International arrivals: widespread bioinvasions in European Seas. *Ethol Ecol Evol.*, *26(2-3)*(0394-9370 (Print)), 152-171. https://doi.org/10.1080/03949370.2014.897651

Garrabou, J., Pérez, T., Sartoretto, S., & Harmelin, J.-G. (2001). Mass mortality event in red coral Corallium rubrum populations in the Provence region (France, NW Mediterranean). *Marine Ecology Progress Series*, *217*, 263-272. https://doi.org/10.3354/meps217263

Gillies, C., McLeod, I., & Creighton, C. (2015). Shellfish reef habitats: a synopsis to underpin the repair and conservation of Australia's environmentally, socially and economically important bays and estuaries. *Report to the National Environmental Science Programme, Marine Biodiversity Hub, Centre for Tropical Water and Aquatic Ecosystem Research (TropWATER) Publication, James Cook University, Townsville, 68 pp*.

Grizel, H. (1996). Quelques exemples d'introdutions et de transferts de mollusques [Some examples of introductions and transfers of mollusks] [Quelques exemples d'introdutions et de transferts de mollusques.]. *International Office of Epizootics) vol. 15,2*(0253-1933 (Print)), 401 - 408.

Hansen, B. W., Dolmer, P., & Vismann, B. (2023). Too late for regulatory management on Pacific oysters in European coastal waters? *Journal of Sea Research*, *191*, 102331. https://doi.org/https://doi.org/10.1016/j.seares.2022.102331

Hansen, B. W., Drillet, G., Pedersen, M. F., Sjøgreen, K. P., & Vismann, B. (2012). Do Acartia tonsa (Dana) eggs regulate their volume and osmolality as salinity changes? *Journal of Comparative Physiology B*, *182*(5), 613-623. https://doi.org/10.1007/s00360-012-0646-y

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome research*, *6 (10)*, 986-994. https://doi.org/10.1101/gr.6.10.986

Herbert, R. J. H., Humphreys, J., Davies, C. J., Roberts, C., Fletcher, S., & Crowe, T. P. (2016). Ecological impacts of non-native Pacific oysters (Crassostrea gigas) and management measures for protected areas in Europe. *Biodiversity and Conservation*, *25*(14), 2835-2865. https://doi.org/10.1007/s10531-016-1209-4

Huang, S., Yoshitake, K., Watabe, S., & Asakawa, S. (2022). Environmental DNA study on aquatic ecosystem monitoring and management: Recent advances and prospects. *Journal of Environmental Management*, *323*, 116310. https://doi.org/https://doi.org/10.1016/j.jenvman.2022.116310

IMO, M. (2004). International convention for the control and management of ships' ballast water and sediments. BWM/CONF./36,

Iwasaki, W., Fukunaga, T., Isagozawa, R., Yamada, K., Maeda, Y., Satoh, T. P., Sado, T., Mabuchi, K., Takeshima, H., Miya, M., & Nishida, M. (2013). MitoFish and MitoAnnotator: a mitochondrial genome database of fish with an accurate and automatic annotation pipeline. *Mol Biol Evol*, *30*(11), 2531-2540. https://doi.org/10.1093/molbev/mst141

Jaspers, C., Huwer, B., Antajan, E., Hosia, A., Hinrichsen, H.-H., Biastoch, A., Angel, D., Asmus, R., Augustin, C., Bagheri, S., Beggs, S. E., Balsby, T. J. S., Boersma, M., Bonnet, D., Christensen, J. T., Dänhardt, A., Delpy, F., Falkenhaug, T., Finenko, G., . . . Woźniczka, A. (2018). Ocean current connectivity propelling the secondary spread of a marine invasive comb jelly across western Eurasia. *Global Ecology and Biogeography*, *27*(7), 814-827. https://doi.org/https://doi.org/10.1111/geb.12742

Jensen, K. (2010). NOBANIS – Invasive Alien Species Fact Sheet – Acartia tonsa – From: Identification key to marine invasive species in Nordic waters – *NOBANIS www.nobanis.org, Date of access 08/05/2023*.

Jensen, K. R., Andersen, P., Andersen, N. R., Bruhn, A., Buur, H., Carl, H., Jakobsen, H., Jaspers, C., Lundgreen, K., Nielsen, R., Strandberg, B., & Stæhr, P. A. U. (2023). Reviewing Introduction Histories, Pathways, Invasiveness, and Impact of Non-Indigenous Species in Danish Marine Waters. *Diversity*, *15*(3), 434. https://www.mdpi.com/1424-2818/15/3/434

Jespersen, P. (1933). Planktonfaunaen i Ringkøbing Fjord og Nymindestrømmen 1914-30. *In: R. Spärck (ed.) 1933-1936. Ringkøbing Fjords Naturhistorie i Brakvandsperioden 1915-31, pp. 49-61.*

Jørgensen, L. L. (2013). NOBANIS – Invasive Alien Species Fact Sheet – Paralithodes camtschaticus. *Online Database of the European Network on Invasive Alien Species – NOBANIS www.nobanis.org, Date of access 13/05/2023*.

Kelly, R. P., Gallego, R., & Jacobs-Palmer, E. (2018). The effect of tides on nearshore environmental DNA. *PeerJ*, *6*, e4521. https://doi.org/10.7717/peerj.4521

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2020a). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271-282. https://doi.org/10.1002/edn3.29

Klymus, K. E., Ruiz Ramos, D. V., Thompson, N. L., & Richter, C. A. (2020b). Development and Testing of Species-specific Quantitative PCR Assays for Environmental DNA Applications. *JoVE*(165), e61825. https://doi.org/doi:10.3791/61825

Knudsen, S. W., Andersen, J. H., Bekkevold, D., Hesselsøe, M., Jensen, S. K. S., & Møller, P. R. (2020a). Tekniske anvisninger for eDNA-baseret overvågning af ikke-hjemmehørende marine arter. *NIVA RAPPORT L.NR. 7455-2020 DK5*.

