Monitoring Groningen Sea Ports

Non-indigenous species and risks from ballast water in Eemshaven and Delfzijl

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Summary

Aim and methods
International shipping comprises an environmental risk: import of ecosystem foreign, and potentially harmful and disease-causing organisms, called non-indigenous species (NIS). One of the main vectors of introduction of NIS in ports is discharged ballast water of ships.
Especially for the Wadden Sea, this can lead to large risks if NIS establish themselves permanently and become invasive.

The Waddenfonds project 'Demonstration of Ballast Water Treatment Barge "- A sustained protection of the ecosystem in the Wadden Sea against invasive alien species and pathogens" was launched in 2013. Damen Green Solutions – coordinator- developed the 'Invasave', a ballast water treatment system on a mobile platform. The project had several additional research components related to the reduction of the introduction of alien species through ballast water in Dutch ports. One of these research components is attributed in this report.

Wageningen Marine Research was asked to perform a baseline study in the port of Eemshaven and the port of Delfzijl, managed by the port authority Groningen Seaports (GSP), in order to describe the present species community, both indigenous and non-indigenous. The results and methodologies should be supportive for any future monitoring program.
In order to evaluate the potential contribution of ballast water with the introduction of NIS, ballast water species composition was identified and compared with the species assemblage in the receiving ecosystems- in this study the harbours of Groningen Seaports (Eemshaven and Delfzijl).
In addition, an assessment of the current risk of untreated ballast water in the ports of GSP and Wadden Sea was conducted. Based on the experiences during monitoring and analyses, best practices were evaluated in order to advise on future monitoring (what, where, when).

The monitoring approach was adapted from available HELCOM/OSPAR protocols and comprised sampling in various relevant habitats (sediments, water and hard substrates, including pontoons, pillars, quays and SETL plates). A variety of techniques was applied for sampling. In June 2016, an inventory was made of the accessibility of the various substrates and a first selective monitoring was conducted. In September 2016, in both harbours replicated sampling was applied at all available substrates in order to account for variation in harbours due to harbour lay-out. Species presence and composition was analysed using classical taxonomical techniques. In a selection of samples, species were detected using DNA metabarcoding (resulting in eDNA profile) techniques.
In addition to the harbour sampling, ballast waters of three ships were sampled and species were analysed both by classical taxonomy and by eDNA profiles. The ballast waters of the ships samples were not treated, and originated from three different regions (Mediterranean, UK east coast, Rotterdam).

Results
A total of 344 species were identified in this survey (harbours and ballast waters combined), using both classical taxonomy and eDNA techniques. In the ballast water of three ships, a total of 88 species was found (both techniques combined), including 12 NIS.

In the harbour of Eemshaven a total of 262 species were found and in Delfzijl 202 species. In both harbours together, 332 unique species were identified, of which 47 are known non indigenous species (NIS). In Delfzijl 31 NIS were found, in Eemshaven 39. Figure B shows the diagrams representing the unique and shared number of species per species status (indigenous/non-indigenous/unknown) per sampled system (Ballast waters, Eemshaven, Delfzijl). A review of all detected established NIS shows that vectors of introduction does not only include ballast water, but also other shipping vectors (hull fouling), as well as fisheries and aquaculture.

Monitoring data showed a difference in species composition between the harbours of Delfzijl and Eemshaven. This can be explained by their different characteristics, both with respect to environmental conditions such as a salinity, and harbour design, such as lay-out and construction materials used. The difference in harbour lay-out also necessitated a difference in sampling intensity

and techniques between harbours, which may have added to the difference. In general, however, Delfzijl is a less biodiverse harbour, compared to Eemshaven.

Comparison and implications

Ballast water and harbours differ largely in species composition. Half of the species found in the ballast water was not observed in the much more intensely sampled harbours. Similarly, of the 12 NIS found, 6 were not yet reported from the Wadden region, or found in the harbours of GSP. The current analysis can, however, not answer the question whether the organisms found in the ballast water samples were viable individuals, eggs or larvae, or only cells (in eDNA) samples). The report provides a risk assessment of these 6 species. Some do have the potential to establish or spread themselves in the region. The Japanese mussel or Green mussel (Arcuatula senhousia), was detected in ballast water, and given its habitats requirements it is not unlikely that the species can establish in the Wadden Sea. It depends on whether the DNA fragments originated from living eggs or larvae, or dead or non-viable cells. Field observations should confirm its presence. Regarding other NIS, such as Hydroides elegans and Amphibalanus amphitrite it is more likely to observe these species near cooling water discharge points first. Depending climate change, these species might established and spread along larger spatial scales, including the Wadden Sea. If these species pose a threat for populations of indigenous species is not known.

Since the limited sampling of three ships already comprised 6 potential new NIS, the risk of species introductions via ballast water is demonstrated. The research thus contributes to a better understanding of the presence of NIS in the sea ports of Delfzijl and Eemshaven, the potential contribution of NIS posed by untreated released ballast water and the potential risk that they may have for the Wadden Sea. As such, the results contribute to the demonstration of the value of ballast water treatment systems in a regional ecological context. This knowledge demonstrates the usefulness and necessity of the use of risk mitigation measures- and thus the use of the Invasave- as ballast water treatment system. These results are consistent with the described goals and activities in the program for the Waddenfonds.

Future monitoring

Given the objective to evaluate the best practices to advise on future monitoring (what, where, when), an overview is provided on the best practices. Results showed that sampling at various habitats and substrates, using multiple techniques complement each other in detecting the variety of species and NIS. Sampling water and hard substrates contributed to highest species detection, sampling sediment is of lesser importance.

Classical taxonomy techniques and eDNA used to identify species yielded a similar number of species but neither technique detected all species. It was the combination of techniques, as well as the sampling of multiple different habitats that resulted in the overall detection of species, both indigenous and non-indigenous. eDNA analysis is a rapidly developing technique, with many possibilities but also knowledge to gain and imperfections to be aware of. This makes it a suitable technique for monitoring species that are easily missed by classical methods and also for identification of life-stages that are hard to identify (eggs, juveniles) and damaged species. The viability of species detected with eDNA was not taken into account, and should be included in upcoming studies using eDNA.

1 Introduction

1.1 Background to the problem

International shipping comprises an environmental risk: import of ecosystem foreign, and potentially harmful and disease-causing organisms, called non-indigenous species (NIS).

One of the main vectors of introduction of NIS in ports is discharged ballast water of ships. Especially for the Wadden Sea, this can lead to large risks if NIS establish themselves permanently and become invasive. Box 1 elaborates on the definitions used. Indigenous (native) species may be displaced and introduced species can disrupt ecological functions. Not only for the ecosystem of the Wadden Sea NIS can be harmful, but also for economic sectors such as mussel / oyster and clam cultivation.

The Waddenfonds project *'Demonstration of Ballast Water Treatment Barge "- A sustained protection of the ecosystem in the Wadden Sea against invasive alien species and pathogens"* was launched in 2013. The project had several research components related to the reduction of the introduction of alien species through ballast water in Dutch ports.

Several parties worked together in this project. Damen Green Solutions – coordinator- developed a mobile ballast water treatment system on a mobile platform, the Invasave. The Invasave has been tested and proven as an effective system to prevent NIS from ballast water to enter ports. The Invasave will be deployed in the ports of Delfzijl and Eemshaven in the course of 2017. Groningen Seaports (GSP) and Waddenfund wanted to demonstrate also whether the Invasave effectively protects the Wadden Sea against introductions of NIS due to ballast water releases.

In order to evaluate the potential contribution of ballast water with the introduction of NIS, it was therefore necessary to identify the organisms contained in ballast water and compare this with the species assemblage in the receiving ecosystems.

Wageningen Marine Research contributed to the project with monitoring and ecosystem knowledge.

Definitions

There are several terms that have been used to name species that are transported out of their native range to become ecological or economic problems.
In this report we use the name non-indigenous species (NIS), being synonymous with introduced, alien, exotic and non-native species. We refer to all species that have been introduced as a result of human activities be it intentionally or unintentionally. These cover both invasive alien species and the not invasive alien species.
NIS can be established (naturalised) in our ecosystems or be only present from time to time (incidental).
The term invasive alien species (IAS) is used for species, which after naturalisation spread and have an effect on native fauna and/or flora.

NIS in this report are defined as a species originally not inhabiting the Dutch coastal zone and Wadden Sea region- whether or not they have become invasive.

Native species (indigenous) are thus species, subspecies or lower taxa, occurring within their natural range and dispersal potential (i.e. within the range it occupies naturally or could occupy without direct or indirect introduction or care by humans) (IUCN Guidelines, 2000).

1.2 Aim of the study

Wageningen Marine Research was asked to perform a baseline study in the port of Eemshaven and the port of Delfzijl, managed by the port authority Groningen Seaports (GSP), in order to describe the present species community, both indigenous and non- indigenous. The results and methodologies should be supportive for any future monitoring program.
In addition, an assessment on the current risk of untreated ballast water in the ports of GSP and Wadden Sea was conducted.

The objectives of the project were:

1. To determine present species within harbour basins Eemshaven and Delfzijl.
   Determine the community and in particular, benthos, plankton and epifauna in Eemshaven and Delfzijl using classical taxonomic and modern molecular techniques. Classify species as indigenous and non–indigenous species (NIS).
2. Determine species in ballast water discharged in Delfzijl and/or Eemshaven:
   The species community within untreated ballast water from a selection of ships is determined via classical taxonomic and modern molecular techniques.
3. Assessment of potential survival of NIS
   A risk assessment for invasion to the Wadden Sea is performed based on ecological profiles of NIS in ballastwater newly recorded to GSP.
4. Evaluate the best practices to advise on future monitoring (what, where, when)

1.3 Application for the client

The research contributes to a better understanding of the presence of NIS in the sea ports of Delfzijl and Eemshaven, the potential contribution of NIS posed by untreated released ballast water and the potential risk that they may have for the Wadden Sea. As such, the results contribute to the demonstration of the value of the “Invasave” ballast water treatment system in a regional ecological context. This knowledge demonstrates the usefulness and necessity of the use of risk mitigation measures- and thus the use of the Invasave as ballast water treatment system. These results are consistent with the described goals and activities in the program for the Waddenfonds.

2 Monitoring approach

2.1 General approach

The baseline monitoring in this study aimed to monitor as many NIS in GSP possible that could form an ecological risk for the Wadden Sea. Site selection and methods were based on the draft HELCOM/OSPAR protocol for harbour sampling (HELCOM/OSPAR, 2013). The HELCOM/OSPAR protocol aims to cover each habitat present in a port, and suggests which monitoring method could be used. Due to time and budget restrictions a selection of the most effective and applicable methods were adopted from the protocol.

It is known, and also described by HELCOM/OSPAR , that a species' affinity to settle on a type of substrate depends on the species. Hence, the types of substrate available in the harbour and ability to include these in the monitoring results in a certain species observation. In Eemshaven and Delfzijl samples were taken from various substrates and ecosystem niches in order to collect as many different species as possible.

Habitats that could be distinguished in the port of Delfzijl and Eemshaven are illustrated in Figure 2. For each habitat, suitable and effective sampling techniques were determined, resulting in various options per substrate type. Harbour size and design was an additional key factor in selection of locations. Not all substrates were present in each harbour or basin.
Species composition of all samples and sites was assessed by means of classical taxonomic identification, and where possible a selection of samples was assessed by means of DNA barcoding.

Relevant habitats included in the study were:

- Sediment: to study infauna
- Water column: to study the plankton community
- Hard substrates: harbour quays, floating docks, dike and pillars to study the (sub-) littoral zone of different substrate types (concrete, wood, steel, ballast)
- SETL: depth integrated fouling plates facilitating new colonisation

2.2 Monitoring design

2.2.1 Location selection

On June 12th and 13th 2016, Eemshaven and Delfzijl were screened in order to design a monitoring strategy for the monitoring in September. In this section a brief overview of methods is presented.

Eemshaven and Delfzijl monitoring designs differed in strategy because of differences in the basic landscape/design of the harbours.

Eemshaven can be described as a relatively open harbour. The harbour basins differ in age, and thus successive state. The harbour has an relatively open structure and the tidal influence reaches all basins. The harbour is connected with the Eemskanaal by the Doekegatkanaal (200*15 m, and depth -15 m NAP).
Beatrixhaven, the basin located most north, contains the most recent constructed quays. Also a floating dock was placed in 2015. The Julianahaven basin with a depth of ~ 12,5 m, has 1200 m long quays and has a ~250m width. Emmahaven basin lays south of Julianahaven and most ancient basin. Its length is 600m, width of 130 m and depth of 8 m. At the south-side of the Emma basin, a floating pontoon of ~225 m is located. The Wilhelminahaven basin was not included in the survey because of planned reclamation activities during this survey.

Eemshaven monitoring design aimed at covering the successive states of the different basins (Emma being most ancient, Beatrix most recent, Juliana middle), covering a variety in observed substrate

types (steel, concrete, basalt), and monitoring depths. Within the harbour, pillars, quays, floating docks and dikes were identified as the most common hard substrates present. These consisted of basalt (dike) and concrete and steel surfaces (docks, quays, pillars).
The water column was assumed to be homogeneous because of the effect of the tide throughout the harbour. Sediment characteristics and successive state were assumed to be homogeneous because of the dredging frequency twice a year. Salinity of Eemshaven is approximately ~28 ppt .

The **Port of Delfzijl** consists of an outer basin and an inner basin. The eastern part of the port is destined for professional shipping and the western part for recreational shipping. The inner basin was not included in this study. The Handelshaven is accessed via the 6km long Zeehavenkanaal. The north side of this canal consists of a breakwater, which could not be accessed for the survey due to exploitation of wind turbines. The south side was accessed via public road.
The inner basin and recreational harbour are influenced by inland freshwater due to operational sluices. Along the Handelskade and Zeehavenkanaal multiple transhipment locations are found. The Zeehavenkanaal is connected to the Eems, and is influenced by tide. Salinity ranges from fresh-brackisch near the sluices, and ~22 ppt near the Eems. Salinity in the channel also varies in depth, depending on the tidal sequence. The Zeehavenkanaal is dredged ~40 weeks a year, and it is assumed this results in an unstable sediment layer in which stable benthic communities cannot establish.
Delfzijl harbour was assumed to be heterogeneous in physical-chemical characteristics, affecting the diversity in species composition that can be found along this spectrum. The monitoring design is adapted accordingly.
Sites were selected along 5 transects along the canal from fresh-brackish to more saline near the Eems.

2.2.2 Applied methods

Sampling was conducted from the shore (Emmahaven pontoon, Delfzijl Jachthaven, dikes) or on board of the Havenschap 1 (harbour vessel of Groningen Seaports) and a RIB of UKMS[1] in order to approach offshore locations. Havenschap 1 facilitated in sediment sampling using the on board available lifting winch and a grap (Van Veen).

2.2.2.1 Water sampling and physico-chemical parameters

At the selected locations water was sampled at ~1m depth (Figure 1). Water was sampled using a depth integrated ball valve water collector. An integrated 1 meter depth water sample is taken containing ~7 liters per dip. In total 20 L was sampled, over 3 dips.
From the collected water, a 100 ml sub sample is taken for phytoplankton (conserved with 1% lugol), a 25 ml sample for UV light transmittance (UVT) (transparency at 254nm). Basic water quality measurements were done directly on site using a Hach HQ40d multimer (O2, salinity, temperature) and Mettler Toledo SevenGo handheld with glass electrode (pH). The remaining water (10 L in June, 20 L in September) was poured over a plankton net of 55 µm in order to collect all zooplankton. The zooplankton was transferred into a 100 ml glass jar and conserved (1% lugol).

2.2.2.2 Sediment sampling

Sediment sampling was conducted using a so called Van Veen grab. Samples of ~2 litres per grab were collected, per grab covering a surface of ~280 cm^2. Per location, a total of 3 grabs were taken, resulting in a sample of ~6 litres, depending on the material.
On deck each grab was placed above a sieve with a mesh size of 1 mm and opened. Prior to rinsing, sediment was mixed by hand (using a spoon) and two sub samples were taken with a spoon to collect samples for metabarcoding (environmental DNA: eDNA). Sub samples from all locations were pooled into one sample per harbour.
The remaining material was rinsed with sea water, to remove sand and clay particles. From the remaining material (biota, shells, stones and other particles) a photograph was taken, after which the sample was stored in a polyethylene container. The sample was preserved with 6-10 % buffered formaldehyde in seawater solution.

[1] Ubels Klus & Maritieme Service

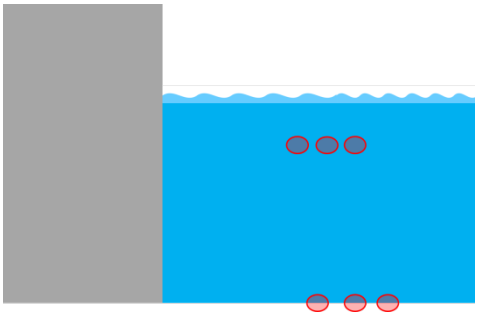

Figure 1. *Overview of sampled habitats. Water column and sediment.*

2.2.2.3 Hard substrate sampling

Hard substrate was sampled in various habitats using various techniques.

SETL plates: In summary, SETL (fouling) plates are grey PVC plates which are usually deployed at a depth of 1 meter and checked for fouling species after three months.

A SETL plate is constructed by rope (ø 0.5 cm), a grey 15 cm x 15 cm PVC plate and a brick. Each plate was sanded prior to deployment to provide a more effective settling substrate for organism. A hole (ø 0.5 cm) was drilled at the centre of each plate for the rope. Plates were secured to the rope. The plates were secured at various rope lengths in order to deploy plates at various depths.
In this survey, at each location, at least three plates were deployed at -1m depth ~15 m apart from each other. Locations were various dock structures where they would not be disturbed by for example port activities. Depending on the location, additional plates were also deployed at -3m, -5m and -10 meter depth (measured from the water level) (Figure 2). If plates could not be attached to floating devices, and thus an assured fixed depth, plates were attached to fixed structures and an average water depth was estimated.

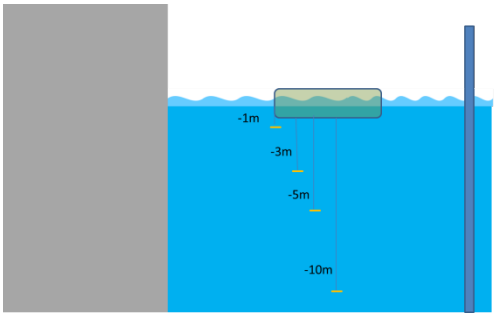

Figure 2*. Hard substrate sampling using SETL plates at various depths.*

SETL plates were deployed in both March and June 2016 and retrieved in June (March deployment) and September (June deployment) 2016. March deployments consisted of -1m plates only. SETL plate data from previous years (2009-2015) in Emmahaven were used to compare fouling species communities over the years.

At retrieval, plates were carefully pulled on the dock to prevent losing organisms such as mobile epifauna. The whole units (plates, bricks and ropes) were placed in plastic white containers and the ropes and bricks were separated from the plates. The plates were gently washed to discard settled organic material. Plates were positioned with the fouled bottom upwards and left for an hour to settle. Each plate was digitally photographed in overview, and every species on a plate was photographed in detail. Smaller algal specimens or unknown species were preserved on 4% formaldehyde and identified in the laboratory at a later time.
Thereafter, a selection of plates was placed in labelled plastic bags and conserved with ethanol in order to conduct DNA analyses. Selections were made based on the inclusion of plates from -1 from each basin and a selection of plates from 1 site comprising plates from various depths (-1, -3, -5m)

Identification of organisms on each photo was done by reviewing the photos on the computer. The overview photos were digitally subdivided in 25 equal grids, and every species per grid was scored. Only taxa up to species level were scored, all other higher levels (family, genus) were not scored.

In total 47 SETL plates were deployed in Eemshaven en 15 plates deployed in Delfzijl. Besides the traditional -1 m deployment, additional plates were deployed at -3, -5 and -10 meters.

Ponton snorkeling:

On the 9th of August 2016 a survey was done by a snorkeler (Figure 3) of the species growing on the floating dock in the Emmahaven. In three inner compartments of the dock, species were scored. While searching, each “new” species that was recorded was photographed and noted (by a colleague on the dock), after which the search continued focusing on finding additional species. Each of the sides of a compartment (north, west, south and east) was searched until less than one extra species was expected to be found on that side within double the search time. Each compartment, of about ~2.5 x 2.5 m, was searched for at least half an hour. Species were digitally photographed with a 21.1 megapixel camera Canon EOS 5D Mark II within an underwater housing.

Figure 3*. Inventory of floating dock by snorkelling.*

Dike littoral zone:

The littoral zones of several dikes were monitored at low tide. On the 9th and 10th of August 2016 the littoral zones on the dikes were searched for species in four locations in the Eemshaven region and in four locations in the Delfzijl region.

