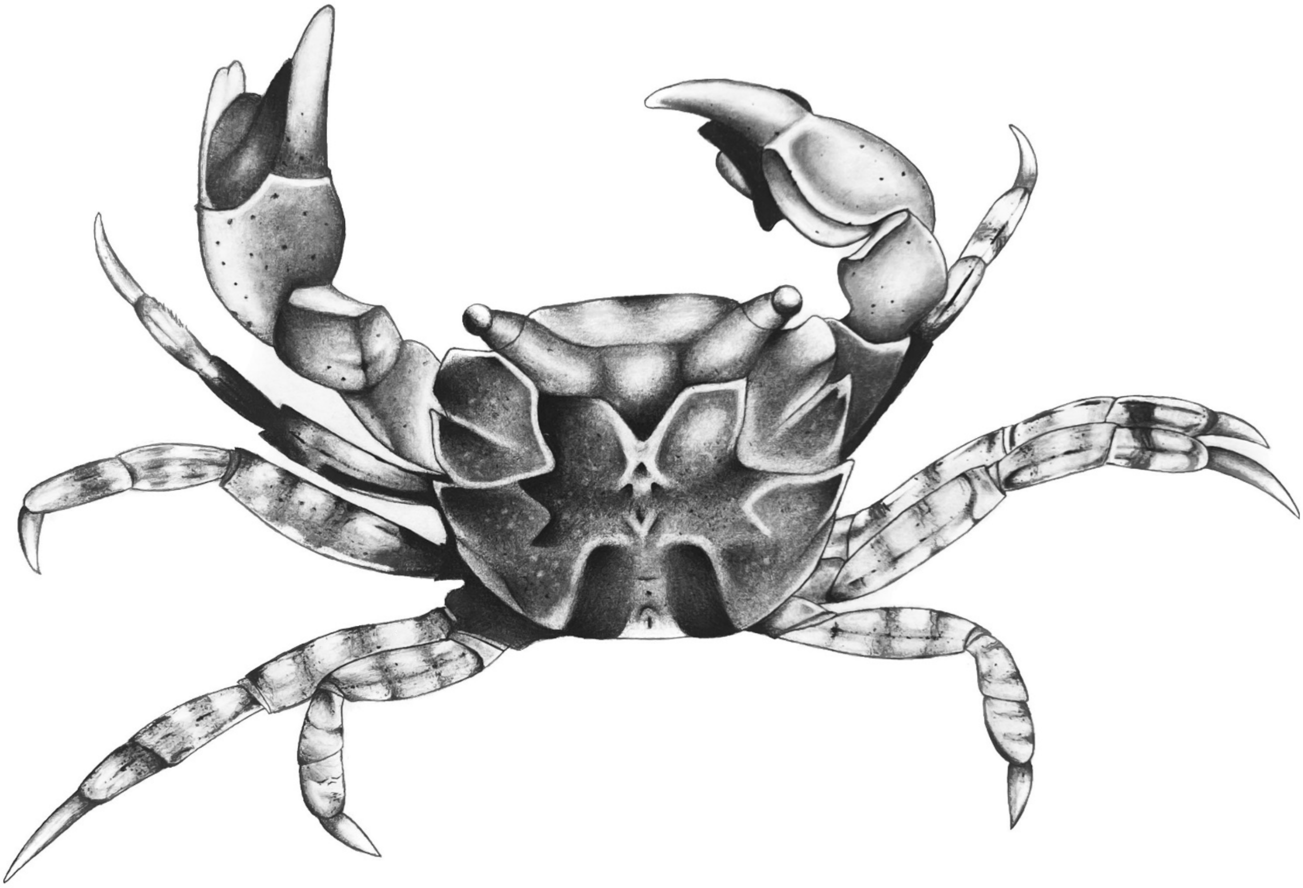

IDENTIFICATION, DISPERSAL, AND POSSIBLE MITIGATION RESPONSES FOR NON-INDIGENOUS SPECIES IN THE DANISH WADDEN SEA AREA

Scientific Report from DCE – Danish Centre for Environment and Energy no. 547 2023

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Data sheet

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Abstract:	Through monitoring using molecular and conventional techniques, novel data on the distribution and number of non-indigenous species (NIS) are provided for the Danish Wadden Sea area. Review of existing data sources, including the national monitoring program for marine species, enabled a description of changes in NIS occurrences over time, and updated information on the total number of NIS in the region, for which we applied an expert-based judgement to identify high impact NIS. Combining a literature review on introduction pathways for the observed NIS with a model tracer-based assessment of NIS dispersal, helped identify the importance of different introduction pathways. This information was finally used to identify the possible and suitable mitigation responses for NIS in the Danish Wadden Sea.

Keywords:	Non-indigenous species, Wadden Sea, Denmark, Monitoring, Detection, Modelling, Mitigation measures, Review
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Contents

Preface

This is a project which through monitoring, review of data and modelling provides information on the identification, dispersal and possible mitigation responses for non-indigenous species in the Danish Wadden Sea area. The project is financed by the Ministry of Environment and developed in agreement with the Danish Environmental Protection Agency. The project has been reviewed by the Danish Environmental Protection Agency.

Sammenfatning

Rapporten frembringer ny viden om antal, udbredelse, introduktion og påvirkning af ikke-hjemmehørende arter (NIS) i den danske del af Vadehavet. Denne viden er relevant for udvikling af det danske indsatsprogram i fht. deskriptor 2 (Ikke-hjemmehørende arter) under Havstrategidirektivet. Gennem et nyt intensivt moniteringsprogram og ved analyse af data fra det nationale overvågningsprogram samt andre datakilder, blev der lavet en opdateret liste over NIS i området. Information om mulige introduktionsveje og påvirkning (impact) for de fundne NIS blev etableret ud fra eksisterende publiceret viden. Opsætning af en hydrodynamisk model for området gjorde det desuden muligt at vurdere sandsynligheden for tilførsel af nye NIS via havstrømme gennem scenarier for frigivelse af partikler og sporing af disses bevægelse i Vadehavsområdet. Design af moniteringsprogrammet samt valg af metoder blev koordineret med en følgegruppe med repræsentanter fra det trilaterale Vadehavssamarbejde, med deltagelse af hollandske og tyske NIS eksperter. Resultaterne og vurderinger af mulige indsatser blev desuden fremlagt og diskuteret med denne følgegruppe.

Den nye monitering identificerede i alt 50 NIS i området ved en kombination af konventionelle samt molekylære metoder. Prøver blev indsamlet i tre større havne samt fire tidevandsområder i Vadehavet. Flest NIS blev fundet i havnene, særligt Esbjerg havn, samt i tilstødende vandområder, mens betydeligt færre blev observeret i sydligere mere afsidesliggende områder. Flest arter blev fundet blandt invertebrater (54%), fytoplankton (21%), makroalgar (14%) med færre blandt zooplankton (7%) og fisk (4%). 14 helt nye NIS blev identificeret for danske farvande, to af disse med konventionelle metoder og 12 med molekylære. Den sidste gruppe vurderes som mere usikre bestemt, og det anbefales, at disse arter placeres på en observationsliste til yderligere verifikation.

Gennemgang af data fra det nationale marine overvågningsprogram samt andre datakilder, resulterede i yderligere 32 arter, hvormed det samlede antal NIS i Vadehavet anslås at være 82 arter, det højeste antal i nogen dansk region. Til sammenligning blev der i 2016 kun indrapporteret 29 NIS i området. Baseret på data fra det nationale marine overvågningsprogram, var det muligt at beskrive den tidslige udvikling (1990 – 2020) for 39 NIS i Vadehavet. Ud af de i alt 82 NIS, vurderes 41 at være kommet til via skibstrafik, primært ballastvand, men også begroning. Sekundær naturlig spredning fra andre farvande i Europa blev identificeret for 39 NIS, og for nogle få arter vurderes udsætning samt udslip fra havbrug at være relevant. Mere end halvdelen af arterne havde flere sandsynlige introduktionsveje og for 11 arter var det ikke muligt at identificere en introduktionsvej. Model-baseret vurdering af spredningsveje sandsynliggjorde at arter (larvestadier og planktoniske arter) frigivet via ballastvand udenfor Vadehavet, kan spredes ind i lavere liggende tidevandsområder og havnene. Sekundær spredning af arter via havstrømme fra sydligere beliggende områder vurderes også at kunne bidrage med nye NIS i den danske del af Vadehavet. Baseret på en ekspertbaseret score af påvirkning, vurderes 11 NIS at kunne have en kraftig påvirkning.

Baseret på data over antal af NIS, deres etablering i området, spredningsveje og mulige påvirkning, vurderedes forskellige mulige indsatser til at reducere tilførsel og påvirkning af NIS i Vadehavet. Etablerede konventioner om

tilførsel via ballastvand og anbefalinger omkring tilførsel af arter via begroning og udsætning bør efterleves for at reducere sandsynligheden for nye introduktioner. Formidling om problemets omfang med tilhørende anbefalinger om mulige forholdsregler bør styrkes, og man kan med fordel overveje at styrke eksisterende beskyttelse og anvendelse af naturgenopretning for at styrke økosystemets modstandskraft over for invasive NIS. Vigtigheden af fortsat intensiv monitering af NIS i Vadehavet for at kunne vurdere mulige påvirkninger fra NIS og effekten af forskellige indsatstyper fremhæves.

Summary

This report provides data and knowledge about non-indigenous species (NIS) established in the Danish part of the Wadden Sea. Through novel intensive monitoring and review of available data sources, comprehensive updated lists of NIS were obtained, along with information on pathways of introduction and assessments of various types of impacts. Dispersal routes of NIS into the Wadden Sea were quantified with a 3D hydrodynamic model. The combination of updated NIS lists, knowledge of pathways of introduction and modelling of dispersal routes were used to identify possible mitigation measures against NIS, including invasive species, in the Danish part of the Wadden Sea. As such, the project provides important information to the Danish Marine Strategy's program of measures related to MSFD Descriptor 2. The project collaborated with an advisory board with representatives from Germany and the Netherlands to ensure compatibility with monitoring methods used in the other parts of the Wadden Sea and facilitate the exchange of knowledge on suitable mitigation measures.

A total of 50 NIS was detected using conventional and molecular techniques applied for four different sampling approaches in both selected harbours and tidal flats and tidal channels. Most NIS occurred in the two largest harbors, followed by water areas with intensive shipping, and with least NIS in the more disconnected water areas. Most NIS were observed within invertebrates (54%), phytoplankton (21%), macroalgae (14%), zooplankton (7%) and fish (4%). Interestingly, 14 NIS appear to be new for Danish marine waters compared to the most recent version of the Danish gross NIS list. Only two of these were detected with conventional techniques, one with qPCR and metabarcoding and the remaining 11 only by metabarcoding. We consider the latter group as potential NIS until validated against conventional sampling techniques.

Combining NIS detected with the novel sampling approaches with historical data from the Danish national monitoring program (NOVANA), previous research projects and records validated by taxonomic experts, a total list of 82 NIS were established in the Wadden Sea area. Of the 50 NIS detected through monitoring in this project, 26 were new to the region and the remaining NIS were primarily detected through the national monitoring program, from which it was possible to describe their annual presence since 1990. Of 82 NIS observed in the Wadden Sea, 41 appear to have been introduced via shipping (mostly ballast water and hull fouling), followed by 39 introduced through secondary spread (unaided), a few via aquaculture and 11 with unknown pathways. More than half of the NIS was assessed to have two or more pathways of introduction. While these numbers are associated with considerable uncertainty, our model-based NIS dispersal assessment supports shipping as a key introduction pathway. In addition, our model suggests that the secondary spread of NIS from populations in more southernly parts of the Wadden sea is of relevance.

Relevant mitigation measures to reduce NIS introductions and impact include enforcement of international agreements related to ballast water, biofouling and biosecurity guidelines, increased public awareness, considerations of improved protection and active restoration of selected sites. Continued monitoring is finally critical for assessments of NIS impact.

1 Introduction

The spread of marine species is a natural process that has introduced new species into neighboring seas with favorable habitat conditions. A range of physical and environmental dispersal barriers has historically limited the long-distance spread. However, human activities, such as trade, travel, and aquaculture, have breached those barriers, leading to a steep increase in the introduction of "non-indigenous species (NIS)" into new areas beyond their natural range (Thomsen et al., 2008). See Figure 1.1.

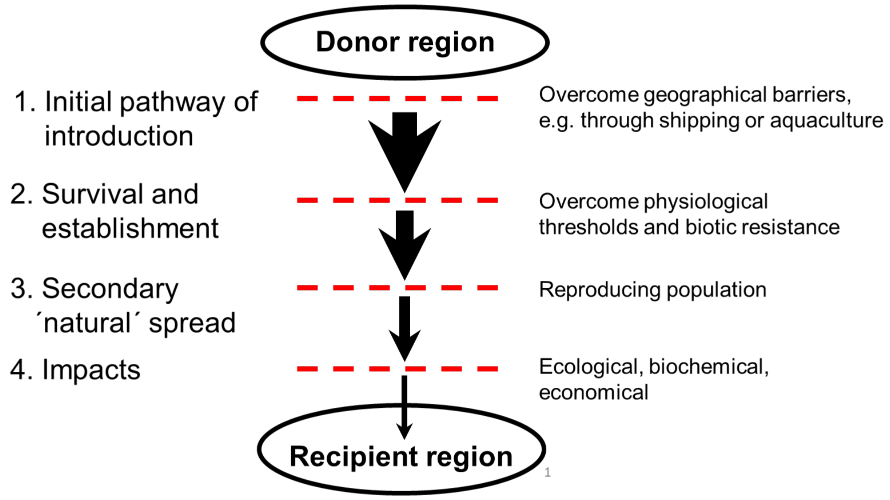


Figure 1.1. The successful introduction of new NIS can be divided into four phases describing important barriers and processes involved from the initial pathway of introduction from a donor region to the ultimate impacts in recipient regions.

Climate change has further contributed to the introduction of more warm-water species (García-Gómez et al., 2020). A NIS is defined by the International Union for Conservation of Nature (IUCN) as species, occurring outside of its natural range and dispersal potential. Meaning that it is outside the range it occupies naturally or could not occupy without direct or indirect introduction or care by humans. Also, it is highlighted that a NIS includes any part, gametes or propagule of such species that might survive and subsequently reproduce (Reise et al., 2023; IUCN, 2000). Certain NIS may be cryptogenic, i.e. of unknown origin, and may not have been directly introduced, but may have arrived due to environmental changes, such as warming (Tsiamis et al., 2019). Recent reports show that Danish marine waters have received an increasing number of new NIS at an increasing rate, especially over the past decades (Staehr et al. 2020, Büttger et al. 2022, Jensen et al. in press) with 123 marine NIS currently recorded in Denmark (The Danish Environmental Protection Agency, 2022). NIS can enter a marine area via multiple pathways, such as shipping, which is considered a significant spreading vector via the discharge of ballast water and hull fouling (e.g. LITEHAUZ 2016; Büttger et al. 2022). Some NIS which establishes large populations in new marine areas, can have harmful effects on native species, the ecosystem functions, leading to a decrease of the conservation and nature value of the habitat. In that case, they are called "invasive species" (Thomsen et al., 2008). Thus, invasive species can cause severe ecological, economic, and health effects (Ruiz et al., 1997; Thomsen 2008). It is estimated that NIS, together with overexploitation of the earth's resources, climate change and pollution, constitute one of the biggest threats to biological diversity (WWF, 2022).

Despite the potentially great and damaging impacts of NIS in the Danish Wadden Sea and available marine monitoring data dating back more than three decades, no studies have previously quantified the NIS and attempted to investigate pathways of introduction and impacts on the Danish Wadden Sea. This is despite recent studies from neighboring Germany suggest that the Wadden Sea potentially host some of the most extensive occurrences of NIS in Denmark (Reise et al., 2023).

The trilateral Wadden Sea area stretches from the Netherlands over Germany and to Denmark. It is a geologically young coastline that has developed since the post-glacial sea level rise deccelerated around 7,000 years ago (Flemming, 2002). It is the largest connected system of intertidal sand flats, mud flats and salt marshes in the temperate world (Baptist et al., 2022). Before diking commenced around 1,000 years ago, the Wadden Sea region continued into the Rhine delta but is now restricted to a 500 km long coastal zone between the Marsdiep in Holland to Grådyb in the Danish waters (Reise, 2005). The Danish part of the Wadden Sea comprises 1.500 km^2 of the most northern region, including 300 km^2 of land, making up about 10 % of the total area (Laursen & Frikke, 2016). Embankments have historically decreased the size of the tidal areas; however, they also extended further seaward, presumably making the current tidal areas in the Wadden Sea close to their size before 1,000 AD (Reise, 2005). In the Danish part of the Wadden Sea, the average tidal amplitude ranges from 1.5-1.8 m (Laursen & Frikke, 2016).

In contrast to the German and Dutch parts of the Wadden Sea, influenced by large riverine outflow, the Danish part of the Wadden Sea is dominated by coastal North Sea water since only small rivers feed into this section. The Wadden Sea is Denmark's largest nature reserve and national park and entered UNESCO's World Heritage List in 2009. This highly protected and dynamic tidal marine ecosystem is a susceptible area serving as a spawning and fry-rearing area for many marine species, including economically important fish species (Tulp et al., 2022). NIS has previous been assessed to pose a large impact on the Wadden Sea area (The Danish Environmental Protection Agency, 2019), although this is debated (Riese et al. 2023). Most of the NIS in the Wadden Sea become established on artificial structures such as harbor walls, pontoons in marinas, and coastal defense structures, but also on mussel and oyster beds, facilitating the sessile life stages of many marine NIS (Buschbaum et al., 2012). In a recent publication, Reise et al. 2023 summarized both the negative and positive impacts of alien species in the Wadden Sea. Hansen et al. (2023) also stressed some positive effects of biogenic reefs formed by invasive Pacific oysters in the Wadden Sea and some in the Limfjord. It is thus of great importance to gather more knowledge regarding NIS in the Danish part of the Wadden Sea to prioritize management actions and identify suitable mitigation measures to reduce the impact of NIS.

Unfortunately, the uptake of management actions to mitigate the negative impacts of NIS in Denmark and worldwide falls short. Once a species has established self-sustaining populations in a new area, its eradication is extremely difficult. Thus, prevention or early intervention is the best and most cost-effective defence against NIS (Haubrock et al., 2020; Buschbaum et al., 2012). Several efforts have therefore been initiated, such as legal measures, including the International Convention for the Control and Management of Ship’s Ballast Water and Sediment (IMO, 2004) and EU Commission's Marine Strategy Framework Directive (MSFD) (DIRECTIVE 2008/56/EC). MSFD aims to achieve Good Environmental Status (GES). Further, NIS are treated as a distinct Descriptor (D2) of GES "Non-indigenous species introduced by human activities are at levels that do not adversely alter the ecosystem". This Descriptor includes one primary criterion examining new NIS introductions (DC2C1), and two secondary criteria, dealing with the abundance and spatial distribution of NIS (D2C2) and their adverse impacts on indigenous species and broad habitat types (D2C3) (COMMISSION DECISION (EU) 2017/848). The EU member states, including Denmark, are obliged to monitor, assess, and achieve GES for these GES-specified criteria for NIS. As part of the Trilateral Wadden Sea Cooperation (TWSC), Denmark has signed the Joint Declaration on the Protection of the Wadden Sea, with the aim to "achieve, as far as possible, a natural and sustainable ecosystem in which natural processes proceed in an undisturbed way". As part of this, Denmark is obliged to monitor and assess the quality of the Wadden Sea ecosystem as a basis for adequate protection and management.

The main objective of this project is to identify possible mitigation measures against NIS, including invasive species, in the Danish part of the Wadden Sea. As such, the project provides information on the Danish Marine Strategy's program of measures related to MSFD Descriptor 2. The project collaborated with an advisory board of Dr. Pedro Martínez Arbizu, Senckenberg, Germany, Dr. Christian Buschbaum, Alfred-Wegener Institute, Germany; and Dr. Saa Kabuta, Senior, Ministry of Infrastructure & Water Management, Netherlands. Their role was to provide important information to 1) ensure that new NIS observations achieved through field investigations were aligned with applied methodologies in the German and Dutch part of the Wadden Sea, 2) gather knowledge about new NIS that we do not know from the Danish part of the Wadden Sea to ensure that we did not overlook relevant species in our analyses, and finally 3) to enhance possible synergy between project findings and activities in Germany and the Netherlands.

To identify possible mitigation measures, the project identifies occurrences and potential pathways of the introduction of NIS (i.e., shipping). To that end, the report provides an overview of known impacts from NIS on the food web and habitats of the Wadden Sea. The project combines information obtained by reviewing available data, new field investigations and dispersal modelling (Figure 1.2).