Knudsen, S. W., Andersen, J. H., & Møller, P. R. (2020b). Development of species-specific eDNA-based test systems for monitoring of non-indigenous Decapoda in Danish marine waters. *NIVA REPORT SNO 7544-2020*. www.niva.no

Knudsen, S. W., Hesselsøe, M., Thaulow, J., Agersnap, S., Hansen, B. K., Jacobsen, M. W., Bekkevold, D., Jensen, S. K. S., Møller, P. R., & Andersen, J. H. (2022). Monitoring of environmental DNA from nonindigenous species of algae, dinoflagellates and animals in the North East Atlantic. *Science of the Total Environment*, *821*. https://doi.org/10.1016/j.scitotenv.2022.153093

Landschoff, J., Lackschewitz, D., Kesy, K., & Reise, K. (2013). Globalization pressure and habitat change: Pacific rocky shore crabs invade armored shorelines in the Atlantic Wadden Sea. *Aquatic Invasions*, *8*(1).

Laubier, L. (2001). Climatic changes and trends and marine invertebrates: a need for relevant observing networks and experimental ecophysiology. *Atti della Associazione Italiana di Oceanologia e Limnologia*.

Leray, M., Knowlton, N., & Machida, R. J. (2022). MIDORI2: A collection of quality controlled, preformatted, and regularly updated reference databases for taxonomic assignment of eukaryotic mitochondrial sequences. *Environmental DNA*, *4*(4), 894-907. https://doi.org/https://doi.org/10.1002/edn3.303

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., Boehm, J. T., & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in zoology*, *10*. https://doi.org/10.1186/1742-9994-10-34

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *R Soc Open Sci*, *2*(7), 150088. https://doi.org/10.1098/rsos.150088

Nielsen, P., Geitner, K., Jakobsen, J., Köppl, C. J., & Petersen, J. K. (2019). *Fagligt grundlag for forvaltningsplan for udvikling af bæredygtige fiskerier af muslinger og østers i Vadehavet*. Institut for Akvatiske Ressourcer, Danmarks Tekniske Universitet. DTU Aqua-rapport No. 334-2018.

Occhipinti-Ambrogi, A. (2007). Global change and marine communities: alien species and climate change. *Mar Pollut Bull*, *55*(7-9), 342-352. https://doi.org/10.1016/j.marpolbul.2006.11.014

OSPAR. (2017). *Intermediate Assessment 2017, D2.1 - Abundance and state characterisation of non-indigenous species, in particular invasive species*. Retrieved from https://oap.ospar.org/en/ospar-assessments/intermediate-assessment-2017/pressures-human-activities/non-indigenous/

OTA, Congress, U. S., & Assessment, O. o. T. (1993). Harmful Non-Indigenous Species in the United States, OTA-F-565 *Washington, DC: U.S. Government Printing Office, September 1993*.

Pawlowski, J., Bruce, K., Panksep, K., Aguirre, F. I., Amalfitano, S., Apothéloz-Perret-Gentil, L., Baussant, T., Bouchez, A., Carugati, L., Cermakova, K., Cordier, T., Corinaldesi, C., Costa, F. O., Danovaro, R., Dell'Anno, A., Duarte, S., Eisendle, U., Ferrari, B. J. D., Frontalini, F., . . . Fazi, S. (2022). Environmental DNA metabarcoding for benthic monitoring: A review of sediment sampling and DNA extraction methods. *The Science of the total environment*, *818, 151783*. https://doi.org/10.1016/j.scitotenv.2021.151783

Peck, N., Peters, J., Diekmann, R., Laakmann, S., & Renz, J. (2015). Interactive effects of temperature and salinity on population dynamics of the calanoid copepod Acartia tonsa. *Journal of Plankton Research*, *37*(1), 197-210. https://doi.org/10.1093/plankt/fbu093

Petersen, K. S., Rasmussen, K. L., Heinemeier, J., & Rud, N. (1992). Clams before Columbus? *Nature*, *359*, 679-679.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glockner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*, *41*(Database issue), D590-596. https://doi.org/10.1093/nar/gks1219

R Core Team. (2021). R: A language and environment for statistical computing. *R Foundation for Statistical Computing, Vienna, Austria.* https://www.R-project.org/

Reise, K., Baptist, M., Burbridge, P., Dankers, N., Fischer, L., Flemming, B., Oost, A. P., & Smit, C. (2010). The Wadden Sea – A Universally Outstanding Tidal Wetland. *Wadden Sea Ecosystem No. 29. Common Wadden Sea Secretariat, Wilhelmshaven, Germany*, 7 - 24.

Reise, K., Buschbaum, C., Lackschewitz, D., Thieltges, D. W., Waser, A. M., & Wegner, K. M. (2023). Introduced species in a tidal ecosystem of mud and sand: curse or blessing? *Marine Biodiversity*, *53*(1), 5. https://doi.org/10.1007/s12526-022-01302-3

Reusch, T. B., & Williams, S. L. (1998). Variable responses of native eelgrass Zostera marina to a non-indigenous bivalve Musculista senhousia. *Oecologia, 113(3), 428–441*(1432-1939 (Electronic)). https://doi.org/10.1007/s004420050395

Riisgård, H. U. (2017). Invasion of Danish and Adjacent Waters by the Comb Jelly Mnemiopsis leidyi—10 Years After. *Open Journal of Marine Science*, *07*, 458-471. https://doi.org/10.4236/ojms.2017.74032

Rius, M., Clusella-Trullas, S., McQuaid, C. D., Navarro, R. A., Griffiths, C. L., Matthee, C. A., von der Heyden, S., & Turon, X. (2014). Range expansions across ecoregions: interactions of climate change, physiology and genetic diversity. *Global Ecology and Biogeography*, *23*(1), 76-88. https://doi.org/10.1111/geb.12105