A methodology for monitoring the littoral zone of the dike is not described in the HELCOM/OSPAR protocol. The method used was based on the monitoring method with scuba-divers that is described in the HELCOM/ OSPAR protocol for the sub-littoral zone of the dike and on a methodology used in three alien species focused surveys that were done throughout the Wadden Sea, including the Eemshaven, in 2009, 2011 and 2014 (Gittenberger et al. 2010, 2012, 2015). In each clearly distinguishable littoral zone on the dike each “new” species that was recorded was photographed and noted, after which the search continued focusing on finding additional species.

In the Eemshaven, each clearly distinguishable littoral zone on the dike was searched. Dikes in the Delfzijl region were very homogeneous and therefore no distinction was made between zones on the dike. Every zone (Eemshaven) and every dike location (Delfzijl) was searched for at least half an hour after which the search was continued until less than one extra species was expected to be found within double the time searched. This was done within a time period of about 1.5 hour before and 1.5 hour after low tide. Most species could be identified in the field.

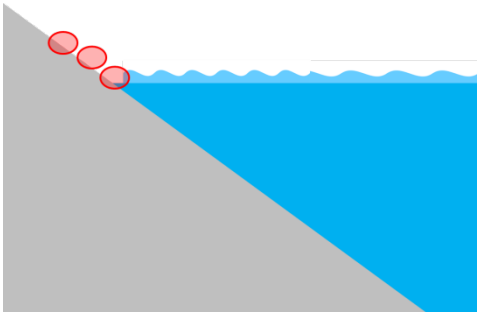

Figure 4. *Overview of samples zone at the dike, littoral zone.*

Figure 5. *Sampled littoral zones in Eemshaven.*

Scraping sub-littoral zone

In June the variety of hard substrate types in each harbour was identified, and included in the monitoring of September. Of each hard substrate type at least 3 samples of the sub-littoral zone were taken if possible 15 meter apart at each location. Sampled hard substrates in Eemshaven included concrete quays, and steel pontons and pillars. Sampled hard substrates in Delfzijl included wooden pillars, steel pillars, concrete quays and steel docks (Figure 6).

Instead of using SCUBA to scrape the substrates at various depths, only the sub-littoral zone up to -1 m is sampled. Depth integrated fouling is included in the SETL plate methodology.

Hard substrates were scraped at low tide to collect organisms. A macrofauna net with a 2-3 mm mesh and a 25 cm frame width, provided with a scraping blade was used to scrape surfaces vertically from a depth of 1 meter up to the waterline. All collected organisms were put in a labelled container and preserved with 8 % buffered formalin. All anemones in the sample were first put in an oversaturated methanol solution in order to keep them “open” to aid identification. When anemones were open, they were also preserved in formalin and identified within 2 days at the laboratory.

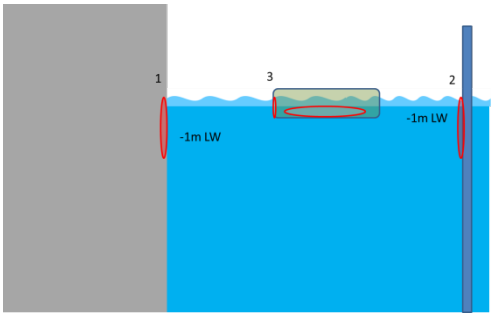

Figure 6. *Quays (1) pillars (2) and floating pontoons (3) by means of scraping the sublittoral zone (-1m).*

Figure 7. *Scraping the large dock in Delfzijl.*

eDNA sampling and conservation
DNA samples were collected during the sampling of the other samples.
Water samples of 1 litre were collected in 1L 10% chlorine and milli-Q desinfected jars and filtered over (pre-rinsed chlorine + Milli-Q) a Life Science Supor filter (450 Grid 47 mm; 0.45 um). Filters were individually stored in greiner-tubes at -20ºC during the field visits, and at -80ºC in the laboratory prior to sequencing.

Hard substrate scrape samples were collected in plastic ziplock bags and preserved with 98% ethanol and stored at -80º in the laboratory prior to further steps.

Sediment subsamples were taken at each (sediment) sampling location and pooled into one sample per harbour. Sediment samples were preserved with 98% ethanol and stored at -80º in the laboratory prior to further steps.

2.3 Sampling effort per harbour

Harbour layout, and availability of substrates resulted in a difference in sampling effort between the two harbours (see rationale 2.1). In Table 1 an overview is presented of the number of samples taken per harbour and sample type. In Figure 8 sampled locations are presented. Availability of substrate types per harbour determined the monitoring design, resulting in variability in scrape samples numbers per harbour and per substrate type. The variability in substrate type results most likely in a higher variability in species. But this also means that differentiation into the substrate type (wood, steel, concrete) results in too limited samples per harbour and basin to conduct proper statistical analyses in order to differentiate per basin, type of substrate and the combination of these factors. However, the main aim was to identify as many species within the whole of the harbour basin, in order to aid the risk assessment of species found in ballast water. For this reason, not only results from the current SETL-deployments were used, but also data collected in the Eemshaven and Delfzijl since 2009 by GiMaRIS during three alien species focused surveys targeting all habitats (Gittenberger et al., 2010, 2012, 2015) and the continuous monitoring project SETL focusing on aliens in fouling communities (Gittenberger et al., 2017).

***Table 1**. Overview of samples taken in both harbours per substrate type, method and month. HS= Hard substrate; FD= Floating Dock; * : Dike samples in Eemshaven are all separated per zone, in Delfzijl all zones per location are pooled.*

Sample type	Month	Eemshaven	Delfzijl
HS FD steel scrape	September	6	0
HS FD steel snorkel	September	12	0
HS Quay concrete scrape	September	6	3
HS Quay steel scrape	September	0	3
HS Pillar wood scrape	September	0	3
HS Pillar steel scrape	September	3	3
HS Dike *	September	10	4
Sediment	September	10	15
SETL	June	15	0
SETL	September	32	15
Water	June	3	5
Water	September	3	5
SUM		**100**	**56**

***Table 2.** Overview of eDNA samples taken.*

	total	Month	Locations
Water	12	June + September	Eemshaven (all 3) Delfzijl (3: 2 outer + 1 middle section)
Sediment	2	September	Pooled sample in each harbour, taken from individual grabs
Scrape	3	September	Eemshaven: Emma basin
SETL plates	6	September	Eemshaven: Emma basin: 2 plates pooled[2] Emma basin: 4 plates pooled[3] Juliana basin: 3 plates pooled Beatrix basin: 4 plates pooled Delfzijl: Yacht harbour: 3 plates pooled Aldel location: 3 plates pooled

[2] Depending availability the number of plates pooled into an eDNA samples varied
[3] Sample with plates from -1/-3/-5 meters. All other pooled samples contained plated from -1 m.

Figure 8. *Sampled locations and matrices/techniques in Delfzijl (upper figure) and Eemshaven (lower figure).*

2.4 Ballast water sampling

Ballast water of three ships was sampled in the period June-September 2016. Selection of vessels was done using the website of Groningen Seaports (https://pc-gsp.com/public/visits/), providing information on all incoming vessels and their origin. Through contact with agencies and captains, vessels with ballast water on board (to be discharged in Eemshaven or Delfzijl) could be addressed. Permission to visit and collect ballast water followed accordingly for some of the contacted vessels. Table 3 presents the specifications of sampled ships. Ship names are not included in this report.

The different origin of the ships' ballast water and various time periods needed to arrive in GSP (holding times) resulted in a variety of detecting possible NIS originating from different regions.

Table 3. *Specifications of sampled ships for ballast water.*

Ship	BW origin	Region	Time underway	Sampling Date
Ship 1	Tunesia- Rades	Mediterranean	Intake 1/7 (14 days)	15/7/2016
Ship 2	UK- Tilbury	North Sea- UK estuary	Intake 25-26/7 (1 day)	26/7/2016
Ship 3	NL- Rotterdam Waalhaven	North Sea	Intake 4/9 (1 day)	5/9/2016

At all three ships a total of three separate ballast water tanks were sampled, resulting in three 20 L water samples per ship, using 20 L chlorine pre-rinsed jerry cans to collect the water. The methodology of sampling depended on the possibilities per ship. Ballast tanks of ship 1 were sampled through the air channels of the tanks, using a small water pump and pre-rinsed water hose with 1 cm diameter. Ballast water of ship 2 was sampled by Marine Eco Analytics (MEA), using direct outlets at the ballast tanks. Ship 3 was sampled by letting down a bottle sampler carousel with 3 pre-rinsed 1 L bottles through man holes.

Photo 1*. Left: sampling vessel 3 using the carousel in manholes; Right: sampling vessel 1 via air channels.*

In the laboratory, phyto- and zooplankton samples were taken from each jerry-can and preserved with lugol. DNA samples were prepared by filtering 350 ml from each jerry-can onto a filter which was stored at -80 °C prior to DNA preparations.
UV Transmission (UVT) and chlorophyl were measured in the laboratory as an indication for respectively the clarity and amount of phytoplankton in the water. Ballast water from ship 1 and ship 2 contained very low chlorophyll levels, indicating almost no presence of algae. This ballast water was, therefore, put onto a marine growth medium to study the ability of phytoplankton present to grow. Based on growth and growth characteristics within a week of culturing these samples, samples of cultured ballast water were sent to be analysed for plankton as well (2 different cultures from the ship1, 1 culture from ship 2).
A total of 9 phytoplankton and zooplankton samples and 3 DNA samples resulted from the ballast water sampling.

2.5 Species analysis and data handling

2.5.1 Hard substrate species

Scrape and sediment samples were processed at the Wageningen Marine Research laboratory. Prior to identification, the formalin preserved samples were sieved over a 500µm mesh sieve and rinsed with seawater. Fauna present was first sorted into major taxonomic groups, and thereafter identified to the lowest possible taxon using a binocular (Zeiss Stereo Discovery V8, max. zoom: 80x). The number of individuals per taxon was noted. Identification was aided by various applicable determination keys and by the World Register of Marine Species (WoRMS Editorial Board 2016) as taxonomic reference. Finally, each sample was preserved in an alcohol solution (70% ethanol, 3% glycerol).

Snorkel inventory:
Although most species were identified in the field and from the photographs, species that could not be identified in the field were collected and preserved on either ethanol 96% (animals) or formaldehyde 4% (algae). They were later identified to the species level in the GiMaRIS laboratory using a digital microscope (DinoLite AM7013, max zoom: 250x), a microscope (Leitz Ortholux II. max zoom: 1000x) and/or a stereo microscope (Wild Heerburgg MS-26, max zoom: 80x). Identification was aided by various applicable determination keys, e.g. “Handbook of the marine fauna of NorthWest Europe” (Hayward & Ryland, 1995), in combination with the Synopses of the British Fauna (New Series), published by The Linnean Society of London and the book series “Seaweeds of the British Isles” published by The Natural History Museum of London. The World register of Marine Species (WoRMS Editorial Board 2016) was used as a taxonomic reference.

Dike inventory
Organisms that could not be identified in the field, were collected and preserved on either ethanol 96% (animals) or formaldehyde 4% (algae). They were identified to the species level in the GiMaRIS laboratory using a digital microscope (DinoLite AM7013, max zoom: 250x), a microscope (Leitz Ortholux II. max zoom: 1000x) and/or a stereo microscope (Wild Heerburgg MS-26, max zoom: 80x). Identification was aided by various applicable determination keys, e.g. “Handbook of the marine fauna of NorthWest Europe” (Hayward & Ryland, 1995), in combination with the Synopses of the British Fauna (New Series), published by The Linnean Society of London and the book series “Seaweeds of the British Isles” published by The Natural History Museum of London. The World register of Marine Species (WoRMS Editorial Board 2016) was used as a taxonomic reference.

2.5.2 Plankton analysis

Plankton samples were analysed at Koeman and Bijkerk Laboratories, using standard protocol MET-001 (phytoplankton) and protocol SPEC_ZP marien analyse ((micro)zoöplankton). Taxonomic nomenclature used is in accordance with the Taxa Waterbeheer Nederland (TWN).

2.5.3 Taxa data handling

Data usability in this study depends on the taxonomic level the species could be identified[4]. Only at the species level, a species can be further tagged as being Indigenous or Non-Indigenous. At genus or family level it becomes less sure whether the taxon is indigenous or not. See Figure 9 for an example of taxonomic classification for Cod.

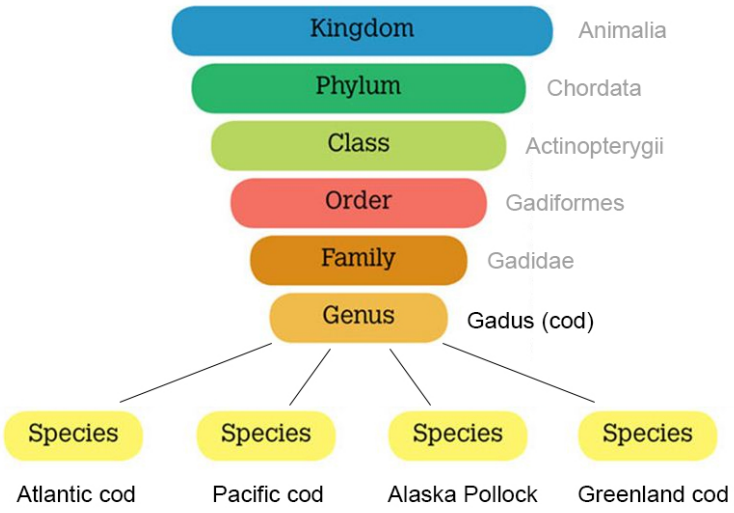

Figure 9. *Example of taxonomic classification for Cod species.*

[4] In biology, a taxon (plural taxa) is a group of an organism or organisms seen by taxonomists to form a unit. One common way of classifying living things is based in the Linnaeus System of classification which puts organisms into Taxonomic Groups that indicate their natural relationships. Depending the level of classification in the laboratory organisms can placed into the eight taxonomic groups: Domain, Kingdom, Phylum, Class, Order, Family, Genus and Species.

Available data presented in this study are by definition all “taxon”, but not all taxa could be identified up to species level. This depends e.g. on the life stage of a specific organisms in the sample or integrety of the organism within the samples[5]. Data from sediment and hard substrate (scraping) are identified including family genus or species levels. Data from the hard substrates that were surveyd, i.e. on SETL plates, platforms (snorkeling) and dikes, are only reported when a taxon could be identified up to the species level.

All taxa classified up to species level are included in the assessment and scored “Indigenous” or “Non-Indigenous”. The latter is done by comparing the benthic species lists with available data sets of Dutch or European Non-Indigenous species. Plankton taxa are also compared with the known data bases. Taxa from ballast water sampling are also compared with available species lists from the Dutch coastal zone (see Table 4 for an overview). Plankton taxa are often classified up to a level higher then species. In this case, the family or genus level is taken and assessed to be present in the Dutch coastal zone. If not, the genus or family is tagged ”Non-Indigenous” for the Netherlands too and presented to plankton experts of Koeman & Bijkerk to evaluate whether or not the taxa are known for Dutch waters.

Table 4. *Overview of screened databases and species lists.*

Database	Reference
Daisy (Delivering Alien Invasive Species Inventories for Europe)	http://www.europe-aliens.org/speciesSearch.do
EASIN (European Alien Species Information Network)	http://alien.jrc.ec.europa.eu/SpeciesMapper
Nederlandse soortenlijst	http://www.nederlandsesoorten.nl/content/voorkomen
species list of microzooplankton in Dutch waters	Verweij et al (2013)
species list of phytoplankton in Dutch waters	Brochard et al (2013)
World Register of Marine Species (WoRMS)	http://www.marinespecies.org/
algaebase	http://www.algaebase.org/

2.6 Statistical analysis

2.6.1 Species accumulation curves

Species accumulation curves (SACs) are graphs presenting the cumulative number of species observed in a particular environment as a function of the cumulative effort expended searching for them. In this study the effort is expressed as the number of collected samples.

The species accumulation curve increases with the number of samples taken. The rate of increase will slow down after a certain number of samples as at that point all common occurring species (present in most samples) are included in the curve while the more rare species (not present in most samples) are still missing. Rare species are more difficult to detect (= requires more samples to grasp them), so at that point forward more samples are required to increase the total number of species with a similar unit. When the curve flattens (becomes a horizontal line, and does not accelerate anymore) all or close to all species are detected that could have been detected during the monitoring effort (time spend). From that point onwards it is less effective to take additional samples. The curve can be extrapolated by fitting a non-linear regression model or by eye to inteprete this effectivity of additional sampling. Here the non-linear model 'Lomolino' was used for the interpolation of the species accumulation curves (Lomolino, 2000, Dengler 2009), but data on additional sampling effort in number of samples are not presented. Analyses were carried out in the software package R (R Core Team, 2016), using functions available in package 'vegan' (Oksanen, 2017).

[5] If an organism is missing certain body parts it cannot always be identified up to species level

In this study, the SACs are used to estimate the effectiveness of each applied sampling method in order to advise on future monitoring design and effort.

2.6.2 Multidimensional scaling

Multidimensional scaling (MDS) is a way of visualizing the level of similarity of individual samples within a dataset. Non-metric multidimensional scaling (nMDS) is an indirect gradient analysis approach which produces an ordination based on a distance or dissimilarity matrix. The nMDS-plot provides a visualization of the information hidden in the data, in particular to display the information contained in a distance matrix.

Data from all samples were analysed to test whether there were differences that may distinguish communities, and if so, whether these are driven by substrate type, technique and season (water only).

2.7 DNA metabarcoding

In this study, we included DNA metabarcoding to contribute to the development of this innovative technique and to assess its added value for monitoring of the species richness of ballastwater and harbour habitats.

Metabarcoding has recently emerged as a potentially significant tool to assess aquatic species compositions. In such montoring, genetic material shed by organisms, hereafter referred to as environmental DNA (eDNA), is collected and analysed in order to identify the species that belongs to it. As long as genetic material (skin fragments for instance) is present in a sample a species may be succesfully identified. The method does not require the collection of the whole species, and the species does not have to be at the exact location of sampling. Therefore, the detection rate of eDNA-based monitoring may be higher than that of classic monitoring methods (e.g. collecting of individuals and visual observations). Therefore, monitoring based on eDNA has been conducted to detect rare and invasive species and also to describe biodiversity (Herder et al., 2014). Identification of species by visual inspection of individuals is hampered when an individual is not fully grown (in larval stage for instance) and species specific characteristics are still absent preventing a 'full' determination up to the species level. Especially NIS are prone to enter a new enviroment in larval or egg stages and by using DNA techniques this does not hamper a succesful identification. These DNA-techniques can not distinguish between the presence of living and dead organisms however, and the material of the species recorded may not occur at the sampling location. It may come from somewhere upstream or locally from dead organisms in ballast water discharged in the harbour. In the discussion session we will elaborate more on this topic.

After extraction of the DNA from a sample the conservative region of a gene is used as a binding site of a man made 'universal' primer sequency. Once the primer is attached to the target region, this pre-selected DNA fragment initiates the Polymerase Chain Reaction (PCR), copying the DNA of the target region. PCR starts from the universal region, continuing into the species specific region of the gene. As the starting site is not species specific, DNA from various species is amplified in this step and the PCR product will now contain a mixture this barcode fragment originated from multiple taxa. A high-throughput sequencing method (next-generation sequencing or NGS) is then applied to simultaneously determine the order of the bases (Adenine, Cytosine, Guanine and Thymine) of each of these thousands of fragments in the mixture. A bioinformatic pipeline is then used to cluster these sequences and match the unique sequences of each fragment against the reference dataset. This reference dataset contains the sequence of known species. By coupling of the sequences contained in the collected sample with the sequences stored in the reference database unknown sequences can be identified and a list of taxa present in the sample, and the number of fragment copies present per taxon can be constructed. A schematic overview of this procedure is provided in Figure 10.