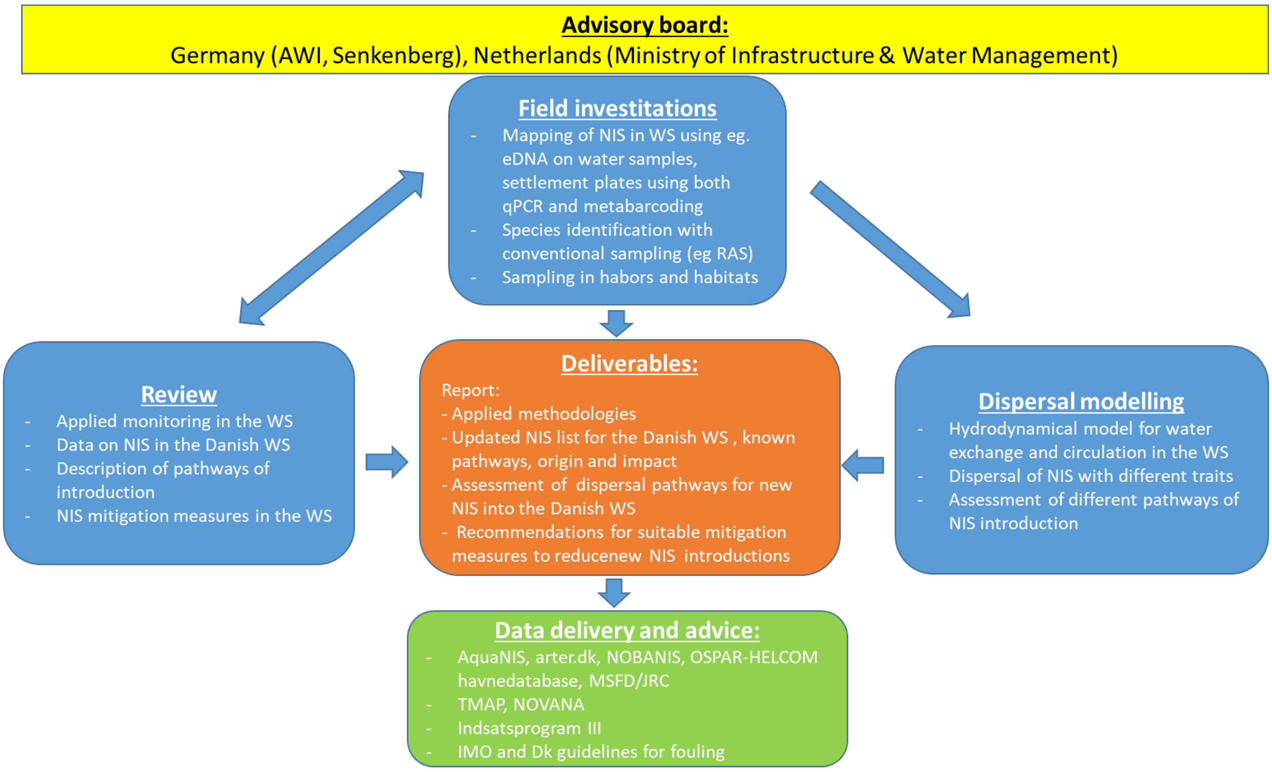


Figure 1.2. Project methodology and deliverables: Through a combination of data review, field investigations and dispersal modelling, the project provides an updated list of NIS for the Danish Wadden Sea (WS) and an assessment of dispersal pathways to recommend suitable mitigation measures. The project involved interaction with an advisory board with participation from Germany and the Netherlands.

2 Methods

2.1 Sampling program for NIS detection

The methods applied for monitoring NIS in the Danish part of the Wadden Sea were chosen to optimize agreement with monitoring practices and results obtained in the German and Dutch parts of the Wadden Sea. To ensure this, we reviewed applied NIS monitoring approaches for the Wadden Sea. During a workshop with the Danish Environmental Protection Agency (EPA), we shared this information with representatives from Germany and the Netherlands. Following this, we wrote a detailed method description which was sent to the Danish EPA for their acceptance before conducting data collection. The following text describes the actual NIS monitoring in terms of sampling stations and the applied techniques. Sampling was performed at nine sampling stations, covering three harbors and four water areas (Figure 2.1).

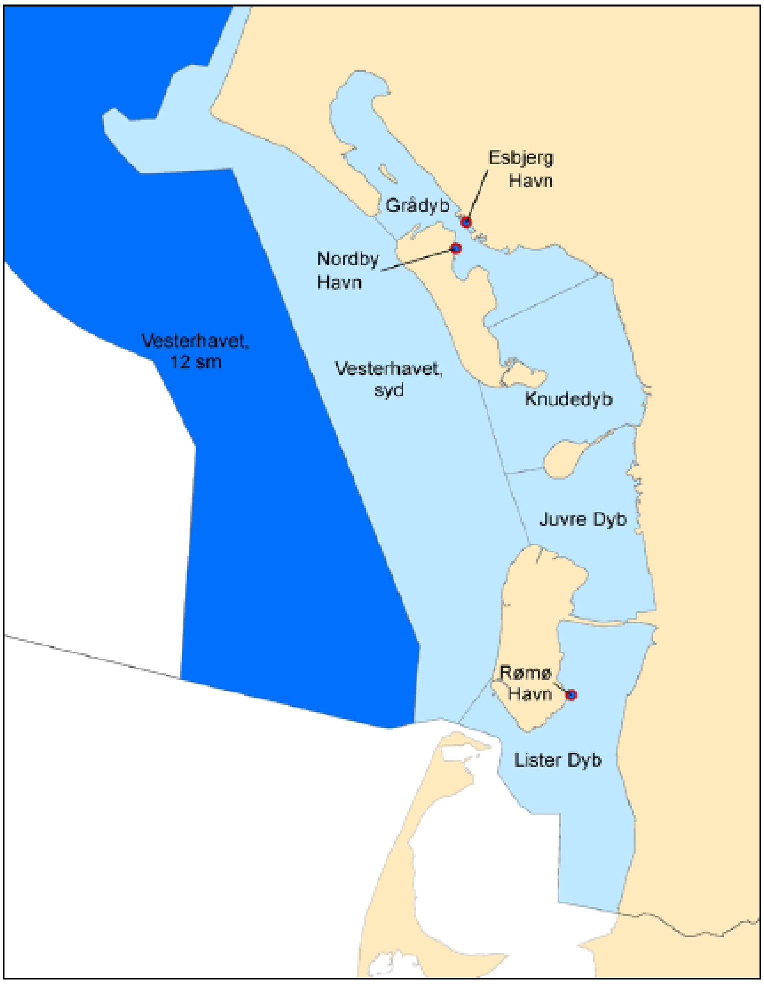


Figure 2.1. Map of the Danish part of the Wadden Sea area with an indication of the three harbors included in the sampling (Esbjerg havn, Nordby Havn and Rømø Havn). Also, the map shows the division into four sub-areas where samples were collected (Grådyb, Knudedyb, Juvre Dyb and Lister Dyb).

In each of the four water areas, we collected samples in different habitats, including tidal channels, tidal flats, biogenic reefs, and scrapings from hard structures such as navigation buoys, large rocks, poles, and piers. Table 2.1 provides an overview of the sampling stations and their coordinates. Figure 2.2 shows the geographical positions.

Table 2.1. Overview of sampling locations, in harbors (H) and water areas (WA). Three stations were placed in Esbjerg harbor.

Location	Type	Sample type	Station	Lattitude	Longitude
Esbjerg	H	Scrape, Sediment, eDNA	A	55,475929	8,414073
Esbjerg	H	Scrape, Sediment, eDNA	B	55,464722	8,432887
Esbjerg	H	Scrape, Sediment, eDNA	C	55,477502	8,419556
Nordby	H	Scrape, Sediment, eDNA	A	55,443422	8,407235
Rømø	H	Scrape, Sediment, eDNA	A	55,087974	8,566713
Grådyb	WA	Sediment	A	55,43758	8,44343
Grådyb	WA	Scrape	A	55,484014	8,375739
Grådyb	WA	Scrape	A	55,477256	8,402186
Grådyb	WA	Scrape	A	55,454877	8,4398
Grådyb	WA	eDNA	A	55,471691	8,422195
Grådyb	WA	Frame and visual inspection	A	55,44142	8,44446
Knudedyb	WA	Sediment	A	55,29447	8,58044
Knudedyb	WA	Scrape	A	55,31017	8,642809
Knudedyb	WA	eDNA	A	55,293562	8,580369
Knudedyb	WA	Frame and visual inspection	A	55,30198	8,5855
Juvredyb	WA	Sediment	A	55,174073	8,574267
Juvredyb	WA	Scrape	A	55,147411	8,606077
Juvredyb	WA	eDNA	A	55,17224	8,57246
Juvredyb	WA	Frame and visual inspection	A	55,1773	8,59289
Listerdyb	WA	Sediment	A	55,090847	8,562758
Listerdyb	WA	Scrape	A	55,06445	8,557717
Listerdyb	WA	Scrape	A	55,045	8,493666
Listerdyb	WA	Scrape	A	55,033	8,496
Listerdyb	WA	eDNA	A	55,085521	8,57177

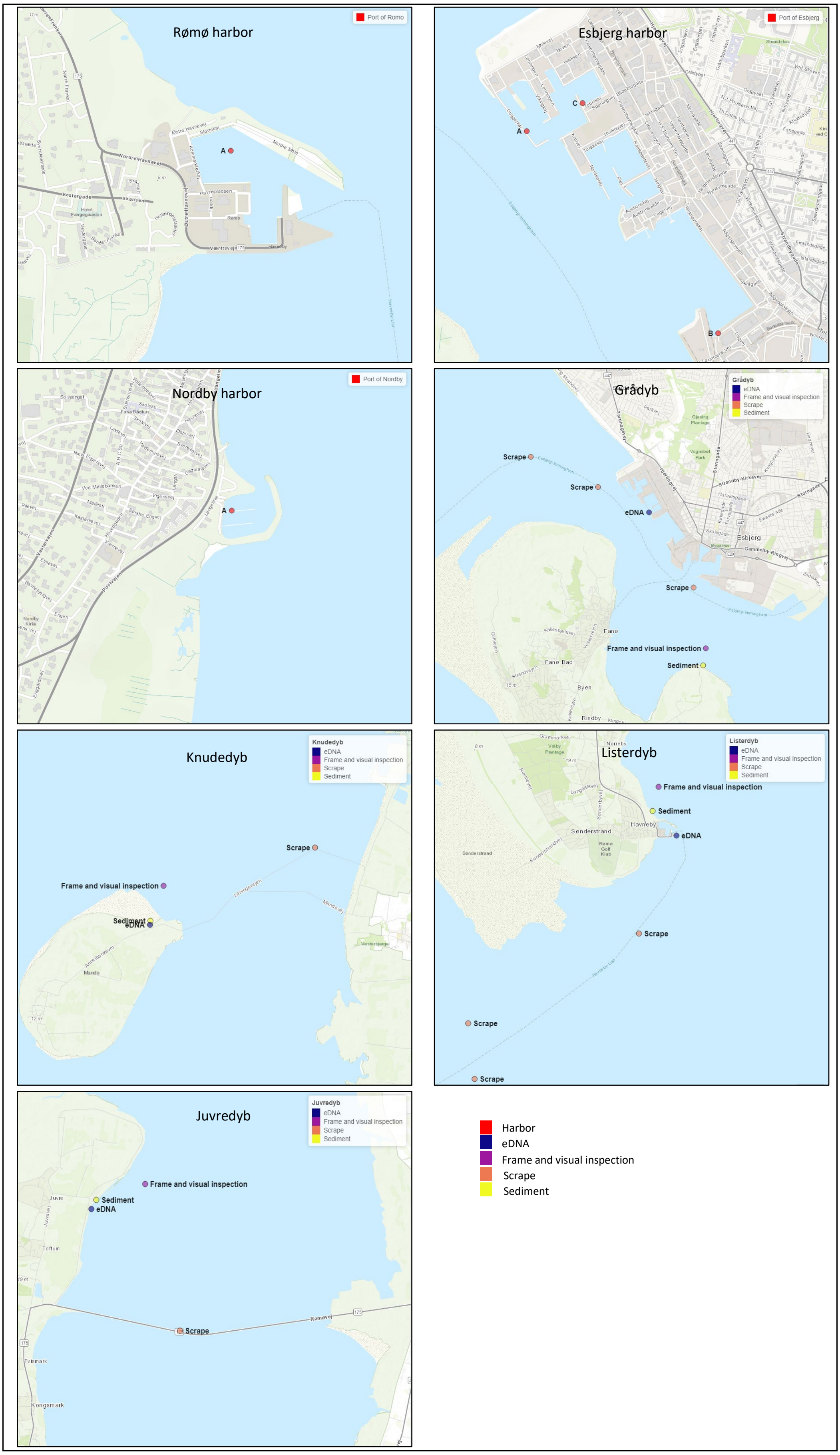


Figure 2.2. Positioning of sampling stations in three harbors (Nordby, Rømø and Esbjerg) and on the tidal flats and channels within the water areas. Scrape samples in Listerdyb and Knudedyb were taken from navigation buoys (See Table 2.2).

NIS were detected by the use of both conventional methods and molecular techniques. The conventional methods included settlement plates, soft sediment samples, scrapings, collection within frames and visual inspections. Molecular (eDNA) techniques involved species-specific detection systems (qPCR) and metabarcoding. The types of samples collected at each station are listed in Table 2.2 and will be explained further in the following text.

Table 2.2. Number of samples collected in the Danish part of the Wadden Sea for detection of NIS. In addition, NIS was identified through visual inspection on biogenic reefs in all four water areas.

			eDNA water	eDNA settlement plates	Sediment samples	Frame samples	Scrape samples	Settlement plates	Samples per area
Harbor	Esbjerg harbor		3	9	3	0	9	9	33
Harbor	Nordby harbor		1	3	1	0	3	3	11
Harbor	Rømø harbor		1	3	1	0	3	3	11
Water area	Grådyb	Tidal canal	3	-	-	-	-	-	13
		Tidal flat	-	-	3	-	-	-	
		Biogenic reef	-	-	-	4	-	-	
		Buoys	-	-	-	-	3	-	
	Knudedyb	Tidal canal	3	-	-	-	-	-	10
		Tidal flat	-	-	3	-	-	-	
		Biogenic reef	-	-	-	4	-	-	
		Buoys	-	-	-	-	-	-	
	Juvre Dyb	Tidal canal	3	-	-	-	-	-	10
		Tidal flat	-	-	3	-	-	-	
		Biogenic reef	-	-	-	4	-	-	
		Buoys	-	-	-	-	-	-	
	Lister Dyb	Tidal canal	3	-	-	-	-	-	13
		Tidal flat	-	-	3	-	-	-	
		Biogenic reef	-	-	-	4	-	-	
		Buoys	-	-	-	-	3	-	
	Samples per method		17	15	17	16	21	15	Total **101**

2.1.1 Conventional morphological sampling

Settlement plates

We based this method on HELCOMs "Guidelines for non-indigenous species monitoring by extended Rapid Assessment Survey" (HELCOM 2019). Settlement plates were deployed in May 21st to 23rd 2022 and collected from 12th to 14th of September 2022. The plates were a hard grey PVC material with 150 x 150 x 5 mm dimensions. A central hole was drilled in each plate, which was sanded gently to enhance the fouling by animals and plants. Plates were distributed evenly in the water column, with a plate ca. 1 m above the sea floor, ca. 1 m below the sea surface and one around the center of the water column. Plates were tied to a 10 mm rope with a brick at the sea floor, a small floating buoy at the surface, and then attached to the pier (Figure 2.3).

Figure 2.3. Settlement plates used for detection of NIS in three harbors

At retrieval, the settlement plates were carefully lifted out of the water, and immediately placed in a container, fixed with a bolt and nuts, using the central hole to protect the attached biota. Plates were preserved using 96 % ethanol diluted with freshwater to a final concentration of ca. 70 %. Back in the laboratory, photos were taken of both sides of the plates, and all species were identified to the lowest taxonomic level possible using a stereoscope.

Soft sediments

This field method was based on the Joint HELCOM/OSPAR Guidelines on the granting of exemptions under the International Convention for the Control and Management of Ships' Ballast Water and Sediments, Regulation A-4 (Joint HELCOM/OSPAR Guidelines), and the technical guideline M19 (Hansen & Josefson, 2014). All soft bottom samples in the harbors were collected from the 12th to 16th of September 2022. We used a hand-held Van Veen grab (0.025 m^2) to take one sample per station (Figure 2.4). Upon retrieval, the sample was sieved through a 1 mm mesh at the pier. The retained material was subsequently preserved in 96 % ethanol and diluted with freshwater to a final concentration of ca. 70 %. Soft bottom sediments were taken at the tidal flats using a so-called "smørstikke" corer with a sampling area of 0.0143 m^2. Sieving and preservation was done in the same way as for the Van Veen Grab samples.

Figure 2.4. Hand-held Van Veen grab and 1 mm sieve used for soft bottom sampling in harbors.

Scrapings

Material associated with hard structures was collected with a handheld 10 cm wide scraping device, and the material was retained in a 1 mm mesh (Figure 2.5). The method was applied in agreement with descriptions in Joint HELCOM/OSPAR Guidelines (HELCOM, 2013). Hard structures were typically pier walls in the harbors and large rocks or poles in the water areas. Three scrapings were conducted at each station, with each scrape taken ca. 10 m apart. Scrapes were collected from 1 m below sea surface up to the surface resulting in ca. 0.1 m^2 per scrape. The collected material was preserved in 96 % ethanol and diluted with freshwater to a final concentration of ca. 70 %. Species were identified using a stereoscope in the laboratory.

Figure 2.5. Scraper used to collect biological material associated with hard structures in the harbors.

Beyond the standardized scrape samples from harbors, we collaborated with the Danish Maritime Authority, which collected scraped samples from marker buoys placed in the four water areas during their inspection visits in March 2022. Samples were collected from a total of six buoys situated in Grådyb and Listerdyb (Figure 2.2, Table 2.2). Biological material was scraped off the buoys using a hand-held spatula. An area of ca. 0.1 m^2 was scraped and samples were then transferred into 2 L plastic freezing bags and stored at -18 °C on the ship until preservation in a 70% ethanol solution and later taxonomic identification in the laboratory.

Frames

In agreement with sampling in other parts of the Wadden Sea region (Nehls et al., 2006), we supplemented our detection of NIS with a frame-based sample on mussel reefs in each of the four water areas. To standardize observations, we applied a quadrate frame of 50x50 cm and mapped three times at each mussel reef, supplemented with one sample at the edge of each reef. Species were collected within each frame for identification by a taxonomic expert.

Visual inspections

Based on recommendations from NIS experts in Germany and the Netherlands, we also had a taxonomic expert conduct a general on-the-ground visual inspection of the mussel reefs.

2.1.2 Molecular (eDNA) detection

eDNA based detection was conducted both on filtered water samples and on bulk samples collected from settlement plates (Table 2.2). The sampling of water for eDNA analyses followed the technical guideline (Knudsen et al., 2020). Water was collected in both harbors and in tidal canals using a two-liter Van Dorn water sampler (KC Denmark A/S) (Figure 2.6). To minimize the risk of contamination, sampling equipment was rinsed thoroughly between sampling stations with water from the next sampling station. At deeper sites (harbors), we conducted a temperature and salinity depth profile to identify possible stratification. From each water sample, 190-600 ml was filtered through a Sterivex filter and two replicates were collected per station, with one sample as a backup. The filtration volumes lower than the recommended ca. 500 ml was experienced in the tidal channels and were due to sediment particles in the water which caused clogging of the filters. The filters were snap frozen in liquid nitrogen and, upon arrival at the lab, stored at -80°C until DNA extraction.

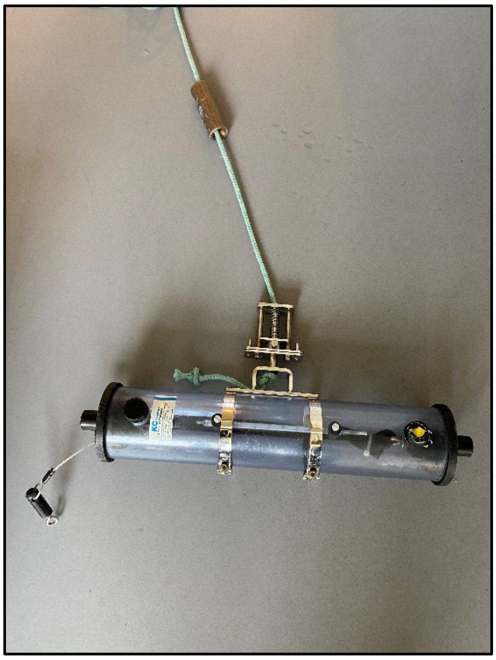


Figure 2.6. Van Dorn water sampler for collection of water for eDNA-analysis.

As the Wadden Sea has a high density of filtrating organisms, we chose a sampling design in the four water areas, which tried to account for the effect of these organisms. To reduce the effect of large mussel reefs on the removal of particle-bound DNA from areas upstream at a given sampling site, we timed the water collection to 1-2 hours after rising tide, thus increasing the chance of collecting DNA not associated with large filtrating organisms such as mussels. We chose 1-2 hours after the beginning of rising tide to allow for some residence time of the water and thus likely release of DNA from NIS around the local sampling sites.

DNA extraction

Tissue DNA extractions: Unicellular and microscopic NIS in growth culture were centrifuged (7000 rpm for 10 m) to concentrate cells and tissues prior to DNA extraction. Larger NIS tissue was incised in the laboratory from the animal parts avoiding gut, mouth, or skin parts, and subsequently subjected to grinding using mortar and pestle using liquid nitrogen. In a similar way, algae material was treated similarly. DNA was then extracted from the collected tissue extraction using DNeasy Blood & tissue kit (QiAGEN), following the manufacturer’s protocol, except the samples were treated with 10 µl Proteinase K (600 U/ml) (QIAGEN) and incubated for at least three hours at 56°C and 1000 rpm before the bead-based homogenization. The DNA concentration was quantified using a Qubit 4.0 fluorometer. All the DNA extracts were stored at −20°C prior to downstream processing.

DNA extraction from water samples: DNA extraction from the filters was carried out using DNeasy Blood & tissue kit (Qiagen) with spin-columns, following the manufacturer's protocol, except proteinase K treatment was added. The filters were opened and processed at sterile conditions in a flow hood. A 720 µL ATL buffer mixture and 80 µL proteinase K (600 U/ml) were used instead of 720 µL ATL buffer. The filter with ATL and proteinase K was incubated on a rotor in a heating cabinet at 55°C (± 1°C) for 4 to 24 hours so that the filtrate was completely lysed. Further steps in the extraction followed the manufacturer's protocol. The DNA concentration was quantified using a Qubit 4.0 fluorometer. Extracted DNA was split into several Eppendorf tubes and stored at -20 °C until used for quantitative PCR.

DNA extraction from settlement plates

The settlement plates were gently removed from the boxes with ethanol and placed at sterile conditions in a flow hood to avoid DNA contamination. DNA was extracted separately from the upper and lower surface of the settlement plates. Samples were collected from five different spots on each side of the individual settlement plates and transferred to a 50 ml tube. Collected samples were centrifuged at 3000 rpm for 5 mins to remove supernatant ethanol. Subsequently, samples were air dried for one to two hours at room temperature to remove traces of ethanol before storage at -20 °C until further processed for DNA extraction. Samples were lyophilized for 3 days and then ground using a bead beater. In total, 10-15 metal beads of 2.4 mm diameter were used to grind each sample in three cycles of 30 s at 4 m s^{-1} speed using a bead mill homogenizer (Bead Ruptor Elite, Omni International). Once grinded and homogenized, 250 mg of each sample was used for DNA extraction using the DNeasy PowerLyser PowerSoil kit (QIAGEN), following the manufacturer's protocol. The DNA concentration was quantified using a Qubit 4.0 fluorometer. The DNA of settlement plates were pooled for the upper and lower side of each of the settlement plates at each station prior to qPCR detection, while the samples were kept separate for metabarcoding detection. This resulted in four settlement samples for qPCR and 18 settlement samples for metabarcoding.