Savini, D., Occhipinti–Ambrogi, A., Marchini, A., Tricarico, E., Gherardi, F., Olenin, S., & Gollasch, S. (2010). The top 27 animal alien species introduced into Europe for aquaculture and related activities. *Journal of Applied Ichthyology*, *26*(s2), 1-7. https://doi.org/https://doi.org/10.1111/j.1439-0426.2010.01503.x

Shrader-Frechette, K. (2001). Non-Indigenous Species and Ecological Explanation. *Biology and Philosophy*, *16*(4), 507-519. https://doi.org/10.1023/A:1011953713083

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T., Sigsgaard, E. E., & Hellström, M. (2017). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*, *8*(5), 635-645. https://doi.org/10.1111/2041-210X.12683

Stæhr, P. A. U., Andersen, N. R., Andersen, K. R., Buur, H., Jakobsen, H. H., Larsen, J., Maar, M., Sapkota, R., Schourup-Kristensen, V., Zimmer, K., & Winding, A. (2023). Identification, dispersal, and possible mitigation responses for non-indigenous species in the Danish Wadden Sea area. *Aarhus University, DCE – Danish Centre for Environment and Energy, 72 pp. Scientific Report No. 547*. http://dce2.au.dk/pub/SR547.pdf

Staehr, P. A. U., Dahl, K., Buur, H., Göke, C., Sapkota, R., Winding, A., Panova, M., Obst, M., & Sundberg, P. (2022). Environmental DNA Monitoring of Biodiversity Hotspots in Danish Marine Waters. *Frontiers in Marine Science*, *8*. https://doi.org/10.3389/fmars.2021.800474

Stoeckle, B. C., Beggel, S., Kuehn, R., & Geist, J. (2021). Influence of stream characteristics and population size on downstream transport of freshwater mollusk environmental DNA. *Freshwater Science*, *40*(1), 191-201. https://doi.org/10.1086/713015

Strasser, M. (1998). Mya arenaria — an ancient invader of the North Sea coast. *Helgoländer Meeresuntersuchungen*, *52*(3), 309-324. https://doi.org/10.1007/BF02908905

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For Biodiversity Research and Monitoring*. Oxford University Press. https://doi.org/10.1093/oso/9780198767220.001.0001

Tendal, O. S., Jensen, K. R., & Riisgård, H. U. (2007). Invasive ctenophore Mnemiopsis leidyi widely distributed in Danish waters. *Aquatic Invasions*, *2*(4), 455-460.

Tiselius, P., & Møller, L. F. (2017). Community cascades in a marine pelagic food web controlled by the non-visual apex predator *Mnemiopsis leidyi*. *Journal of Plankton Research*, *39*(2), 271-279. https://doi.org/10.1093/plankt/fbw096

Torti, A., Lever, M. A., & Jørgensen, B. B. (2015). Origin, dynamics, and implications of extracellular DNA pools in marine sediments. *Marine Genomics*, *24*, 185-196. https://doi.org/https://doi.org/10.1016/j.margen.2015.08.007

UNESCO World Heritage. https://whc.unesco.org/en/list/1314/

van Aken, H. M. (2008). Variability of the salinity in the western Wadden Sea on tidal to centennial time scales. *Journal of Sea Research*, *59*(3), 121-132. https://doi.org/10.1016/j.seares.2007.11.001

van Beusekom, J. E. E., & de Jonge, V. N. (2002). *Long-term changes in Wadden Sea nutrient cycles: importance of organic matter import from the North Sea* Springer Netherlands. https://doi.org/10.1007/978-94-017-2464-7_15 (Nutrients and Eutrophication in Estuaries and Coastal Waters: Proceedings of the 31st Symposium of the Estuarine and Coastal Sciences Association (ECSA), held in Bilbao, Spain, 3--7 July 2000)

van der Meeren, G., Støttrup, J., Ulmestrand, M., Øresland, V., J.A., K., & Agnalt, A.-L. (2010). NOBANIS – Invasive Alien Species Fact Sheet – *Homarus americanus*. *From: Online Database of the European Network on Invasive Alien Species - NOBANIS www.nobanis.org, Date of access 08/05/2023*. https://doi.org/

Vogensen, T. K., Nielsen, M. B., & Jensen, K. T. (2020). Klippekrabber i Vadehavet: en trussel mod den lokale flora og fauna? *Flora og Fauna 125: 15-23*.

Waser, A. M., Deuzeman, S., Kangeri, A. K. w., van Winden, E., Postma, J., de Boer, P., van der Meer, J., & Ens, B. J. (2016). Impact on bird fauna of a non-native oyster expanding into blue mussel beds in the Dutch Wadden Sea. *Biological Conservation*, *202*, 39-49. https://doi.org/https://doi.org/10.1016/j.biocon.2016.08.007

Weigand, H., Beermann, A. J., Čiampor, F., Costa, F. O., Csabai, Z., Duarte, S., Geiger, M. F., Grabowski, M., Rimet, F., Rulik, B., Strand, M., Szucsich, N., Weigand, A. M., Willassen, E., Wyler, S. A., Bouchez, A., Borja, A., Čiamporová-Zaťovičová, Z., Ferreira, S., . . . Ekrem, T. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *The Science of the total environment, 678, 499–524.* https://doi.org/10.1016/j.scitotenv.2019.04.247

Weisse, R., & Plüß, A. (2005). Storm-related sea level variations along the North Sea coast as simulated by a high-resolution model 1958–2002. *Ocean Dynamics*, *56*(1), 16-25. https://doi.org/10.1007/s10236-005-0037-y

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS ONE*, *8 (3)*. https://doi.org/10.1371/journal.pone.0059520