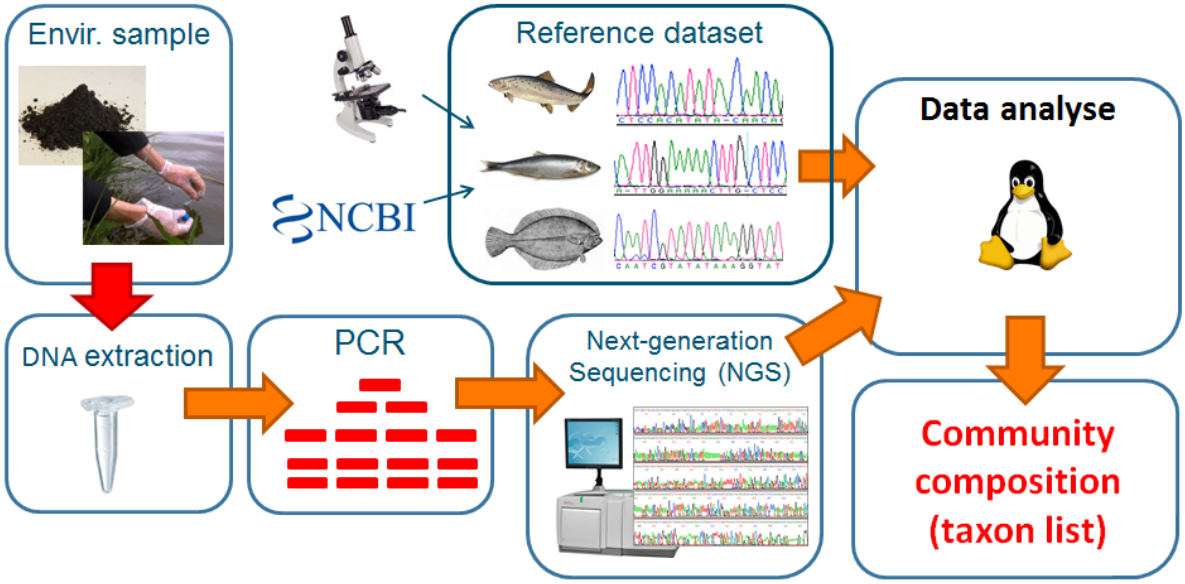

Figure 10. *Overview of the DNA metabarcoding procedure.*

2.8 Lab procedure metabarcoding samples

2.8.1 DNA extraction

DNA was extracted from 16 water samples, 2 sediment samples, 3 hard substrate samples and 6 SETL-plates, using different procedures per sample type. The whole DNA extraction process was performed at a extraction laboratory dedicated to processing of environmental DNA (eDNA) samples, available at Wageningen Environmental Research.
1L water samples were filtrated on the boat directly after sampling. In June 2016, Pall Super-450 filter membranes were used. Due to a very small pore size of these filters (0.45 µm), filters rapidly got cloaked, and up to three filters had to be used to filter the total volume of water. In September, this problem was avoided by switching to Whatman Cyclopore polycarbonate filter membranes with a pore size of 1.0 µm. Filters were stored in 2ml tubes at -80 °C upon DNA extraction. DNA extraction was performed for each individual filter using the DNeasy Blood & Tissue Kit (Qiagen) in combination with Qiashredder homogenizer columns (Qiagen). In case DNA extracts from multiple filters were available for the same 1L water sample, these DNA extracts were pooled, resulting in one pooled extract per water sample for further processing in the PCR amplification.

Sediment samples, SETL plates and hard substrate samples were stored in 96% ethanol at -80 °C, moved to -20 °C the day before DNA extraction and allowed to thaw at room temperature just before DNA extraction. In the case of sediment and hard substrate samples, all material was transferred to a glass flask. In the case of SETL plates, all tissue material was scraped off the surface of both sides of the plate and transferred to a flask. An additional volume of 100ml ethanol was then added to the flask, after which the total content was homogenized using a titanium grinder. Flasks were kept on ice throughout the procedure. After processing of each sample, the grinder was thoroughly cleaned, submerged in bleach and then rinsed with demineralized water. 8 gram of homogenized material was then transferred to a 50ml tube, and subjected to DNA extraction using the PowerMax Soil Isolation kit (MoBio). All DNA extraction steps were performed on ice. All DNA extracts were then subjected to an extra cleaning step to remove remaining substances that might inhibit PCR amplification. For this purpose, we used the OneStep™ PCR Inhibitor Removal Kit (Zymo Research).

2.8.2 PCR amplification

In the current analyses, we used two different barcode regions (DNA fragments). The first is a fragment located in the V4-section of the 18S region of the mitochondrial DNA. Primers for this region (TAReuk454FWD1 and TAReukREV3, developed by Stoeck *et al.*, 2010), amplify a fragment of 416 base pairs, and target all eukaryote organisms. This allows a broad screening of the diversity of all fauna, plants, algae, protists and fungi in the samples. The discriminatory power within these groups

is, however, relatively low. Therefore, we also used a second barcoding region, targeting only the faunal groups, and allowing discrimination of faunal taxa at a higher taxonomic resolution (up to species level). This concerns a fragment of the cytochrome oxidase 1 gene (CO1). Primers for this region (mlCI1intF and jgHCO2198) were developed by Leray *et al.* (2015) and target a fragment of 313 base pairs.
PCR amplification of both markers was performed using largely the same PCR protocol. PCRs were carried out in a volume of 25 µL, using the Supreme NZYTaq 2x Green Master Mix (NZYTech). The reaction mixture was incubated as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C (18S) or 58 °C (COI) for 1 min, 72 °C for 30 s, and a final extension step at 72 °C for 10 minutes. A negative control that contained no DNA was included to check for contamination during library preparation.

2.8.3 Sequencing

Next-generation sequencing of the PCR products was performed by AllGenetics (www.allgenetics.eu; A Coruña, Spain). Before the actual sequencing, a second round of PCR was performed on the PCR product of each sample, to attach a unique index sequence to the DNA fragments, allowing separation of data from different samples in the later bio-informatic analysis. The resulting indexed products were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). Then, DNA concentrations of all products were determined using a Qubit dsDNA HS Assay, after which all samples were pooled in equimolar amounts. The resulting pool was sequenced on a single was sequenced in a single MiSeq PE300 run (Illumina).

2.9 Literature review

Comparison with literature and previous monitoring in the Wadden Sea region was conducted in order to provide additional information on the observed NIS concerning their origin, establishment and invasive potential. Additoin review was performed to discuss the detection techniques (classical taxonomy versus eDNA).

2.10 Result presentation

Obtained data in this survey resulted in a large amount of possible comparisons, possibilities in detailed elaborations of data presentations and analysis. Only a selection of analysis is presented in this report aiming at providing an overview of:

- Number of species found, number of NIS found, in both the harbours and ballast waters
- Comparison of harbour species list and ballast waters to provide information on the differences
- Providing an overview of NIS found in harbours, contributing NIS found in ballastwater, and identification of the risks of these “new” NIS

The above is done based on species lists obtained from both classical taxonomy and eDNA together. In addition, data from classical taxonomy only were used to analyse the effectivity of monitoring techniques.

3 Survey results

3.1 Summary

In this section, an overview is provided on the total of species observed, by the both techniques applied (classical and eDNA). In Annex 1- Table 10, all detected species (including NIS) are listed in more detail. Information on harbour, detection method, type of habitat, are provided. In Annex 1- Table 11 an overview is provided on all detected NIS, including information on vectors, presence in Waddensea, year of introduction, and origin.

3.1.1 Harbours

A total of 386 species were identified in this survey (harbours and ballast waters combined), using both classical taxonomy and eDNA techniques.
In the harbour of Eemshaven 262 species were found and in Delfzijl 204 species. In both harbours together, 332 unique species were identified, of which 47 are known non-indigenous species (NIS). In Delfzijl, 31 NIS were found, in Eemshaven 39 NIS were found. Concerning NIS, classical techniques and eDNA yielded a similar number of species (32 and 30 resp.), but neither technique found all species. It was the combination of techniques, as well as the sampling of multiple different habitats (water, sediment, hard substrates) that resulted in the highest number of species, indigenous and non-indigenous.

Monitoring data showed a difference in species composition between the harbours of Delfzijl and Eemshaven, which can be explained by their different characteristics, both environmental conditions such as a salinity gradient for instance, and by harbour design (layout and construction materials used). The difference in harbour lay out also necessitated a difference in sampling intensity and techniques between harbours. Although additional analyses indicated that this may have slightly influenced the results, the difference in species diversity was mostly explained by the environmental conditions and harbour design. Delfzijl is known to be less biodiverse in general compared to Eemshaven and our data are in line with previous observations (Gittenberger et al., 2010).

3.1.2 Ballast water

In the ballast water sampled in three ships, a total of 89 species was found (both techniques combined), including 12 NIS. Out of the 86 taxa, only 20 could be identified up to species level using classical taxonomy, the other species were identified using eDNA.

3.1.3 Comparison and implications

Ballast water and harbours differ largely in species composition. Sampling opportunities and intensity do play a role, however, even the less intensive monitoring in ballast waters already proved that almost 1 out of 2 species in the ballast water was not found in the much more intensely sampled harbours. The current analysis cannot answer the question whether the organisms found in the ballast water samples were viable and able to establish themselves.

3.2 Harbours: species and NIS observed

In Eemshaven, a total of 262 species were found of which 39 are known to be NIS. NIS thus account for 15% of the total observed species richness in Eemshaven. This data is based upon the combination of techniques (classical and eDNA). With classical taxonomy alone,144 species were identified, including 29 known NIS (20% of the observed species).

In Delfzijl, a total of 202 species were found of which 31 are known to be NIS. NIS thus also account for 15% of the total observed species richness in this harbour. This data is also based upon the

combination of techniques. With classical taxonomy alone, 88 species were observed, including 20 known NIS (23 % of the observed species).

Previous regional studies based upon classical taxonomy showed comparable fractions of NIS (Gittenberger et al. 2010, 2012, 2015).

Looking into more detail (classical taxonomy data only) the group of algae (Ochrophyta, Rhodophyta, Chlorophyta) comprised most species in general (Figure 11, Table 5). The most dominant group of Ochrophyta mainly consists of microalgae sampled from the water column, but Rhodophyta and some of the Chlorophyta are macroalgae (sea weeds) that cover most hard substrates in Eemshaven. The only Chlorophyte found in Delfzijl (*Pyramimonas longicauda)* appeared to be non-indigenous. Overall the group of algae contained 16% NIS, which is less than average based on classical taxonomy. More NIS were found in the invertebrate fauna, most notably the Arthropoda like the crabs *Hemigrapsus sanguineus* and *Hemigrapsus takanoi*, and the barnacles (two species of *Amphibalanus),* and the Chordata (mostly tunicates).

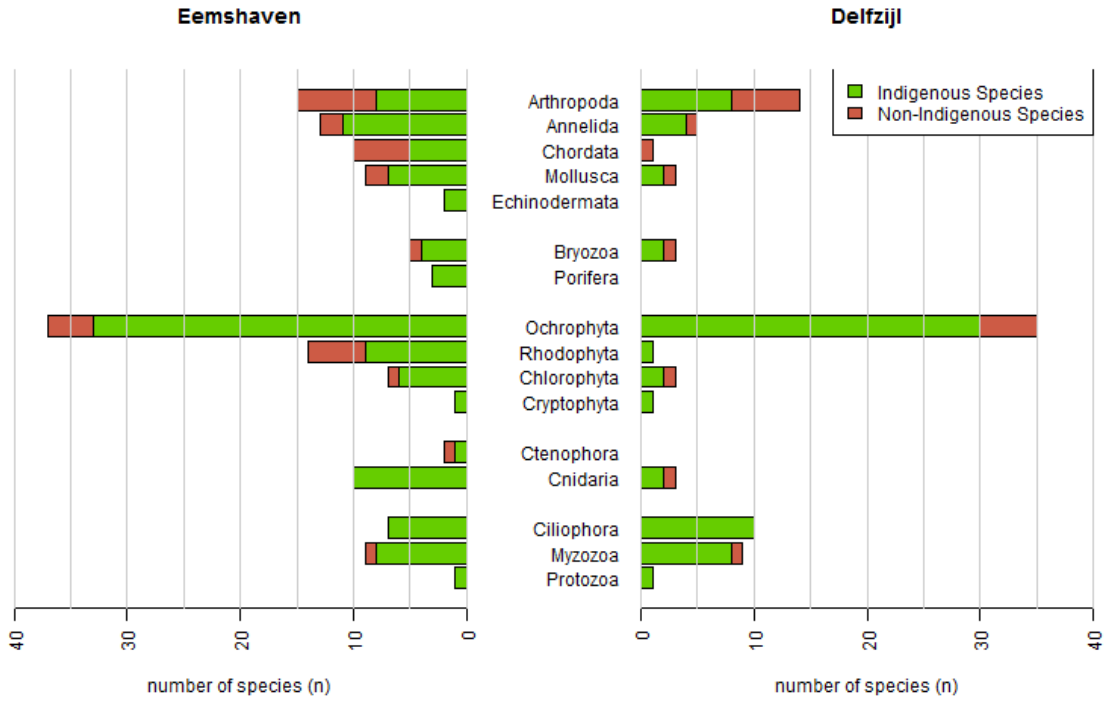

Figure 11. *Number of indigenous and non-indigenous species per phylum found in Eemshaven and Delfzijl, using classical taxonomic analysis.*

***Table 5**. Overview of total species observed per harbour, and % NIS, presented per species group and phylum.*

		Eemshaven				Delfzijl			
	Phylum	total	indigenous	non-indigenous	%NIS	total	indigenous	non-indigenous	% NIS
worms, crustaceans, sponges, etc	Arthropoda	15	8	7	47%	14	8	6	43%
	Annelida	13	11	2	15%	5	4	1	20%
	Chordata	10	5	5	50%	1	0	1	100%
	Mollusca	9	7	2	22%	3	2	1	33%
	Echinodermata	1	1	0	0%	0	0	0	
	sum	**48**	**32**	**16**	**33%**	**23**	**14**	**9**	**39%**
	Bryozoa	5	4	1	20%	3	2	1	33%
	Porifera	3	3	0	0%	0	0	0	
	sum	**8**	**7**	**1**	**13%**	**3**	**2**	**1**	**33%**
weeds/algae	Ochrophyta	37	33	4	11%	35	30	5	14%
	Rhodophyta	13	9	4	31%	0	0	0	
	Chlorophyta	6	5	1	17%	1	0	1	100%
	Cryptophyta	1	1	0	0%	1	1	0	0%
	sum	**57**	**48**	**9**	**16%**	**37**	**31**	**6**	**16%**
Yellyfish anemones	Ctenophora	2	1	1	50%	0	0	0	
	Cnidaria	10	10	0	0%	3	2	1	33%
	sum	**12**	**11**	**1**	**8%**	**3**	**2**	**1**	**33%**
Protists	Ciliophora	7	7	0	0%	10	10	0	0%
	Myzozoa	9	8	1	11%	9	8	1	11%
	Protozoa	1	1	0	0%	1	1	0	0%
	sum	**17**	**16**	**1**	**6%**	**20**	**19**	**1**	**5%**

3.3 Ballast water

3.3.1 Species and NIS observed

Ballast water of three ships was sampled and analysed for planktonic species composition (fytoplankton, zooplankton) using classical taxonomic methods and for total species composition with eDNA.

Both analysis techniques combined, a total of 88 species were found, including 12 NIS (Table 7, Annex 1-Table 10).

Based upon classical taxonomy, a total of 86 taxa were identified of which 69 were phytoplankton (microalgae), 1 cyanobacteria and 16 were zooplankton taxa. However, only 20 of these 86 taxa could be identified down to species level. Species identified were mainly microalgae (Ochrophyta)- such as *Skeletonema potamos* (fresh/brackish water diatom), *Chaetoceros subtilis* and *Ditylum brightwellii* (both marine diatoms)-, followed by zooplanktonic arthropoda, mainly consisting of freshwater crustacean species belonging to taxa as Bosmina and Daphnia. The presence of fresh water species can be explained by the origin of the ballast water (Rotterdam).

Using eDNA, the number of species identified in the ballast water amounted to 69 (Annex 1-Table 10). The difference with the species number identified with classical taxonomy can partially be explained by the fact that 'difficult species' (e.g. ciliates) that need special expertise and/or special sample treatment are also detected and partially because juvenile stadia, unidentifiable by classical means, are identified. In addition, with the eDNA method organisms can be identified that have only left genetic traces in the water including minor fragments that invisible to the eye.
This illustrates that how it is possible that species which were detected by metabacording have already died. The speed of eDNA degradation can vary greatly between days to weeks and sometimes months depending on the taxa concerned, the medium (e.g. water samples or sediments) and environmental conditions described by parameters like temperature, salinity and acidity (Thomsen et al., 2012, 2015).

3.3.2 Comparing ballastwater species composition with harbours

Species composition of the ballast waters significantly differed from the species compositions found in both harbours (Figure 12 and Figure 13).
All three ballast waters together accounted for 89 species of which 42 species were not observed in the harbours (Table 10). eDNA revealed 31 of these species. Species in ballast water which were not observed in the harbours, were mainly micro-algae (diatoms) and flagellates, bivalves and crustaceans. Whether these species were present in viable state is not known, eDNA in this study only recorded presence and absence of DNA fragments, not the state of the organism.

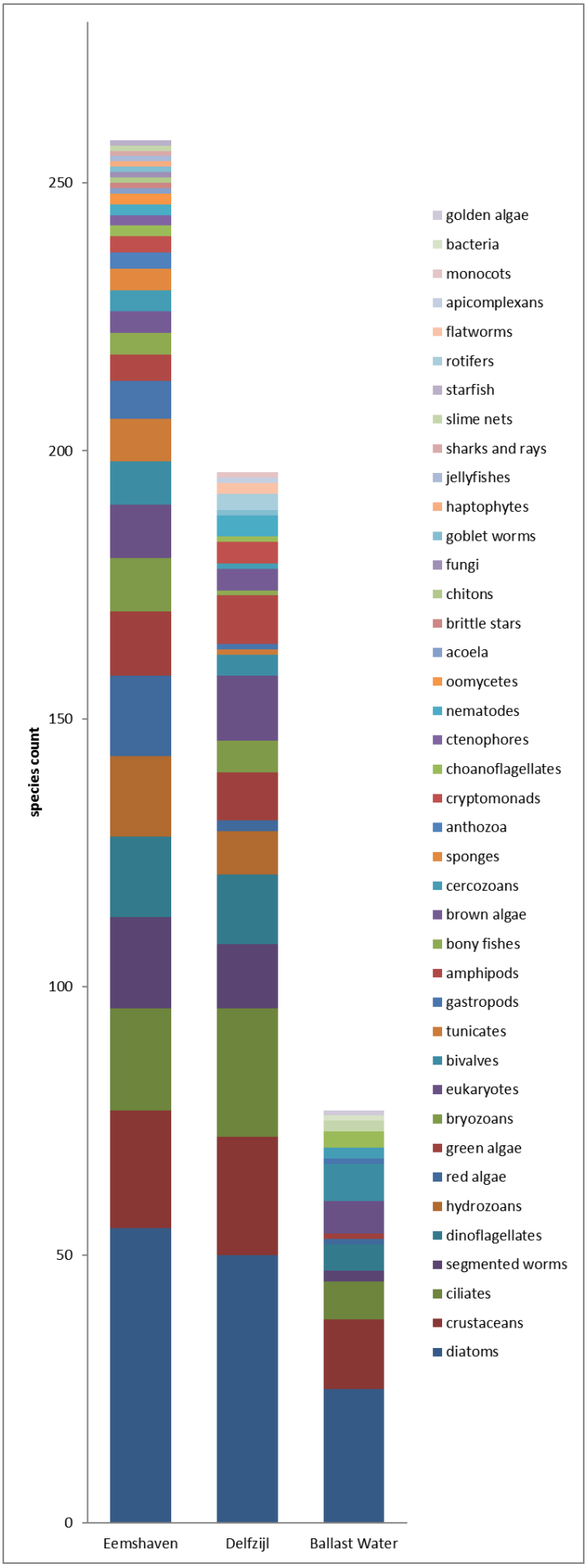

Figure 12. *Species count per species group in the harbours of Eemshaven and Delfzijl, and three sampled ballast waters (combined).*

Between ships, ballast waters differed in species numbers (Table 6) and composition. Ship 1 (origin Tunesia) differed most from harbour waters, contained 41 species of which a total of 28 species were not found in GSP harbours during this study. A total of 5 NIS were found in this ballast water, 2 found with classical taxonomy, and 3 with eDNA. All 5 were not found in the harbours and could be newly introduced species.
Ship 2 (origin UK) brought 44 species, but the ballast water was much more similar to the harbour composition. Only 7 species were not observed in the harbours, and all of the NIS present in the ballast water of ship2 were already established NIS in the harbours.
With only 21 species, ship 3 (origin Rotterdam, NL) brought the least number of species, of which 8 were not observed in the harbours. A total of 4 NIS were identified, 1 new for Eemshaven and Delfzijl.

Table 6. *Overview of species numbers per ship.*

Ship	Total N species	Not found in harbour	Total NIS	"new" NIS
1 (Rades- Tunesia)	41	28	5	5
2 (Tilbury- UK)	44	7	5	0
3 (Rotterdam- NL)	21	8	4	1

Figure 13 is based on only the planktonic (classical taxonomy) determination, and clearly shows the dissimilarity of plankton samples of harbours, and the three sampled plankton communities within the ships ballast waters. eDNA plots show similar deviation between the samples (figure not shown).