Quantitative PCR

TaqMan qPCR was used for the detection and quantification of NIS in eDNA in water samples and on settlement plates. Amplification reactions were performed in a BioRAD Real-time PCR system (Life Technologies) using 96-well plates. Primers and probes developed and described earlier were used for detection and quantification (Andersen et al., 2018; Knudsen et al., 2020, 2022). Of the 25 detection systems described the detection system for #9 *Neogobius melanostomus* has later been reported to be less specific (Andersen et al., 2021) and was omitted from the present investigation. Furthermore, the two detection systems developed for the *Acispenser* species (#21 and 22) will react against eDNA from either species, and therefore only one of the two systems was used thus resulting in a total of 23 species for qPCR detection.

A total reaction mixture of 25 µl was used, containing 3 µl of the DNA template (1-5 ng/µl), 1 µl each of forward and reverse primers (10 µM stock), 0.5 µl of probe (5 µM stock), 7 µl of water, and 12.5 µl of qPCRBio Probe Mix Lo Rox-Cobio (PCR Biosystems). For the negative controls and standard curves, 3 µl of sterile water and serial dilutions of NIS tissue DNA PCR products were used, respectively. Thermal cycles in the qPCR consisted of an initial denaturation at 95°C for 10 min followed by 50 cycles of 95°C for 30 s and 60°C for 45 s. Three technical replicates were prepared for each sample. A standard curve was obtained by plotting the quantification of the cycle (Cq) values as a function of log10 of the amount of NIS PCR product DNA added in a 10-fold serial dilution (10^{-4} to 10^{-11}).

The PCR products of the individual NIS DNA were obtained via a PCR reaction mixture of 25 µl containing 4 µl of the tissue DNA template (1-10 ng/µl), 0.5 µl each of forward and reverse primers (10 µM stock), 14.25 µl of water, and 5 µl of PcrBio HiFi buffer, PCRBIO HiFi Polymerase (2U/µl) (PCR Biosystems). PCR thermal cycles consisted of an initial denaturation at 95°C for 1 min followed by 35 cycles of 95°C for 30 s, 60°C for 45 s and 72 60 s, and final elongation 72 °C for 5 m. The PCR products obtained were purified using a QIAquick PCR Purification Kit (Qiagen, catalogue number 28104). We used Gel and PCR clean-up column for NIS with an amplicon size of less than 100 bp (Macherey-Nagel).

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

qPCR data analysis

According to Knudsen et al. (2020), for a qPCR result to be considered as a detection of the NIS, the assay should include a standard series from which Limit of Detection (LOD) and Limit of Quantification (LOQ) are defined. Hence, qPCR results with Ct values below LOD can only be considered weak traces of the target DNA. In this report, we report values above LOD as the species being detected, while we consider values below LOD as the species being identified but not detected. Appendices 1A-C provides detailed information on the certainty of the qPCR detections.

Metabarcoding

In total, 17 water filter samples and four samples from settlement plates with two technical replicates of each sample were used for DNA metabarcoding. Invertebrates, eukaryotes and fish sequencing libraries were generated by a two-step dual indexing strategy for Illumina MiSeq sequencing. We used three different primers targeting 18S rDNA, 12S rDNA and COI region of mitochondrial DNA to study eukaryote, fish, and invertebrate communities, respectively (Table 2.3). PCR amplicons were performed in a 25 µl reaction mixture consisting of 12.5 µl KaPa HiFi HotStart ReadyMix 2x (Roche), 1 µl of 10 mM forward and reverse primers, 0.5 µl of bovine serum albumin 20 mg µL–1, and 2 µL (10–20 ng) of DNA template. For 18S rDNA, the PCR cycle program included initial denaturation at 98 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final elongation at 72 °C for 10 min. Thermal cycles were performed similarly for invertebrates and fish, except the annealing temperatures were 48°C for COI and 65°C for 12S rDNA. A 15-cycle indexing PCR followed this, called a second PCR (PCR2), during which unique index combinations (i7 and i5) and adaptors were added. For PCR2, thermocycler conditions were 95 °C for 5 min, 13 cycles of 95 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min, and final elongation at 68 °C for 10 min. The amplicon size of PCR products was confirmed by visualization in a 1.5% agarose gel using SYBR staining. Subsequently, the amplicon product was cleaned using HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, USA), according to the manufacturer's instructions. The amplicon concentration was quantified using a Qubit 4.0 fluorometer. Finally, amplicons were equimolarly pooled for equal representation in the sequencing library, and sequencing was carried out using the Illumina MiSeq platform at DCE, Aarhus University.

Table 2.3. Target genomic region, primer sets, and their references used in this study

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

Bioinformatics (metabarcoding)

Our bioinformatical analysis follows the DCE pipeline as described in Sapkota et al. (2023). The DNA reads obtained from the Illumina MiSeq runs were analyzed using QIIME2 (Bolyen et al., 2019). The DADA2 (Callahan et al., 2016) plugin in QIIME2 was used with default parameters except reads trimmed for primer sequence and truncated after 230 bp. For 18S and COI, the resulting amplicon sequence variants were classified using the QIIME2 naive Bayes classifier trained on 99% Operational Taxonomic Units from the SILVA rRNA database (v. 138) and MIDORI2 database v. GB253 merged with GEANS reference database version 4 containing 1993 COI sequences from 565 species from North Sea region, respectively, after trimming to the primer region (Leray et al., 2022; Quast et al., 2012). In addition, COI amplicons were nucleotide blasted (blastn) against the BOLD database using the sequence-id tool (www.gbif.org). ASVs were assigned at the species level with coverage and identity (>97%) were used, if not assigned by MIDORI database. Similarly, 12S ASVs were blasted against Mitofish database (Iwasaki et al., 2013). Blast taxa with high similarity and coverage (>97%) at species were used.

The ASV table and taxonomy files were imported into the statistical software R and statistical analyses and data visualizations were performed in v.4.2.1 (R-Core-Team, 2019) using 'phyloseq' package (McMurdie et al., 2013). The ASV table was merged at species level using the tax_glom function from 'phyloseq' and species names were checked and corrected using WoRMs Taxon match tool (https://www.marinespecies.org/).

2.2 Review of NIS records

2.2.1 NIS definitions

In the present study, a gross list of marine non-indigenous species (NIS) and cryptogenic species found in the Danish Wadden Sea was collated based on the following definitions:

<u>Non-indigenous species (NIS):</u> "Alien species" (non-native, non-indigenous, foreign, exotic) means a species, subspecies, or lower taxon occurring outside of its natural range (past or present) and dispersal potential (i.e. outside the range it occupies naturally or could not occupy without direct or indirect introduction or care by humans) and includes any part, gametes or propagule of such species that might survive and subsequently reproduce. (IUCN, 2000).

Cryptogenic species: "Cryptogenic species" refers to a "species with no definite evidence of their native or non-indigenous status (due to unknown origin or due to unclear mode of introduction from the native range: natural spread vs human-mediated)" (Tsiamis et al., 2019).

NIS included: Different considerations are applied when defining which species to include (see Tsiamis et al. 2021). In this report, we align with the official Danish terminology of NIS. Thus, we had both strictly NIS (primary introduction), cryptogenic species, and NIS introduced through natural (secondary) dispersal. Also, we included unicellular algae (phytoplankton) but excluded parasites. Using these definitions, we also found that our NIS list became comparable with observations from the German and Dutch reports on NIS in the Wadden Sea.

2.2.2 NIS data sources

Besides data collected during field investigations in this study, multiple other data sources were used to compile a comprehensive list of marine NIS and cryptogenic species in the Danish Wadden Sea.

NOVANA: The National Monitoring and Assessment Programme for the Aquatic and Terrestrial Environments (NOVANA) is a program run by the Danish Environmental Protection Agency (EPA) for the monitoring of the Danish environment and is based on a number of international and national obligations and needs (The Danish Environmental Protection Agency, 2022b). The program is carried out by institutions/topic centers within the Ministry of the Environment (including the Marine Topic Centre (M-FDC) placed in the Department of Ecoscience, Aarhus University) and the regional authorities (Hansen & Høgslund, 2021). The Danish Marine Topic Centre conducts overall coordination for Environment and Energy (DCE), at Aarhus University. NOVANA was implemented on the 1st of January 2004 and replaced the Danish Aquatic Monitoring and Assessment Program (NOVA-2003), which had been running since 1998. In NOVANA, a greater priority has been accorded to aquatic species and habitats, where the marine monitoring registers, among other components, phytoplankton and zooplankton species composition, coverage, and species composition of angiosperms, macroalgae, and species composition of benthic invertebrates (Svendsen et al., 2005). Monitoring data are stored and quality assured in a number of joint public databases, including the "surface water database" (Overfladevandsdatabase, in Danish) (ODA). The phytoplankton database is currently under reconstruction, and for this study, all relevant and available phytoplankton species records were used.

For the present study, ODA data from the four sub-areas in the Danish Wadden Sea area (Grådyb, Knudedyb, Juvre Dyb and Lister Dyb) were analysed for the presence of NIS and cryptogenic species in five different "groups"/"systems" within the database (benthic fauna, benthic vegetation, phytoplankton, stone reef and zooplankton). Data were extracted in February 2023. All species names were matched against WORMS to ensure taxonomic comparability against the list of NIS for European waters (described in section 2.2.3). The methodology used for monitoring those five different groups can be found in the M-FDC's technical instructions (tekniske anvisninger–TA): https://ecos.au.dk/forskningraadgivning/fagdatacentre/marint-fagdatacenter/

Table 2.4 provides an overview of the Danish monitoring programmes which we extracted data from to obtain information on marine species, including NIS for the Danish Wadden Sea area.

Table 2.4. Danish monitoring programs investigated to obtain information on NIS in the Danish Wadden Sea area. The programs are described in technical guidelines (TA).

Monitoring groups	**TA**	**References**
Benthic fauna – soft substrate	M18	Hansen & Josefson, 2014; Rasmussen et al., 2013
Benthic fauna – hard substrate (incl. stone reef)	M17	Lundsteen & Dahl, 2016
Benthic vegetation – soft substrate	M19	Bruhn et al., 2013
Benthic vegetation – hard substrate (incl. stone reef)	M14, M12	Dahl & Lundsteen, 2018; Høgslund et al., 2013
Plankton (phytoplankton & zooplankton)	M01, M09, M10, M11	Fossing et al., 2017; Jakobsen & Fossing, 2015a; Jakobsen & Fossing, 2015b; Jakobsen & Møller, 2016
NIS	M30	Fossing & Stæhr, 2017

Danish marine NIS gross list: The Danish marine NIS and cryptogenic species gross list developed by the Danish EPA (The Danish Environmental Protection Agency, 2022a), as mentioned above, was also used to feed into the present marine NIS and cryptogenic species gross list for the Danish Wadden Sea. If the observation of a given species was noted as first observed in the Wadden Sea, it was included in the current gross list. Information about the first observation date in Danish waters, the status of the given species (NIS or cryptogenic), as well as the introduction pathways were also extracted from this list.

Common Wadden Sea Secretariat (CWSS): CWSS is a coordinating body of the Trilateral Wadden Sea Cooperation (TWSC). It supports and facilitates research, monitoring and protection measures of the Wadden Sea states (Denmark, Germany, and the Netherlands). TWSC also produces Wadden Sea Quality Status Reports (QSR) describing and evaluating the ecological status of the Wadden Sea (CWSS, 2017), including alien species (Büttger et al., 2022). The Danish records from the trilateral list of the Wadden Sea alien species, which displays the latest status of alien marine species recorded in the Wadden Sea Area, were used to identify NIS for the present gross list. The data registered by CWSS is extracted from the "Alien species of the Wadden Sea database" and is primarily based on literature studies, including for the Danish par Buschbaum et al. (2012), Lackschewitz et al. (201), Jensen & Knudsen (2005), Stæhr et al. (2016), Andersen et al. (2014) and Andersen et al. (2022).

MONIS 4: In 2022, NIVA Denmark published their fourth MONIS report, "MONIS 4 - A baseline study of the occurrence of non-indigenous species in Danish harbours" (Andersen et al., 2022). The data presented in the report is based on conventional and biomolecular (eDNA) sampling methods carried out in 16 Danish harbors, including Esbjerg, located in the Danish Wadden Sea area. Therefore, NIS data from Esbjerg were used to feed into the Danish Wadden Sea's current marine NIS gross list.

Harbor investigation 2021: A new report on the occurrence of NIS in Danish harbors has just been published by Aarhus University (Andersen et al., 2023), which is based on conventional and biomolecular (eDNA) sampling methods carried out in six Danish harbors, including Esbjerg during 2021. The eDNA samples from this study were further analyzed by metabarcoding for the identification of multiple species within the same sample, without having to focus on one specific organism (Sapkota et al. 2023). NIS data from Esbjerg were used to feed into the present marine NIS gross list in the Danish Wadden Sea.

Arter.dk: The Danish Environmental Protection Agency (EPA), in collaboration with the Natural History Museum of Denmark and the Natural History Museum Aarhus, is running a Danish national species registration portal, which is a database containing observations of organisms mainly from Denmark. Users continuously add new observations through the registration portal https://arter.dk. Arter.dk aims to gather all available information about observations of Danish biodiversity and make the data available to the public (Møller et al., 2020). Arter.dk also uses another database called Taxonbasen, which constitutes the taxonomic backbone of the Danish national species registration portal. This database aims to present a list of all known taxa native to Denmark supplemented with non-native taxa. Furthermore, the list contains a number of taxa, which are not considered Danish - casual introductions, taxonomic or geographically unresolved species, incorrectly stated taxa etc. (Skipper et al., 2020). All species included in the present gross list were checked in Arter.dk for their occurrence in the Danish Wadden Sea, by looking at their geographical distribution. An additional search was executed by filtering for marine and brackish organisms found in the Wadden Sea area using the "Fund" function in Arter.dk.

Fiskeatlas: The Fish Atlas (Fiskeatlas, in Danish) is a national mapping of Danish fish developed and maintained by the National History Museum at the University of Copenhagen. Data on fish populations in all Danish waters are continuously collected. The mapping of the fish distribution is built on information from various sources, including public reports. The primary data sources of the Atlas are commercial fishermen, anglers, sports divers, amateur fishermen and other nature enthusiasts; fisheries surveys; scientific collections; historical sources/literature; targeted fieldwork. Through verbal communication with Henrik Carl (02-11-2022), the Project Coordinator of the Fish Atlas, we obtained information about one NIS recorded through the Fish Atlas in the Wadden Sea - the rainbow trout (*Oncorhynchus mykiss*). In 1982, a rainbow trout was caught and photographed on the Danish West coast near Esbjerg. Through the Fish Atlas, we were, therefore, able to add the rainbow trout to the current gross list.

2.2.3 NIS list used to match up against

For all groups, our search for NIS and cryptogenic species in ODA relied on a new list of NIS records recently published by Zenetos et al. (2022). In addition to the published list here, we chose to include phytoplankton and cryptogenic species as these are currently being included in the official Danish NIS list. The Danish NIS list was updated in January 2023 and included 123 species. The final EU list, including recent (January 2023) updates to the Danish list of NIS, covers 934 species, including the 123 NIS known to occur in Danish seas. This list of marine NIS and cryptogenic species was developed and is regularly updated by the Danish EPA and can be found HERE.

2.2.4 Scoring of invasive species

Assessing the environmental impacts of specific NIS is, in Denmark and other European countries, carried out by scoring NIS to identify those of most concern for mitigation actions by using the so-called Belgian Harmonia system, which is based on the environmental impact assessment protocol

(ISEIA) (Branquart, 2009). The Harmonia system consists of four sections: 1) dispersion potential or invasiveness; 2) colonization of high conservation value habitats; 3) adverse impacts on native species, and 4) alteration of ecosystem functions. A given NIS is, for each section, given a score from 1 to 3, where 1 is low impact, 2 is medium, and 3 is high impact. In addition to assessing the environmental risks, Denmark also assesses the impact of NIS on the socio-economy and human health (Strandberg, 2017). In addition to score parameters 1,2,3 and 4 (described above), the Danish assessment also scored for possible human health and socio-economic impact. However, to align with the assessment of impact performed in other parts of the Wadden Sea, we focused on the scores used in the Harmonia assessment only (parameters 1,2,3 and 4). Consistent with the Global ISEIA scoring system, the total score is calculated as the sum of risk rating scores. Following the ISEIA guidelines, we allocated each of the assessed species into three different risk categories:

Score 11-12: (A) High risk / Black list
Score 9-10: (B) Medium risk / Watch list
Score 4-8: (C) Low risk

Several sources of information regarding the score parameters 1,2,3, and 4 were investigated to provide information for as many NIS as possible. Priority was given to a recent study by Strandberg et al. (in press.), where experts from Aarhus University have carried out updated NIS scorings for the Danish EPA. Here they, however, suggested changing the 1-3 scale to 0-3 to discriminate between 'no impacts' (score 0) and 'negligible impacts' (score 1), which in the past were combined into score 1, which covered 'no or negligible impacts'. Thus, to make these new scores comparable with the previous impact scores, 0-values were replaced with 1-values.

However, not all NIS from the gross list were given a score in the Strandberg et al. (in press) report, in which case, we used older literature (Strandberg, 2017; The Danish Environmental Protection Agency 2017a, b and c) as well as lists from EPA (The Danish Environmental Protection Agency, 2022) and the CWSS to score the remaining NIS. The scores carried out by CWSS were also based on the ISEIA guidelines. The available scoring reports and their references for the individual NIS scores are noted in Appendix 2.

2.2.5 NIS dispersal pathways

To assess the importance of different dispersal pathways, we applied the system used in both OSPAR and HELCOM, which defines 20 categories within seven major pathway types: Release in nature, Escape from confinement, Transport contaminant, Stowaway contaminant, Corridor, Unaided, Unknown. Information on the relevant categories for each of the identified NIS were either obtained from the Danish Gross NIS list, or from the World register of invasive marine species (WRiMS).

2.3 NIS dispersal modelling

To assess the spreading of NIS, a common modelling approach is to apply a 3-dimensional hydrodynamical model to an area of interest, e.g. the North Sea or the Wadden Sea (Androsov et al., 2019; Berg & Poulsen, 2012). Hydrodynamical models provide values for, e.g., salinity, temperature, current velocity and water mixing for all points in the model domain.

To assess the spreading of NIS, discrete individuals are represented by adding a Lagrangian particle tracking model to the hydrodynamical setup. Here, each discrete particle represents one or more individuals. This approach is also called an Individual-Based Model (IBM) (Goodwin et al., 2001). An IBM can be used to, e.g., identify dispersal corridors of NIS in Danish waters (Hansen, et al., 2021a) and is thus a powerful tool for management purposes.

The simplest IBMs consist of particles released at a given place and time in the model domain and subsequently passively advected with the currents in a hydrodynamical model setup. It is further possible to add traits to the particles, such as the duration of the pelagic larvae stage and a gravitational sinking speed, or one may add sensitivity to the environment, e.g., salinity or temperature tolerance. Adding a condition for a specific type of settling substrate is also common, e.g. (Hansen et al., 2021b).

2.3.1 Previous Wadden Sea model studies

While a number of modelling studies have focused on the hydrodynamics of the German part of the Wadden Sea, e.g., Androsov et al. (2019), including studies combining observations with modelled tracking of eukaryotes and plastic litter (Ricker et al., 2022; Sprong et al., 2020), only a few model studies have been performed for the Danish part of the Wadden Sea.

The MIKE hydrodynamical model has been used to address specific questions in the Danish Wadden and North Sea areas. One study used the model to assess the possibility of re-opening Sønderho Port (Lumborg, 2008). This study was based on a model domain covering part of the Danish Wadden Sea with a relatively short run time of one month. In another study, the MIKE model was employed to assess the impact of dumping sand in the North Sea, a study in which the Danish Wadden Sea was represented, though with a coarse resolution (NIRAS, 2015). Hansen, Gabellini, Lindegren, et al. (2021) performed a study of dispersal corridors for NIS in Danish waters and identified the Wadden Sea as critical for NIS invasions.

2.3.2 Modelling of NIS dispersal in the Wadden Sea

Model setup

FlexSem is a coastal 3-dimensional hydrodynamical model which solves the standard Navier-Stokes equations under the Boussinesq approximation. It has been applied to managing Danish fjords and coastal waters, e.g. (Maar et al., 2021; Schourup-Kristensen et al., 2021). FlexSem has been coupled with an individual-based module (Pastor et al., 2021), which was used to represent NIS in the current study. To reproduce the tidal environment of the Wadden Sea, wetting and drying of tidal flats have been incorporated into the current setup of FlexSem.

The domain of the multi-resolution hexagonal mesh created for the Danish Wadden Sea setup (Figure 2.7a) covers the area from the southern tip of the German island of Sylt (54.8N) to the southern part of Ringkøbing fjord in Denmark (55.9N). The resolution of the computational mesh ranges from 250 m in tidal channels to 3 km on the North Sea boundary of the mesh.

Boundary conditions for the open sea and initial conditions for salinity, temperature and sea surface height were provided by The CMEMS North-West European Shelf Ocean forecasting system (Crocker et al., 2020) made available through the E.U. Copernicus Marine Services. FlexSem was forced by atmospheric data from the Weather Research and Forecast model, version 3 (Frohn et al., 2021; Skamarock, 2008). Daily freshwater runoff from land to the coastal sea (Figure 1a) was given by the DK-QNP model (Windolf et al., 2011). The model was run for the year 2021. The modelled sea surface temperature, salinity and sea surface height are validated in Schourup-Kristensen (2023) and appendix 3.