Wolff, W. J., & Zijlstra, J. J. (1980). Management of the Wadden Sea. *Helgoländer Meeresuntersuchungen*, *33*(1), 596-613. https://doi.org/10.1007/BF02414783

Wood, S. A.-O., Pochon, X., Laroche, O., von Ammon, U., Adamson, J., & Zaiko, A. (2019). A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. *Molecular ecology resources, 19(6), 1407–1419.* https://doi.org/10.1111/1755-0998.13055

Wrange, A.-L., Valero, J., Harkestad, L., Strand, Ø., Lindegarth, S., Christensen, H., Dolmer, P., Kristensen, P., & Mortensen, S. (2010). Massive settlements of the Pacific oyster, *Crassostrea gigas*, in Scandinavia. *Biological Invasions*, *12*, 1145-1152. https://doi.org/10.1007/s10530-009-9535-z

Xia, Z., Johansson, M. L., Gao, Y., Zhang, L., Haffner, G. D., MacIsaac, H. J., & Zhan, A. (2018). Conventional versus real-time quantitative PCR for rare species detection. *Ecology and evolution, 8(23), 11799–11807*(2045-7758 (Print)).

Yang, J., Zhang, X. A.-O., Zhang, W., Sun, J., Xie, Y., Zhang, Y., Burton, G. A., Jr., & Yu, H. (2017). Indigenous species barcode database improves the identification of zooplankton. *PloS one, 12(10), e0185697.* https://doi.org/10.1371/journal.pone.0185697

Appendix

The following tables 9 to 18 show the used compositions for the master mixes as well as the protocols and used primers for the metabarcoding analyses and species-specific qPCR assays. The tables 19 to 21 are displaying the results of the qPCR analyses with approach 1 to calculate LOD and LOQ on the different sample types (water samples from the harbor, tidal channels, and settlement plate samples). The tables 22 to 24 are showing the qPCR results from using approach 2 to determine LOD and LOQ.

Table 9: Composition of Master Mix for PCR 1 (Metabarcoding)

Samples	***1***
	Volume [µl]
KaPa HiFi master	12.5
Forward-primer (10 µM)	1
Reverse-primer (10 µM)	1
BSA (20 mg/ml)	0.5
PCR H_2O	8
Total Master Mix	**23**
+ DNA template	2

Table 10: Protocol for PCR 1 (metabarcoding)

Temperature [°C]	**Time**	**Cycles**
98°	2 min	1
98°	40 sec	35
COI: 48°, 12 S: 65°, 18 S: 57°	30 sec	
72°	30 sec	
72°	5 min	1
4°	∞	

Table 11: Dilution of cleaned PCR 1 products for PCR 2

DNA concentration cleaned PCR 1 product [ng/µl]	**Dilution**	
	Volume cleaned PCR 1 product [µl]	**Volume PCR water [µl]**
> 10	5	10
10 - 1	10	5
< 1	5	0

Table 12: Composition of Master Mix for PCR 2 (Metabarcoding)

Samples	***1***
	Volume [µl]
KaPa mix 2x	12.5
Index 1	2.5
Index 2	2.5
PCR H_2O	2.5
Total Master Mix	20
+ Cleaned PCR1 product	5

Table 13: Protocol for PCR 2 (Metabarcoding)

Temperature [°C]	**Time**	**Cycles**
95°	3 min	1
95°	30 sec	10
55°	30 sec	
72°	30 sec	
75°	5 min	1
4°	∞	

Table 14: Composition of Master Mix for PCR (Standards for qPCR)

	Undiluted DNA	*Diluted DNA*
	Volume [µl] for 1 sample	**Volume [µl] for 1 sample**
PCR Ultra mix 2x	12.5	12.5
Forward-primer (10 µM)	0.5	0.5
Reverse-primer (10 µM)	0.5	0.5
BSA (20 mg/ml)	0.5	0.5
PCR H_2O	9	6
Total Master Mix	**23**	**20**
+ DNA template	2	5

Table 15: Protocol for PCR (Standards for qPCR)

Temperature [°C]	**Time**	**Cycles**
95°	1 min	1
95°	30 sec	35
60°	30 sec	
72°	1 min	
72°	5 min	1
4°	∞	

Table 16: Composition of Master Mix for qPCR

Samples	***1***
	Volume [µl]
2x qPCRBIO Probe Mix Lo-ROX	12.5
Forward-primer (10 µM)	1
Reverse-primer (10 µM)	1
Probe (5 µM)	0.5
PCR H_2O	7
Total Master Mix	**22**
+ Cleaned PCR product (standard)	3