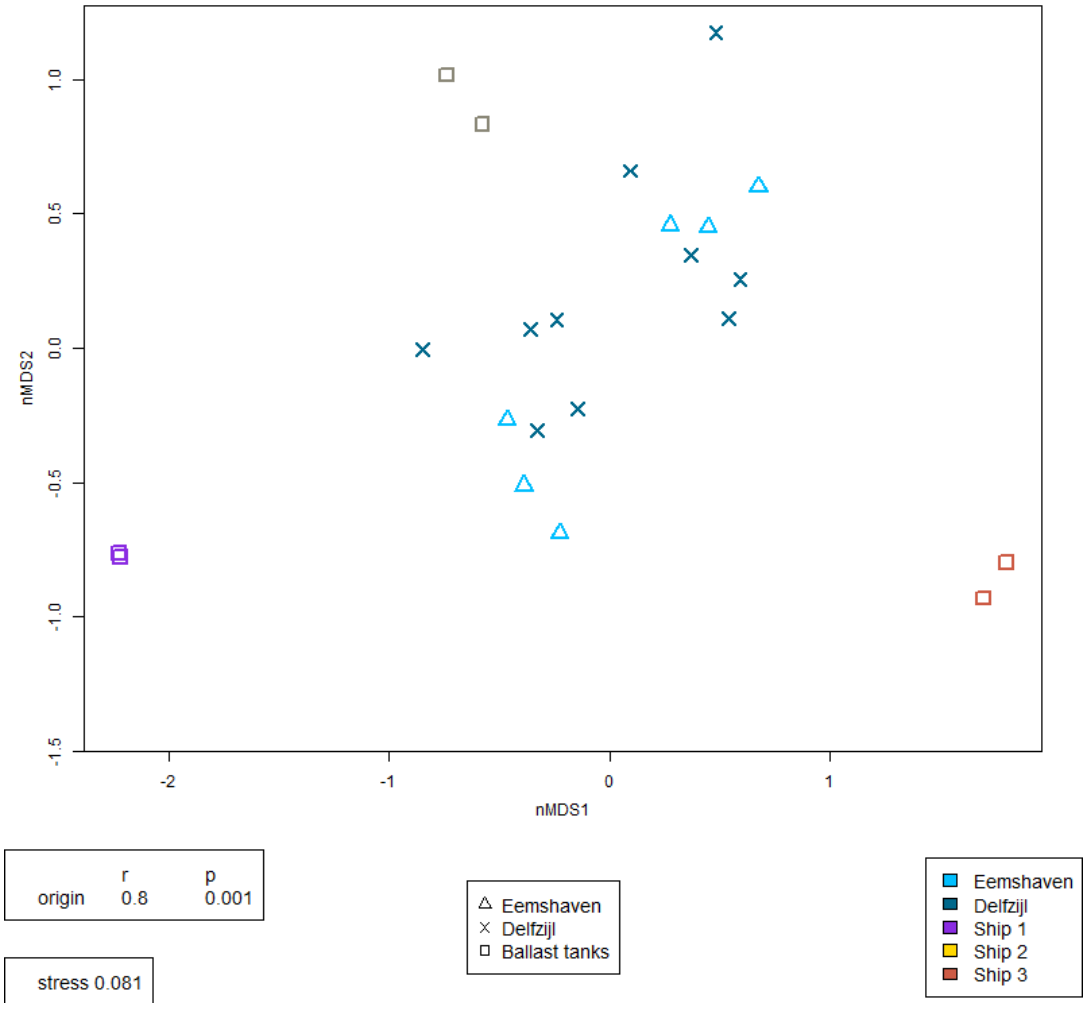

Figure 13. *nMDS of plankton samples of ballast water (ship1-3) and harbours.*

A total of 12 NIS were found in ballast water (BW) (classical taxonomy and eDNA combined). In total six species in the ballast water would concern new NIS for the Dutch northern coastal zone if they would manage to settle. They are not yet reported in earlier studies (**Table 6**).

Using classical taxonomic analysis, 2 NIS in ballast water (ship 1) were found. Neither of them (algae *Pauliella taeniata* and *Pronoctiluca pelagica*) were found in the harbours of GSP, nor reported from the North Sea area before (Table 7, Table 10).
Using eDNA, 10 other species of NIS were found in the ballast water. Of these two (*A. amphitrite* and *H. elegans*, found in ship 1) were not found in the current harbours inventory, but are known to be present in Dutch waters, but were reported decades ago, in the southern waters of the Netherlands, not in the Wadden sea region.
Two bivalve species (*D. rostriformis* and *M. senhousia*) were unknown for the Wadden Sea (www.nederlandsesoorten.nl). *Dreissena rostriformis* is known to occur in freshwater rivers and lakes throughout the Netherlands. It is not expected to settle in the Wadden Sea because of the relatively high salinities. Musculista senhousia could possible settle however as it concerns a bivalve that is known to occur in marine waters (Crooks, 1996).
The diatom *C. guillardii* but was not only found in the ballast water from Tilbury (ship 2), but also in the water sampled in both harbours. This indicates that this NIS has probably already established itself. It was however not reported as a species and NIS in the Netherlands before.

Table 7. *List of all NIS found in ballast water in the three ships. * is NIS new to Dutch Wadden sea and GSP harbours. Year of introduction based on Wolf (2005) and nederlandsesoorten.nl.*

Division	species	Ship1	Ship2	Ship3	Present in EH/DZ	First record north sea or Wadden Sea	technique
crustaceans	*Amphibalanus improvisus*		x	x	EH +DZ	1827 (North Sea/common)	eDNA
crustaceans	*Amphibalanus amphitrite*	x				1962 (Vlissingen)	eDNA
zooplankton	*Acartia tonsa*		x	x	EH+ DZ	1934 (Estuaries)	eDNA
segmented worms	*Hydroides elegans*	x				1973 (Vlissingen)	eDNA
segmented worms	*Ficopomatus enigmaticus*		x		EH + DZ	1968 (Veerse meer) Wadden Sea: 2009 (Gittenberger)	eDNA
bivalves	*Mya arenaria*		x		EH	1765 (North Sea/common)	eDNA
bivalves	*Rangia cuneata*			x	DZ	2005 (Noordzeekanaal, Groningen)	eDNA
bivalves	*Dreissena rostriformis*			x		This report	eDNA
bivalves	*Musculista senhousia*	x				This report	eDNA
algae	*Pauliella taeniata*	x				This report	classic
algae	*Pronoctiluca pelagica*	x				This report	classic
diatoms	*Conticribra guillardii*		x		EH+ DZ	This report	eDNA

4 Non-indigenous Species (NIS) in context

4.1 NIS in Wadden Sea

In addition to two aliens species focused surveys in the Wadden Sea in 2009 and 2011 (Gittenberger et al., 2010, 2012), a rapid assessment of marine algae and macrofauna of hard and soft substrates was done by Gittenberger *et al.* (2015) between August and October 2014. They visited 242 sampling stations, including artificial habitats (harbours) and natural habitats (mussel beds, mudflats). A variety of sampling methods was used, similar to the current study. In total, 254 species were found of which 48 are probably non-indigenous (or 40 species, excluding the 'cryptogenics' [6]). This fraction (16-19%, depending on definition) corresponds with the 20% NIS found in our monitoring of 2016.

In total, 74 different NIS were found in total in the studies of 2015 (Gittenberger *et al.* 2015) and current study (Annex 1- **Table 13** and **Table 14**)
However, only 19 NIS overlap among both studies, indicating that both studies detected unique NIS. Spatial and temporal scales, and differences in study design account for these differences. Hereby Gittenberger et al. (2015) only recorded living organisms that had settled in the Wadden Sea. For example, only macro-algae that were attached to the substrate were recorded. The ones that were only found washed ashore were specifically excluded, regardless of whether or not they were alive. In the present study eDNA techniques were used that can not distinguish between living or dead organisms. Gittenberger et al., 2015 sampled much more locations, including musselbeds, accounting for specific species composition, different from the habitats in current study. The additional species found in this study, resulted from scrapings, snorkelling and eDNA samplings.

When comparing the current sampling to previous (2009, 2011, 2014) dike sampling in the Eems harbour it can be concluded that more or less the same number of species was found (data not shown). Data comparison showed that species compositions were the same.

Some of the established NIS in the Wadden region were also found in this survey and included for example now-a-days- common species such as crabs *Hemigrapsus sanguineus* and *Hemigrapsus takanoi (*Figure 14*)* , tunicates *Styela clava and Botrylloides violaceus (***Figure 15***), and barnacles and worms Austrominius modestus, A. improvisus, and Ficopomatus enigmaticus* (**Figure 16**).

Figure 14. *Crab species and established NIS since 1999/2000: Hemigrapsus sanguineus (left), Hemigrapsus takanoi (right). Pictures by Gittenberger.*

[6] A cryptogenic species is a species whose origins are unknown. It may be either a native species or an introduced species, clear evidence for either origin being absent.

Figure 15. *Tunicates* Styela clava *(left) and* Botrylloides violaceus *(right) observed at the floating dock during snorkelling. Pictures by Gittenberger.*

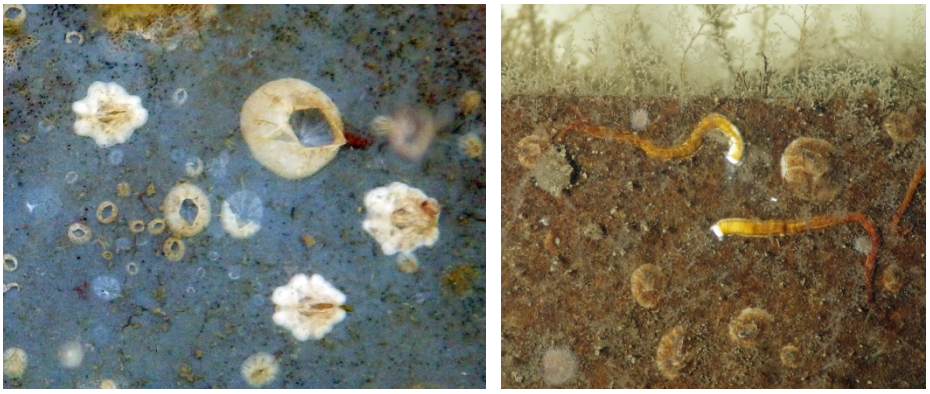

Figure 16. Austrominius modestus *and (empty)* A. improvisus *(left piture) and* Ficopomatus enigmaticus *and* A. improvisus *found on plates. Pictures by Gittenberger.*

4.2 Vectors

Of the 52 detected NIS in this study (in harbours and ballast water combined), most species are related to multiple vectors of introduction. Only 23 species are primarily associated with ballast water, but most often more vectors are described (Table 11). Only three are uniquely associated with ballast water and distribution via waterways.

4.3 Invasivity, origin and habitats preferences of NIS

Based on the data provided by Naturalis Biodiversity Centre through their website www.nederlandsesoorten.nl, specifications of the NIS was reviewed (see ANNEX 1- Table 11). For most species Naturalis has created a 'NIS-passport', that provides details on the origin, vectors, the invasiveness and other characteristics. However, not all species are included and the passports are not always complete. The invasivity of the NIS detected varies, or is unknown. Up to ~55% of the NIS detected is invasive or potentially invanse (Figure 17), depending the circumstances.
For 43% of the species the origin is not clear, since the species was not in the database or the passport had not been made yet. About 22% of the species has been imported from the Pacific, 16% from the Atlantic region. The remaining NIS originate from other regions in Europe, the arctic or asia (Table 11, Figure 18). Most species relate to marine and brackish environments (Table 11, Figure 19), but some are found in freshwater habitats and will not pose a direct risk to the Wadden Sea. For 1 out of 4 species its preference is not clear.

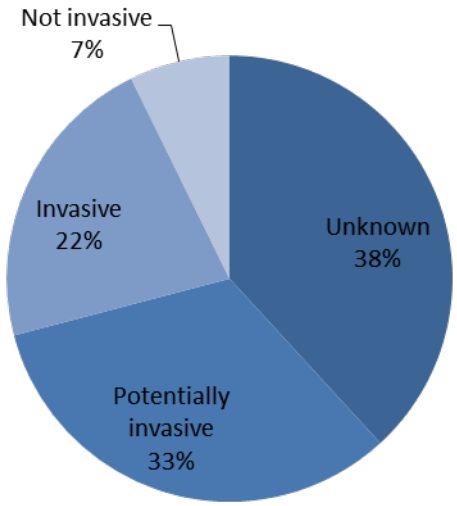

***Figure 17**. Invasivity of NIS found in this study.*

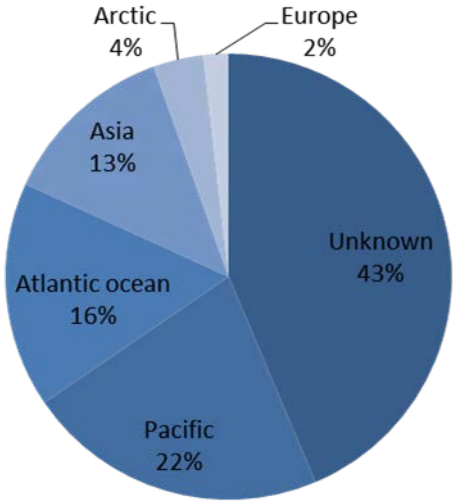

***Figure 18.** Origin of non-indigenous species (NIS) per ocean.*

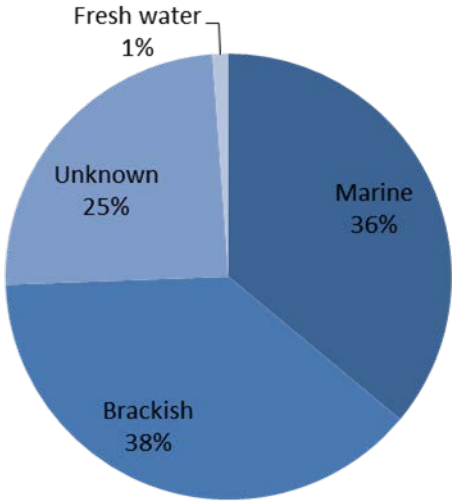

***Figure 19**. Origin of habitat preferences of non-indigenous species (NIS).*

4.4 Ballastwater NIS profiles and risk assessment

In this chapter we describe the six NIS found in the ballast water tanks and that were not reported before for the Dutch coastal waters.

4.4.1 Pauliella taeniata

P. taeniata is a marine diatom that normally occurs in oligotrophic and oligosaprobic environments (Stachura-Suchoples 2001).

Although this species is reported to be found throughout the world (Guiry& Guiry, 2017), it is described to be an arctic species (Smol and Stoermerm, 2010). This cold-water species was common in the Gulf of Gdańsk (Witkowski & Pempkowiak 1995, Stachura-Suchoples 1999, Hallförs 2004, Witak *et al.* 2006, Leśniewska & Witak 2008, Witak 2010) and other basins of the Baltic Sea (Andrén *et al.*

1999, 2000; Olli *et al.* 2008; Tuovinen *et al.* 2009). According to Guiry & Guiry(2017) it was first reported for the Netherlands by Van Veen *et al.* in 2015.

The species is not recorded as harmfull algal by UNSECO and the database on Harmful Algal Information System.

Due to limited specification on the species, it cannot be assessed whether it can establish itself in the temperate waters of the Dutch coast.

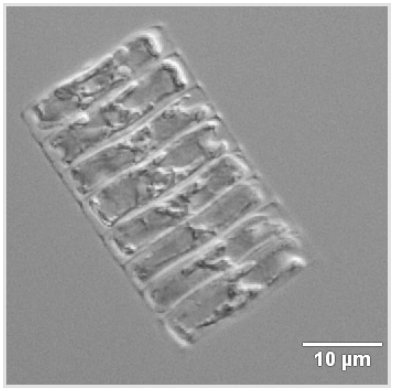

Figure 20. Pauliella taeniata *(picture taken from http://www.iopan.gda.pl/~wiktor/diatom/taeniata.html).*

4.4.2 Pronoctiluca pelagica

P. pelagica is a marine dinoflagellate belonging to the class of Noctiluca.
This species is reported to be found throughout the world (Guiry& Guiry(2017). Guiry & Guiry (2017) indicate that this species was first reported for the Netherlands by Van Veen *et al.*, (2015). However,it was recorded in Dutch waters for the first time in 1996 (Koeman *et al.*, 2009). Since 2006 it has been recorded repeatedly in various years in the Dutch North Sea off Terschelling (Brochard *et al.*, 2006; Koeman *et al.*, 2009).

Due to limited specific information on the species, it cannot be assessed whether it can establish itself in the temperate waters of the Dutch coast. The species is not recorded as a harmful algae by UNSECO and the database on Harmful Algal Information System.

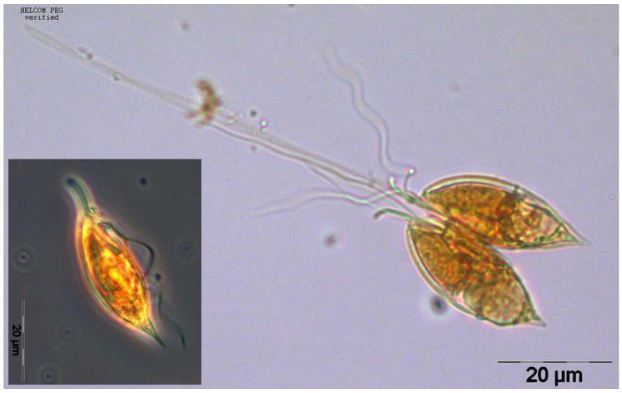

Figure 21. Pronoctiluca pelagica *(picture from Susanne Busch, retrieved from http://nordicmicroalgae.org/taxon/pronoctiluca%20pelagica).*

4.4.3 Hydroides elegans

Hydroides is a genus of tube-forming serpulid worms found in many coastal areas around the world. Serpulid polychaetes produce calcareous tubes and aggregate to form dense populations both on natural and man-made structures, such as piers and ship hulls (Çinar 2013). Their dense populations increase weight, maintenance costs, and fuel consumption as the fouling reduces the hydrodynamics of ships (Schwindt et al. 2001). *Hydroides elegans* is recognized as an invasive species in many harbour areas of the world, growing mainly on man-made structures such as pier pilings, where

diversity is low. In addition, because they build large aggregations of calcareous tubes, this increases the maintenance and fuel costs of vessels (Dos Santos Schwan *et al.*, 2016).

Hydroides elegans has a short larval stage (Hadfield *et al.* 1994; Carpizo-Ituarte & Hadfield 1998) and reaches sexual maturity early (Paul 1937). Given these characteristics it quickly colonizes hard substrata (Unabia & Hadfield 1999) and has invaded many parts of the world, including Africa, Atlantic and Pacific coasts of America, Southern Europe and the west Pacific (Zibrowius 1972, Zibrowius H. 1973, Zibrowius H. 1992, Bastida-Zavala & ten Hove 2003, Çinar 2013).

Wolf (2005) reported the species for the Dutch North Sea as temporarily established. Ten Hove (1974) found this species near the Keersluisbrug at Vlissingen in the Kanaal door Walcheren in September 1973. At that time the canal was thermally polluted by the power station at Vlissingen (Ten hove, 1974). After the first observation in 1973 it was not found again, although it was looked for several times (Ten Hove & Lucas, 1996). The species originates from tropical waters, and given the fact that the species was found in the ballast water of ship 1, originating in the Mediterranean, this observation matches well. The most likely spot to find the species is near the cooling water discharges in the harbors of Eemshaven and Delfzijl. It can be a potential risk for cooling water discharge locations since it can cause severe fouling.

Figure 22. Hydriodes elegnas *(picture © Brian Nedved via http://taxondiversity.fieldofscience.com/2016/07/hydroides.html).*

4.4.4 (*Amphi)balanus amphitrite*

Amphibalanus amphitrite is a species of acorn barnacle in the Balanidae family. Its common names include Striped barnacle, Purple acorn barnacle and Amphitrite's rock barnacle. It is found in warm and temperate waters worldwide. Its origin is uncertain but may have been the Indian Ocean or southwestern Pacific Ocean (Cohen, 2005). Gollasch (2002) calls it a warm-temperate species of Japan and Korea. It has now spread to most of the warm and temperate seas of the world. A. *amphitrite* is a common coastal and estuarine organism found on hard natural surfaces such as bedrock, boulders, mollusc shells. It is also found on artificial surfaces such as the hulls of ships, pilings and seawalls. *A. amphitrite* is a hermaphrodite and individuals have both male and female reproductive organs. Free-swimming larvae are released into the water column where they become part of the zooplankton. In temperate areas, spawning occurs mainly in the spring and summer, but in warmer waters it may continue throughout the year (Pillai 1958). Individuals can release up to ten thousand eggs per brood and there may be many broods per year (Masterson 2007). It can tolerate low salinity levels in estuaries, but appears to need higher salinity in order to breed (Vaas (1978). It can also tolerate temperatures as low as 12°C to grow, but needs temperatures of at least 15°C to breed which limits its northerly spread (Bishop, 1950).