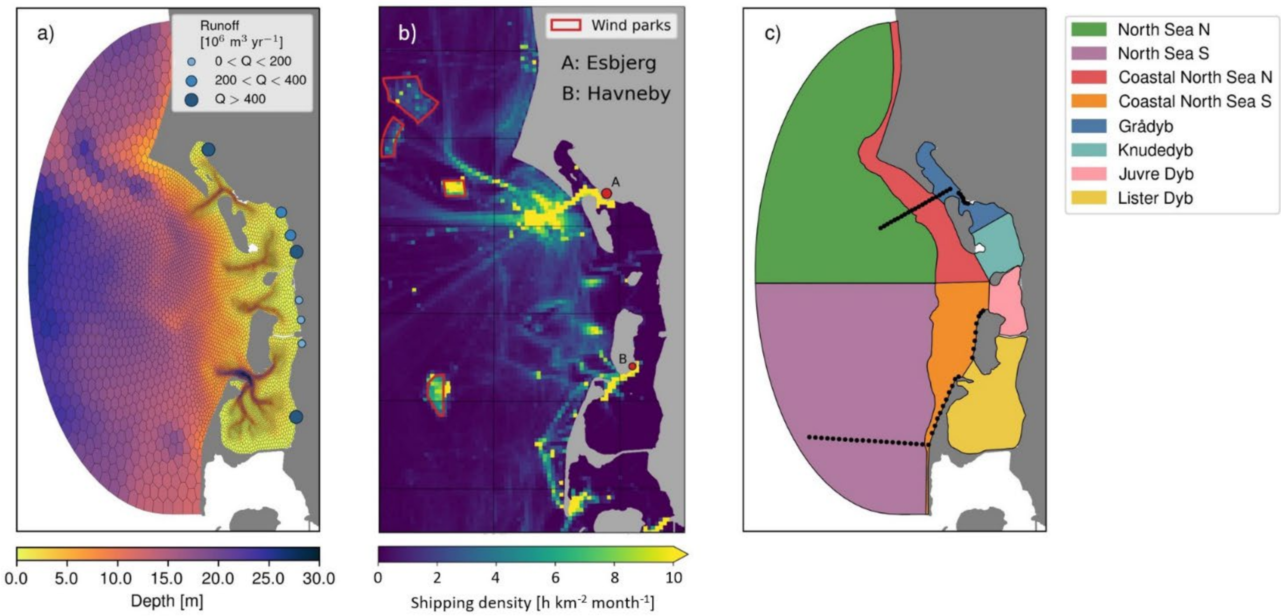


Figure 2.7. a) Model grid for the Wadden Sea setup of FlexSem. The polygons of the computational grid are marked in black, while the background colour shows the depth of the model domain. Blue dots show the location and magnitude of freshwater sources. **b)** Mean shipping density in the Wadden Sea area. Wind parks are marked by red contours (EMODnet, 2020). c) Subdivision of the Wadden Sea for connectivity analysis. Black dots mark locations of NIS release in the model.

An individual-based module (IBM) in a Lagrangian framework (Larsen et al., 2020) was set up to be forced by the Wadden Sea FlexSem hydrodynamics. One IBM represents a group of NIS. We are assuming that the spreading of NIS occurs during the pelagic larval stage of the organisms, during which larvae are dispersed in the Wadden Sea and the coastal North Sea of the model domain.

Simulations

North Sea NIS species typically have a pelagic duration (PD) ranging from 0 to 21 days (e.g., the veined rapana whelk (Savini et al., 2007) and Japanese skeleton shrimp (Cook et al., 2007). Consequently, the six simulations performed here employ a pelagic duration of 1, 3, 5, 7, 14 and 21 days, thus potentially covering a range of species.

To illustrate the release of NIS from the ballast tank of cargo ships, the modelled NIS were released in the surface water along the high-density track of ship traffic leading to the port of Esbjerg (Figure 2.7b and 2.7c), as well as in the port itself. Additionally, to assess the risk of northward migration, particles were released along the coast of Sylt and Rømø, as well as in a pelagic line from Sylt into the North Sea, south of the Danish-German border (Figure 2.7c). Particles were released once per day from the 1st of June to the 1st of October.

Post-processing

The model domain was divided into four Wadden Sea sub-areas based on morphology (Grådyb, Knudedyb, Juvre Dyb and Lister Dyb, Figure 2.7c) to analyze the movement of particles in the Wadden and North Sea areas. This division follows the boundaries used for implementing the water framework directive in Danish waters. The North Sea was divided into a northern and southern part, divided in the middle of the domain by the island of Mandø (55.3N). To assess the number of individuals ending up close to the coast, an additional boundary followed the 10-meter depth contour was applied (Figure 2.7c).

We created a connectivity matrix to quantify the degree of connectivity between the different sub-areas. The x-axis of the matrix represents the five donor areas (Grådyb, the northern and southern coastal North Sea, and the northern and southern deep North Sea). The receiver sites on the y-axis include all eight sub-areas (Figure 2.7c). Additionally, some particles are advected out of the model domain during the simulation, and we thus add two receiver areas: the North Sea area north of the model domain and the area south of the domain, adding up to a total of ten receiver areas. The percentage of particles each receiver area receives is calculated and illustrated in the connectivity matrix for each release area. The release positions represent input from transport vectors. However, the connectivity matrix does not represent the general connectivity between the sub-basins; rather, it aims to illustrate the fate of particles released in these chosen positions.

Given the plasticity in the pelagic phase of potential NIS species, we show density plots of the particle trajectories, thus illustrating the most common pathways for NIS in the model domain. This further gives an overview of areas where NIS will likely be found during sampling. The dispersal density is calculated on a 1x1 km grid by summing the number of NIS passing through each grid cell during the simulation from June 1st to October 21st with a time step of one hour.

Finally, settling density is shown on a 1x1 km grid, illustrating the number of particles ending up in each cell.

2.4 Review of NIS mitigation measures

The review aimed to identify relevant and implemented mitigation practices to reduce the pressures asserted by NIS on the Wadden Sea area. Our study included four components:

1) An overview of existing legislation and directives regarding required regulation of NIS in European marine waters.
2) Identification and ranking of the pathways of NIS introduction into the Wadden Sea area. This was based on information collected from our own data review (See section 2.2).
3) Overview of relevant mitigation measures. We initially consulted the Danish National Park Wadden Sea, and their recent report on invasive species (Invasive arter i Nationalpark Vadehavet, 2022). In addition, we searched the literature and contacted NIS experts in the Wadden Sea region and OSPAR to obtain information on mitigation measures taken to reduce the pressure asserted by NIS on the Wadden Sea.
4) Finally, we discuss the relevance of mitigation measures available and implemented to successfully reduce the impact of NIS into the Wadden Sea, given the identified pathways of introduction and information gained from NIS dispersal modelling.

3 Results

3.1 NIS identified through monitoring

Overall, we detected 50 NIS in the Wadden Sea area using conventional, qPCR and metabarcoding methods (Figure 3.1).

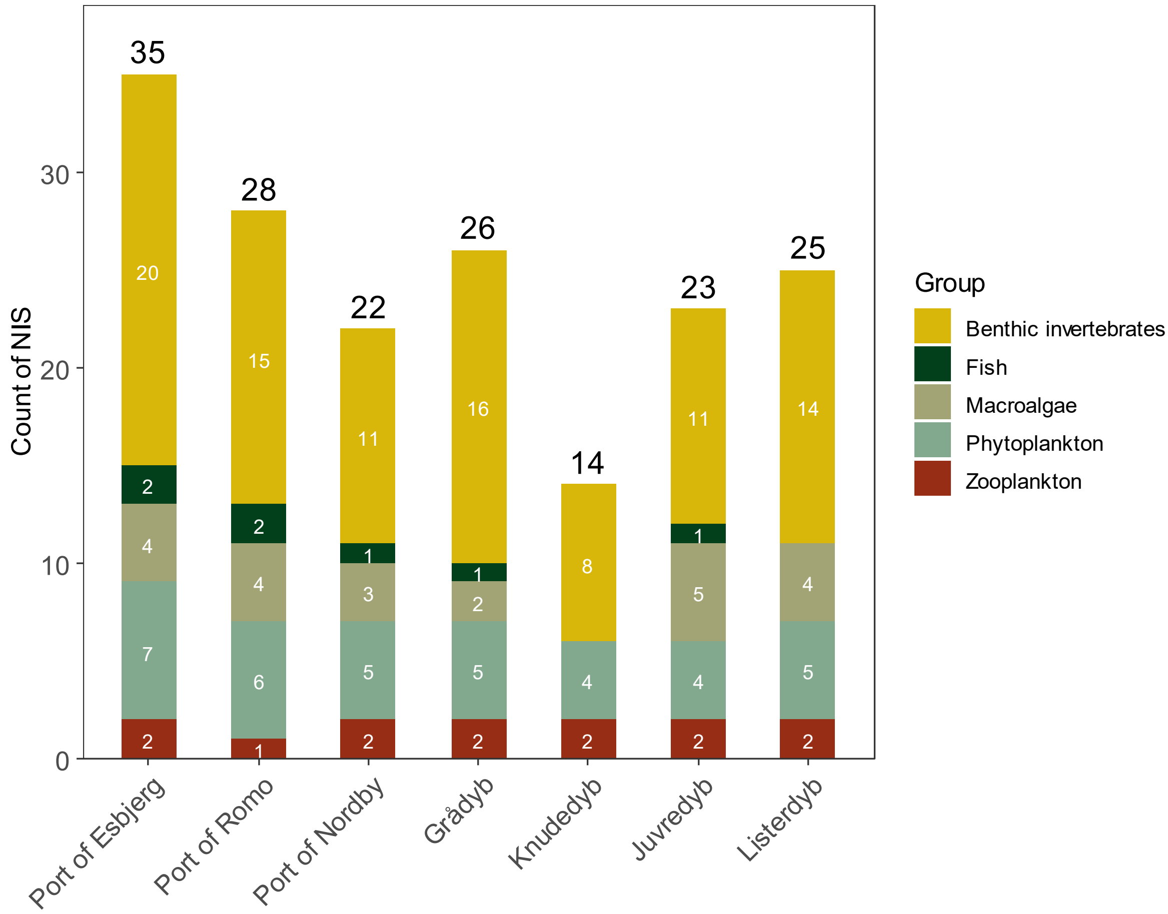


Figure 3.1. Total number of NIS detected through monitoring in three harbors (ports) and four water areas. The colors refer to major taxonomical groups.

Combining data from different sampling types, the highest number of NIS were detected in Esbjerg and Rømø harbors. Benthic invertebrates dominated in all sampling areas (54%), followed by phytoplankton (21%), macroalgae (14%), zooplankton (7%) and fish (4%).

Multivariate analysis was used to identify similarities (Bray-Curtis) in the composition of NIS among sampling areas showed that the three ports clustered in a group separate from Grådyb and Listerdyb. Knudedyb and Juvredyb were the most dissimilar areas concerning NIS composition (Figure 3.2). Grådyb and Listerdyb are located in the main shipping lane connecting the Wadden Sea with the open North Sea. With the high number of NIS found here and the closer similarity in species composition to the three ports, NIS composition within the Wadden Sea area is likely influenced by shipping.

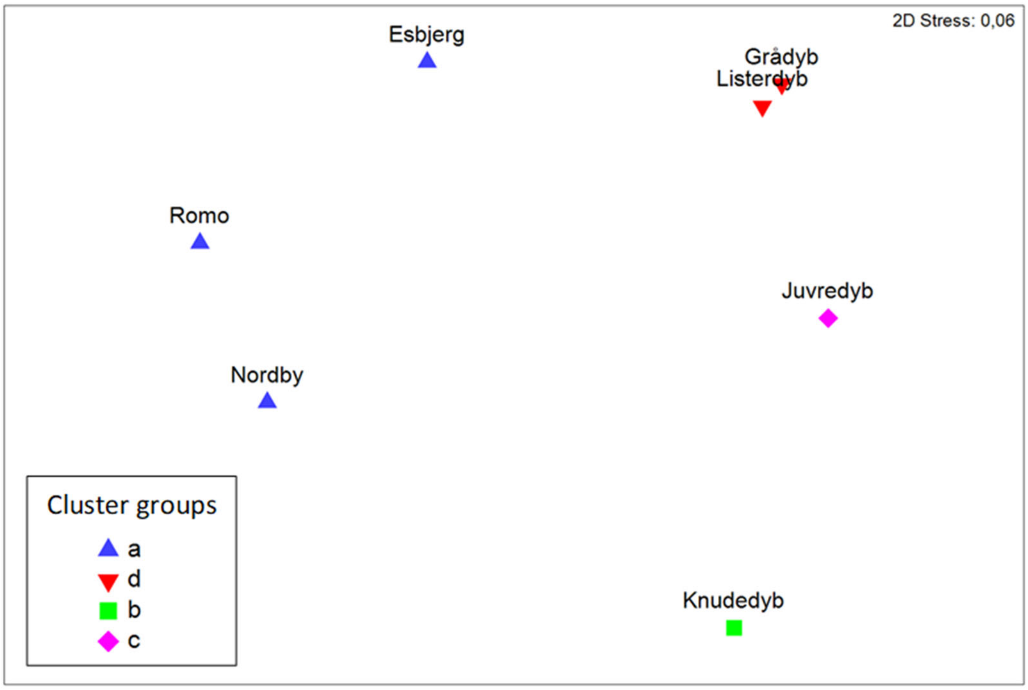
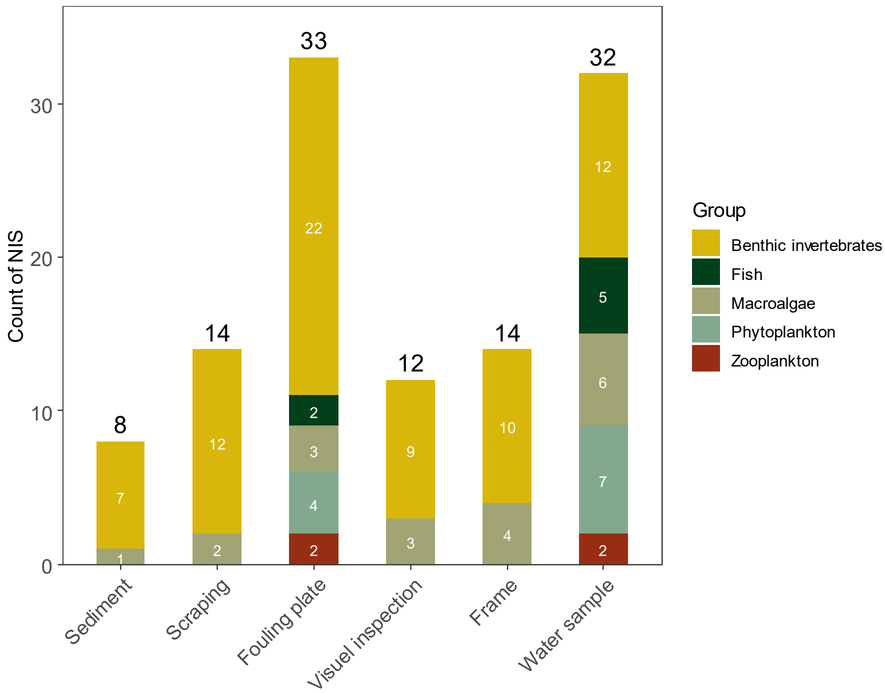


Figure 3.2. Non-metric multidimensional ordination plot of the similarity of species composition for the three ports and four sampling areas. The analysis was based on the presence/absence data of the 52 NIS detected through monitoring in the overall seven areas. The ordination used a Bray-Curtis similarity index, and the analysis was performed using Primer software which automatically clustered the seven areas into four groups according to levels of similarity.

Comparing the number of NIS detected with the different sampling techniques showed that most NIS were detected in water samples and on settlement plates (Figure 3.3). In water samples, all NIS were detected using qPCR and metabarcoding. For settlement plates, 29 of the 33 detected NIS were observed with eDNA, nine with visual identification methods and five NIS were detected with both molecular and visual identification methods.

Figure 3.3. Total number of NIS detected per sampling method. The colors refer to major taxonomical groups.



To distinguish NIS detected with conventional and molecular techniques, we performed a more detailed comparison of the seven sampling sites according to the applied detection technique (Figure 3.4).

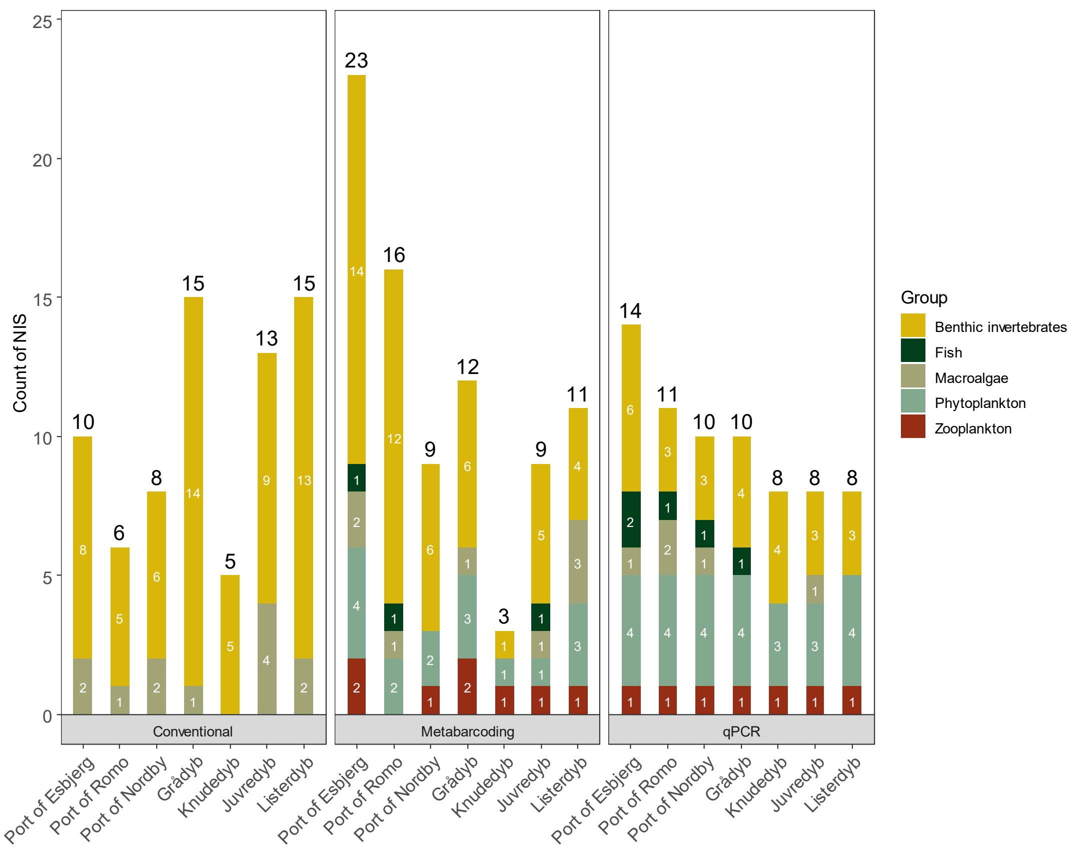
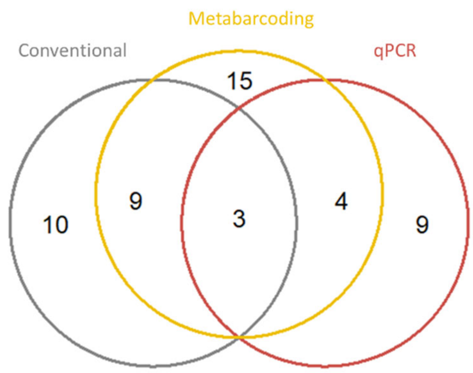


Figure 3.4. Total number of NIS observed through monitoring with different detection techniques (conventional, metabarcoding and qPCR) in each of the seven sampling sites. The colors refer to major taxonomical groups.

The detection techniques are not directly comparable as they are optimized towards different habitats and/or selected species (Sapkota et al. 2023). The conventional methods (sediment cores, scraping, visual identification on tidal flats, and morphological identification on settlement plates) do not include fish and planktonic species. The qPCR technique is developed for 23 selected NIS. In comparison, the metabarcoding technique should, in principle, be able to detect all species in all habitats. The overlap between detection techniques used in this study is shown in Figure 3.5.

Figure 3.5. Venn diagram comparing the NIS in common between the three detection techniques applied.



Overall, conventional techniques detected 22 NIS, compared to 16 for qPCR and 33 for metabarcoding. The three NIS identified by all three detection techniques were the pacific oyster (*Magallana gigas*), the soft-shell clam (*Mya arenaria*) and the Asian shore crab (*Hemigrapsus takanoi*) (Figure 3.6).

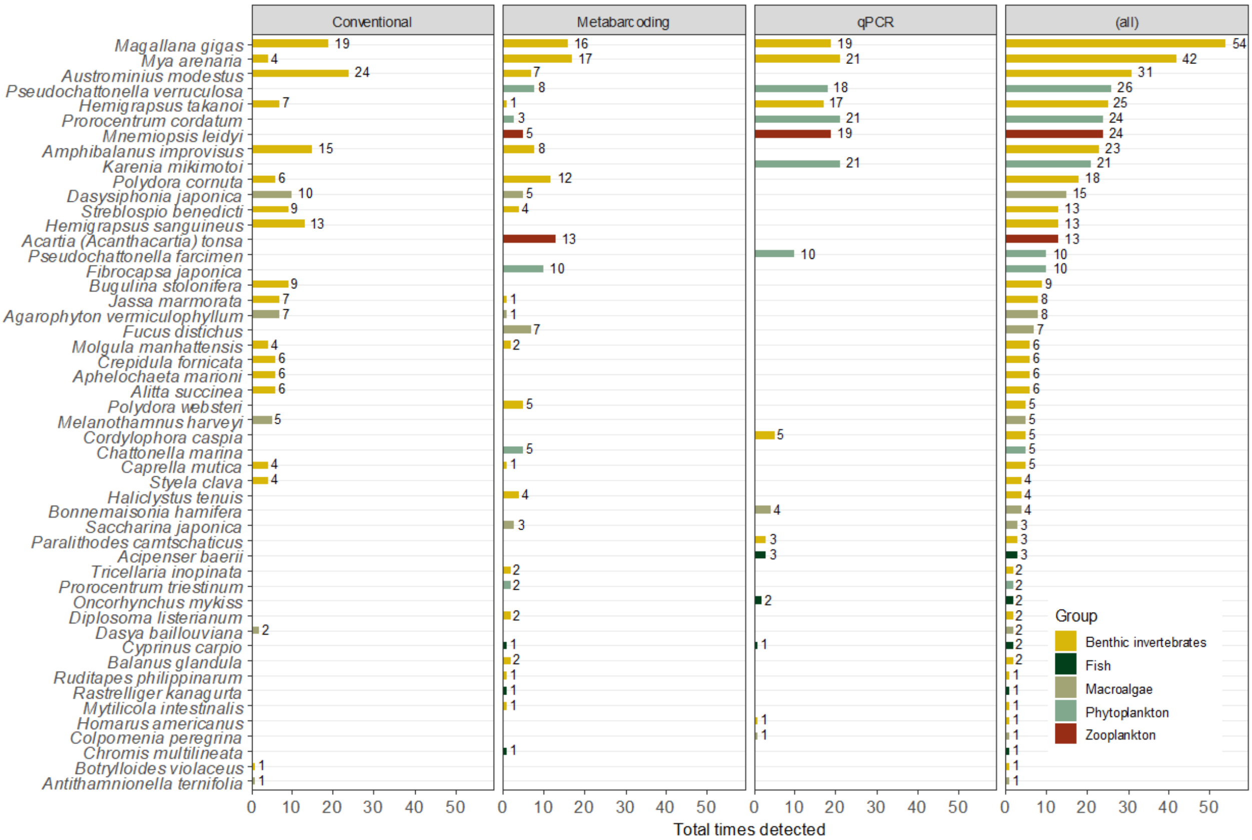


Figure 3.6. NIS detected by conventional, metabarcoding and qPCR techniques in the Wadden Sea. Color codes identify major taxonomic groups. Species are sorted according to the total number of observations per NIS.