Table 17: Protocol for qPCR

Temperature [°C]	**Time**	**Cycles**
95°	10 min	1
95°	30 sec	50
60°	45 sec	

Table 18: Sequences of species-specific primers and probes used for qPCR analyses. Order of listed sequences: forward primer (F), reverse primer (R) and probe (P). Primers and probes after Andersen et al. (2018); Knudsen et al. (2020b); Knudsen et al. (2022). *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Nr.	**Species**	**Primer and probe name (R and F)**	**Sequences of species-specific primers and probes for qPCR (5‘-3‘)**	**PM 5'-end**	**PM 3'-end**
1	*Bonnemaisonia hamifera*	Bon_ham_rbcL_F02	CAATTACTAGATTACCTGGGCAAT		
		Bon_ham_rbcL_R02	CTTCTTTTACAAAGTCCCGACCT		
		Bon_ham_rbcL_P01	TCGTGCCATAACCATAGACTCTAAAGCC	FAM	BHQ-1
2	*Prorocentrum minimum*	Pro_min_28S_F03	CTTGGCAAGATTGTCGGGT		
		Pro_min_28S_R03	TATTCACTCACCCATAGACGA		
		Pro_min_28S_P03	ACACACAAGGCAAGAGACGATCAAGC	FAM	BHQ-1
3	*Pseudochattonella farcimen*	PsefarF	GGGAGAAATTCTTTGGAACAAGG		
		PsefarR	GCAACTCGACTCCACTAGG		
		PseP	TCAGAGAGGGTGACAATCCCGTCT	FAM	BHQ-1
4	*Pseudochattonella verruculata*	PseverF	GGGAGAAGTCCTTTGGAACAAGG		
		PseverR	GCAACTCGACTCCATTAGC		
		PseP	TCAGAGAGGGTGACAATCCCGTCT	FAM	BHQ-1
5	*Karenia mikimotoi*	KarmikF3	CCGAGTGACTGAATGTCCTC		
		KarmikR3	GATCGCAGGCAAGCACATGA		
		KarmikP3	GCAGTGCTACCAGACACACAGAG	FAM	BHQ-1
6	*Carassius auratus*	Caraur_COI_F01	TTCTTCCCCCATCATTCCTGT		
		Caraur_COI_R01	GTATACTGTCCATCCGGAGG		
		Caraur_COI_P02	TAGCTTCCTCTGGTGTTGAAGCCGGAG	FAM	BHQ-1

Nr.	Species	Primer and probe name (R and F)	Sequences of species-specific primers and probes for qPCR (5‘-3‘)	PM 5'-end	PM 3'-end
7	*Cyprinus carpio*	CCcytbF	CTAGCACTATTCTCCCCTAACTTAC		
		CCcytbR	ACACCTCCGAGTTTGTTTGGA		
		CCcytbP	CCCTCTAGTTACACCACC	FAM	TAMRA
8	*Colpomenia peregrine*	Col_per_COX_3_F01	GCAAGCTTTTGAATATGCTAATG		
		Col_per_COX_3_R01	CAGCTAAAAATATTGTACCGATT		
		Col_per_COX_3_P01	TTCAGTTTTTTACATGGCTACAGGCTTC	FAM	TAMRA
10	*Oncorhynchus mykiss*	Onc_myk_CytB_F01	ACCTCCAGCCATCTCTCAGT		
		Onc_myk_CytB_R01	AGGACGGGGAGGGAAAGTAA		
		Onc_myk_CytB_P01	TGAGCCGTGCTAGTTACTGCTGTCCTT	FAM	BHQ-1
11	*Oncorhyncus gorbuscha*	Oncgor_CO1_F09	TCCTTCCTCCTCCTCCTTTC		
		Oncgor_CO1_R06	TGGCCCCTAAAATTGATGAG		
		Oncgor_CO1_P06	CAGGGGCATCCGTCGACTTAACTAT	FAM	BHQ-1
12	*Magallana gigas*	Cragig_CO1_F07	TTGAGTTTTGCCAGGGTCTC		
		Cragig_CO1_R09	ACCAGCAAGGTGAAGGCTTA		
		Cragig_CO1_P06	AACATTGTAGAAAACGGAGTTGGGGC	FAM	BHQ-1
13	*Mya arenaria*	Mya_are_CO1_F01	CCCTCCGTTGTCGAGAAATA		
		Mya_are_CO1_R02	ACGCATGTTACCCCAAGTTC		
		Mya_are_CO1_P06	TATCCCTTCATATTGGAGGGGCTTCAT	FAM	BHQ-1
14	*Rhithropanopeus harrisii*	Rhihar_co1_F03	GTCAACCTGGTACTCTCATTGGT		
		Rhihar_co1_R03	ACGAGGAAATGCTATATCAGGGG		
		Rhihar_co1_P03	TGTTGTAGTAACAGCTCACGCCTTTGT	FAM	BHQ-1
15	*Paralithodes camtschaticus*	Parcam_co1_F02	GGGCTTGAGCTGGAATAGTG		
		Parcam_co1_R05	CAATTTCCAAACCCTCCAAT		
		Parcam_co1_P02	ATTCGAGCTGAACTAGGACAACCAGGT	FAM	BHQ-1
16	*Eriocheir sinensis*	Erisin_cytb_F02	ACCCCTCCTCATATCCAACCA		
		Erisin_cytb_R02	AAGAATGGCCACTGAAGCGG		
		Erisin_cytb_P02	TTTGCTTACGCTATTTTACGATCAATTCCT	FAM	BHQ-1
17	*Homarus americanus*	Homame_co1_F06	TTACAGCAGTTCTTTTACTACTCTCG		
		Homame_co1_R08	ACTGGGTCTCCACCTCCAG		
		Homame_co1_P08	TCGAAATTTAAATACTTCATTCTTCGATCCA	FAM	BHQ-1
18	*Cordylophora caspia*	Cor_cas_COI_F01	TCATCTGTACAAGCACATTCTGG		
		Cor_cas_COI_R01	TTGAAGAAGCTCCTGCACAGT		
		Cor_cas_COI_P01	CCTTCTGTAGACATGGCTATATTTAGTC	FAM	BHQ-1
19	*Mnemiopsis leidyi*	Mnelei_its2_F04	ACGGTCCCTTGAAGTAGAGC		
		Mnelei_its2_R06	TCTGAGAAGGCTTCGGACAT		
		Mnelei_its2_P06	GTGCCTCTCGGTGTGGTAGCAATATCT	FAM	BHQ-1
20	*Acipenser baerii*	Acibae_CR_F02	CAGTTGTATCCCCATAATCAGCC		
		Acibae_CR_R03	TTATTCATTATCTCTGAGCAGTCGTGA		
		Acibae_CR_P01	ATGCCGAGAACCCCATCAACATTTGGT	FAM	BHQ-1
21/ 22	*Acipenser* spp.*	Acibae_cytb_F11	TTCCACCCGTACTTCTCATAC		
		Acibae_cytb_R11	CCTAATGCTAGTCGGACTCACCTCCGT		
		Acibae_cytb_P16	GGCGTAGGCGAAGAGAAAGTA	FAM	BHQ-1