Fofonoff et al. (2014) and Wolf (2005) report that the species is known for the North Sea. The species is probably introduced on ships' hulls (Darwin, 1854; Boschma et al., 1961), but ballast water could have been a vector too. According to Wolf (2005) the species is permanently established in the Netherlands. Established specimens were found in a cooling water discharge canal at Vlissingen in 1962, 1965, and 1967 (Borghouts-Biersteker, 1969). Vaas (1975) mentions that the species was found in the Veerse Meer first in 1970, in 1975 it occurred all over this lake. Wolf (2005) reports later

observations by several observers mostly in the southern coastal areas of the Netherlands and Belgium, however not in the northern regions.

Most tropical non-indigenous species such as A. amphitrite either occur in thermally polluted waters or have a doubtful origin (Wolf, 2005). Also for this barnacle, the risks for invasion is restricted to cooling water discharge pipelines or in close vicinity of these.

Figure 23. *Striped acorn barnacle,* Amphibalanus amphitrite*. Image courtesy of Melissa Frey, Royal BC Museum, taken from https://invasions.si.edu/nemesis/calnemo/SpeciesSummary.jsp?TSN=89616.*

4.4.5 Dreissena rostriformis

Dreissena rostriformis (Figure 24)*,* also known as the quagga mussel, is a small freshwater bivalve mollusc, indigenous to the Dneiper River drainage of Ukraine and Ponto-Caspian Sea. The mussel has probably been able to reach The Netherlands via the Main-Danube canal. It is a characteristic mussel with a marked pattern on the shell. In 2006, the first shells were discovered in a soil sample from the Hollands Diep and on settlement plates in the Haringvliet (Schonenberg & Gittenberger, 2008). In 2007 in the Rhine near Wageningen, almost half of the Dreissena population was already replaced by the quaggamussel (Nederlandsesoorten.nl). Since 2007, the species is also found in Ijsselmeer (RWS 2009)

These species are prodigious water filterers, removing substantial amounts of phytoplankton and suspended particulate from the water. As such, their impacts are similar to those of the zebra mussel. By removing the phytoplankton, they decrease the food source for zooplankton, altering the food web. Impacts associated with the filtration of water include increases in water transparency, decreases in mean chlorophyll a concentrations, and accumulation of pseudofeces (Claxton *et al.* 1998). Water clarity increases light penetration, causing a proliferation of aquatic plants that can change species dominance and alter the entire ecosystem. For the Wadden Sea no impact is expected as the species distribution is restricted to fresh water habitats. Inland however, the species can spread and have a distinct impact on the ecosystem by for example increasing the water transparency.

Figure 24. Dreissena rostriformis*. Picture by J. E. Marsden (http://www.northeastans.org/online-guide/species-information.html?SpeciesID=23).*

4.4.6 Musculista senhousia

Arcuatula senhousia (also known as *Musculista senhousia*), commonly known as the Asian (date) mussel, Japanese mussel or Green mussel, is a small marine bivalve mollusk species in the family Mytilidae.
It is native to the Pacific Ocean, but it has been introduced and become an invasive species in numerous other areas worldwide. It prefers soft substrates, and can be found in the intertidal or shallow subtidal zones, but also down to twenty metres below the surface (Edgar, 1997). *Musculista senhousia* is thought to have been introduced into Australia and New Zealand by ship fouling, in ships' seawater systems, or in ballast water. Shellfish may have played a role in its introduction and spread in the Mediterranean, including oysters imported from Japan (http://www.exoticsguide.org/musculista_senhousia).
The Asian date mussel can have a variety of effects on various ecosystems. Reported impacts are increase in the biomass of benthic macro-organisms in general (Slack-Smith & Brearley 1987), and the decrease in species richness and abundance of indigenous species, or even completely outcompeting indigenous species (Crooks, 2001). Competition with indigenous species is the primary cause of concern in areas the Asian date mussel has invaded (Creese *et al.*, 1997).
One of several negative impacts of this invasive species is that it has a detrimental effect on eelgrass. The mussel shares its habitat with eelgrass and the presence of the mussel has been shown to negatively affect rhizome growth in the eelgrass. This decreases the ability of established patches to spread. The Asian date mussel has the most detrimental effect on rhizome growth in areas where the eelgrass is sparse and patchy. This is a cause of concern for conservationists, because beds of eelgrass are already degraded and sparse as a result of anthropogenic forces. The presence of the mussel can only worsen the situation (Reusch *et al.*, 1998).
The species is not yet reported in the Wadden Sea or North sea region. Given its habitat requirements (Euryhaline (17-37 ppt, optimum range 20-25 ppt) and tolerance of a wide range of temperatures (5 – 30 °C)) it is not unlikely that the species can establish in the Netherlands.
Ship 1 contributed with the eDNA of this species, originated from the Mediterranean. The species is established in this region. If the eDNA originates from eggs or larvae, it may have survived and settled in the Netherlands.

: 

Figure 25. Arcuatula senhousia*. Picture taken from Bachelet et al (2009).*

5 Monitoring effectivity

5.1 Summary

In order to advise what techniques are most effective for use in upcoming monitoring programs, the effectivity of the monitoring is discussed in this section. Sampling techniques are only discussed in detail using the species information derived by classical taxonomy. The use of eDNA techniques is discussed separately.

The marine life in Eemshaven is more diverse than that in Delfzijl, as was also found in previous studies (Gittenberger *et al.* 2010, 2012, 2015). As the number of species found is strongly dependent on the number of samples taken, this difference may be aggravated by the higher sampling effort in Eemshaven (Table 1). However, species accumulation curves based upon the individual samples indicate that completeness of the sampling effort was comparable in both areas.

The variation in samples habitats, applied sample techniques to collect species and methodologies to identify species (eDNa and classical taxonomy) complement each other. Data show that the combination of sampled habitats, sample techniques and identification methods resulted in much more detected species and NIS then when a selection was applied.

5.2 Where and how to sample: Species per habitat and method effectivity

In order to evaluate the number of species per habitat, and compare the effectivity of different methods, additional analyses were performed focussing on the substrate type and methods applied. Species accumulation curves (SAC) and nMDS plots are suitable analysis techniques in this evaluation. **Note that only data from detected species using the classical taxonomy are included in this section**. The number of samples taken for eDNA-analyses was not sufficient for this type of analysis.

Species accumulation curves represent the cumulative number of species recorded as a function of sampling effort (*i.e.* number of individuals collected as function of the cumulative number of samples). nMDS plots represent the (dis-)similarity between samples in an ordination grid.

Figure 26 shows the (dis)similarity among samples in both harbours derived from hard substrate samples using various sampling techniques. A clear separation of samples obtained by different techniques indicate that the techniques complement each other in collecting different species compositions. While overlapping of samples from different methods indicate a higher similarity. The SETL-plates, diving results and dike samples can be identified as separate groups, yielding complementary results. Scraping pillars and quays, and in Eemshaven also scraping floating docks, resulted in more similar data, showing overlap of community structure. As a group they are complementary to the first three techniques. SETL plate species compositions at samples deployed at different depths (-1, -3, 5, -10m) in Eemshaven were similar (Annex 3- Figure 30), indicating that deployment at one depth will be sufficient.
Similar plots representing the (dis)similarity among water samples show a clear separation of samples taken at different locations and seasons (Annex 2, Figure 29). In Delfzijl, species composition in water among locations is less similar than in Eemshaven, indicating clear gradients in water quality. In Eemshaven locations showed to be more similar, probably because of the strong influence of the tides. Sampling in spring and autumn also resulted in different species composition indicating that the variety in species composition is both determined by location and season.

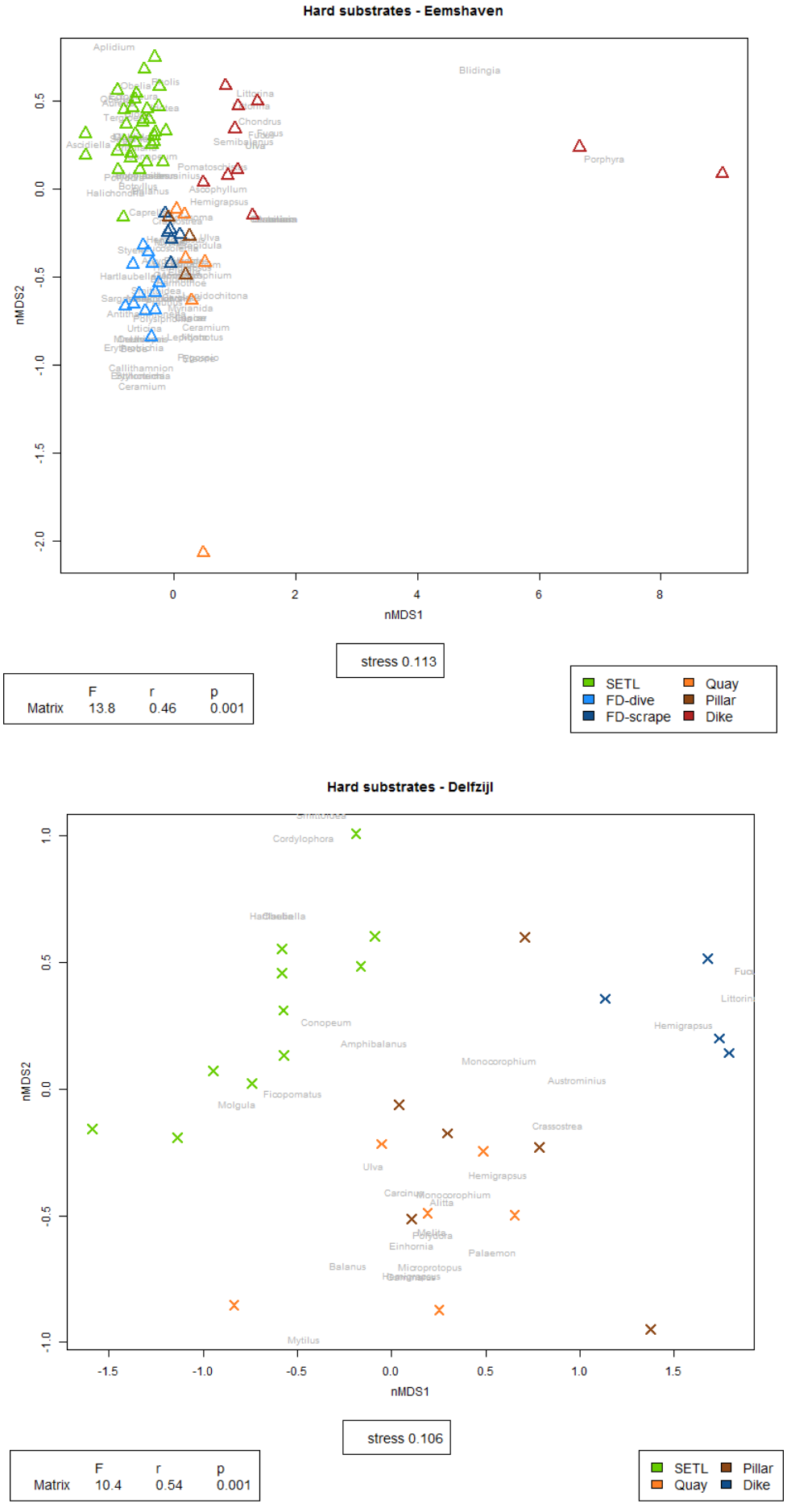

***Figure 26**. nMDS plots representing hard substrate samples using various techniques.*

In Figure 27 species accumulation curves (SACs) for Eemshaven en Delfzijl are presented. The accelleration rate (steepness and extent of smoothing) of the curves related to the number of samples, indicates the potential number of additional species that might be discovered with increasing effort.
To the right of the curves, the total species and collected species per habitat are visualized in column graphs, including information on NIS and indigenous species numbers. The upper white part of the column shows the (very theoretical) estimate of the number of potentially missed species in this survey.

As mentioned in the previous section, species diversity is higher in Eemshaven compared to Delfzijl. The SACs for both areas collecting species by means of hard substrates are very horizontal, indicating that any additional sample only contributed a minor portion of new species to the total. For water samples, these SACs are still very steep, indicating that additional effort may result in many more species. However, the diversity in water samples was very comparable in both harbours. In addition, the detection chance of NIS in water samples was expressed as the number of samples taken, and the % of samples in which the NIS is detected (annex 3, Table 15). This showns clearly the variability of the chance of detecting a species (10-100%) which is depends both on its abundance and number of samples taken. The same is observed for NIS in hard substrate samples, and technique applied (annex 3-Table 16). Chage of detecting NIS varies on the species with 2-100 %, depending the habitat, technique and species abundance. Nothwithstanding the difference in total species found, both harbours showed a similar contribution of NIS (Figure 27).

In conclusion, additional effort in sampling a larger number of sediment samples will hardly improve species detection. Doubling the effort of sampling hard substrates might increase the number of species species detected by ~30% (40 additional species). Most new species will probably be detected by additional water sampling, especially when more seasonal variation is covered (Figure 27). NIS are, however, mostly found on hard substrates and, therefore, the best strategy for detecting more NIS is probably to increase hard-substrate sampling.

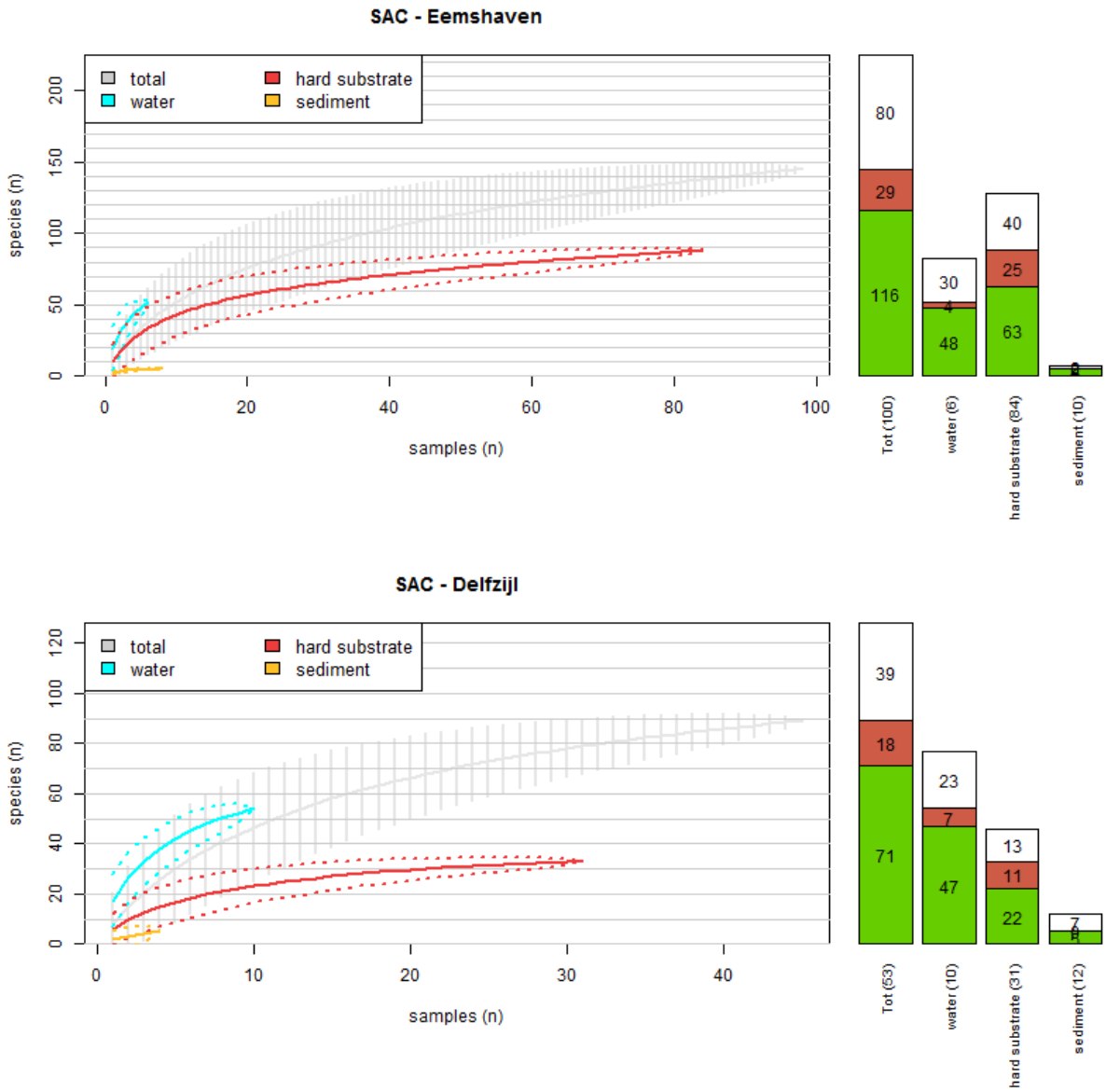

Figure 27. *Species accumulation curve for species collection methods in Eemshaven (top) and Delfzijl (bottom). Green: collected indigenous species ; red: collected non-indigenous species; white: expected number of species in additional samples. (n) represents the total number of samples.*

5.3 NIS per substrate type and monitoring effectivity

The results presented in the previous section showed that hard substrate sampling yielded a relatively high number of NIS.
In this section the contribution of the type of substrate and sampling technique towards the observations of NIS is described in more detail. Also in this section, only data from the classical taxonomy are included. The results of eDNA analyses are left out here, because these represent only a limited number of samples.

NIS were found in both harbours and with all applied techniques and types of substrates. Some hard substrate related NIS were found on almost all hard substrate samples (e.g. arthropod *A. modestus* and mollusc *C. gigas*) (data not shown). Some were however only found using a specific technique or type of substrate, such as *M. leidy* during snorkelling the dock, and *C. caspia* and *A. glabrum* using SETL plates.

Although NIS were found using all techniques and in various substrates (22-42%), most NIS were observed during snorkelling (15 out of 25 NIS, see Figure 28). It is important however to realise, not to focus solely on the number (quantification) of NIS found within a technique or habitat. Techniques

complement eachother because they focus on different habitat charecteristics, or the method targets different species communities. SETL plates are empty substrates which are deployed, and “new” species compositions will be observed after several months. This new community will include pioneer species too and SETL plates serve as an early detection method for these prioneer species and NIS. Fully grown pillars or dock will less easy “capture” pioneer species because of lack of space. In time, pioneer species that establish, will be found on the docks or pillar too.