50 NIS were detected across the seven sampling areas covered in this project. The occurrence of each NIS in these seven areas are listed in Table 3.1. Interestingly, 14 NIS appears to be new for Danish marine waters compared with the most recent version of the Danish gross NIS list. These are highlighted in Table 3.1 with notes on their know occurrences.

Table 3.1. List of NIS detected in Esbjerg (Esb), Rømø (Ro), Nordby (No), Knydedyb (Knu), Juvredyb (Juv) and Listerdyb (List) using metabarcoding (1), conventional (2) and qPCR (3). New NIS for Danish waters are highlighted in bold with notes on their know occurrences. Zooplankton (Zoo), phytoplankton (Phy) and Benthic invertebrates (BI).

Species	Grp	Esb	Ro	No	Grå	Knu	Juv	List	Method	Notes on occurrences
			Ports			Water areas				
Acartia (Acanthacartia) tonsa	Zoo	x		x	x	x	x	x	2	
Acipenser baerii	Fish	x			x				3	
Agarophyton vermiculophyllum	Ma				x		x	x	1,2	
Alitta succinea	BI		x		x		x	x	1	
Amphibalanus improvisus	BI	x	x	x	x		x	x	1,2	
Antithamnionella ternifolia	Ma			x					1	
Aphelochaeta marioni	BI				x	x	x	x	1	
Austrominius modestus	BI	x	x	x	x	x	x	x	1,2	
Balanus glandula	**BI**	**x**	**x**						**2**	**Barnacle found in Belgium 2016**
Bonnemaisonia hamifera	Ma	x	x	x			x		3	
Botrylloides violaceus	**BI**			**x**					**1**	**Small ascidian found in Holland since 2009**
Bugulina stolonifera	**BI**	**x**		**x**	**x**				**1**	**Bryozoan found in Holland in 1993**
Caprella mutica	BI	x			x			x	1,2	
Chattonella marina	**Phy**	**x**	**x**	**x**		**x**	**x**		**2**	**Pacific NIS found in most of Europe**
Chromis multilineata	**Fish**						**x**		**2**	**Reef fish from American Atlantic coast**
Colpomenia peregrina			x						3	
Cordylophora caspia	BI	x			x	x			3	
Crepidula fornicata	BI				x		x	x	1	
Cyprinus carpio	Fish	x							2,3	
Dasya baillouviana	Ma						x		1	
Dasysiphonia japonica	Ma	x					x	x	1,2	
Diplosoma listerianum	**BI**	**x**			**x**				**2**	**Ascidian found in the Wadden Sea**
Fibrocapsa japonica	**Phy**	**x**	**x**		**x**			**x**	**2**	**Pacific NIS found in most of Europe**
Fucus distichus	Ma	x			x			x	2	
Haliclystus tenuis	**BI**	**x**	**x**	**x**					**2**	**Ctenophore found in Helgoland in 2010**
Hemigrapsus sanguineus	BI	x			x	x	x	x	1	
Hemigrapsus takanoi	BI	x	x	x	x		x	x	1,2,3	
Homarus americanus	BI	x							3	
Jassa marmorata	BI		x		x			x	1,2	
Karenia mikimotoi	Phy	x	x	x	x	x	x	x	3	
Magallana gigas	BI	x	x	x	x	x	x	x	1,2,3	
Melanothamnus harveyi	BI	x	x	x			x		1	
Mnemiopsis leidyi	Zoo	x	x	x	x	x	x	x	2,3	
Molgula manhattensis	BI	x	x	x					1,2	
Mya arenaria	BI	x	x	x	x	x	x	x	1,2,3	
Mytilicola intestinalis	BI		x						2	
Oncorhynchus mykiss	Fish		x	x					3	

Table 3.1 continued										
Species	**Grp**	**Esb**	**Ro**	**No**	**Grå**	**Knu**	**Juv**	**List**	**Method**	**Notes**
			Ports			**Water areas**				
Paralithodes camtschaticus	**BI**	**x**				**x**			**2,3**	**Red king crab, not likely to occur**
Polydora cornuta	BI	x	x	x	x	x	x	x	1,2	
Polydora websteri	**BI**	**x**	**x**					**x**	**2**	**Polychaete associated with M. gigas**
Prorocentrum cordatum	Phy	x	x	x	x	x	x	x	2,3	
Prorocentrum triestinum	Phy	x							2	
Pseudochattonella farcimen	Phy	x	x	x	x			x	3	
Pseudochattonella verruculosa	Phy	x	x	x	x	x	x	x	2,3	
Rastrelliger kanagurta	**Fish**		**x**						**2**	**Indian mackerel**
Ruditapes philippinarum	**BI**		**x**						**2**	**Small mussel found in Europe**
Saccharina japonica	**Ma**		**x**					**x**	**2**	**Laminarian found in France 2017**
Streblospio benedicti	BI	x			x		x	x	1,2	
Styela clava	BI	x	x	x					1	
Tricellaria inopinata	**BI**	**x**							**2**	**Bryozoan found in the Dutch Wadden Sea**

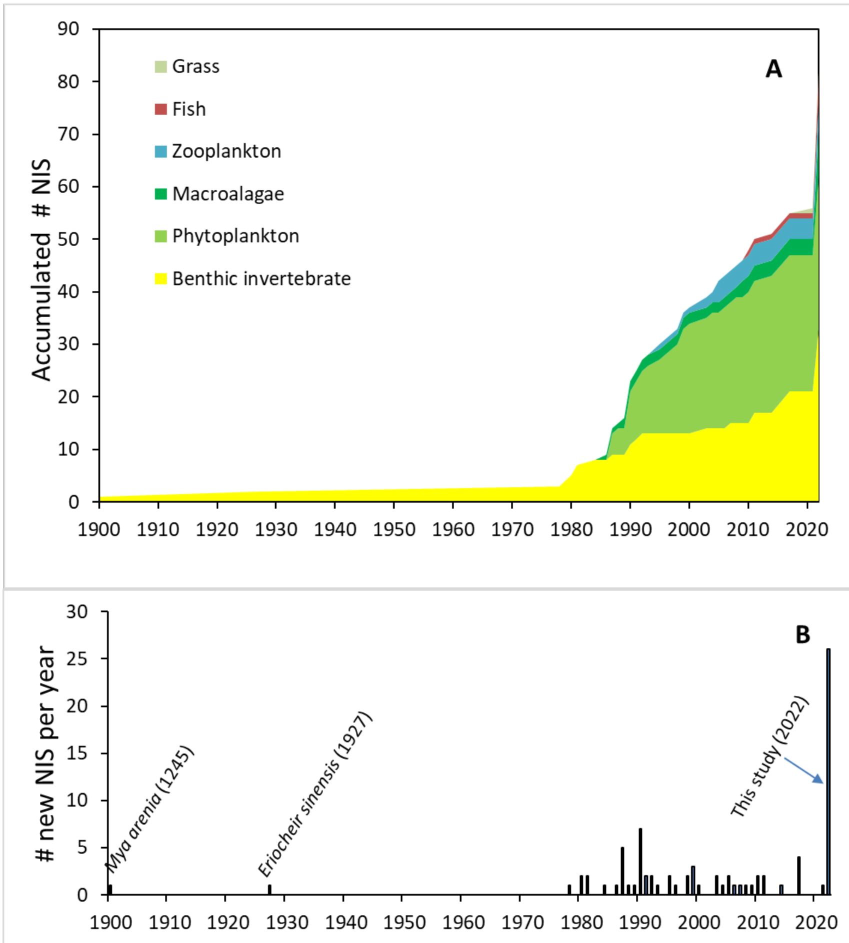
3.2 NIS identified through a review of data archives

Combining observations of NIS detected in this study with evidence of NIS recorded as part of the Danish national monitoring program and with observations published in different reports and official websites made it possible to collate and update the number of NIS in the Danish part of the Wadden Sea area. We found evidence of 82 NIS in the Wadden Sea area, of which 26 were detected during our sampling in 2022. The names of these NIS, their taxonomical associations, years of first and last observation, assessment of introduction pathways and assessment of impact are shown in appendix 2. In the following text, we summarize results imbedded in this appendix.

3.2.1 Historical trends in NIS observations

Beyond a few observations during 1900 to ca. 1980, most NIS have been recorded in the Wadden Sea since the initiation of the Danish marine monitoring program (Figure 3.7). To evaluate changes in the annual rate of NIS introduction, we calculated this as the slope coefficient using linear regression models of accumulated NIS vs. year for three periods, 1900 to 1989, 1990 to 2009 and 2010 to 2022. The respective rates were 0.1, 1.2 and 1.9 NIS per year.

Figure 3.7. Records of NIS in the Danish Wadden Sea area. A) accumulated trend indicating the contribution from different taxonomical groups; B) Number of new NIS records per year. We have highlighted two early NIS records observed before the onset of the monitoring program. Also, the high number of new NIS recorded in this study is marked.



Considering the entire data set (appendix 2, Figure 3.7), we recorded 33 NIS benthic invertebrates, 29 phytoplankton, nine macroalgae, five zooplankton, five fish and one grass plant (*Spartina anglica*). Of the 82 species, 17 were assigned as cryptogenic of unknown origin, and the remaining 65 as NIS. Of the 50 NIS detected during this study, 26 species were new NIS for the Wadden Sea area (this study, Figure 3.7).

3.2.2 NIS occurrences since 1990

By extracting information on species occurrences reported in the Danish National marine monitoring program (NOVANA), we obtained comprehensive lists of marine species for both benthic invertebrates, marine vegetation, phytoplankton, and zooplankton. While extensive, these programs do not cover all species, therefore, we cannot assess trends in jellyfish and fish in this study. Also, our data extraction was limited to the Danish Wadden Sea region, for which not all species have been monitored since 1990. Hence, there were years with no monitoring data available, and for zooplankton, we did not find data on NIS in this region. To identify NIS in the monitoring data sets, these were matched against the European NIS + cryptogenic list (See section 2.2.3).

As the number of monitoring sites varies from year to year, we reduced the records to the simple presence (1) and absence (0) for the entire region per year (Table 3.2).

Table 3.2. Annual records of NIS observations within the Danish Wadden sea area. Data were obtained from the Danish marine monitoring program (NOVANA). Present (1), absent (0), no monitoring data available (NA). Years of first and last observation are noted.

NIS scientific name	First	Last	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Agarophyton vermiculophyllum	2009	2010	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Akashiwo sanguinea	1990	2016	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	0	0	1	0	1	NA	NA	NA	NA
Alexandrium ostenfeldii	1996	2009	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	NA	NA	NA	NA
Alexandrium pseudogonyaulax	1999	2016	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	NA	NA	NA	NA
Alexandrium tamarense	1990	1991	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	NA	NA	NA
Alitta succinea	1980	2020	0	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	0	0	1	1	1
Amphibalanus improvisus	1992	2019	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
Aphelochaeta marioni	1984	2019	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	0	1	1	1	0
Bacteriastrum hyalinum	1990	2016	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	0	1	1	1	0	1	1	0	1	1	1	NA	NA	NA	NA
Biddulphia rhombus	1987	2016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	NA	NA	NA	NA
Biddulphia sinensis	1987	2016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	NA	NA	NA	NA
Chaetoceros circinalis	2006	2010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	NA	NA	NA	NA
Coscinodiscus wailesii	2014	2014	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	NA	NA	NA
Crepidula fornicata	1990	2010	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
Dipolydora quadrilobata	1980	2010	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Ensis leei	1981	2011	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Ethmodiscus punctiger	1999	2016	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	1	0	1	0	1	1	0	1	0	1	0	1	NA	NA	NA	NA
Hemigrapsus takanoi	2015	2015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Heterosigma akashiwo	1995	2014	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	NA	NA	NA
Karenia mikimotoi	2000	2016	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	NA	NA	NA	NA
Lauderia pumila	2008	2014	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	NA	NA	NA	NA
Lepidodinium chlorophorum	1999	2007	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	NA	NA	NA	NA
Magallana gigas	2007	2020	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1
Marenzelleria viridis	1990	2018	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	0	0	1	0	0
Mediopyxis helysia	2010	2016	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	NA	NA	NA	NA
Molgula manhattensis	1991	2004	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mya arenaria	1980	2020	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1
Peridiniella danica	2004	2009	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0	NA	NA	NA	NA
Petricolaria pholadiformis	1987	2008	0	1	1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Phaeocystis pouchetii	1987	2016	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	NA	NA	NA	NA
Polydora cornuta	1981	2015	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0
Prorocentrum cordatum	1988	2016	1	0	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1	NA	NA	NA	NA
Prorocentrum gracile	1990	2014	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	NA	NA	NA
Prorocentrum lima	1990	2009	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	NA	NA	NA	NA
Prorocentrum triestinum	1992	2016	0	0	1	0	0	0	1	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	NA	NA	NA	NA
Pseudochattonella spp.	1998	2016	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	NA	NA	NA	NA
Thalassiosira nordenskioeldii	1993	2016	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	NA	NA	NA	NA
Tripos arietinus	1987	2009	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	NA	NA	NA	NA
Tripos macroceros	1991	2013	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	NA	NA	NA

We obtained information on 39 NIS, covering 29 phytoplankton species, nine benthic invertebrates and one macroalga. The NIS varied considerably in occurrences from two observations to 27 for the entire 30-year period. On average, the NIS were present for 11 years from 1990 to 2020. For several species, the monitoring data sets extended further back, making it possible to extract information on the first year of observation all the way up to 1990.

3.2.3 NIS introduction pathways

Of the 82 NIS recorded, most were assessed to be introduced via different types of shipping, with ballast water and hull fouling as the dominant pathways. Unaided dispersal from neighboring areas was another major pathway, according to the available sources of information. At the same time, intentional release or escape from aquaculture seems to be of minor importance (Figure 3.8). A minor group (11 species) of NIS could not be related to any specific pathway (unknown). Several NIS (48 species) were mentioned with more than one pathway, with unaided dispersal being the most common additional pathway.

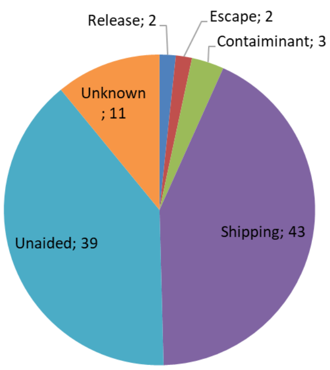
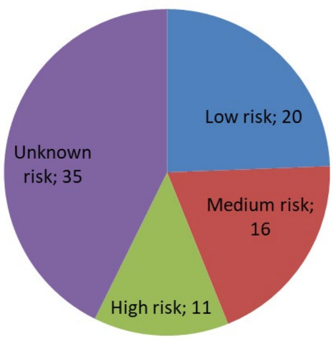


Figure 3.8. Pathways of introduction of NIS into the Danish part of the Wadden Sea area.

Within the shipping pathway, ballast water accounted for 39 NIS, whereas hull fouling was assigned to 11 NIS. And for four NIS, both ballast water and hull fouling were assessed as possible pathways. Hitchhikers on boats (entanglement) were also considered for one NIS.

3.2.4 NIS impact

Assessment of NIS impact was based on available expert-based judgements using the principles of the Harmonia score (Figure 3.9).



Species – high risk	Group	First obs	Last Obs
Agarophyton vermiculophyllum	Macroalagae	2009	2022
Heterosigma akashiwo	Phytoplankton	1995	2014
Karenia mikimotoi	Phytoplankton	2000	2022
Magallana gigas	Benthic invertebrate	2007	2022
Mnemiopsis leidyi	Zooplankton	2005	2022
Molgula manhattensis	Benthic invertebrate	1991	2022
Prorocentrum cordatum	Phytoplankton	1988	2022
Pseudochattonella farcimen	Phytoplankton	1998	2022
Pseudochattonella verruculosa	Phytoplankton	1998	2022
Sargassum muticum	Macroalagae	1989	2021
Spartina anglica	Grass	2021	2022

Figure 3.9. Assessment of NIS impact based on available expert-based assessments using the Harmonia score. Here we reclassified the scores into three impact categories per the ISEIA guideline. The table shows the NIS assessed to have a potentially high impact.

We aggregated the summed Harmonia scores into high, medium, and low-risk groups to simplify risk assessment. The group of high-risk NIS includes several common NIS for Danish waters (Figure 3.9).

3.3 NIS dispersal assessed through modelling

3.3.1 Importance of water movement

The spreading of NIS depends on the dispersal by ocean currents and mixing. The modelled current speed and direction during summer are illustrated for the coastal North Sea and the Wadden Sea by rose plots summing up the conditions in each sub-areas of the model domain (Figure 3.10a). The current is consistently northward in the deep North Sea areas due to the Jutland Current. Figures 3.10a and b show the mean and standard deviation (STD) of the surface current on the 21st of August 2021, a day with a relatively strong flow. The North Sea branch of the Jutland current is visible here (Figure 3.10b), illustrating how the current is following the bathymetry northward and does not flow as strongly close to the Wadden Sea barrier islands. Periods of southward flow occasionally occur, depending on the wind strength and direction. In our simulation, southward currents mainly occurred during winter months.

In the coastal areas of the North Sea (orange and red, Figure 3.10a), where the depth is less than 10 meters, the current direction is more variable than in the deeper water, partly due to a tidal impact. The speed of the current is slower, but the flow still predominantly follows the coastline in a northerly direction. In the two closed Wadden Sea basins in the south (Listerdyb and Juvredyb), the tidal signal dominates the current (Figure 3.10a). In the northern Wadden Sea, the coastal water flows northeastward into Knudedyb. The boundary between Knudedyb and Grådyb is very shallow, thus to some degree inhibiting the exchange of water between the two basins on a tidal scale, though some exchange still takes place (Figure 3.10c).

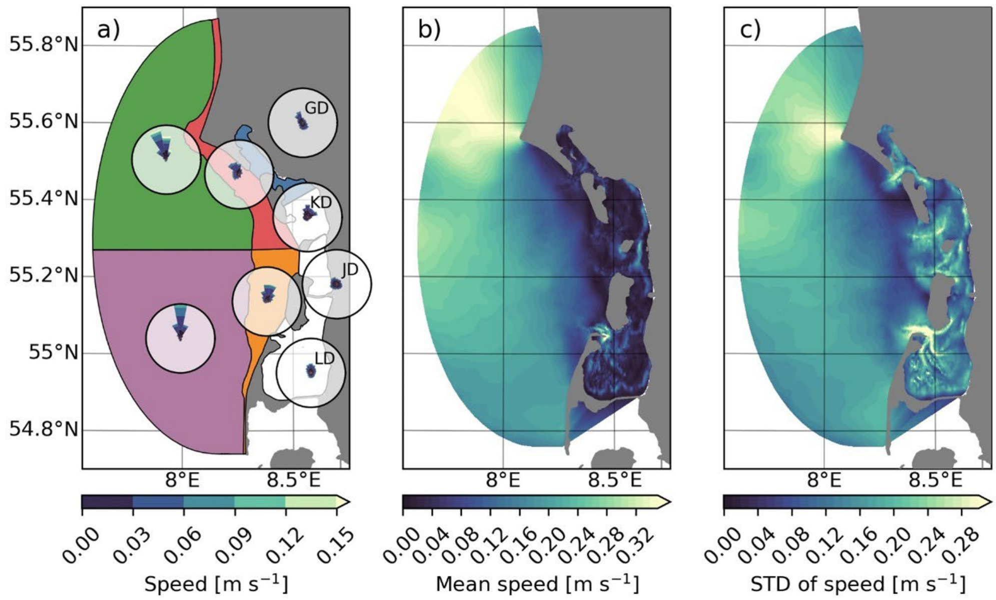


Figure 3.10. a) Rose plots showing the direction and speed of the current in each of the water bodies of the study area between the June 1st and October 1st of 2021. The edge of the rose plots denotes a frequency of 30%. The North Sea current roses are located in the center of the corresponding water body, while the Wadden Sea current roses are located in or close to the water bodies and marked with the names; GD: Grådyb, KD: Knudedyb, JD: Juvredyb, and LD: Listerdyb. **b)** Mean speed on the 1st of August 2021. **c)** STD of the speed on the 21st of August 2021.

3.3.2 Particle dispersal

To illustrate the movement of particles in the North and Wadden Seas, density plots of the particles released in each water body were created for a particle dispersal (PD) of 3 days and 21 days (Figure 3.11). These PDs could represent spreading scenarios of, e.g., *C. mutica* and *R. venosa*, respectively. As the simulation is seven times longer for a PD of 21 days than 3 days, subplots f-j of Figure 3.11 entail 7 times as many positions.

The PD of a species logically makes a significant difference in the distances transported. With a PD of three days, particles stay close to the water body in which they were released. However, the main current directions can be seen in the direction of the transport, especially in the deep North Sea water bodies (Figure 3.11a, f, d and i). When the PD is increased from three to a maximum of 21 days, the specific direction of the transport becomes visible, along with the track variability over the summer and with the release position.