Nr.	Species	Primer and probe name (R and F)	Sequences of species-specific primers and probes for qPCR (5‘-3‘)	PM 5'-end	PM 3'-end
23	*Callinectes sapidus*	Calsap_co1_F01	GGGCCTCAGTTGATCTTGGT		
		Calsap_co1_R01	GTAGAGAACAGGGTCGCCTC		
		Calsap_co1_P01	ATACCTCATTCTTCGACCCAGCTGGAG	FAM	BHQ-1
24	*Hemigrapsus sanguineus*	Hemsan_COI_F01	CCTGGGCCGGTATAGTAGGT		
		Hemsan_COI_R01	GGGGCTCCGAGTATAAGTGG		
		Hemsan_COI_P01	CGAGCAGAATTAAGACAACCAGGAAGC		
25	*Hemigrapsus takanoi*	Hemtak_co1_F05	AGGTTTTGACTTCTTCCTCCTTCT		
		Hemtak_co1_R05	CTGCGAGTGGAGGGTAAACG		
		Hemtak_co1_P05	TAGAAAGAGGTGTAGGTACAGGATGGA	FAM	BHQ-1

Table 19: Detected NIS with qPCR in the harbors. LOD and LOQ were determined with approach 1. The triplicates from every sample were categorized in “No Cq” / “Signal with Cq above than LOD” / “Signal with Cq between LOD and LOQ” / “Signal with Cq lower than LOQ”. Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	Esbjerg harbor 1	Esbjerg harbor 2	Esbjerg harbor 3	Esbjerg harbor extra	Fanø harbor	Rømø harbor
Pseudochattonella verruculosa	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/1/2
Pseudochattonella farcimen	0/0/1/2	0/0/1/2	0/0/2/1	0/0/1/2	0/2/1/0	0/1/2/0
Prorocentrum cordatum	0/0/1/2	0/0/2/1	0/0/2/1	0/0/3/0	0/0/3/0	0/0/0/3
Karenia mikimotoi	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Colpomenia peregrina	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	1/1/0/1
Bonnemaisonia hamifera	2/0/0/1	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0
Magallana gigas	0/0/2/1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/1/2	0/0/1/2
Mya arenaria	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/1/2/0	0/0/0/3
Cordylophora caspia	3/0/0/0	2/1/0/0	2/0/0/1	3/0/0/0	3/0/0/0	3/0/0/0
Mnemiopsis leidyi	0/0/0/3	0/0/0/3	0/0/0/3	0/0/1/2	0/0/0/3	0/0/3/0
Rhithropanopeus harrisii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Eriocheir sinensis	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Hemigrapsus takanoi	2/0/1/0	2/0/1/0	1/0/1/1	1/1/1/0	0/2/1/0	1/0/2/0
Hemigrapsus sanguineus	3/0/0/0	3/0/0/0	1/2/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Callinectes sapidus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Paralithodes camtschaticus	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Homarus americanus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	2/0/0/1