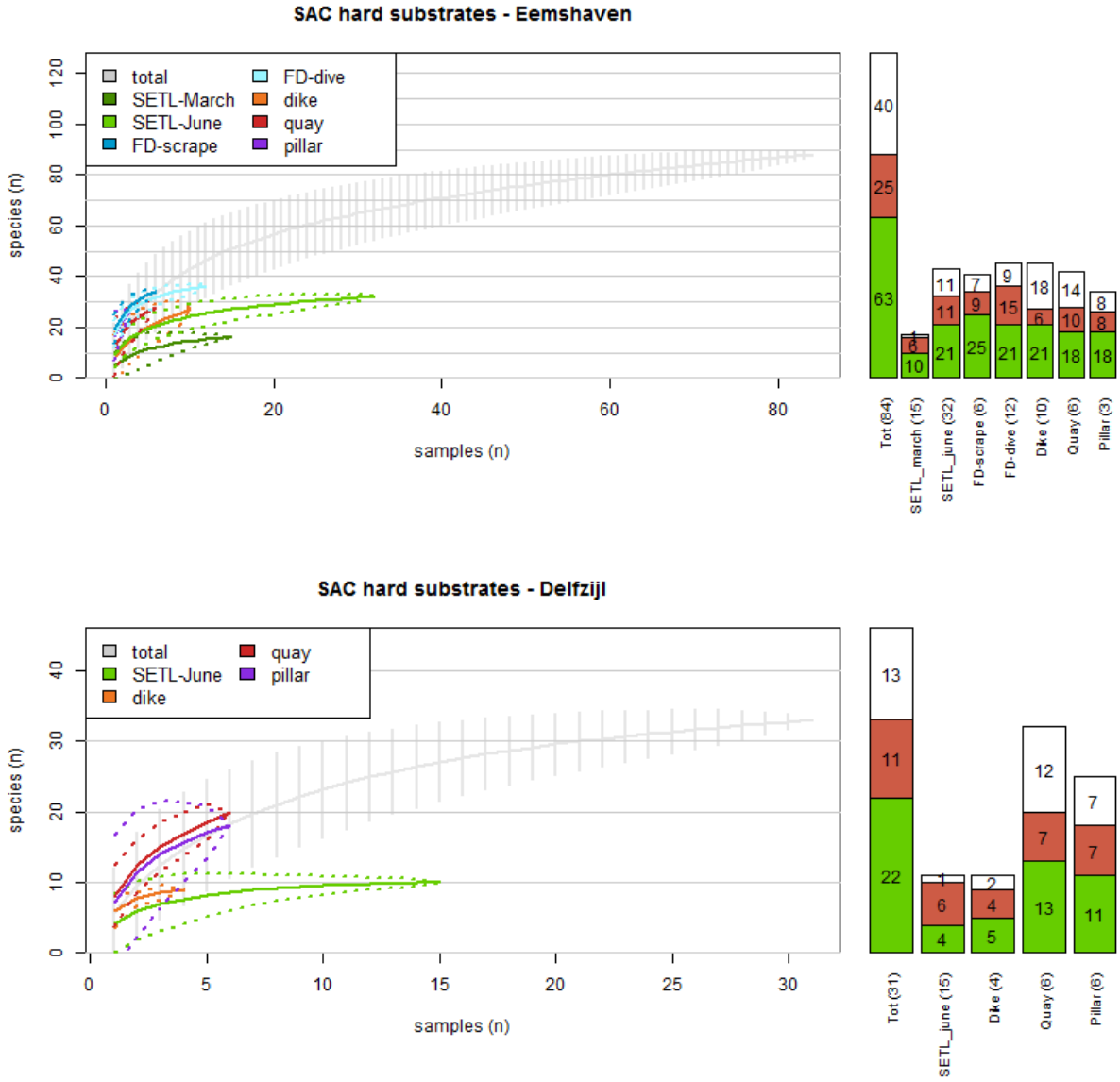

Figure 28. *Species accumulation curve for hard substrate collection methods in Eemshaven (top) and Delfzijl (bottom). Green: collected indigenous species ; red: collected non-indigenous species; white: expected number of species in additional samples. (n) represents the total number of samples.*

The results for NIS observed can be summarized as follows:

Eemshaven:

- A total of 28 NIS were observed using classical taxonomy: 13 of these were uniquely found in Eemshaven, 15 were also observed in Delfzijl.
- 4 NIS were exclusively found in water and are planktonic species. On hard substrate 24 NIS were observed, of which 17 were found on the floating dock. In sediment samples, no NIS were observed.
- NIS on hard substrates were found by a variety of techniques (Figure 26, Figure 27, Figure 28).
    - 4 species were exclusively found by scraping quays and pillars.
    - 1 species was exclusively observed on SETL plates

    - 5 species were exclusively found by snorkelling observations on the floating dock.
    - 1 species was exclusively observed on the dike.
    - The remaining 12 hard substrate NIS were found by more than one technique.
- NIS within the group of weeds were mainly found by snorkelling, except for one NIS within the groups of weeds that was found exclusively on the dike. Microalgae are mainly found using water sampling. Seasonal sampling for algal species yielded different NIS. All other species were found using a variety of techniques.

Delfzijl:

- A total of 18 NIS were observed in total, of which 6 were only found in Delfzijl. The other 12 were also found in Eemshaven.
- 7 NIS are planktonic species and were exclusively found in water samples, while the other 11 NIS were observed on hard substrates. In the sediment samples, no NIS were observed.
- NIS on hard substrate were found by a variety of techniques (Figure 26, Figure 27, Figure 28).
    - 4 species were exclusively found on hard substrates by scraping quays and pillars.
    - 1 species was exclusively observed on SETL plates
    - The other species were found by more than one technique.
- 3 (arthropod) species were exclusively found by scraping pillars and quays, 2 NIS were exclusively found on SETL plates.
- None of the observed NIS were found exclusively on the dike.

The above results implicate that the applied sampling techniques complement each other in monitoring NIS and should be applied in combination in order to collect the diversity of species present. Sampling hard substrates in spring might increase the variety in NIS too as this has been observed for the planktonic NIS as well but added value should be studied in upcoming surveys.

5.4 eDNA

The results of the eDNA analyses are presented here separately. Firstly because the number of samples used for eDNA analyses was considerably lower than the number of samples analysed by classical taxonomy. An other important reason is that DNA is a very stable molecule, that may remain present in environmental samples for a long time. This makes it a suitable technique for monitoring more cryptic species that are easily missed by classical methods and also for identification of life-stage that are hard to identify (eggs, juveniles, etc) and damaged species. However, special techniques, not used for this project, are needed to separate DNA in living organisms, from ‘ancient’ DNA.

5.4.1 Results per habitat/matrix

Table 8 shows an overview of number of samples, species and NIS per harbour, habitat/matrix, and analysing technique. Hard substrate includes combined results from SETL plates, snorkelling, dike and scraping samples.

From each sediment sample taken for classical taxonomy, a sub sample was taken for eDNE. The sub samples were pooled into 1 sediment sample per harbour to be analysed by eDNA. Classical taxonomy revealed a limited species numbers due to the focus of identification (only macrobenthic species) and the difficulties in identifying incomplete and/or juvenile individuals. The eDNA analyses revealed traces of much more species, including planktonic species or microscopic small species. In addition,the observed species could have been both dead and alive during sampling. Benthic species in sediment found with classical taxonomy were however not found by eDNA. A clear explanation for this is lacking- only that the subsamples did not contain DNA of the species found in the complete sediment samples. Techniques complemented each other in listing species and NIS.

This observation also holds for water samples and hard substrate samples. More species and NIS were found with eDNA compared to classical taxonomy in the same (number) of water samples. In addition, classical taxonomy also detected species which could not be identified using eDNA.

The number of hard substrate samples used in classical taxonomy was much higher compared to the number of samples used in eDNA analysis. Samples from hard substrate scraping resulted in relatively low amount of eDNA that could be processed. We assume this could be a consequence of the presence of anemones. Anemone enzymes to inhibit the PRC step in the metabarcoding proces, resulting in

lower quality sequence results. Although samples were stored at -80 °C and processed as quickly as possible to reduce this risk, this was not effective enough. An additional extraction step had to be included to discard these inhibiting compounds, but was not done in this survey.

In summary, eDNA analyses resulted in a marked increase in identified species in sediment and water samples, probably due to the fact that unidentifiable fragments and juveniles can still be identified using their DNA. Even in hard substrate samples, the number of identified species was much higher (if the number of samples analysed is taken into consideration), but this is probably a gross underestimation due to the presence of anemones in the sample.
Underestimation of the number of species may also be caused by the fact that only 100% matches were used to identify a species. Often 96% is used as (rather arbitrary) limit. And of course, only species that have been sequenced, can be matched to the sequences found in the samples. This may be (one of) the resons(s) that (some) species found using classical taxonomy, were not identified using eDNA analysis in the same sample matrix.

On the other hand, the number of species actually present may also be grossly overestimated. For this research, all DNA was amplified (using a few general primers). As DNA is very stable, this will also concern 'free' DNA of species that were already dead, or in remnants of individuals (scales, bones, slime) were not present. It may be expected that real old remnants will mostly contain DNA that is damaged and will, therefore, not yield a 100% match. For a description of the species composition of the harbour areas this is not crucial. However, it can make a significant difference when evaluating ballast water that is introduced into the harbour.

Table 8. *Number of samples, species per habitat/matrix and harbour. Hard substrate includes combined results from SETL plates, snorkelling, talud and scraping samples.*

		eDNA			classical			total	
origin	Matrix	N sample	N species	N NIS	N sample	N species	N NIS	N species	N NIS
EH	sediment	1	37	5	10	5	0	42	5
EH	water	6	111	14	6	52	4	152	17
EH	hard substrate	7	57	14	84	88	23	122	28
DZ	sediment	1	53	4	13	5	0	58	4
DZ	water	6	102	13	6	54	7	144	19
DZ	hard substrate	2	33	12	31	33	10	59	18
Ship1	water	2	36	3	3	6	2	41	5
Ship2	water	1	36	5	3	9	0	44	5
Ship3	water	1	13	4	3	9	0	21	4

5.4.2 eDNA: some remarkable species and results

The survey did not aim to collect fishes, but with eDNA 2 fishes (*Abramis brama, Merluccius merluccius* (common bream and hake) and a shark (*Scyliorhinus canicula*- small-spotted catshark) were detected in water samples of Eemshaven. More fish species should be found using eDNA, but specific primers for fish were not chosen in this pilot study.
One fish was detected in water of Delfzijl (Bream). Bream is a remarkeble species to be found in Eemshaven since it is a freshwater species. Most probably, DNA of bream is transported via bird feaces or fresh water discharge into or near Eemshaven, such as the Eemskanaal.

Sediment samples revealed much more species using eDNA, of which most species belonged to other species groups than looked for in the classical benthic analysis. Most species detected via eDNA were not reported in classical analysed sediment samples and belong to algal or flagellates groups. This

species are no part of classical sediment benthic taxonomy, and eDNA complements the sediment species list with additional species.
Other reported species in sediment using eDNA could have been too small or incomplete to be reported by classical taxonomy techniques.

It should also be noted, that sediments act as “sinks” and they are known to contain and release “ancient” DNA from sedimented particles of organisms (Turner *et al.*, 2015). eDNA derived from sediments is fairly persistent and ancient eDNA thus adds to the eDNA of the actual present species into the overall species composition detected. eDNA from these sediments thus reveal an accumulated species composition over time, and not necessarily actual species presence. Data from this study show this aspect clearly. The interpretation of eDNA results is more complicated than classical taxonomy results. To correct for this accumulated eDNA it is possible to separate live and dead DNA prior to sequencing (Nocker *et al.*, 2007). This may especially be appropriate for the assessment of ballast water, as this may not only contain many propagules (f.i. resting eggs) that cannot be identified by classical taxonomic techniques, but also a lot of unbound DNA, released from decaying bodies of dead organisms.
During this pilot study, these novel techniques to specifically target DNA in living cells were not applied.

6 Discussion and monitoring advise

6.1 Aim of the study

The description of the species community of Eemshaven and Delfzijl, based upon both indigenous and non- indigenous species, was the main goal of the current study. In addition, an assessment of the current risk of species introductions in the ports of GSP and Wadden Sea by untreated ballast water was conducted.

The objectives of the project were:

- To determine species present within the harbour basins of Eemshaven and Delfzijl.
  Determine the community and in particular, benthos, plankton and epifauna in Eemshaven and Delfzijl using classical taxonomic and modern molecular techniques. Classify species as indigenous and non–indigenous species (NIS).
- Determine species in ballast water discharged in Delfzijl and/or Eemshaven:
  The species community within untreated ballast water from a selection of ships is determined via classical taxonomic and modern molecular techniques.
- Assessment of potential survival of NIS
  A risk assessment for invasion to the Wadden Sea is performed based on ecological profiles of NIS in ballastwater newly recorded to GSP.
- Evaluate the best practices to advise on future monitoring (what, where, when)

6.1.1 Species composition

The species community was described for the harbours and three ballast waters, using classical taxonomic and modern molecular techniques, which showed to be complementory in detecting species.
From the 344 species detected, 1 out of 7 species in GSP harbours are found to be non-indigenous, and originate from other regions.

Sampling and detection techniques differed in effort and/or surface sampled. The number of samples and quantification of species found per sample is, therefore, hard to compare. E.g. whereas snorkelling obtained most NIS out of all hard substrates, this is inherent to the sampling technique in which sampling continues untill no additional species are found. The snorkelling, therefore, consisted of several hours of monitoring, continuously searching specifically for species that were not recorded yet. This resulted in a variable surface sampled, depending on the richness of the study area, and (theoretically) an optimal number of species. This technique, however, strongly depends on the ability to identify species in the field. Only a limited number of samples of unidentifyable species was taken, mainly comprising macro-algae. This survey methodology differs from sample based methodologies, where samples have to be taken for further processing in the laboratory. In this case a fixed number of samples has to be taken, using a predefined sampling scheme. Previous research may be used to identify an optimal number of samples (using SAC analysis) for this type of sampling.

In the current research, the main question is, however, whether the different sampling techniques and intensities, as well as the different identification techniques used (classical vs. molecular) have resulted in a sufficient exhaustive characterisation of the species (indigenous and NIS) in both harbour areas and ballast water alike. This was presented in chapter 5.3, indicating that depending on the habitat and technique, the survey detected many species, but that any additional water or hard substrate sample would have resulted in additional species detection too. The use of eDNA complemented the classical taxonomy species list, resulting in a more exhaustive list.
Variation in species detection over the years however occurs due to varation in species composition in time and space, and due to differences in study design. This variation was applicable to this study as well compared to previous inventories in the Wadden region (Gittenberger et al (2009, 2011, 2014)).

6.1.2 Risk of introduction of NIS by ballast water

A risk assessment for invasion to the Wadden Sea is performed based on ecological profiles of NIS in ballast water newly recorded to GSP in chapter 4. In this study, 6 new NIS to GSP and the Wadden Sea region were deteted.
Some of these newly detected NIS, can establish themselves in the Wadden Sea. The Japanese mussel or Green mussel (*Arcuatula senhousia*, also known as *Musculista senhousia*), was detected in ballast water, and given it habitat requirements it is not unlikely that the species can establish in the Wadden Sea. It depends on whether the DNA fragments originated from living eggs or larvea, or dead or non-viable cells. Field observations should confirm its presence. Regarding the other species, such as *Hydroides elegans* and *Amphibalanus amphitrite* it is most likely to observe these species near cooling water discharge points first. Depending climate change, these species might established and spread along larger spatial scales, including the Wadden Sea. It is not known if these species pose a threat for populations of indigenous species.

Given the objective to evaluate the best practices to advise on future monitoring (what, where, when), chapter 6.3 will provide an advise on best practises based on the results in chapter 5.

Given the number of species detected in ballast water of only three ships, and the number of new NIS found in these samples, treatment of the ballast water is urgent. Ballast water treatment will reduce the risk of introducing new NIS, and potentially invasive spieces to the harbours of GSP and the nearby located Wadden Sea largely.

6.2 eDNA versus classical taxonomy

The eDNA technique is a relatively new approach used to identify species in the environment. Using this method it is possible to detect species without actually seeing or catching them (in this report: the classical taxonomy technique). The method uses DNA-based identification, also called barcoding, to detect species. Extracellular DNA, or cell debris, which a species leaves behind in the environment is sampled in water, sediment or scrapes and processed into species lists. Prior to the analysis, a “false” species check was run and obvious false species were discarded (e.g. ants, butterflies, terrestrial (inland) plants of which DNA could easily be spread by wind, but were obviously not our target species). Due to contrains in time, not all 242 species detected via eDNA were checked for their ecological profiles in this study.

As already discussed in Chapter 5, there are several alternative explanations for the presence of DNA in a water habitat, without viable organisms present. Predators like piscivorous birds, for example herons, could spread DNA by eating a fish at one location and excreting the remains at other locations (Amberg *et al.*, 2013). In our study, this could explain the presence of Bream DNA in Eemshaven because it is obvious this freshwater species would not be a common part of the marine ecosystem. In practice, even monitoring equipment such as nets and boats, could serve as a vector for DNA from one location to another. This example illustrates that ecological meaningful interpretation of results will always need ecologists.

Nothwithstanding the above mentioned limitations, research has shown that in water, eDNA breaks down within a few days to a month (Thomson *et al.*, 2015). Therefore, the detection of a species’ DNA in the water confirms its potential presence. A water sample thus provides a relative actual and potential species composition. In other habitats, such as sediments, the persistence of eDNA can be much longer, under specific conditions even several millennia. Therefore, in those environments it is more difficult to confirm current presence of a species based on eDNA (Herder *et al.*, 2014). The species profile much more provides an accumulated species composition. This was also seen in this study.

eDNA metabarcoding is proven to be a very powerful approach, allowing the detection of many different species without any prior knowledge of species distribution in the study area. This makes the method highly applicable to study the presence or early establishment of non-indigenous species in habitats with little prior knowledge of possible species composition, e.g. ballast water, or in poorly investigated habitats (Herder *et al.*, 2014). In **Table 9** an overview if provided of most advantages and disadvantaged of both techniques based on Herder *et al*. (2014) which apply also to this study.

In this project, eDNA was used as a pilot/case study in order to evaluate its additional value in monitoring NIS in the harbours of GSP and in ballast waters to screen potential introductions of NIS. As discussed, the detection of a species' DNA in the water confirms its potential presence, but not it's actual viability. eDNA in ballast water, therefore, only serves as an early warning signal, but tells nothing yet about the viability of the cells present in ballast water. Additional monitoring focussing on the visual detection of the species confirms its actual establishment in the environment.

Monitoring and species detection using eDNA was proven to be of value to detect additional NIS which were not detected using the classical approaches. In turn, the classical approach also found species and NIS which were not detected with eDNA. As such, we conclude that the techniques complement each other and were both very valuable.

Table 9. *Summary of pros and cons of both monitoring and species detection techniques after Herder* et al. *(2014).*

Monitoring approach	Advantages	Disadvantages	References
Classical monitoring using marine experts	+ taxonomic resolution currently often higher than with molecular techniques (dependent on expert, species, DNA database) + able to estimate local species diversities and infer population dynamics + able to find newly invaded species aside from the ones on a metabarcoding alert list + widely deployable	- experts have specialised taxonomic knowledge, thus success in finding species dependent on expertise - high workload, possibly resulting in fewer visits to bioinvasion hotspots - (very) small species might be overlooked and not all stages are identifiable (eggs, juveniles) - not many persons with taxonomic expertise, loss of expertise due to expert retirement - negative result does not imply that organism was not in area, just that there was no individual in sample	i.a. Buschbaum *et al.* (2012) Herder *et al.*, 2014
eDNA via metabarcoding	+ taxonomically comprehensive + relative quick to produce + less reliant on taxonomic expertise + editable by third parties + can uncover morphologically cryptic species (complexes) and unidentifiable stages (eggs, juveniles) + can collect DNA of difficult-to-trap taxa + able to census vagrant species + very sensitive, high specificity	- presence and location of a particular species still needs to be verified by fieldwork - negative result does not imply that organism was not in area, just that there was no eDNA in sample - metabarcode data sets subject to error and loss of information - necessary to generate and maintain individuals barcoded to be able to link metabarcoding sequences to species: ongoing process. Many marine NIS have their origin in SE Asian marine waters and species descriptions and DNA in databases are incomplete - effects of sea currents and wave action on dispersion and dilution of eDNA, and pH, temperature and salinity impacts preservation and extraction of eDNA - Water bodies influence eachother by overflows (e.g. freshwater discharge into coastal areas: detection of fresh water species in marine environment - only semi- quantification of abundance of organisms possible - cannot give real-time information on organism's location - it does not provide information regarding factors such as the life stage, reproduction or fitness (live/death) of a species - live/death differentiation not included on regular basis: actual vs cumulated eDNA. New approaches to be tested	i.a. Ji *et al.* (2013) Herder *et al.* (2014) This study

6.3 Lessons learned for future monitoring in Groningen Seaports

The results of this study imply that

- Hard substrates were the most effective habitat to collect species in general and to collect NIS in particular, based upon classical taxonomy. The eDNA results for hard substrates were of lesser quality. Additional progress in sampling and preservation protocols is needed to obtain better results.
- Sampling hard substrates was most effective using scraping technique, SETL plates and snorkelling inventory. Although dikes inventory does not add many species, all techniques seemed complementary in yielding species and NIS.
- With relatively limited sampling effort in hard substrates, a relatively high number of species is found
- Sampling water resulted in the highest number of species, with a relatively low sampling effort. This was, however, primarily based upon detection with eDNA. Classical taxonomy detected a relatively low number of species.
- Sediments yielded no NIS using classical taxonomy techniques. The effort taken (need of an extra boat and grab facilities, boatmen and two persons on deck) to collect and (classical) analyse sediment samples seems out of balance compared to the other habitats and techniques. Only when using eDNA as additional identification technique, collection of sediment samples seems worth the effort. However, due to the cumulative nature of eDNA in sediment samples, it is important to take into account the cumulative temporal dimensions of these results, or to develop techniques to separate “ancient” DNA from recent (living) DNA..
- Techniques to separate ancient from living DNA are also relevant for identifying potential NIS in ballast water samples.

To optimise to monitoring, the following monitoring design is suggested:

- Put effort in continuation of hard substrate sampling to sample most NIS. All applied techniques compliment the list of NIS found. Excluding a technique will result in less species and NIS found. Currently classical taxonomy is the most suitable technique to identify species, as eDNA techniques need to be improved for these substrates.
- Species compositions among SETL plates deployed at various depths were similar. Additional plates do yield more species, independent of depth. Deployment at -1m will be sufficient in future monitoring.
- Put effort in additional water samples, and analyse these with both classical taxonomy and eDNA. The samples should be taken over a wider seasonal span.
- Start a survey of an area with a “quick” eDNA study based on water samples and sediment samples. After the results are known, plan and do the survey of the area with classical monitoring techniques that focus not only on sampling the habitats, but also on searching for the presence of settled individuals of the NIS scored by the eDNA techniques. Based on NIS profiles the classical monitoring designs can be adapted. E.g. additional habitats or techniques can be selected in order to detect and confirm certain species (such as species detection near cooling water stations)
- Do not put effort in sediment sampling, hardly any species and NIS will be identified using classical taxonomy. Applying eDNA on sediment sample will increase species identification, but results are difficult to inteprete as this is likely to be sedimented DNA instead of actual present species.
- If time and budget is limited, prioritise monitoring design on SETL plates, scrape samples, snorkelling and water samples to collect most species and NIS. Snorkelling is however only possible at certain locations, and depends other activities in the area[7].
- Add sampling locations near cooling water discharge pipes for early detection of NIS.