Few of the particles released in the deep North Sea (Figure 3.11a, f, d and i) pass through the Wadden Sea despite the release sites' proximity to the coast. This indicates a strong division between particles released within and outside the Jutland current. The tracks of particles released in the coastal North Sea, close to the entrance to Grådyb, and in Grådyb itself (Figure 3.11b, c, g and f) show considerable track variability. Here, the tidal signal is strong and dominates over the northward transport. After three days, these particles have passed through the coastal North Sea and Grådyb (Figure 3.11b and c), and after 21 days, the tracks go through all water bodies except the two southern Wadden Sea basins (Figure 3.11g and h). The general trend is northward, but periods of southward current direction occur during September near the North Sea coast and in the deep North Sea, causing a southward track for some particles. All particles released in Grådyb leave the area through the northern entrance, showing that the shallow water between Grådyb and Knudedyb creates an effective barrier between the two water bodies. However, some particles originating in Grådyb subsequently pass through Knudedyb from the North Sea (Figure 3.11h).

The particles released in the Jutland Current in the southern deep North Sea are consistently transported northward in the North Sea and only enter the Wadden Sea to a very small degree (Figure 3.11d and i). For particles released in the coastal southern North Sea, the tracks are variable. However, many particles enter the two southern Wadden Sea basins, Juvre and Listerdyb, with tidal oscillations (Figure 3.11e and j). After 21 days, a tendency of transport out in the Jutland current and northward appears, while northward transport within the coastal water does not occur (Figure 3.11j).

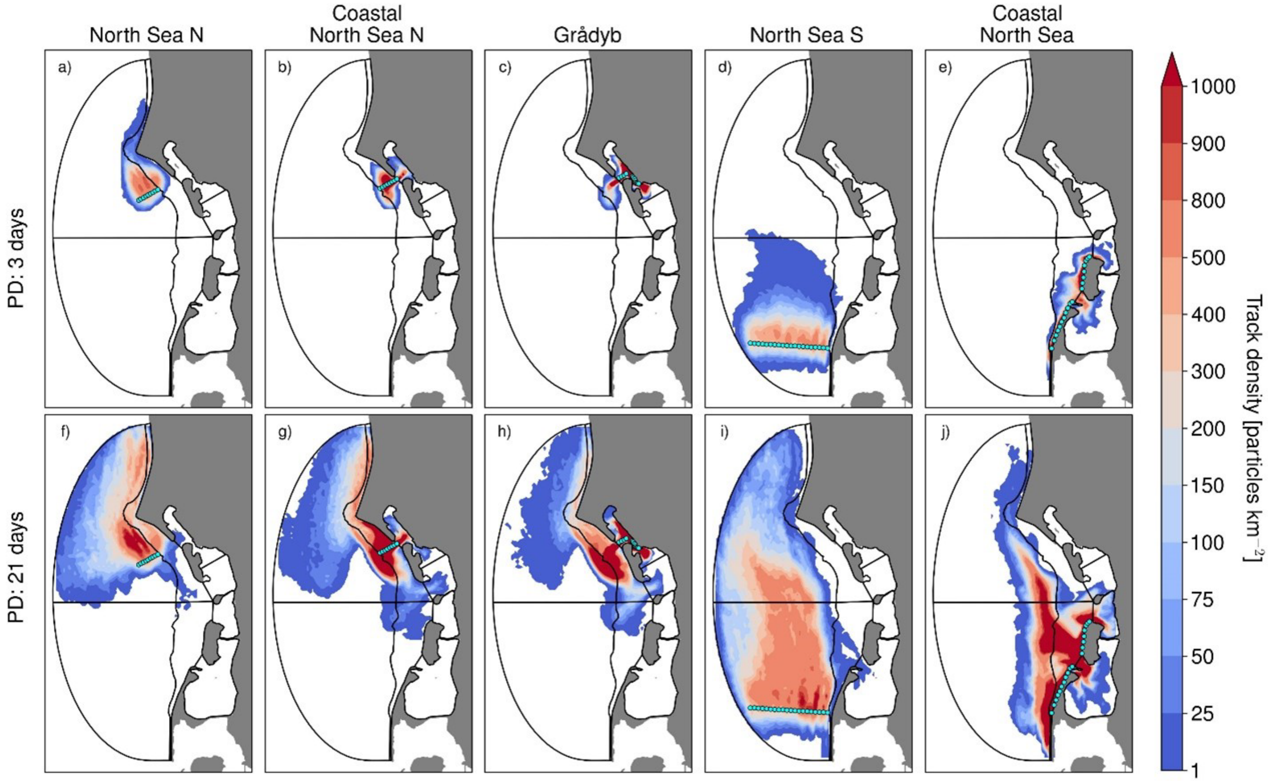


Figure 3.11. Density of particle tracks for a pelagic duration of 3 and 21 days, respectively. The subplots show the density of particles released in each of the water bodies. The cyan-colored circles mark the initial ABM position in each plot: a and f) Released in the deep parts of the northern North Sea. b and g) Released in the coastal northern North Sea. c and h) Released in Grådyb. d and i) Released in the southern deep North Sea. e and j) Released in the southern coastal North Sea.

3.3.3 Connectivity between sub-areas

When a particle reaches the end of its pelagic duration, we assumed that settling occurs if it is in an area with a depth of fewer than 10 meters. Settlement in the deep North Sea areas (marked in muted colors in Figure 3.12) thus does not take place. In the simulation with a pelagic duration of 1 day, the particles do not travel far from their initial position, and most of them thus settle in the area in which they were released (Figure 3.12a).

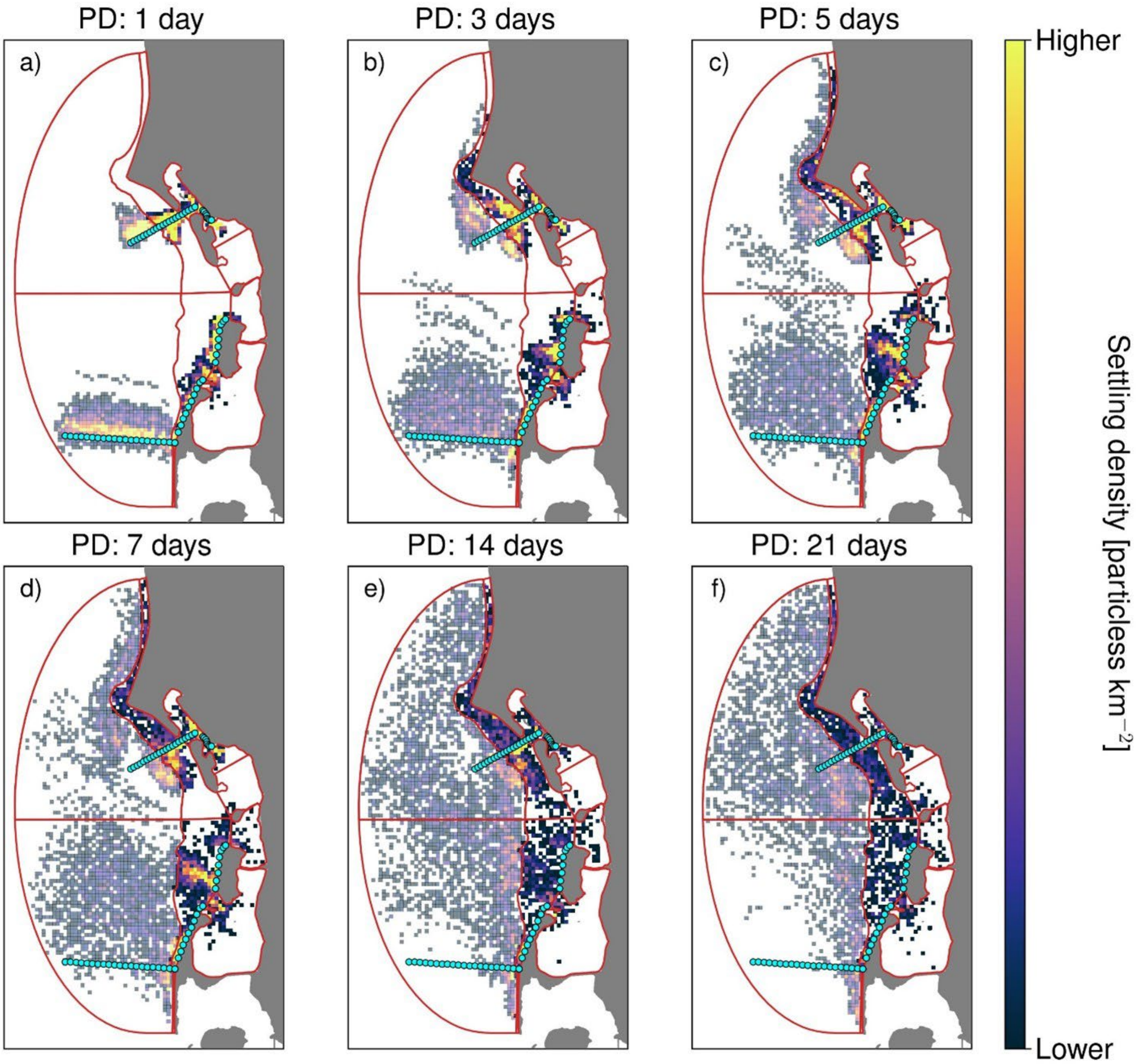


Figure 3.12. Settling density calculated on a 1x1 km grid for all particles released in simulation with varying pelagic duration. In areas deeper than 10 meters, the water is considered too deep for settling, and the colors of the settling density have been lightened. Cyan dots mark release points. Red polygons mark the sub-areas used to produce the connectivity matrix.

However, even after one day, several particles released in the North Sea reached three Wadden Sea sub-areas. The percentage of particles reaching the Wadden Sea increases when the pelagic duration is increased to 3 and 5 days, after which it decreases (Figure 3.12). This can be explained by the steady inflow of freshwater to the Wadden Sea from land, causing a small net flow out of the Wadden Sea, on time scales larger than those of the tidal oscillation and the intermittent large-scale inflow caused by changes in the wind patterns, thus eventually acting to push particles out. Similarly, a more significant percentage of the particles released to Grådyb (GD) in the Wadden Sea leave the area with increasing pelagic duration (Figure 3. 12). Only a small percentage of the particles released in Grådyb end up in the neighboring Knudedyb. These entries are from the coastal North Sea and do not take the direct route within the Wadden Sea (Figure 3.11g and h), thus explaining why the fraction received in Knudedyb increases with pelagic duration.

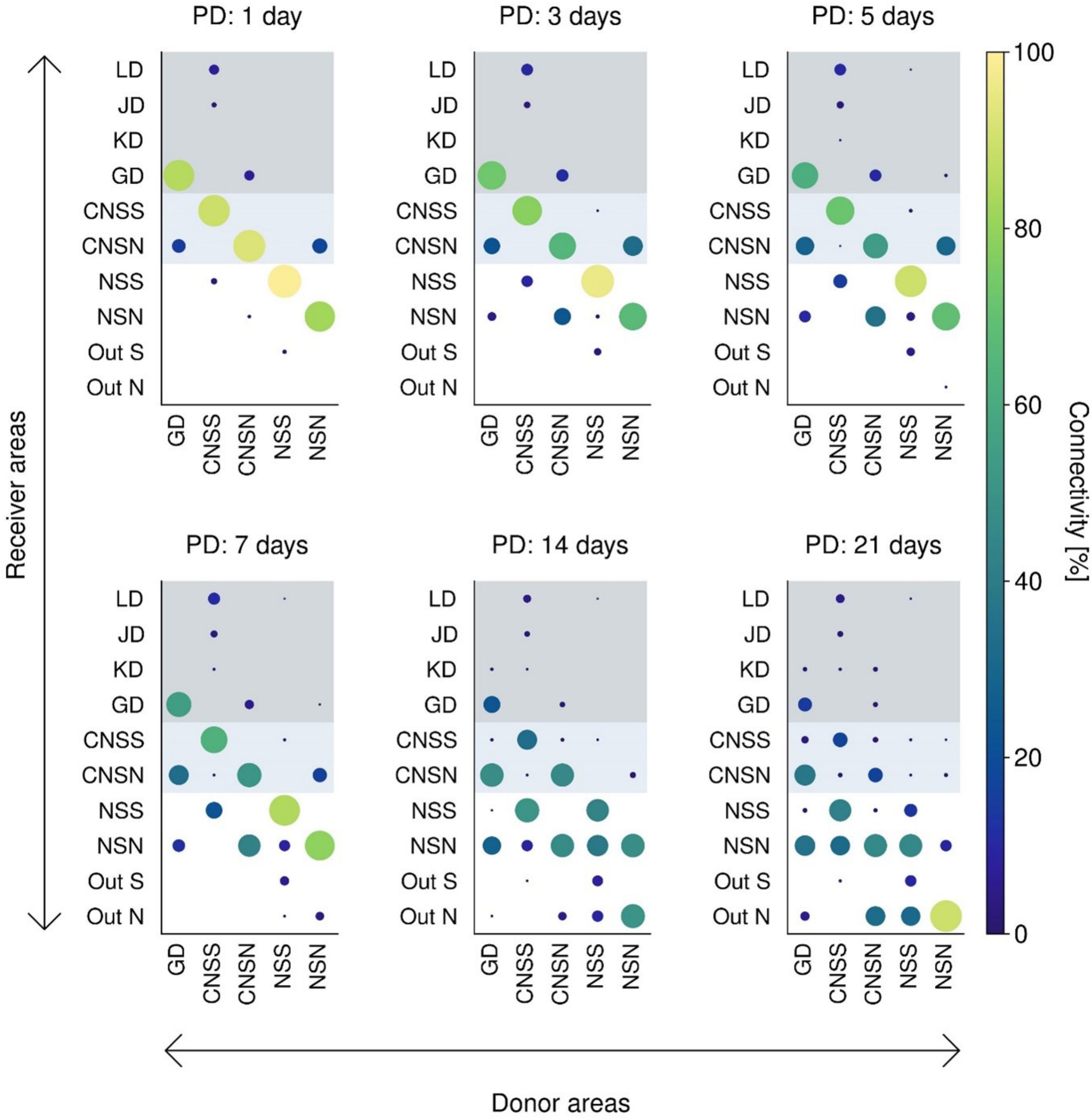


Figure 3.13. Connectivity matrices for each of the simulations show how the particles released in the donor areas (x-axis) are distributed spatially at the end of the simulation, including the particles that have left the model domain on the southerly and northerly border. Receiver areas are on the y-axis. The color and size of the scatter denote the percentage. Dark grey background mark receiver areas in the Wadden Sea, while light grey background mark receiver areas in the coastal North Sea, shallow enough for settlement. White background marks receiver areas, which are considered too deep for settlement. A map of the sub-areas and the initial position of the particles can be seen in Figure 1. GD: Grådyb, KD: Knudedyb, JD: Juvredyb, LD: Listerdyb, CNSS: Coastal North Sea South, CNSN: Coastal North Sea North, NSS: North Sea South, NSN: North Sea North, Out S: Particles have left the domain in a southerly direction, Out N: Particles have left the model domain in a northerly direction.

While all areas are mainly self-recruiting in simulations with a pelagic duration of one day, increasing pelagic duration leads to an increasing percentage of particles ending in the deep North Sea, and ultimately transported out of the model domain as the Jutland Current move them rapidly northwards (Figure 3. 10a). In the simulation with a pelagic duration of 21 days, nearly all particles initialized in the Northern North Sea have left the model domain in a northerly direction, and so have approximately 25% of the particles initialized in the southern North Sea and the coastal northern North Sea (Figure 3. 10a). Conversely, only particles released in the southern North Sea leave the model domain in a southerly direction.

3.4 NIS mitigations

The following overview of NIS mitigations largely builds on information gathered in a Thematic assessment for NIS in the OSPAR quality status report (Staehr et al. in press).

3.4.1 NIS legislation

Marine NIS and Invasive Aquatic Species (IAS) are both addressed by European Union (EU) policies, including the EU Biodiversity Strategy 2020 (COM (2011) 244) target 5; the European Water Framework Directive (WFD) (2000/60/EC); the EU Marine Strategy Framework Directive (MSFD) (2008/56/EC) with a dedicated descriptor (D2 "Non-indigenous species introduced by human activities are at levels that do not adversely alter the ecosystems") and the IAS Regulation (No 1143/2014). NIS is one of the 11 descriptors in the MSFD that refer to anthropogenic pressures on the marine environment of the EU (Commission, 2017).

The MSFD descriptor D2 on marine NIS has two criteria. The primary criterion D2C1 states that: "The number of non-indigenous species which are newly introduced via human activity into the wild, per assessment period (6 years), measured from the reference year (2011) as reported for initial assessment under Article 8(1) of Directive 2008/56/EC, is minimized and where possible reduced to zero". In addition, two secondary indicators address the abundance and spatial distribution of established NIS (D2C2) and the impact of invasive NIS (D2C3) on species and habitats (Commission, 2017). Efforts to make these secondary indicators more quantitative are ongoing (HELCOM, 2018; OSPAR, 2022; UNEP, 2021b). Recently a quantitative measure for D2C1 has been encouraged by Target 6 of the first draft of the Convention on Biological Diversity (CBD) Post-2020 Global Biodiversity Framework, which stipulates at least a 50% reduction in the rate of new introductions (UNEP, 2021b). This target has also been recommended by Tsiamis et al. (2021). However, to date, only the Baltic Marine Environment Protection Commission (Helsinki Convention, HELCOM) has set a numerical threshold of zero new NIS introductions through anthropogenic activities in the Baltic Sea (HELCOM, 2018). The following description presents mitigation measures identified to minimize the number of new NIS introduced into the Wadden Sea area. To determine the importance of these measures, we initially summarize the link between human activities and pathways of NIS introduction and then describe the mitigation measures associated with these pathways with the identification of possible improvements.

3.4.2 Activities and pathways responsible for NIS introduction to the Wadden Sea

A series of human activities may affect the rate of NIS introduction and, eventually, the impact of NIS on marine biodiversity. The human activities of relevance have recently been described in an OSPAR NIS thematic assessment report, and the most important ones are summarized in table 3.2 with notes on their significance for introduction pathways.

Table 3.2. Linkages between human activities, effects on the marine environment relevant for related pathways of NIS introduction and list of available mitigation measures.

Human activity	Description of effect	Related pathway of NIS introduction	Mitigation measure
Extraction of oil and gas	Installation of structures → steppingstones for NIS	Unaided (secondary spread)	Marine protected areas Nature restoration
Renewable energy (wind, wave, tidal power)	Installation of structures → steppingstones for NIS	Unaided (secondary spread)	Marine protected areas Nature restoration
Extraction of minerals	Creating corridors of expansion	Corridor: Interconnected seas	Marine protected areas Nature restoration
Marine aquaculture	Release of NIS and structures as steppingstones for NIS dispersal	Escape from confinement	National actions
Fisheries		Release in nature	Marine protected areas
Shipping	Transport and release of NIS	Transport via ballast water and hull fouling	BWM convention / IMO
Recreational boating	Transport and release of NIS	Transport	National Biosecurity plans
Angling	Transport and release of NIS	Release in nature	National Biosecurity plans
Climate change	Facilitating NIS northward expansion through warming	Unaided (secondary spread)	National climate action plans

Human activities associated with these pathways are distributed widely across the North-East Atlantic. Still, the intensity of activities and their pressures on the marine environment and NIS vary greatly between OSPAR Regions and sub-divisions. For the Danish part of the Wadden Sea, all the human activities, related effects and pathways seem relevant.

3.4.3 NIS mitigation measures

Shipping

BWM convention: The International Convention for the control and management of Ships Ballast water and sediments, 2004 (BWM convention) is a global response to prevent the effects of the spread of invasive species carried by ships' ballast water and sediments, which entered into force globally on 8 September 2017. This is a crucial step towards reducing the spread of non-indigenous species regionally and worldwide. The BWM convention requires ships in international traffic to apply ballast water management measures, such as ballast water exchange (D-1) or to fulfil a certain discharge standard (D-2). The latter requires installing a certified ballast water treatment device, which enables sterilization and avoids transfers of ballast water mediated species. Existing ships must initially meet the D-1 standard but by 2024, all must meet the D2 standard. Every ship is required to have a Ballast Water Management Plan and International Ballast Water Management Certificate and to keep a Ballast Water Record Book. The International maritime organization (IMO) has also issued guidelines on aspects of implementation and continues to review the operation of the Convention during the current experience-building phase. Several amendments to the Convention and the Code for Approval of Ballast Water Management Systems (BWMS Code), replacing the former G8 Guidelines, came into force in October 2019; amendments and revised guidance were also adopted in 2020. As of 15 September 2020, 88 states and IMO associate members had ratified the Convention (IMO, 2020), including all OSPAR Contracting Parties other than the United Kingdom. In 2020, Denmark and Sweden established a bilateral agreement to implement the BWC Regulation A-4 and create a Same Risk Area designation for the Öresund. Something similar could be considered for the Wadden Sea region.

Biofouling: The IMO Biofouling Guidelines (resolution MEPC.207(62) are intended to provide a globally consistent approach to managing biofouling, which is the accumulation of various aquatic organisms on ships' hulls. They were adopted by the Marine Environment Protection Committee (MEPC) at its sixty-second session in July 2011 and resulted from three years of consultation between IMO Member States.

Exemptions: One of the principal responses undertaken by OSPAR to address NIS has been through the "Joint HELCOM/OSPAR Harmonized Procedure for the Contracting Parties of OSPAR and HELCOM on the granting of exemptions under the International Convention for the Control and Management of Ship's Ballast Water and Sediments, Regulation A-4" (JHP) is based on the Guidelines for Risk Assessment under Regulation A-4 of the Ballast Water Management (BWM) Convention (G7) (Resolution MEPC.289(71), and was originally agreed by HELCOM and OSPAR Contracting Parties in 2013 (OSPAR Agreement 2020-01). The JHP procedure aims to ensure that exemptions are granted in a coherent manner that does not impair or damage the environment, human health, property, or resources. Main users of this procedure include shipowners/operators, port State administrations and relevant experts and researchers. Based on the Regulation A-4 of the Ballast Water Management Convention (the Convention), exemptions from ballast water management requirements described in the JHP can be issued to a ship on voyages between specified ports or locations for a maximum of five years. A port State may grant such an exemption if the risk is acceptably low based on the results of a risk assessment. In the initial transitional period of the BWM Convention (2017-2024), the JHP is to be implemented flexibly and practically to gain experience and enable further development and improvements. Alignment of procedures for exemptions within the Wadden Sea regions should be considered.