Species	Esbjerg harbor 1	Esbjerg harbor 2	Esbjerg harbor 3	Esbjerg harbor extra	Fanø harbor	Rømø harbor
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	0/2/1/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Carassius auratus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser baerii	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser spp.*	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Table 20: Detected NIS with qPCR on settlement plates. LOD and LOQ were determined with approach 1. The triplicates from every sample were categorized in "No Cq" / "Signal with Cq above than LOD" / "Signal with Cq between LOD and LOQ" / "Signal with Cq lower than LOQ". Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	Esbjerg harbor 2	Esbjerg harbor 3	Fanø harbor	Rømø harbor
Pseudochattonella verruculosa	3/0/0/0	0/2/1/0	3/0/0/0	2/1/0/0
Pseudochattonella farcimen	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Prorocentrum cordatum	0/0/3/0	0/0/3/0	0/0/3/0	0/0/2/1
Karenia mikimotoi	0/0/0/3	2/0/0/1	0/0/0/3	1/0/0/2
Colpomenia peregrina	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Bonnemaisonia hamifera	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0
Magallana gigas	1/0/2/0	0/0/3/0	1/0/2/0	3/0/0/0
Mya arenaria	0/0/0/3	0/0/0/3	0/0/1/2	0/2/1/0
Cordylophora caspia	0/0/2/1	0/0/2/1	2/1/0/0	3/0/0/0
Mnemiopsis leidyi	0/0/0/3	1/2/0/0	1/1/1/0	1/2/0/0
Rhithropanopeus harrisii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Eriocheir sinensis	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Hemigrapsus takanoi	1/1/1/0	0/0/0/3	1/1/1/0	3/0/0/0
Hemigrapsus sanguineus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Callinectes sapidus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Paralithodes camtschaticus	3/0/0/0	0/0/0/3	2/1/0/0	3/0/0/0
Homarus americanus	3/0/0/0	0/2/1/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	2/1/0/0	0/3/0/0	0/0/0/3	2/1/0/0
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	1/2/0/0	2/1/0/0	3/0/0/0
Carassius auratus	0/3/0/0	1/2/0/0	2/1/0/0	3/0/0/0
Acipenser baerii	2/0/1/0	1/0/1/1	3/0/0/0	3/0/0/0
Acipenser spp.*	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Table 21: Detected NIS with qPCR in the tidal channels. LOD and LOQ were determined with approach 1. The triplicates from every sample were categorized in "No Cq" / "Signal with Cq above than LOD" / "Signal with Cq between LOD and LOQ" / "Signal with Cq lower than LOQ". Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	Lister Dyb 1	Lister Dyb 2	Lister Dyb 3	Juvre Dyb 1	Juvre Dyb 2	Juvre Dyb 3	Grådyb 1	Grådyb 2	Grådyb 3	Grådyb 16:30	Grådyb 17:30	Knude-dyb 1	Knude-dyb 2	Knude-dyb 3
Pseudochattonella verruculosa	0/0/3/0	0/0/3/0	0/0/3/0	0/0/3/0	0/0/3/0	0/0/2/1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	1/0/0/2	0/2/1/0	0/1/2/0	1/0/2/0
Pseudochattonella farcimen	1/0/2/0	0/1/2/0	1/2/0/0	2/1/0/0	2/1/0/0	2/1/0/0	0/0/2/1	0/0/3/0	0/0/2/1	0/0/0/3	0/0/2/1	3/0/0/0	3/0/0/0	2/1/0/0
Prorocentrum cordatum	1/0/1/1	0/0/0/3	0/0/3/0	0/0/3/0	1/0/2/0	0/0/3/0	0/0/1/2	0/0/0/3	0/0/3/0	0/0/0/3	0/0/3/0	0/0/0/3	0/0/0/3	0/0/3/0
Karenia mikimotoi	0/0/0/3	0/0/0/3	0/0/0/3	1/1/0/1	1/0/0/2	2/0/0/1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/1/0/2	1/0/0/2	2/0/0/1
Colpomenia peregrina	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Bonnemaisonia hamifera	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Magallana gigas	0/0/1/2	0/0/3/0	0/0/3/0	0/0/0/3	0/0/1/2	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	1/0/0/2	1/0/2/0	3/0/0/0	1/0/2/0
Mya arenaria	0/0/1/2	0/1/0/2	0/0/2/1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Cordylophora caspia	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/0/0/1	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0
Mnemiopsis leidyi	0/0/2/1	0/0/0/3	0/0/1/2	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/1/2/0	0/1/2/0	0/0/2/1	0/0/3/0
Rhithropanopeus harrisii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Eriocheir sinensis	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Hemigrapsus takanoi	1/0/2/0	1/1/1/0	0/1/2/0	1/1/1/0	0/1/0/2	0/0/3/0	2/0/1/0	0/0/1/2	0/0/3/0	1/2/0/0	1/0/1/1	3/0/0/0	3/0/0/0	2/1/0/0
Hemigrapsus sanguineus	2/1/0/0	1/2/0/0	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Callinectes sapidus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Species	Lister Dyb 1	Lister Dyb 2	Lister Dyb 3	Juvre Dyb 1	Juvre Dyb 2	Juvre Dyb 3	Grådyb 1	Grådyb 2	Grådyb 3	Grådyb 16:30	Grådyb 17:30	Knude-dyb 1	Knude-dyb 2	Knude-dyb 3
Paralithodes camtschaticus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	1/0/0/2	3/0/0/0	1/0/0/2	0/1/0/2	1/2/0/0
Homarus americanus	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	3/0/0/0	2/1/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	2/0/0/1	3/0/0/0	2/0/0/1	3/0/0/0	3/0/0/0
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	0/3/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Carassius auratus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser baerii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0	2/0/1/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser spp.*	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Table 22: Detection of NIS with qPCR in the harbors. LOD and LOQ were determined with approach 2 after Klymus et al. 2020. The triplicates from every sample were categorized in "No Cq" / "Signal with Cq above than LOD" / "Signal with Cq between LOD and LOQ" / "Signal with Cq lower than LOQ". Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	Esbjerg harbor 1	Esbjerg harbor 2	Esbjerg harbor 3	Esbjerg harbor extra	Fanø harbor	Rømø harbor
Pseudochattonella verruculosa	0/0/3/0	0/0/1/2	0/0/3/0	0/0/1/2	0/0/3/0	0/1/2/0
Pseudochattonella farcimen	0/1/0/2	0/1/0/2	0/2/0/1	0/1/0/2	0/3/0/0	0/3/0/0
Prorocentrum cordatum	0/1/0/2	0/2/0/1	0/2/0/1	0/3/0/0	0/3/0/0	0/0/0/3
Karenia mikimotoi	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Colpomenia peregrina	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	1/1/1/0
Bonnemaisonia hamifera	2/0/0/1	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0
Magallana gigas	0/2/0/1	0/0/0/3	0/0/0/3	0/0/0/3	0/1/0/2	0/1/0/2
Mya arenaria	0/0/0/3	0/0/0/3	0/0/3/0	0/0/0/3	0/3/0/0	0/0/0/3
Cordylophora caspia	3/0/0/0	2/1/0/0	2/0/1/0	3/0/0/0	3/0/0/0	3/0/0/0
Mnemiopsis leidyi	0/0/0/3	0/0/0/3	0/0/0/3	0/1/0/2	0/0/0/3	0/3/0/0
Rhithropanopeus harrisii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Eriocheir sinensis	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Hemigrapsus takanoi	2/1/0/0	2/1/0/0	1/1/1/0	1/2/0/0	0/3/0/0	1/2/0/0
Hemigrapsus sanguineus	3/0/0/0	3/0/0/0	1/2/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Callinectes sapidus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Paralithodes camtschaticus	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Homarus americanus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	2/0/1/0
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	0/3/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Carassius auratus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser baerii	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser spp.*	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Table 23: Detection of NIS on settlement plates with qPCR. LOD and LOQ were determined with approach 2 after Klymus et al. 2020. The triplicates from every sample were categorized in "No Cq" / "Signal with Cq above than LOD" / "Signal with Cq between LOD and LOQ" / "Signal with Cq lower than LOQ". Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	**Esbjerg harbor 2**	**Esbjerg harbor 3**	**Fanø harbor**	**Rømø harbor**
Pseudochattonella verruculosa	3/0/0/0	0/3/0/0	3/0/0/0	2/1/0/0
Pseudochattonella farcimen	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Prorocentrum cordatum	0/3/0/0	0/3/0/0	0/3/0/0	0/2/0/1
Karenia mikimotoi	0/0/3/0	2/0/0/1	0/0/1/2	1/0/1/1
Colpomenia peregrina	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Bonnemaisonia hamifera	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Magallana gigas	1/2/0/0	0/3/0/0	1/2/0/0	3/0/0/0
Mya arenaria	0/0/0/3	0/0/0/3	0/1/2/0	0/3/0/0
Cordylophora caspia	0/2/1/0	0/2/1/0	2/1/0/0	3/0/0/0
Mnemiopsis leidyi	0/0/0/3	1/2/0/0	1/2/0/0	1/2/0/0
Rhithropanopeus harrisii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Eriocheir sinensis	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Hemigrapsus takanoi	1/2/0/0	0/0/3/0	1/2/0/0	3/0/0/0
Hemigrapsus sanguineus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Callinectes sapidus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Paralithodes camtschaticus	3/0/0/0	0/0/3/0	2/1/0/0	3/0/0/0
Homarus americanus	3/0/0/0	0/3/0/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	2/1/0/0	0/3/0/0	0/0/3/0	2/1/0/0
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	1/2/0/0	2/1/0/0	3/0/0/0
Carassius auratus	0/3/0/0	1/2/0/0	2/1/0/0	3/0/0/0
Acipenser baerii	2/1/0/0	1/1/0/1	3/0/0/0	3/0/0/0
Acipenser spp.*	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Table 24: Detection of NIS in tidal channels with qPCR. LOD and LOQ were determined with approach 2 after Klymus et al. 2020. The triplicates from every sample were categorized in "No Cq" / "Signal with Cq above than LOD" / "Signal with Cq between LOD and LOQ" / "Signal with Cq lower than LOQ". Yellow highlighted samples have at least one positive signal, which has a Cq above LOD. Samples with at least one positive signal between LOD and LOQ are highlighted orange and samples with one or two positive signals with a lower Cq than LOQ are colored red. Black colored samples have three positive signals with Cq below LOQ. A species is considered as detected, if at least one sample has a Cq value below LOD. *No distinction was possible between the primers and probes for species 21 *Acipenser gueldenstaedtii* and species 22 *Acipenser ruthenus*, which is why this primer- and probe set was used to detect *Acipenser* spp. in general.