Eemshaven:

- Sample hard substrates and floating docks <u>in all basins</u> using SETL plates and scrape sampling at all possible substrates (concrete and steel).

[7] Depending approval of harbour authorities

- Deploy SETL plates in June, collect in September. Deployment in March does not yield additional species
- Sample all dikes at all zones
- Collect water samples in all basins in (winter,) spring, summer and autumn

Delfzijl:

- Sample hard substrates along the canal and yacht harbour using SETL plates and scraping all possible substrates (steel, concrete, wood)
- Sample water along a transects from inside to entrance in spring, summer and autumn
- Sample all dikes at all zones if possible.

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8 Quality Assurance

Wageningen Marine Research utilises an ISO 9001:2008 certified quality management system (certificate number: 187378-2015-AQ-NLD-RvA). This certificate is valid until 15 September 2018. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V.

The above controls are described in Wageningen Marine Research working instruction ISW 2.10.2.105. If desired, information regarding the performance characteristics of the analytical methods is available at the chemical laboratory at IJmuiden.

If the quality cannot be guaranteed, appropriate measures are taken.

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Justification

Report C045/17
Project Number: 431.51000.42

The scientific quality of this report has been peer reviewed by a colleague scientist and a member of the Management Team of Wageningen Marine Research

Approved: Dr. Klaas Kaag
Researcher

Signature:

Date: 16th June 2017

Approved: Drs. J. Asjes
Manager Integration

Signature:

Date: 16th June 2017

Annex 1 Observed species

Table 10. *List of observed species in 2016, including information on origin (NIS, unknown or ind= indigenous), information on the detection method (18S en CO1 refer to eDNA primers used) , and where the species was found (Eemshaven, Delfzijl or Ballastwaters).*

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
Bacteria		Bacteria	Cyanobacteria	*Planktothrix agardhii*	Ind.	classic			x
eukaryotes		Protozoa		*Ebria tripartita*	Ind.	classic	x	x	
acoela	Invertebrates	Animalia	Xenacoelomorpha	*Paramecynostomum diversicolor*	?	18s	x		
amphipods	Invertebrates	Animalia	Arthropoda	*Apocorophium lacustre*	Ind.	COI		x	
amphipods	Invertebrates	Animalia	Arthropoda	*Caprella mutica*	NIS	classic	x		
amphipods	Invertebrates	Animalia	Arthropoda	*Caprella mutica*	NIS	COI	x	x	
amphipods	Invertebrates	Animalia	Arthropoda	*Corophium volutator*	Ind.	COI		x	
amphipods	Invertebrates	Animalia	Arthropoda	*Gammarus locusta*	Ind.	classic	x	x	
amphipods	Invertebrates	Animalia	Arthropoda	*Gammarus tigrinus*	NIS	COI		x	
amphipods	Invertebrates	Animalia	Arthropoda	*Melita nitida*	NIS	COI	x	x	
amphipods	Invertebrates	Animalia	Arthropoda	*Melita palmata*	Ind.	classic		x	
amphipods	Invertebrates	Animalia	Arthropoda	*Microprotopus maculatus*	Ind.	classic		x	
amphipods	Invertebrates	Animalia	Arthropoda	*Monocorophium acherusicum*	NIS	classic	x	x	
amphipods	Invertebrates	Animalia	Arthropoda	*Monocorophium acherusicum*	NIS	COI	x		
amphipods	Invertebrates	Animalia	Arthropoda	*Monocorophium insidiosum*	Ind.	classic	x	x	
amphipods	Invertebrates	Animalia	Arthropoda	*Monocorophium insidiosum*	Ind.	COI	x	x	
Anthozoa	Invertebrates	Animalia	Cnidaria	*Metridium dianthus*	Ind.	classic	x		
Anthozoa	Invertebrates	Animalia	Cnidaria	*Sagartia elegans*	Ind.	classic	x		
Anthozoa	Invertebrates	Animalia	Cnidaria	*Urticina felina*	Ind.	classic	x		
apicomplexans	Invertebrates	Chromista	Myzozoa	*Lecudina tuzetae*	?	18s		x	
bivalves	Invertebrates	Animalia	Mollusca	*Cerastoderma edule*	Ind.	18s	x		x
bivalves	Invertebrates	Animalia	Mollusca	*Cerastoderma glaucum*	Ind.	COI			x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
bivalves	Invertebrates	Animalia	Mollusca	*Corbicula fluminea*	NIS	18s		x	
bivalves	Invertebrates	Animalia	Mollusca	*Crassostrea gigas*	NIS	classic	x	x	
bivalves	Invertebrates	Animalia	Mollusca	*Crassostrea gigas*	NIS	COI	x	x	
bivalves	Invertebrates	Animalia	Mollusca	*Dreissena rostriformis*	NIS	18s			x
bivalves	Invertebrates	Animalia	Mollusca	*Ensis directus*	NIS	COI	x		
bivalves	Invertebrates	Animalia	Mollusca	*Limecola balthica*	?	COI	x		
bivalves	Invertebrates	Animalia	Mollusca	*Macomangulus tenuis*	Ind.	classic	x		
bivalves	Invertebrates	Animalia	Mollusca	*Musculista senhousia*	NIS	18s			x
bivalves	Invertebrates	Animalia	Mollusca	*Mya arenaria*	NIS	COI	x		x
bivalves	Invertebrates	Animalia	Mollusca	*Mytilus edulis*	Ind.	classic	x	x	
bivalves	Invertebrates	Animalia	Mollusca	*Mytilus edulis*	Ind.	COI		x	
bivalves	Invertebrates	Animalia	Mollusca	*Petricolaria pholadiformis*	NIS	COI	x		
bivalves	Invertebrates	Animalia	Mollusca	*Polititapes aureus*	Ind.	COI			x
bivalves	Invertebrates	Animalia	Mollusca	*Rangia cuneata*	NIS	COI		x	x
brittle stars	Invertebrates	Animalia	Echinodermata	*Ophiura ophiura*	Ind.	classic	x		
brittle stars	Invertebrates	Animalia	Echinodermata	*Ophiura ophiura*	Ind.	COI	x		x
bryozoans	Invertebrates	Animalia	Bryozoa	*Alcyonidioides mytili*	Ind.	classic	x		
bryozoans	Invertebrates	Animalia	Bryozoa	*Alcyonidioides mytili*	Ind.	18s	x		
bryozoans	Invertebrates	Animalia	Bryozoa	*Alcyonidium verrilli*	?	COI	x		
bryozoans	Invertebrates	Animalia	Bryozoa	*Amathia gracilis*	NIS	COI/18s	x		
bryozoans	Invertebrates	Animalia	Bryozoa	*Amathia tertia*	?	COI	x	x	
bryozoans	Invertebrates	Animalia	Bryozoa	*Bugulina stolonifera*	NIS	COI	x	x	
bryozoans	Invertebrates	Animalia	Bryozoa	*Conopeum reticulum*	Ind.	classic	x	x	
bryozoans	Invertebrates	Animalia	Bryozoa	*Conopeum reticulum*	Ind.	18s	x		
bryozoans	Invertebrates	Animalia	Bryozoa	*Conopeum tenuissimum*	?	COI	x	x	
bryozoans	Invertebrates	Animalia	Bryozoa	*Crisularia plumosa*	Ind.	classic	x		
bryozoans	Invertebrates	Animalia	Bryozoa	*Einhornia crustulenta*	Ind.	classic	x	x	
bryozoans	Invertebrates	Animalia	Bryozoa	*Smittoidea prolifica*	NIS	classic	x	x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
cercozoans	Invertebrates	Chromista	Cercozoa	*Cryothecomonas aestivalis*	?	18s	x		
cercozoans	Invertebrates	Protozoa		*Ebria tripartita*	Ind.	18s	x	x	x
cercozoans	Invertebrates	Chromista	Cercozoa	*Massisteria marina*	?	18s			x
cercozoans	Invertebrates			*Minorisa minuta*	?	18s			x
cercozoans	Invertebrates			*Phagomyxa bellerocheae*	?	18s	x		
cercozoans	Invertebrates			*Trachyrhizium urniformis*	?	18s	x		
cercozoans	Invertebrates			*Ventrifissura artocarpoidea*	?	18s	x	x	
Chitons	Invertebrates	Animalia	Mollusca	*Lepidochitona cinerea*	Ind.	classic	x		
choanoflagellates	Invertebrates	Protozoa	Choanozoa	*Acanthocorbis unguiculata*	?	18s		x	
choanoflagellates	Invertebrates	Protozoa	Choanozoa	*Bicosta minor*	?	18s	x		x
choanoflagellates	Invertebrates	Protozoa	Choanozoa	*Crinolina isefiordensis*	?	18s	x		
choanoflagellates	Invertebrates	Protozoa	Choanozoa	*Didymoeca costata*	?	18s			x
choanoflagellates	Invertebrates	Protozoa	Choanozoa	*Hartaetosiga gracilis*	?	18s			x
ciliates	Invertebrates	Chromista	Ciliophora	*Acineta flava*	?	18s			x
ciliates	Invertebrates	Chromista	Ciliophora	*Acineta tuberosa*	?	18s			x
ciliates	Invertebrates	Chromista	Ciliophora	*Anteholosticha scutellum*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Carchesium polypinum*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Cyclotrichium cyclokaryon*	?	18s	x		
ciliates	Invertebrates	Chromista	Ciliophora	*Dysteria semilunaris*	?	18s			x
ciliates	Invertebrates	Chromista	Ciliophora	*Hemigastrostyla enigmatica*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Holosticha diademata*	?	18s	x		
ciliates	Invertebrates	Chromista	Ciliophora	*Kiitricha marina*	?	18s	x		
ciliates	Invertebrates	Chromista	Ciliophora	*Laboea strobila*	Ind.	classic	x		
ciliates	Invertebrates	Chromista	Ciliophora	*Leegaardiella sol*	Ind.	classic	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Levicoleps taehwae*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Lohmanniella oviformis*	Ind.	classic	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Lynnella semiglobulosa*	?	18s	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Mesodinium rubrum*	Ind.	classic	x	x	x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
ciliates	Invertebrates	Chromista	Ciliophora	*Metanophrys sinensis*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Oxytricha saltans*	?	18s			x
ciliates	Invertebrates	Chromista	Ciliophora	*Parabirojimia similis*	?	18s	x		
ciliates	Invertebrates	Chromista	Ciliophora	*Parastrombidinopsis shimi*	?	18s	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Pelagostrobilidium paraepacrum*	?	18s	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Pseudoamphisiella lacazei*	?	18s			x
ciliates	Invertebrates	Chromista	Ciliophora	*Pseudocohnilembus hargisi*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Rimostrombidium veniliae*	?	18s	x		
ciliates	Invertebrates	Chromista	Ciliophora	*Stentor muelleri*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidinopsis acuminata*	Ind.	classic	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidium biarmatum*	?	18s	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidium conicum*	Ind.	classic	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidium conicum*	Ind.	classic		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidium elongatum*	Ind.	classic		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidium minor*	Ind.	classic			x
ciliates	Invertebrates	Chromista	Ciliophora	*Strombidium paracalkinsi*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Tintinnopsis beroidea*	Ind.	classic	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Tintinnopsis lobiancoi*	Ind.	classic		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Tintinnopsis minuta*	?	18s	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Uronychia sinica*	?	18s		x	
ciliates	Invertebrates	Chromista	Ciliophora	*Zoothamnium alternans*	?	18s	x	x	
ciliates	Invertebrates	Chromista	Ciliophora	*Zoothamnium duplicatum*	?	18s	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Acanthocyclops americanus*	?	COI		x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Acartia bifilosa*	?	COI/18s	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Acartia clausii*	?	COI/18s	x	x	x
crustaceans	Invertebrates	Animalia	Arthropoda	*Acartia tonsa*	NIS	COI/18s	x	x	x
crustaceans	Invertebrates	Animalia	Arthropoda	*Ameira scotti*	?	18s	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Amphibalanus amphitrite*	NIS	COI			x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
crustaceans	Invertebrates	Animalia	Arthropoda	*Amphibalanus improvisus*	NIS	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Amphibalanus improvisus*	NIS	COI	x	x	x
crustaceans	Invertebrates	Animalia	Arthropoda	*Austrominius modestus*	NIS	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Austrominius modestus*	NIS	COI	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Balanus balanus*	NIS	COI	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Balanus crenatus*	Ind.	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Balanus crenatus*	Ind.	18s	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Bosmina (Bosmina) longirostris*	Ind.	classic			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Bosmina (Eubosmina) coregoni*	Ind.	classic			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Cancer pagurus*	Ind.	classic	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Carcinus maenas*	Ind.	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Carcinus maenas*	Ind.	COI	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Crangon crangon*	Ind.	COI		x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Cyclops kikuchii*	?	COI	x		x
crustaceans	Invertebrates	Animalia	Arthropoda	*Daphnia cucullata*	Ind.	classic			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Diaphanosoma brachyurum*	Ind.	classic			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Eubosmina coregoni*	?	COI		x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Eudiaptomus gracilis*	?	COI		x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Eurytemora affinis*	Ind.	COI/18s	x	x	x
crustaceans	Invertebrates	Animalia	Arthropoda	*Eurytemora carolleeae*	?	COI			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Harpacticus flexus*	Ind.	COI	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Hemigrapsus sanguineus*	NIS	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Hemigrapsus takanoi*	NIS	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Idotea linearis*	Ind.	classic	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Isias clavipes*	?	18s			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Neomysis americana*	NIS	18s	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Neomysis integer*	Ind.	COI/18s		x	x
crustaceans	Invertebrates	Animalia	Arthropoda	*Palaemon elegans*	Ind.	classic		x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
crustaceans	Invertebrates	Animalia	Arthropoda	*Paracalanus parvus*	?	COI			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Praunus flexuosus*	Ind.	classic	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Pseudodiaptomus marinus*	?	COI		x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Rhithropanopeus harrisii*	NIS	COI		x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Schlerochilus oshoroensis*	?	18s	x		
crustaceans	Invertebrates	Animalia	Arthropoda	*Semibalanus balanoides*	Ind.	classic	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Splanchnotrophus angulatus*	?	COI			x
crustaceans	Invertebrates	Animalia	Arthropoda	*Temora longicornis*	Ind.	COI/18s	x	x	
crustaceans	Invertebrates	Animalia	Arthropoda	*Tisbe cf. tenera CCUMP 44*	?	18s	x		
ctenophores	Invertebrates	Animalia	Ctenophora	*Beroe gracilis*	Ind.	classic	x		
ctenophores	Invertebrates	Animalia	Ctenophora	*Mnemiopsis leidyi*	NIS	classic	x		
ctenophores	Invertebrates	Animalia	Ctenophora	*Mnemiopsis leidyi*	NIS	COI	x	x	
eukaryotes	Invertebrates			*Flamella arnhemensis*	?	18s		x	
eukaryotes	Invertebrates	Protozoa	Loukozoa	*Jakoba libera*	?	COI			x
eukaryotes	Invertebrates	Protozoa	Picozoa	*Picomonas judraskeda*	?	18s	x	x	x
eukaryotes	Invertebrates			*Pseudoperkinsus tapetis*	?	18s	x		
eukaryotes	Invertebrates	Protozoa	Amoebozoa	*Squamamoeba japonica*	?	COI	x	x	
flatworms	Invertebrates	Animalia	Platyhelminthes	*Pseudomonocelis agilis*	?	18s		x	
flatworms	Invertebrates	Animalia	Platyhelminthes	*Strongylostoma elongatum*	?	18s		x	
gastropods	Invertebrates	Animalia	Mollusca	*Crepidula fornicata*	NIS	classic	x		
gastropods	Invertebrates	Animalia	Mollusca	*Crepidula fornicata*	NIS	COI	x		
gastropods	Invertebrates	Animalia	Mollusca	*Facelina bostoniensis*	Ind.	COI	x		
gastropods	Invertebrates	Animalia	Mollusca	*Gibbula magus*	Ind.	18s	x		
gastropods	Invertebrates	Animalia	Mollusca	*Littorina littorea*	Ind.	classic	x	x	
gastropods	Invertebrates	Animalia	Mollusca	*Littorina littorea*	Ind.	COI	x		
gastropods	Invertebrates	Animalia	Mollusca	*Littorina saxatilis*	Ind.	classic	x		
gastropods	Invertebrates	Animalia	Mollusca	*Peringia ulvae*	Ind.	classic	x		
gastropods	Invertebrates	Animalia	Mollusca	*Peringia ulvae*	Ind.	COI/18s	x		x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
gastropods	Invertebrates	Animalia	Mollusca	*Tergipes tergipes*	Ind.	classic	x		
gastrotrichs	Invertebrates	Animalia	Gastrotricha	*Urodasys calicostylis*	?	COI			x
goblet worms	Invertebrates	Animalia	Entoprocta	*Barentsia benedeni*	Ind.	18s	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Blackfordia virginica*	NIS	COI		x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Bougainvillia muscus*	Ind.	COI	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Clytia Clytia*	Ind.	classic	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Cordylophora caspia*	NIS	classic		x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Ectopleura crocea*	?	COI	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Ectopleura larynx*	Ind.	classic	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Eucheilota maculata*	Ind.	COI/18s	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Gonothyraea loveni*	Ind.	18s	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Hartlaubella gelatinosa*	Ind.	classic	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Hartlaubella gelatinosa*	Ind.	COI	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Hydra oligactis*	?	COI	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Nemopsis bachei*	Ind.	COI	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Obelia bidentata*	Ind.	COI/18s	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Obelia dichotoma*	Ind.	classic	x	x	
hydrozoans	Invertebrates	Animalia	Cnidaria	*Obelia dichotoma*	Ind.	COI/18s	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Obelia geniculata*	Ind.	classic	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Obelia longissima*	Ind.	classic	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Opercularella lacerata*	Ind.	18s	x		
hydrozoans	Invertebrates	Animalia	Cnidaria	*Tubularia indivisa*	Ind.	COI	x		
jellyfishes	Invertebrates	Animalia	Cnidaria	*Aurelia aurita*	Ind.	classic	x		
jellyfishes	Invertebrates	Animalia	Cnidaria	*Aurelia aurita*	Ind.	COI	x		
nematodes	Invertebrates	Animalia	Nematoda	*Chromadorita tentabundum*	?	18s		x	
nematodes	Invertebrates	Animalia	Nematoda	*Litoditis aff. marina PmIV*	?	COI	x		
nematodes	Invertebrates	Animalia	Nematoda	*Panagrolaimus paetzoldi*	?	18s		x	
nematodes	Invertebrates	Animalia	Nematoda	*Pellioditis marina*	?	18s	x		