OSPAR and HELCOM have established a joint task group for the management of NIS relating to ballast water management exemptions, and the management of ballast water and biofouling (JTG BALLAST & Biofouling). The joint work includes the development of a system for managing information about priority species of NIS and their occurrence in the region to be used as a basis for decision-making when considering whether an exchange of ballast water by a ship between, for example, two port areas, is of particular concern, for example.

Marine Protected Area network

Within OSPAR, Marine Protected Area network (MPAs) are understood as areas for which protective, conservation, restorative or precautionary measures have been instituted for the purpose of protecting and conserving species, habitats, ecosystems, or ecological processes of the marine environment (as defined in Recommendation 2003/3, implementing Annex V of the OSPAR Convention). The potential for NIS to negatively impact MPAs such as the Wadden Sea, is of particular concern, given the importance of the ecosystems they encompass and the statutory requirements to protect them.

On the other hand, MPAs are highlighted as a management approach to reduce the impact of NIS. There are, unfortunately, very little data documenting the effect of MPAs on invasive species and determining if MPAs can increase resilience to biological invasions (Giakoumi et al. 2017). The few studies so far showed contradictory effects (either positive or negative or non-significant) on NIS, although NIS density was usually greater outside than inside MPAs (Giakoumi et al. 2017). MPAs are generally not a response to address the spread or establishment of NIS. However, there may be important management actions within protected areas, such as seabird nesting grounds through predator eradication programs, or measures to manage other NIS pathways within protected areas to reduce the risk of introduction and spread of NIS within the protected areas.

Restoration and NIS

A Nature Restoration Law is currently being formulated, which includes binding targets to restore ecosystems including marine, aligning with ambitions in the UN Decade for Ecosystem Restoration. The implementation of this ambition is articulated within OSPAR and by 2023 OSPAR will identify habitats suitable for restoration and develop a common knowledge base on the most appropriate and effective methods for restoring degraded habitats. Restoration is considered a measure to strengthen marine ecosystems.

Aquaculture - marine, including infrastructure

At the EU level, Council Regulation (EC) No 708/2007 regulation concerning the use of alien and locally absent species in aquaculture, aims to optimize benefits associated with introductions and translocations while at the same time avoiding alterations to ecosystems, preventing negative biological interaction, including genetic change, with indigenous populations and restricting the spread of non-target species and detrimental impacts on natural habitats. It is noted that this response does not apply to the Pacific Oyster, which is identified as a species of concern regarding the OSPAR listed flat oyster.

National Biosecurity plans

Biosecurity measures are highlighted as important for reducing the introduction and spread of marine NIS, and includes measures to prevent their introduction and secondary spread (Mustow 2021). Ideally the applied approaches should be tailored to the relevant stakeholders and should consider policy interventions, including laws or voluntary agreements, and social incentives for good behavior (Shannon et al. 2020). Examples of biosecurity measures to control marine NIS are listed in Mustow (2021) describing measures to 1) prevent new NIS, 2) detect and rapid response, 3) control and containment. Under the CBD Decision VI/23 and European-wide implementation of this through EU Regulation 1143/2014, countries are obliged to develop national biosecurity plans. A biosecurity action plan for the Wadden Sea should preferably be developed within framework of the Trilateral Wadden Sea collaboration.

National climate plans

Climate change, primarily ocean temperature increases, may facilitate the introduction and establishment of NIS. In addition, climate change may increase the magnitude of impacts associated with invasive NIS, for example, by reducing the resilience of native ecosystems and habitats. In addition to the impacts of climate change, higher rates of between-continent dispersal events associated with increasing levels of international trade and human travelling are expected (Hewitt et al., 2018; Sardain et al., 2019). Denmark has committed to an ambitious national climate plan to reduce greenhouse gas emissions to reduce the rate of climate warming. Irrespective of this, model predictions indicate continued warming, which, combined with especially international shipping, maintains a high risk of new NIS introductions.

4 Discussion

4.1 NIS records in the Danish Wadden Sea area

4.1.1 NIS monitoring in this study

Monitoring of NIS using conventional and molecular techniques provided extensive information on NIS in different harbors and habitats within the shallow tidal zone of the Wadden Sea. A total of 50 NIS was detected including 26 new NIS for the area, and 14 new NIS for Danish seas. The monitoring techniques applied were chosen to optimize detection of NIS bearing in mind the complementarity of the techniques recently evaluated by Sapkota et al. (2023). Furthermore, our choice of techniques and sampling efforts (locations, timing, and number of samples) were discussed with an advisory board from Germany and the Netherlands, to optimize detection and ensure compatibility with monitoring in other parts of the Wadden Sea area. Hence, we assume that our monitoring and detection of NIS in the Danish part of the Wadden Sea provided a representative documentation of the NIS abundance and their distribution in different habitats.

Special effort was made to assess and ensure high quality in the NIS detected using different techniques. Conventional sampling was performed by a highly trained taxonomist with expertise in marine NIS. The two new NIS detected in Danish waters with this technique was confirmed by experts in Holland to verify species identification. For the NIS detected using metabarcoding, there was overall a good agreement with both conventional detection and the species specific (qPCR) detections. 11 new NIS for Danish waters were identified with metabarcoding and for these we obtained information on their known occurrence to evaluate the likelihood that these can occur in the Danish Wadden Sea. Regarding these NIS, we consider the species to be "highly likely to occur", but recommend that they currently are allocated to a watch list awaiting further documentation, preferably with conventional taxonomic identification. Monitoring of NIS using conventional and molecular techniques provided extensive information on alien species in different harbors and habitats within the shallow tidal zone of the Wadden Sea.

4.1.2 NIS recorded through national monitoring

The Danish national monitoring program (NOVANA) provided important data on first years of observations and annual records for 39 NIS in the Danish Wadden Sea area. As the NOVANA program was not originally intended to be used for NIS monitoring, some groups such as fish and jellyfish are not covered (Fossing and Stæhr, 2017). For these, we relied on information from other sources including citizen science and recent eDNA based observations (Andersen et al. 2022). The NOVANA data makes it possible to track changes in the abundance of species over time, thereby providing information useful for assessment of how well established a certain NIS is, and its potential impact on the indigenous species (e.g., Stæhr et al. 2000, Stæhr et al. 2019, Staehr et al. 2020). A robust analysis of trends and impacts should preferably take into account changes in monitoring effort to rule out that changes in abundance of a given species is merely a result of changes in number of sampling sites or frequencies. While such normalization is possible within each sampling program, it's complicated when comparing different types of sampling. Therefore, and for the sake of simplicity we chose to only discuss the overall presence and absence of each NIS within the entire Danish Wadden Sea area. Such normalization of data has previously been applied when comparing data obtained from different types of sampling (Staehr et al. 2020). Our results showed that most of the NOVANA based detections of NIS were observed during more than 10 years on average during the 30 years (1990 to 2020) investigated. Several species had been recorded earlier than 1990, and many occurred almost on a yearly basis suggesting that these have become an important and well-established component of the Wadden Sea ecosystem.

4.1.3 Total number of NIS and long-term trends

Combining the 50 NIS detected in this study, with observations through the national monitoring program, and other data sources with information on the Danish Wadden Sea, provided a NIS list of 82 species. Compared to recent national reports this is below the 123 NIS recorded for all of Denmark, which similarly includes NIS observations using molecular techniques and reports from intensive harbor investigations along with the NOVANA monitoring data. The Wadden Sea and national NIS data should therefore be comparable. Interestingly, the number of NIS observed in the Wadden Sea is much higher than any other Danish region (Jensen et al., 2023), suggesting that the Wadden Sea is a hot spot for NIS introductions. Alternatively, the lack of comprehensive investigations in other regions, severely underestimates the number of NIS reported here. Comparing the new Danish Wadden Sea record (82 NIS), with recent reports in other parts of the Wadden Sea, however shows a good agreement with both the German (92 NIS) and Netherlands (90 NIS), and is by far higher than the previous Danish estimate of 29 NIS from 2016 (Kloepper et al. 2022). Calculating the annual rate of new NIS introductions for different time periods, showed that for the Danish data, this increased from 0.1, to 1.1, and 1.9 per year when comparing the periods 1900-1989, 1990-2010 and 2010-2022. A similar increase in the rate of new NIS was also observed when comparing the entire Wadden Sea where the rates changed from 0.5 to 1.5 and 2.7 per year (Kloepper et al. 2022). A similar tendency with increasing rates of new NIS were observed for the entire Danish marine seas (Jensen et al. 2023) and is assumed to be linked to a combination of increased international shipping and raised monitoring effort (Stæhr and Jakobsen, 2023).

4.1.4 Assessment of pathways of dispersal and impact

In agreement with recent reports for the Wadden Sea (Kloepper et al. 2022), shipping (hull fouling and ballast water) was considered important along with secondary spread (unaided or natural distribution) and with some species being introduced via release of species (e.g., Oyster and other shellfish). Large uncertainties, however, remain on this mostly expert based assessment which should therefore be used with care. To guide future mitigation of NIS, assessment of pathways should therefore be evaluated using detailed information of the native distributions of the observed NIS, and information on probability of pathways (e.g., Tsiamis et al. 2018). Such information will be useful for tailored management of specific primary pathways per marine subregion, supporting prioritization efforts.

Our assessment of impact applied the expert-based Harmonia scoring system for which we obtained information for 47 out of the 82 detected NIS. For species with a Harmonia score > 11, a high impact risk score was assigned for 11 NIS. Some of these have occurred for many years in the Danish Wadden

Sea (since the 1980's) while others are recent introductions. Although some NIS have a high impact score, there is no evidence that alien species have caused the extinction of native species in the Wadden Sea (Kloepper et al. 2022). However, it cannot be out ruled that some of the identified high impact NIS have the potential to alter dominance structures, habitats, and trophic regimes. Recent analysis of NIS impact in the German part of the Wadden Sea, however, highlights that no resident populations have gone extinct because of an introduced species. Rather than degrading the ecosystem, the establishment of introduced species seems to have raised the capacity to follow environmental change (Reise et al. 2023). To evaluate potential impacts, further continued monitoring using standardized methods such as in this study, should be prioritized in the Danish Wadden Sea.

4.2 Modelling NIS dispersal in the Danish Wadden Sea area

The Wadden Sea's unique ecosystem requires a holistic conservation and management strategy (Fath, 2015; Carey et al., 2014). For other coastal areas, large-scale models with several interacting compartments, e.g., hydrodynamics, biogeochemistry and food webs, have been applied to quantify fluxes in the ecosystem (Longo et al., 2015; Samhouri et al., 2009). Such complex model setups require a significant degree of knowledge of the system, which is hard to obtain for all variables and introduces considerable uncertainty. Here we have chosen a simple model approach to provide first knowledge of likely dispersal patterns of NIS in the Wadden Sea and coastal North Sea, applying a coastal hydrodynamical model setup for the area, with individual passive particles representing groups of NIS.

The realism of currents and mixing in the hydrodynamical setup is a crucial first step for realistic particle dispersal modelling. Here we assessed the model performance by comparison of measured and modeled sea surface height (SSH) and found a good agreement (appendix 3). SSH is regarded as a main indicator of the model's ability to reproduce current velocities and directions. Salinity and temperature were furthermore validated in Schourup-Kristensen et al. (2023) supporting the realism of the physical model. The setup with a high spatial resolution of dynamically important features, such as the tidal channels, combined with a small-time step of 3 minutes, is an advantage when simulating the complicated water flow of the Wadden Sea. However, the effect of sub-grid-scale features, such as eddies and internal waves, is not resolved. These features would likely add heterogeneity to the particle dispersal as observed and modelled elsewhere (Samuelsen et al., 2012). Following Mayorga-Adame et al. (2022), the passive particles were released in the surface layer, where the current is fastest, and the potential for spreading is highest. The only trait applied to the particles was PD. The choice of PDs was based on two specific target species (The veined rapana whelk (*Rapana venosa*) and the Japanese skeleton shrimp (*Caprella mutica*), respectively). However, the range of PDs, from 1 to 21 days, represent a large range of potential NIS, e.g., Asian kelp (*Undaria pinnatifida*) or the bryozoan *Schizoporella japonica*. Thus, the results broadly apply to other NIS species in the area with similar PDs.

NIS dispersal is affected by a range of factors which are not included in our study. Larvae behaviour can potentially alter the dispersal by, e.g., changing their vertical swimming behaviour (Gary et al., 2020; Fox et al., 2016). The mechanism behind such behaviour is poorly understood (e.g,. James et al., 2019). However, a recent model study showed that for longer PDs, models not including behaviour overestimated the dispersal, while there was no difference for PDs on the order of one day (James et al., 2023). Predation of larvae further affects the dispersal (White et al., 2014), while habitat suitability plays a role in settlement ability (Leis and Carson-Ewart, 1999). In the Wadden Sea, most of the substrate consists of a mix of mud and sand (EMODnet, 2022), while hard substrate, such as mussel beds, oyster reefs, stone reefs and man-made structures, are distributed on a scale smaller than the model resolution, making it complicated to add to the model setup. In the current study, we thus only assess the dispersal of particles but do not include the suitability of the settlement substrate. Combining these species-dependent factors controls the particle's drift scale (Gawarkiewicz et al., 2007). Our simplified approach of passive drift in surface water means that not all scenarios are included. Yet, it gives a direct first knowledge of the role of the physical transport direction over time and applies to several species.

Quantitative information about current NIS distribution in European waters, including the Wadden Sea, is uncertain and likely biased due to, e.g., surveillance programs often monitoring in the vicinity of hot spots such as ports. Further, surveillance programs are not equally implemented in all countries, and the number of countries, including Denmark, have until recently had no baseline studies for NIS distribution (Zenetos et al., 2022). For the current study, the choice of particle release points could potentially significantly impact the particles' dispersal. However, we have based the release positions on qualitative understanding due to the limited quantitative knowledge of NIS distribution and introduction rates. Our monitoring showed that the port of Esbjerg is a hot spot for NIS introduction. This also aligns with recent monitoring of NIS in harbors (Andersen et al. 2023) and model-based studies (Hansen et al., 2020). Northward migration along Jutland's west coast is a concern (Wrange et al., 2010; Schiller et al., 2018) for NIS dispersal into the Wadden Sea. Our tracer-based study of release points does indicate that northward migration of NIS is a potential new vector into the Danish part of the Wadden sea. It is essential to acknowledge that this observation is somewhat biased by our choice of release positions.

The importance of different dispersal pathways for the introduction rate is generally associated with high uncertainty (Zenetos et al., 2022). Some studies have directly measured the number of fouling NIS and NIS in ballast water (Chan et al., 2015). However, quantifying the rate of ship-based NIS introduction into the Wadden sea requires a detailed reconstruction of individual introduction events, currently not available. Although our model-based tracer study indicates that ships can be an important dispersal vector, our modelling does not represent several species but possible scenarios of spreading under the conditions in the model. Further, the fate of NIS released from ships depends not only on currents and mixing at the release site, but also on the type and life stage of the NIS, which is not possible to predict as the residence time of a given NIS in ballast water or as hull fouling may vary widely (Chan et al., 2015). The use of pelagic durations ranging from 1 to 21 days means that our simulation covers a range of possible life stages. Pelagic release points in the southern part of the model domain gained general knowledge about possible dispersal routes of pelagic NIS species. In contrast, bottom-dwelling species, such as the two species considered in the current study, are likely to be better represented by the release points along the coasts of Sylt and Rømø. As we do not know introduction rates, particles were released at the same time every day from June to October, thus including a large range of large-scale current patterns as well release of particles throughout the tidal cycle. However, the study provides qualitative rather than quantitative knowledge about the possible dispersion of NIS in the Wadden Sea. It is an important first step in understanding the dispersal of larvae in the Wadden Sea, but it does not represent a complete dispersal view.

4.3 NIS mitigation responses

There are limited practical and cost-effective means of eradicating or controlling NIS in the marine environment following their establishment (Mustow 2021). Preventing the introduction of NIS is therefore considered the only feasible management option in the marine environment (Lehtiniemi et al 2015). This results from limited practical and cost-effective means of eradicating or controlling NIS in the marine environment, as shown by the minimal number of successful eradication attempts (Ojaveer et al. 2015). Efforts under the MSFD are therefore focused on limiting the environmental pressure of NIS by reducing the rate of their introduction and spread by managing pathways through which NIS move. A similar approach is also the main driver behind the alien species regulations (EC regulations 1143/2014).

Our review of human activities, effects and relevant mitigation measures highlighted the importance of enforcing and strengthening existing efforts. Here we relate these to the dominant pathways of NIS introduction assessed in this report. These were shipping (ballast water, hull fouling and entanglement) followed by unaided transfer (secondary spread) and intended or accidental introduction via aquaculture. Our assessment of these pathways is associated with high uncertainty. It should only be regarded as the best guess of important pathways. We based the assessment on evidence from available literature, often from neighboring countries reporting to databases such as the World Register of Introduced Marine Species or the AquaNIS website. Very few NIS have well-documented evidence of introduction pathways into Danish waters, and even less so for introduction into the Danish part of the Wadden Sea.

Nevertheless, our rough assessment of introduction pathways aligns well with results from our dispersal modelling, which highlighted that larvae from species introduced via ballast water in the proximity of the Wadden Sea area are likely to enter this area. However, the probability decreases as particles (i.e. ballast water) are released in deeper waters outside the Wadden Sea region. In addition, our tracer-based study indicated that northward migration of NIS is a potential vector into the Danish part of the Wadden sea, thus supporting that unaided (secondary dispersal) from southern locations e.g. via bottom trawling shrimp boats, could be an important pathway of introduction. Our dispersal modelling cannot be used to evaluate the importance of hull fouling as a pathway of introduction. This pathway is, however, recognized as essential for other parts of the Wadden Sea (Gittenberger et al. 2017).

Concerning these main pathways of NIS introduction, a series of mitigation measures is highlighted in this report. Here we briefly discuss the usefulness of these with respect to reducing NIS impact in the Wadden Sea.

Shipping:

- Ballast water: The International Maritime Organization (IMO) has adopted a range of measures concerning ballast water and biofouling to reduce the risk of transfer of non-indigenous species through enforcement of the International Convention for the control and management of Ships Ballast

water and sediments, 2004 (BWM convention). This targets the reduction of ballast water exchange (D-1) and the fulfilment of discharge standards (D-2). Denmark has approved the Ballast Water Management Systems (BWMS Code) since 2020. Proper enforcement is key in reducing further NIS introductions into the Wadden Sea area.
- Biofouling: The IMO launched in 2018, the "GloFouling" initiative with the aim of implementing the IMO guidelines for the control and management of biofouling (www.glofouling.imo.org). These represent a decisive step towards reducing the transfer of invasive aquatic species by ships, which currently are being developed.

Biosecurity, awareness, and monitoring:

- The most feasible and efficient measures are those to prevent the introduction of NIS in the first place, for example, through biosecurity or pathway management, awareness raising and education. Denmark should develop and enforce a national biosecurity action plan for improved management in the Wadden Sea. This should provide awareness and guidance on reducing the release and transfer of alien species, both for commercial shipping and the general public.
- Continued monitoring of NIS in the Danish part of the Wadden Sea should be established. Monitoring is also crucial to aid the early detection of new NIS, and thus, rapid response and containment. Also, monitoring is essential to evaluate the impact of NIS on the Wadden Sea ecosystem and evaluate the success of implemented management measures to reduce NIS impact. Currently, we have very little knowledge of the local impacts of NIS in the Wadden Sea, and recent reports from the German part (Reise et al. 2023) suggest that although alien marine species are very abundant in the Wadden Sea, they appear to have limited negative impact. A similar analysis for the Danish part of the Wadden Sea requires continued monitoring to facilitate a quantitative impact assessment.
- In addition to a harmonized monitoring effort, it is recommended to collaborate with local stakeholders on gathering species observation data, which may provide important data on taxonomic groups not monitored regularly such as fish and other mobile fauna.

Marine protected areas and Nature restoration:

- MPAs are created to protect marine ecosystems, processes, habitats and species, as well as provide resilience or resistance to a variety of threats. Among these are invasions by NIS (Caselle et al., 2017). It is assumed that MPAs, owing to their high species diversity and rich abundance of predators/competitors/parasites, are resistant to invasion (Francour et al., 2010; Galil et al., 2017; Gestoso et al., 2017). However, studies on the effects of MPAs as a barrier on invasion by NIS show ambiguous results (Giakoumi and Pey, 2017; Parretti et al. 2020). To the extent that the Wadden Sea can be protected better against human pressures such as pollution from eutrophication, this will likely strengthen the resilience of the ecosystem against invasive NIS.
- No general restoration plan has been developed for Danish seas, and current discussions do not consider how restoration can help mitigate NIS reductions. Restoration is, however, considered a suitable measure to strengthen biodiversity by restoring habitats, with the potential to reduce the impacts of invasive species (Gordon et al. 2020). It should therefore be considered to develop a best practice in restoration to avoid the potential

introduction or spread of NIS as related to these initiatives, for example, building on current guidance, such as the European Guidelines on Biosecurity in Native Oyster Restoration (zu Ermgassen et al., 2020).

5 Conclusions

Through a novel monitoring review of data sources, we provided a much-needed update on the number of NIS in the Danish Wadden Sea, their distribution among different habitats and ports in the area, and changes over more than 3 decades. The highest number of NIS were found in the ports and associated waterways, having a similar composition of NIS, significantly different from more enclosed areas to the south. 26 new NIS were detected for the region, with 14 species potentially new to the entire Danish waters. Most of these (12) were detected with molecular techniques and are recommended to be added to a watch list for further validation. Combining new monitoring data with data from other sources provided 82 NIS in the Danish Wadden Sea region. Half (39 species) of these were identified through the national monitoring program, and for most of these, they appeared over several years, indicating that most NIS entering the Wadden Sea develop more permanent populations there. Assessment of NIS impact based on the expert judgment from previous projects within the Danish and Wadden Sea regions suggests that 11 NIS are potentially associated with a high impact risk. Compared with the long-term observations, several of these NIS appears to be well established in the region. However, a more rigorous assessment of impact requires continued monitoring using the novel techniques applied in this study to provide quantitative and comparable data.