Species	Lister Dyb 1	Lister Dyb 2	Lister Dyb 3	Juvre Dyb 1	Juvre Dyb 2	Juvre Dyb 3	Grådyb 1	Grådyb 2	Grådyb 3	Grådyb 16:30	Grådyb 17:30	Knude-dyb 1	Knude-dyb 2	Knude-dyb 3
Pseudochattonella verruculosa	0/3/0/0	0/3/0/0	0/3/0/0	0/3/0/0	0/3/0/0	0/2/1/0	0/0/3/0	0/0/3/0	0/0/3/0	0/0/3/0	1/0/2/0	0/3/0/0	0/3/0/0	1/2/0/0
Pseudochattonella farcimen	1/2/0/0	0/3/0/0	1/2/0/0	2/1/0/0	2/1/0/0	2/1/0/0	0/2/0/1	0/3/0/0	0/2/0/1	0/0/0/3	0/2/0/1	3/0/0/0	3/0/0/0	2/1/0/0
Prorocentrum cordatum	1/1/0/1	0/0/0/3	0/3/0/0	0/3/0/0	1/2/0/0	0/3/0/0	0/1/0/2	0/0/0/3	0/3/0/0	0/0/0/3	0/3/0/0	0/0/0/3	0/0/0/3	0/3/0/0
Karenia mikimotoi	0/0/0/3	0/0/0/3	0/0/1/2	1/1/0/1	1/0/0/2	2/0/1/0	0/0/2/1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/1/2	0/1/0/2	1/0/0/2	2/0/0/1
Colpomenia peregrina	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Bonnemaisonia hamifera	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Magallana gigas	0/1/0/2	0/3/0/0	0/3/0/0	0/0/0/3	0/1/0/2	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	1/0/0/2	1/2/0/0	3/0/0/0	1/2/0/0
Mya arenaria	0/1/1/1	0/1/2/0	0/2/1/0	0/0/0/3	0/0/0/3	0/0/0/3	0/0/3/0	0/0/3/0	0/0/2/1	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Cordylophora caspia	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0
Mnemiopsis leidyi	0/2/0/1	0/0/0/3	0/1/0/2	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/3/0/0	0/3/0/0	0/2/0/1	0/3/0/0
Rhithropanopeus harrisii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Eriocheir sinensis	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Hemigrapsus takanoi	1/2/0/0	1/2/0/0	0/3/0/0	1/2/0/0	0/1/2/0	0/3/0/0	2/1/0/0	0/1/2/0	0/3/0/0	1/2/0/0	1/1/1/0	3/0/0/0	3/0/0/0	2/1/0/0
Hemigrapsus sanguineus	2/1/0/0	1/2/0/0	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0

Species	Lister Dyb 1	Lister Dyb 2	Lister Dyb 3	Juvre Dyb 1	Juvre Dyb 2	Juvre Dyb 3	Grådyb 1	Grådyb 2	Grådyb 3	Grådyb 16:30	Grådyb 17:30	Knude-dyb 1	Knude-dyb 2	Knude-dyb 3
Callinectes sapidus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Paralithodes camtschaticus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	1/0/2/0	3/0/0/0	1/0/2/0	0/1/2/0	1/2/0/0
Homarus americanus	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Oncorhynchus mykiss	3/0/0/0	2/1/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	2/0/1/0	3/0/0/0	2/0/0/1	3/0/0/0	3/0/0/0
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	0/3/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Carassius auratus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Acipenser baerii	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	3/0/0/0