Dispersal modelling and review of NIS dispersal pathways for the NIS established in the Wadden Sea suggested that shipping via ballast water is an important vector for NIS introductions. Our monitoring further supported this, which identified the largest harbor, Esbjerg port, as a hot spot for NIS establishment. The model study suggests that a slow but steady settling of NIS will likely occur due to species migrating from the south and from NIS released in the coastal zone close to Grådyb or in the port of Esbjerg. However, the risk of NIS introduction from ballast water is significantly reduced if release is done outside of the 10-meter depth contour. Regarding species migration from the south, this aligns well with the identification of secondary (unaided) spread as an important pathway for more than half of the 82 NIS observed in the Danish Wadden Sea area.

Regarding management measures to reduce the rate of NIS introduction and its negative impact on the Wadden Sea ecosystem, several mitigation measures were identified. While the key measures (IMO ballast water and biofouling) are already adopted, it is worth considering if these can be enforced better and supplemented with enhanced protection and possible restoration of restricted habitats of special conservation value within the Wadden Sea area. Increased awareness of the dispersal pathways, with information to both commercial shipping and leisure boats, along with continued monitoring, are highlighted as important measures to reduce the rate of NIS introduction and assess NIS impact and effectiveness of management efforts.

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7 Appendices

Appendix 1A- C. Detailed accounts of molecular data based on *qPCR* with three technical replicates and standard rows based on *PCR produc*ts. Numbers separated with / show number of *te*chnical replicates giving*:* no *Cq, /*under *LOD / over LOD* but, *under LOQ / over LOQ*. Color code*:* white*:* no *Cq,* so no ampification in any of the samples*;* yellow*:* amplification in min. 1 < LOD; orange: min 1 sample > LOD, but < LOQ; red: min. 1 sample > LOQ; Dark grey: all samples > LOQ. LOD: Limit of detection; LOQ: Limit of quantification (Knudsen et al. 2020). *Neogobius melanostomus* not included in this study. The detection systems of *Acispenser gueldenstaedtii* and *A. baerii* could not separate the two species and they are here reported as *A. bearii.*

Appendix 1A: eDNA from water samples in tidal chanels

Species	Listerdyb_1	Listerdyb_2	Listerdyb_3	Juvredyb_1	Juvredyb_2	Juvredyb_3	Grådyb_1	Grådyb_2	Grådyb_3	Knudedyb_1	Knudedyb_2	Knudedyb_3
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
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	0/0/0/3	0/0/3/0
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	3/0/0/0	3/0/0/0	2/1/0/0
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	0/2/1/0	0/1/2/0	1/0/2/0
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	0/1/0/2	1/0/0/2	2/0/0/1
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
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
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	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	3/0/0/0	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	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	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
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	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	1/0/0/2	0/1/0/2	1/2/0/0
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
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
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	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	0/1/2/0	0/0/2/1	0/0/3/0
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	3/0/0/0	3/0/0/0
Acipenser ruthenus	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
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
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	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	3/0/0/0	3/0/0/0	2/1/0/0

Appendix 1B: eDNA from water samples in harbors

Species	Port of Esbjerg_1	Port of Esbjerg_2	Port of Esbjerg_3	Port of Nordby_1	Port of Romo_1
Bonnemaisonia hamifera	2/0/0/1	3/0/0/0	3/0/0/0	2/0/1/0	3/0/0/0
Prorocentrum cordatum	0/0/1/2	0/0/2/1	0/0/2/1	0/0/3/0	0/0/0/3
Pseudochattonella farcimen	0/0/1/2	0/0/1/2	0/0/2/1	0/2/1/0	0/1/2/0
Pseudochattonella verruculosa	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/1/2
Karenia mikimotoi	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3
Carassius auratus	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
Colpomenia peregrina	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	1/1/0/1
Oncorhynchus mykiss	2/1/0/0	3/0/0/0	3/0/0/0	2/1/0/0	2/0/0/1
Oncorhynchus gorbuscha	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Magallana gigas	0/0/2/1	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	0/1/2/0	0/0/0/3
Rhithropanopeus harrisii	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
Eriocheir sinensis	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0	2/1/0/0
Homarus americanus	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Cordylophora caspia	3/0/0/0	2/1/0/0	2/0/0/1	3/0/0/0	3/0/0/0
Mnemiopsis leidyi	0/0/0/3	0/0/0/3	0/0/0/3	0/0/0/3	0/0/3/0
Acipenser baerii	3/0/0/0	3/0/0/0	2/1/0/0	3/0/0/0	3/0/0/0
Acipenser ruthenus	3/0/0/0	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	3/0/0/0
Hemigrapsus sanguineus	3/0/0/0	3/0/0/0	1/2/0/0	3/0/0/0	2/1/0/0
Hemigrapsus takanoi	2/0/1/0	2/0/1/0	1/0/1/1	0/2/1/0	1/0/2/0

Appendix 1C: eDNA from bulk samples collected using settlement plates

Species	Port of Esbjerg_2	Port of Esbjerg_3	Port of Nordby_1	Port of Romo_1
Bonnemaisonia hamifera	3/0/0/0	3/0/0/0	3/0/0/0	2/0/1/0
Prorocentrum cordatum	0/0/3/0	0/0/3/0	0/0/3/0	0/0/2/1
Pseudochattonella farcimen	1/2/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Pseudochattonella verruculosa	3/0/0/0	0/2/1/0	3/0/0/0	2/1/0/0
Karenia mikimotoi	0/0/0/3	2/0/0/1	0/0/0/3	1/0/0/2
Carassius auratus	0/3/0/0	1/2/0/0	2/1/0/0	3/0/0/0
Cyprinus carpio	3/0/0/0	1/2/0/0	2/1/0/0	3/0/0/0
Colpomenia peregrina	2/1/0/0	3/0/0/0	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
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
Rhithropanopeus harrisii	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
Eriocheir sinensis	3/0/0/0	3/0/0/0	3/0/0/0	3/0/0/0
Homarus americanus	3/0/0/0	0/2/1/0	3/0/0/0	3/0/0/0
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
Acipenser baerii	2/0/1/0	1/0/1/1	3/0/0/0	3/0/0/0
Acipenser ruthenus	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
Hemigrapsus sanguineus	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

Appendix 2. Total list of non-indigenous species (NIS), including cryptogenic (CRY), with information on pathways of introduction, impact score and sources of observation. Pathway numbers refer to Release (1,2), Escape (3-7), Contaminant (8-11), Shipping (12-17), Unaided (18) and Unknown (20). Observation sources refer to A) CWSS, B+C) Danish official NIS list, D) NOVANA monitoring, E) NISAR project, F) MONIS4, G) Arter.dk, H) Fiskeatlas, I) qPCR this report, J) metabarcoding this report, K) Conventional this report, L) qPCR Harbor report, M) metabarcoding Harbor report, N) Conventional Harbor report. An extended table is available here

Species name	AphiaID	Group	Year of first observation in WS	Year of latest observation in WS	Pathways of introduction	NIS status	Harmonia score
Acartia (Acanthacartia) tonsa	345943	Zoo	1995	2022	19	NIS	9
Acipenser baerii	233942	Fish	2022	2022	5	NIS	4
Agarophyton vermiculophyllum	1327786	Mac	2009	2022	15, 19	NIS	12
Akashiwo sanguinea	232546	Phy	1990	2017	14, 19	NIS	
Alexandrium ostenfeldii	109712	Phy	1996	2009	14, 19	NIS	
Alexandrium pseudogonyaulax	109713	Phy	1999	2016	14, 19	Cry	
Alexandrium tamarense	109714	Phy	1990	1991	14, 19	NIS	10
Alitta succinea	234850	BI	1980	2022	20	Cry	
Amphibalanus improvisus	421139	BI	1992	2022	20	Cry	8
Antithamnionella ternifolia	163275	Mac	2022	2022	4, 13, 15	NIS	
Aphelochaeta marioni	129938	BI	1984	2022	20	Cry	10
Austrominius modestus	712167	BI	1978	2022	19	NIS	8
Bacteriastrum hyalinum	149119	Phy	1990	2016	14, 19	Cry	
Balanus glandula	394848	BI	2022	2022	15	NIS	
Biddulphia rhombus	149324	Phy	1987	2016	14, 19	NIS	
Biddulphia sinensis	148969	Phy	1987	2016	14, 19	NIS	
Bonnemaisonia hamifera	144442	Mac	2022	2022	14, 15	NIS	8
Botrylloides violaceus	148715	BI	2022	2022	4, 15	NIS	
Bugulina stolonifera	834018	BI	2022	2022	4, 15	NIS	
Caprella mutica	146768	Zoo	2005	2022	8, 14, 15, 19	NIS	7
Chaetoceros circinalis	163019	Phy	2006	2010	14, 19	NIS	
Chattonella marina	233778	Phy	2022	2022	14, 19	NIS	9
Chromis multilineata	273743	Fish	2022	2022	20	NIS	
Colpomenia peregrina	145856	Mac	2022	2022	4, 19	NIS	8
Cordylophora caspia	117428	BI	2022	2022	20	NIS	7
Coscinodiscus wailesii	156632	Phy	2014	2014	14, 19	Cry	9
Crepidula fornicata	138963	BI	1990	2022	4, 19	NIS	8
Cyprinus carpio	154582	Fish	2022	2022	1,2	NIS	6
Dasya baillouviana	144714	Mac	2022	2022	4, 14	NIS	8
Dasysiphonia japonica	836896	Mac	2022	2022	4, 19	NIS	8
Diadumene lineata	395099	BI	2017	2017	14, 15	NIS	6
Diplosoma listerianum	103579	BI	2022	2022	15, 4	NIS	9
Dipolydora quadrilobata	131121	BI	1980	2010	15	NIS	
Ensis leei	876640	BI	1981	2022	14, 19	NIS	10
Eriocheir sinensis	107451	BI	1927	?	20	NIS	8
Ethmodiscus punctiger	148941	Phy	1999	2016	14, 19	NIS	
Fibrocapsa japonica	233761	Phy	2022	2022	14, 19	NIS	9
Fucus distichus	145544	Mac	2022	2022	20	Cry	9
Haliclystus tenuis	287231	BI	2022	2022	20	NIS	
Hemigrapsus sanguineus	158417	BI	2011	2022	14	NIS	10

Appendix 2. Continued

Species name	AphiaID	Group	Year of first observation in WS	Year of latest observation in WS	Pathways of introduction	NIS status	Harmonia score	Observation source
Hemigrapsus takanoi	389288	BI	2011	2022	19	NIS	10	A,B,C,D,E,G,I,J,K,L
Heterosigma akashiwo	160585	Phy	1995	2014	14, 19	Cry	11	C,D,E
Homarus americanus	156134	BI	2022	2022	2	NIS	6	I
Hypereteone heteropoda	333652	BI	2017	2017	20	NIS		A,C,E,F
Jassa marmorata	102433	BI	2003	2022	20	Cry	4	A,C,E,J,K
Karenia mikimotoi	233024	Phy	2000	2022	14, 19	NIS	11	C,D,E,F,I,L
Lauderia pumila	163226	Phy	2008	2014	14, 19	NIS		D,E
Lepidodinium chlorophorum	345481	Phy	1999	2007	14, 20	Cry		C,D
Magallana gigas	836033	BI	2007	2022	2	NIS	12	A,C,D,E,F,G,I,J,K,L,M, N
Marenzelleria viridis	131135	BI	1990	2018	14, 19	NIS	10	A,C,D,E
Mediopyxis helysia	345485	Phy	2010	2016	14, 19	NIS		D
Melanothamnus harveyi	1027787	Mac	1986	2022	19	NIS	8	C,E,F,K
Mnemiopsis leidyi	106401	Zoo	2005	2022	20	NIS	12	A,C,E,F,G,I,J,L
Molgula manhattensis	103788	BI	1991	2022	19	Cry	11	A,C,D,E,F,J,K
Mya arenaria	140430	BI	1245	2022	19	Cry	7	A,C,D,E,G,I,J,K,L
Mytilicola intestinalis	128900	Zoo	2022	2022	11	Cry	5	J
Oncorhynchus mykiss	127185	Fish	2010	2022	4, 19	NIS	5	C,E,G,H,I
Palaemon elegans	107614	Zoo	2003	2022	20	Cry		C,G,N
Paralithodes camtschaticus	233889	BI	2022	2022	14	NIS	10	I
Peridiniella danica	233369	Phy	2004	2009	14, 19	NIS		C,D,E
Petricolaria pholadiformis	156961	BI	1987	2021	3	NIS	7	A,B,C,D,E,G,M
Phaeocystis pouchetii	115088	Phy	1987	2016	14, 19	NIS		D
Polydora cornuta	131143	BI	1981	2022	14, 15	Cry		C,D,E,F,J,K,N
Polydora websteri	153847	BI	2022	2022	14, 16	NIS		J
Prorocentrum cordatum	232376	Phy	1988	2022	14, 19	Cry	11	C,D,E,F,I,J,L
Prorocentrum gracile	110300	Phy	1990	2014	14, 19	NIS		C,D,E,M
Prorocentrum lima	110301	Phy	1990	2009	14, 19	NIS		C,D,E
Prorocentrum triestinum	110316	Phy	1992	2022	14, 19	Cry		C,D,E,J
Pseudochattonella farcimen	531467	Phy	1998	2022	14, 20	NIS	11	F,I
Pseudochattonella verruculosa	531446	Phy	1998	2022	14, 19	NIS	11	E,F,I,J,L
Rastrelliger kanagurta	127020	Fish	2022	2022	1, 19	NIS		J
Ruditapes philippinarum	231750	BI	2022	2022	9, 15	NIS		J
Saccharina japonica	377084	Mac	2022	2022	14, 19	NIS		J
Sargassum muticum	494791	Mac	1989	2021	9, 19	NIS	12	C,G
Schizoporella japonica	470388	BI	2022	2022	19	NIS		N
Spartina anglica	234043	Grass	2021	2022	19	NIS	12	A,B,C,G
Streblospio benedicti	131191	BI	2017	2022	9, 14	NIS	9	A,C,F,J,K,M
Styela clava	103929	BI	2017	2022	9, 15	NIS	10	A,C,E,F,K
Thalassiosira nordenskioeldii	148931	Phy	1993	2016	14, 19	NIS		D
Tricellaria inopinata	111254	BI	2022	2022	1, 12	NIS	9	J
Tripos arietinus	841182	Phy	1987	2009	14, 15	NIS		D,E
Tripos macroceros	841260	Phy	1991	2013	14, 19	NIS		D,E

Appendix 3. Model validation of sea level.

With a boundary towards the North Sea and a tidal range of 1.4 to 1.8 meters (Jacobsen, 1990), the water flow in the Danish Wadden Sea is heavily influenced by the large-scale circulation of the surrounding waters as well as by tides. To assess model's ability to capture the sea surface height (SSH), and thus the movement of the water in the area, observations from seven stations in the Wadden Sea (Figure A1) were obtained from a collaboration between the Danish harbor authorities and the Danish Meteorological Institute (https://confluence.govcloud.dk/display/FDAPI).

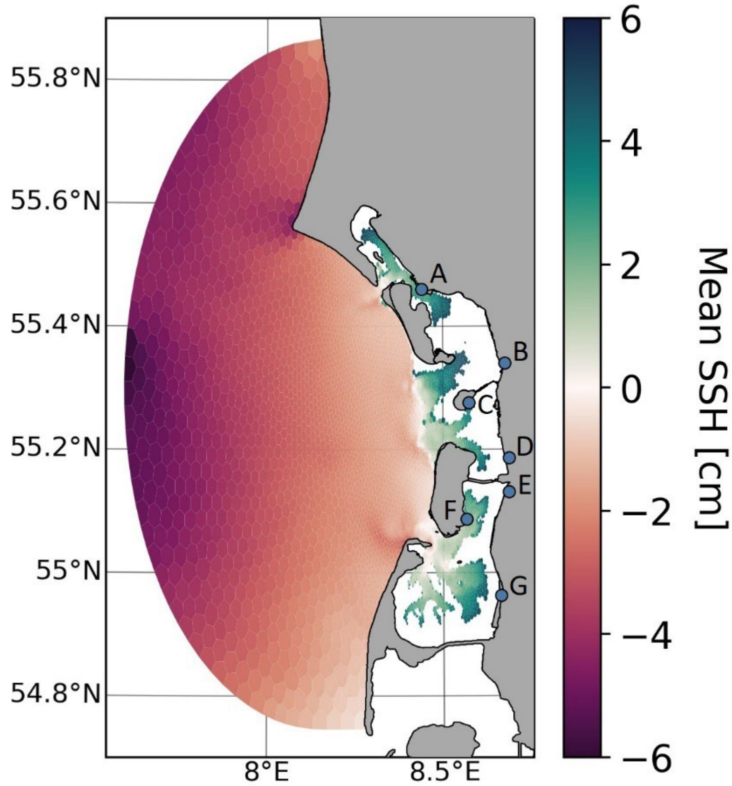

Figure A1. Mean modelled sea surface height in the Danish Wadden Sea. The dots mark the seven stations with observations of SSH: A) Port of Esbjerg, B) Kammer lock, C) Mandø, D) Brøns lock, E) Ballum lock, F) Port of Havneby and G) Vidå lock. White areas are dry areas.

The mean SSH over the year 2021 is lowest in the North Sea, and higher close to the coast where sources from land continuously supply freshwater to the area (Figure A1). The largest fresh water source in the DanishWadden Sea, Varde Å, has the outlet in the north, with the remainder scattered along the coastline. The correlation between the mean modelled and observed SSH at the seven stations is 0.731 when calculated over the spatial domain. This calculation is complicated by the stations being in dug-out waterways close to locks, which are too narrow to be resolved by the model.

To visualize the temporal evolution of the modelled sea surface height at the stations in the Wadden Sea, we have plotted the running mean calculated over a window of two days at four stations (Figure A2). The running mean removes the tidal signal and leaves the pattern of the large-scale circulation, showing oscillations with a frequency of a few weeks. The modelled SSH follows the observations with respect to both the mean and the standard deviation (Figure A2), indicating a good representation of the water movement in the area. However, the extreme peaks of high and low water tend to be underestimated at station A in Esbjerg Port and station D at Brøns Lock (Figure A2), showing that these winter events are underestimated to some degree. Statistics have been calculated for the time series at each station, with all correlations between modelled and observed values above 0.8, with the lowest (R = 0.81) being at station D: Brøns Lock and the highest (R = 0.98) at Havneby.

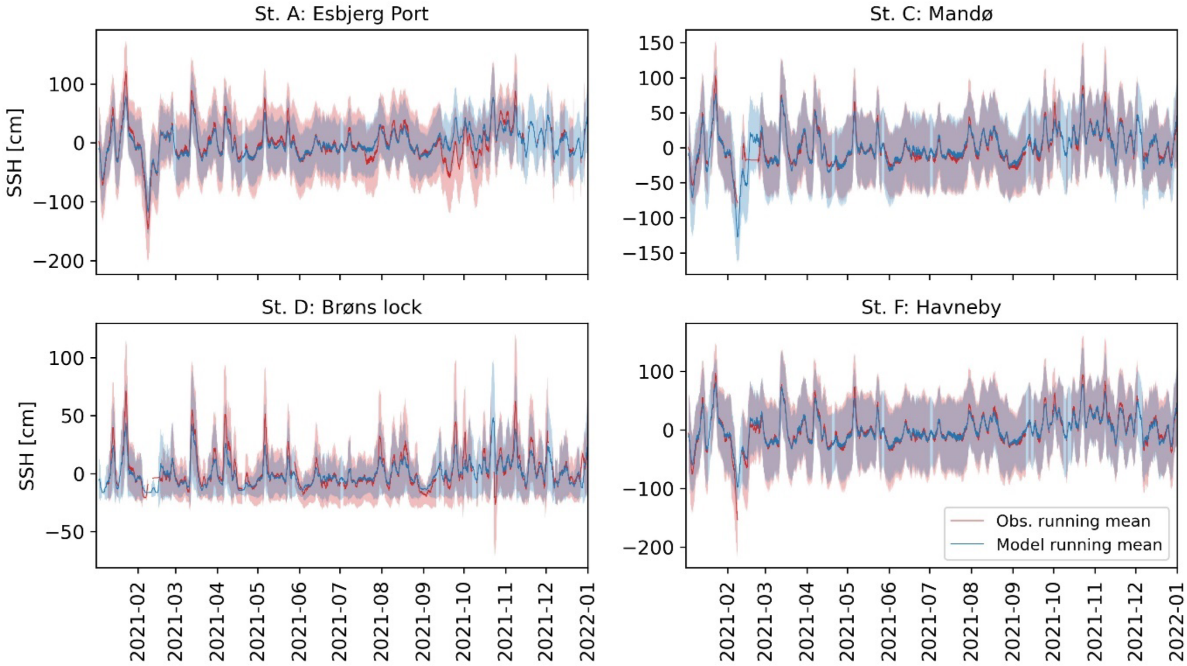

Figure A2. Seasonal development of SSH at four stations in the Wadden Sea for model output and observations. The SSH data is given with a frequency of 30 minutes, but to remove the tidal influence, it is plotted as the running mean over 48 hours with the standard deviation marked.

The normalized standard deviation is somewhat higher than 1 at station C, where the modelled change in the tides is slightly larger than the observed, while the reminder of the stations have a slight underestimation in the SSH. At station B and G, which are located close to locks on the mainland, the difference in standard deviation can partly be explained by the model not resolving the narrow channels of deeper water, allowing the location of the stations to dry out at low tide, while the observations still show lower water levels.

IDENTIFICATION, DISPERSAL, AND POSSIBLE MITIGATION RESPONSES FOR NON-INDIGENOUS SPECIES IN THE DANISH WADDEN SEA AREA

Through monitoring using molecular and conventional techniques, novel data on the distribution and number of non-indigenous species (NIS) are provided for the Danish Wadden Sea area. Review of existing data sources, including the national monitoring program for marine species, enabled a description of changes in NIS occurrences over time, and updated information on the total number of NIS in the region, for which we applied an expert-based judgement to identify high impact NIS. Combining a literature review on introduction pathways for the observed NIS with a model tracer-based assessment of NIS dispersal, helped identify the importance of different introduction pathways. This information was finally used to identify the possible and suitable mitigation responses for NIS in the Danish Wadden Sea.

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