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
nematodes	Invertebrates	Animalia	Nematoda	*Punctodora ratzeburgensis*	?	18s		x	
nematodes	Invertebrates	Animalia	Nematoda	*Sabatieria pulchra*	?	18s		x	
rotifers	Invertebrates	Animalia	Rotifera	*Filinia longiseta*	?	18s		x	
rotifers	Invertebrates	Animalia	Rotifera	*Proales reinhardti*	?	18s		x	
rotifers	Invertebrates	Animalia	Rotifera	*Rotaria rotatoria*	?	COI		x	
segmented worms	Invertebrates	Animalia	Annelida	*Alitta succinea*	Ind.	classic	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Alitta succinea*	Ind.	COI/18s	x	x	x
segmented worms	Invertebrates	Animalia	Annelida	*Amphitrite ornata*	?	18s		x	
segmented worms	Invertebrates	Animalia	Annelida	*Aphelochaeta marioni*	Ind.	classic	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Arenicola marina*	Ind.	COI/18s	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Dero obtusa*	?	COI		x	
segmented worms	Invertebrates	Animalia	Annelida	*Eteone longa*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Eulalia viridis*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Eulalia viridis*	Ind.	18s	x		
segmented worms	Invertebrates	Animalia	Annelida	*Ficopomatus enigmaticus*	NIS	classic		x	
segmented worms	Invertebrates	Animalia	Annelida	*Ficopomatus enigmaticus*	NIS	COI/18s	x	x	x
segmented worms	Invertebrates	Animalia	Annelida	*Harmothoe imbricata*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Heteromastus filiformis*	Ind.	18s	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Hydroides elegans*	NIS	18s			x
segmented worms	Invertebrates	Animalia	Annelida	*Hypereteone heteropoda*	?	COI	x	x	x
segmented worms	Invertebrates	Animalia	Annelida	*Lanice conchilega*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Lepidonotus squamatus*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Myrianida prolifera*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Mysta picta*	NIS	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Polydora ciliata*	NIS	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Polydora cornuta*	Ind.	classic	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Polydora cornuta*	Ind.	COI	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Polydora websteri*	?	COI		x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
segmented worms	Invertebrates	Animalia	Annelida	*Protodrilus adhaerens*	?	COI	x		
segmented worms	Invertebrates	Animalia	Annelida	*Pygospio elegans*	Ind.	classic	x		
segmented worms	Invertebrates	Animalia	Annelida	*Streblospio benedicti*	Ind.	classic	x	x	
segmented worms	Invertebrates	Animalia	Annelida	*Stylaria lacustris*	?	COI		x	
segmented worms	Invertebrates	Animalia	Annelida	*Tubificoides brownae*	?	COI	x		
sponges	Invertebrates	Animalia	Porifera	*Halichondria (Halichondria) bowerbanki*	Ind.	classic	x		
sponges	Invertebrates	Animalia	Porifera	*Halichondria (Halichondria) panicea*	Ind.	classic	x		
sponges	Invertebrates	Animalia	Porifera	*Halichondria panicea*	?	COI	x		
sponges	Invertebrates	Animalia	Porifera	*Leucosolenia variabilis*	Ind.	classic	x		
starfish	Invertebrates	Animalia	Echinodermata	*Asterias rubens*	Ind.	classic	x		
starfish	Invertebrates	Animalia	Echinodermata	*Asterias rubens*	Ind.	COI	x		
tunicates	Invertebrates	Animalia	Chordata	*Aplidium glabrum*	NIS	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Ascidiella aspersa*	Ind.	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Botrylloides violaceus*	NIS	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Botrylloides violaceus*	NIS	COI	x	x	
tunicates	Invertebrates	Animalia	Chordata	*Botryllus schlosseri*	Ind.	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Botryllus schlosseri*	Ind.	COI	x		
tunicates	Invertebrates	Animalia	Chordata	*Ciona intestinalis*	Ind.	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Ciona intestinalis*	Ind.	18s	x		
tunicates	Invertebrates	Animalia	Chordata	*Diplosoma listerianum*	NIS	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Molgula manhattensis*	NIS	classic	x	x	
tunicates	Invertebrates	Animalia	Chordata	*Molgula manhattensis*	NIS	COI	x	x	
tunicates	Invertebrates	Animalia	Chordata	*Styela clava*	NIS	classic	x		
tunicates	Invertebrates	Animalia	Chordata	*Styela clava*	NIS	18s	x		
brown algae	Plants and Fungi	Chromista	Ochrophyta	*Ascophyllum nodosum*	Ind.	classic	x	x	
brown algae	Plants and Fungi	Chromista	Ochrophyta	*Fucus spiralis*	Ind.	classic	x	x	
brown algae	Plants and Fungi	Chromista	Ochrophyta	*Fucus vesiculosus*	Ind.	classic	x	x	
brown algae	Plants and Fungi	Chromista	Ochrophyta	*Hecatonema maculans*	Ind.	COI		x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
brown algae	Plants and Fungi	Chromista	Ochrophyta	*Sargassum muticum*	NIS	classic	x		
cryptomonads	Plants and Fungi	Chromista	Cryptophyta	*Teleaulax acuta*	?	18s	x	x	x
cryptomonads	Plants and Fungi	Chromista	Cryptophyta	*Teleaulax amphioxeia*	?	18s	x	x	x
cryptomonads	Plants and Fungi	Chromista	Cryptophyta	*Teleaulax gracilis*	?	18s	x	x	x
cryptomonads	Plants and Fungi	Chromista	Cryptophyta	*Urgorri complanatus*	?	18s		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Actinocyclus normanii*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Actinoptychus octonarius*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Actinoptychus senarius*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Actinoptychus splendens*	Ind.	classic	x		x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Asterionellopsis glacialis*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Asterionellopsis lenisilicea*	?	COI	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Asterionellopsis thurstonii*	?	COI	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Brockmanniella brockmannii*	?	18s	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Campylosira cymbelliformis*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Cerataulina pelagica*	?	18s	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros danicus*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros debilis*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros debilis*	Ind.	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros didymus*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros elegans*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros socialis*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros socialis*	Ind.	COI	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Chaetoceros subtilis*	Ind.	classic	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Conticribra guillardii*	NIS	18s	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Coscinodiscus granii*	Ind.	classic			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Coscinodiscus radiatus*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Cylindrotheca closterium*	?	COI	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Delphineis minutissima*	Ind.	classic			x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Detonula pumila*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Ditylum brightwellii*	Ind.	classic	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Ditylum brightwellii*	Ind.	COI/18s	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Eucampia zodiacus*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Eunotogramma dubium*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Grammatophora marina*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Guinardia delicatula*	Ind.	classic	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Guinardia striata*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Gyrosigma fasciola*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Haslea crucigera*	?	COI			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Haslea nipkowii*	?	18s	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Lauderia annulata*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Lennoxia faveolata*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Leptocylindrus danicus*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Leptocylindrus minimus*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Leptocylindrus minimus*	Ind.	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Lithodesmium undulatum*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Lithodesmium variabile*	?	COI	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Mediopyxis helysia*	NIS	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Melosira moniliformis*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Melosira nummuloides*	Ind.	classic	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Minutocellus polymorphus*	?	COI	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Minutocellus scriptus*	Ind.	classic	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Nitzschia incerta*	Ind.	classic			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Nitzschia longissima*	?	18s			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Odontella aurita*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Odontella longicruris*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Odontella sinensis*	NIS	classic	x	x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Paralia sulcata*	Ind.	classic		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Paralia sulcata*	Ind.	18s		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Pauliella taeniata*	NIS	classic			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Plagiogrammopsis vanheurckii*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Pleurosigma planktonicum*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Pseudo-nitzschia fraudulenta*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Pseudo-nitzschia pungens*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Rhaphoneis amphiceros*	Ind.	classic	x		x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Rhizosolenia delicatula*	?	18s		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Rhizosolenia imbricata*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Rhizosolenia setigera*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Rhizosolenia setigera*	Ind.	COI	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Skeletonema dohrnii*	?	COI	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Skeletonema menzellii*	?	COI			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Skeletonema potamos*	Ind.	classic	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Skeletonema potamos*	Ind.	COI/18s	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Skeletonema pseudocostatum*	?	COI	x		x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Tenuicylindrus belgicus*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassionema nitzschioides*	Ind.	classic	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassionema nitzschioides*	Ind.	COI	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira gessneri*	?	18s			x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira gravida*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira hendeyi*	?	18s	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira lundiana*	?	18s	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira nodulolineata*	?	18s		x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira nordenskioeldii*	?	COI	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira profunda*	?	18s	x	x	x
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira pseudonana*	?	COI	x	x	x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Thalassiosira punctigera*	?	COI	x	x	
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Trigonium alternans*	Ind.	classic	x		
diatoms	Plants and Fungi	Chromista	Ochrophyta	*Tryblionella apiculata*	?	18s		x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Akashiwo sanguinea*	?	18s	x		
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Gymnodinium galeatum*	Ind.	classic		x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Gymnodinium impudicum*	?	18s			x
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Gyrodinium dominans*	?	18s	x	x	x
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Gyrodinium spirale*	Ind.	classic	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Heterocapsa lanceolata*	Ind.	classic	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Katodinium glaucum*	?	18s	x		
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Nematopsides vigilans*	Ind.	classic	x		
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Noctiluca scintillans*	Ind.	classic	x	x	x
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Noctiluca scintillans*	Ind.	18s	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Oblea rotunda*	Ind.	classic		x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Paragymnodinium shiwhaense*	?	18s	x		
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Paulsenella vonstoschii*	?	18s	x		
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Peridinium achromaticum*	Ind.	classic	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Polykrikos kofoidii*	?	18s	x	x	x
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Pronoctiluca pelagica*	NIS	classic			x
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Prorocentrum cordatum*	NIS	classic	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Prorocentrum triestinum*	Ind.	classic	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Protoperidinium bipes*	Ind.	classic	x	x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Protoperidinium punctulatum*	?	18s		x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Protoperidinium thorianum*	?	18s		x	
dinoflagellates	Plants and Fungi	Chromista	Myzozoa	*Torodinium robustum*	Ind.	classic	x		
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Apedinella radians*	NIS	classic	x	x	
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Apedinella radians*	?	18s		x	
eukaryotes	Plants and Fungi	Chromista	Bigyra	*Bicosoeca kenaiensis*	?	18s			x

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
eukaryotes	Plants and Fungi	Chromista		*Bicosoeca vacillans*	?	18s			x
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Fibrocapsa japonica*	NIS	classic	x	x	
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Fibrocapsa japonica*	NIS	COI	x		
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Heterosigma akashiwo*	NIS	classic		x	
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Heterosigma akashiwo*	NIS	18s	x	x	
eukaryotes	Plants and Fungi	Chromista		*Incisomonas marina*	?	18s		x	
eukaryotes	Plants and Fungi	Chromista		*Katablepharis japonica*	?	18s	x	x	x
eukaryotes	Plants and Fungi	Chromista	Cryptophyta	*Leucocryptos marina*	Ind.	classic	x	x	x
eukaryotes	Plants and Fungi	Chromista	Cryptophyta	*Leucocryptos marina*	Ind.	COI	x	x	x
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Nannochloropsis gaditana*	?	COI		x	
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Pseudochattonella verruculosa*	?	COI	x	x	
eukaryotes	Plants and Fungi	Chromista	Ochrophyta	*Pseudopedinella elastica*	?	18s	x	x	
fungi	Plants and Fungi			*Pandora neoaphidis*	?	18s	x		
golden algae	Plants and Fungi	Chromista	Ochrophyta	*Paraphysomonas bandaiensis*	?	18s			x
green algae	Plants and Fungi	Plantae	Chlorophyta	*Bathycoccus prasinos*	?	COI/18s	x	x	x
green algae	Plants and Fungi	Plantae	Chlorophyta	*Blidingia marginata*	Ind.	classic	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Blidingia minima*	Ind.	18s	x	x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Cymbomonas tetramitiformis*	Ind.	classic	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Mantoniella squamata*	?	18s	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Micromonas pusilla*	?	COI/18s	x	x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Pseudendoclonium fucicola*	?	18s		x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Pyramimonas disomata*	?	18s	x	x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Pyramimonas longicauda*	NIS	classic		x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Pyramimonas obovata*	?	18s	x	x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Trebouxia aggregata*	?	COI		x	
green algae	Plants and Fungi	Plantae	Chlorophyta	*Ulva australis*	NIS	classic	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Ulva compressa*	Ind.	classic	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Ulva prolifera*	Ind.	classic		x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
green algae	Plants and Fungi	Plantae	Chlorophyta	*Ulva pseudocurvata*	Ind.	classic	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Ulva rigida*	Ind.	classic	x		
green algae	Plants and Fungi	Plantae	Chlorophyta	*Ulva rotundata*	Ind.	classic	x	x	
haptophytes	Plants and Fungi	Chromista	Haptophyta	*Phaeocystis globosa*	?	COI	x		
monocots	Plants and Fungi	Plantae	Tracheophyta	*Stuckenia pectinata*	?	18s		x	
oomycetes	Plants and Fungi	Chromista	Oomycota	*Lagenisma coscinodisci*	?	18s	x		
oomycetes	Plants and Fungi			*Salilagenidium thermophilum*	?	18s	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Antithamnionella spirographidis*	NIS	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Callithamnion corymbosum*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Ceramium deslongchampsii*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Ceramium rubrum*	?	18s	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Ceramium sungminbooi*	NIS	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Ceramium tenuicorne*	NIS	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Ceramium virgatum*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Chondrus crispus*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Erythrotrichia bertholdii*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Erythrotrichia carnea*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Gracilaria vermiculophylla*	NIS	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Gracilaria vermiculophylla*	NIS	COI	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Neosiphonia subtilissima*	?	COI	x	x	
red algae	Plants and Fungi	Plantae	Rhodophyta	*Polysiphonia fucoides*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Polysiphonia stricta*	Ind.	classic		x	
red algae	Plants and Fungi	Plantae	Rhodophyta	*Porphyra umbilicalis*	Ind.	classic	x		
red algae	Plants and Fungi	Plantae	Rhodophyta	*Pyropia haitanensis*	?	COI			x
red algae	Plants and Fungi	Plantae	Rhodophyta	*Stylonema alsidii*	Ind.	classic	x		
slime nets	Plants and Fungi			*Stellarchytrium dubum*	?	18s	x		x
slime nets	Plants and Fungi	Chromista	Bigyra	*Thraustochytrium aureum*	?	COI			x
bony fishes	Vertebrates	Animalia	Chordata	*Abramis brama*	?	COI	x	x	

Division	category	Kingdom	Phylum	Species	origiin	Tecgnique	Eemshaven	Delfzijl	Ballast waters
bony fishes	Vertebrates	Animalia	Chordata	*Merluccius merluccius*	Ind.	COI	x		
bony fishes	Vertebrates	Animalia	Chordata	*Pholis gunnellus*	Ind.	classic	x		
bony fishes	Vertebrates	Animalia	Chordata	*Pomatoschistus microps*	Ind.	classic	x		
sharks and rays	Vertebrates	Animalia	Chordata	*Scyliorhinus canicula*	Ind.	COI	x		

Table 11 Overview of NIS found, divided on detection method (classical or eDNA). Empty cell = no information/ Status: est= Established. Vectors of introduction, first record, invasivity, origin and habitat preference are mentioned. Est= Established

Species name	known in Wadden Sea	In NSR	Exoten passport available	Vector: fisheries & aquaculture	Vector: ballast water	Vector: fouling on ship hulls	Vector: connected waterways	Status	First record	Invasivity	Origin	Habitat
Species with classical methods:												
Amphibalanus improvisus	yes	1	1	1	1	1	1	Est.	1827	potentially invasive	N and S Atlantic Ocean	marine, brackish
Antithamnionella spirographidis	yes	1	1	1	0	1	1	Est.	1999	not invasive	Southern Pacific Ocean	marine, brackish
Apedinella radians	?											
Aplidium glabrum	yes	1	1	1	1	1	1	Est.	1977	potentially invasive	N Atlantic Ocean and/or Artic region	marine, brackish
Austrominius modestus	yes	1	1	1	0	1	1	Est.	1948	invasive	Southern Pacific Ocean	marine, brackish
Botrylloides violaceus	yes	1	1	1	1	1	1	Est.	2000	potentially invasive	N Pacific	marine, brackish
Caprella mutica	yes	1	1	1	1	1	1	Est.	1994	potentially invasive	Asia	marine, brackish
Ceramium sungminbooi	?											
Ceramium tenuicorne	?											
Cordylophora caspia	yes	1	1	0	1	1	1	Est.	1874	potentially invasive	Europe (Black Sea, Caspian Sea)	marine, brackish

Species name	known in Wadden Sea	In NSR	Exoten passport available	Vector: fisheries & aquaculture	Vector: ballast water	Vector: fouling on ship hulls	Vector: connected waterways	Status	First record	Invasivity	Origin	Habitat
Crassostrea gigas	yes	1	1	1	1	1	1	Est.	1928	invasive	Asia, N Pacific	marine, brackish
Crepidula fornicata	yes	1	1	1	1	1	1	Est.	1942	invasive	N Atlantic Ocean	marine, brackish
Diplosoma listerianum	yes	1	1	1	0	1	1	Est.	1977	potentially invasive	unknown	marine, brackish
Fibrocapsa japonica	yes	1	1	0	1	0	1	Est.	1991	potentially invasive	unknown	marine, brackish
Ficopomatus enigmaticus	yes	1	1	0	1	1	1	Est.	1968	invasive	Southern Pacific Ocean	brackish
Gracilaria vermiculophylla	?	1										
Hemigrapsus sanguineus	yes	1	1	1	1	1	1	Est.	1999		Asia, N Pacific	marine, brackish
Hemigrapsus takanoi	yes	1	1	1	1	1	1	Est.	2000	not invasive	Asia, N Pacific	marine, brackish
Heterosigma akashiwo	yes	1	1	0	1	0	1	?	1993	potentially invasive	unknown	marine, brackish
Mediopyxis helysia	?											
Mnemiopsis leidyi	yes	1	1	0	1	0	1	Est.	2006	invasive	N America, S America	marine, brackish
Molgula manhattensis	yes	1	1	1	0	1	1	Est.	1934	potentially invasive	N America, N Atlantic	marine, brackish
Monocorophium acherusicum	yes	1										
Mysta picta	?	1										
Odontella sinensis	yes	1	1	0	1	0	1	Est.	1906	potentially invasive	Asia, Africa	marine, brackish
Pauliella taeniata	?											
Polydora ciliata	yes	1										
Pronoctiluca pelagica	?											
Prorocentrum cordatum	?											
Pyramimonas longicauda	?											
Sargassum muticum	yes	1	1	1	0	1	1	Est.	1977	invasive	N Pacific	marine, brackish
Smittoidea prolifica	yes	1	1	1	0	1	1	Est.	2004	potentially invasive	N Pacific	marine, brackish

Species name	known in Wadden Sea	In NSR	Exoten passport available	Vector: fisheries & aquaculture	Vector: ballast water	Vector: fouling on ship hulls	Vector: connected waterways	Status	First record	Invasivity	Origin	Habitat
Styela clava	yes	1	1	1	1	1	1	Est.	1974	potentially invasive	N Pacific	marine, brackish
Ulva australis	yes	1	1	1	0	1	1	Est.	1993	invasive	N Pacific, S Pacific	marine, brackish
species with eDNA:												
Acartia tonsa	no	1	1					Est.				
Amathia gracilis	yes	1	1	0	0	0	1	Est.	1936	not invasive	unknown	marine, brackish
Amphibalanus amphitrite	no	1	1	0	0	0		Est.	1963	potentially invasive	asia, S pacific	marine, brackish
Balanus balanus	?											
Blackfordia virginica	no	1	1	1	1	1	1	Est.	2014	potentially invasive	N America, N Atlantic	brackish, estuaria
Bugulina stolonifera	yes	1	1	1	0	1	1	Est.	1885	potentially invasive	N atlantic	marine, brackish
Conticribra guillardii	?											
Corbicula fluminea	no	1	1	0	1	0	1	Est.	1990	invasive	Asia, africa	fresh
Dreissena rostriformis	?											
Ensis directus	yes	1	1	1	1	0	1	Est.	1983	invasive	N atlantic	marine
Gammarus tigrinus	no	1	1	0	1	1	1	Est.	1961	potentially invasive	N atlantic, America	brackish, estuaria
Hydroides elegans	no	1										
Melita nitida	yes	1	1	0	1	1	1	Est.	1998	invasive	N pasific	marine, brackish
Musculista senhousia	?											
Mya arenaria	yes	1	1	0	0	0	1	Est.	1762	not invasive	NE atlantic, arctic	marine, brackish
Neomysis americana	yes	1	1	1	1	1	1	?	2010	potentially invasive	N atlantic, America	marine, brackish
Petricolaria pholadiformis	yes	1	1	1	0	0	1	Est.	1932	invasive	N atlantic, America	marine, brackish
Rangia cuneata	yes	1	1	0	1	0	1	Est.	2007	potentially invasive	N atlantic, America	marine, brackish
Rhithropanopeus harrisii	no	1	1	1	1	1	1	Est.	1874	invasive	N atlantic, America	marine, brackish

***Table 12**. Links to the Netherlands Species Register (www.nederlandsesoorten.nl) showing species descriptions, NIS passports and background information per NIS-species.*

Species name	Species info (Nederlands Soortenregister)
Amphibalanus improvisus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=144200
Antithamnionella spirographidis	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=116493
Apedinella radians	
Aplidium glabrum	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138816
Austrominius modestus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=144207
Botrylloides violaceus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138825
Caprella mutica	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143305
Ceramium sungminbooi	
Ceramium tenuicorne	NA
Cordylophora caspia	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=140488
Crassostrea gigas	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=137373
Crepidula fornicata	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=137435
Diplosoma listerianum	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138819
Fibrocapsa japonica	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=178871
Ficopomatus enigmaticus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138015
Gracilaria vermiculophylla	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=116645
Hemigrapsus sanguineus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143705
Hemigrapsus takanoi	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143704
Heterosigma akashiwo	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=178873
Mediopyxis helysia	
Mnemiopsis leidyi	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=136523
Molgula manhattensis	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138831
Monocorophium acherusicum	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143297
Mysta picta	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=176235
Odontella sinensis	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=178891
Pauliella taeniata	NA
Polydora ciliata	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138035
Pronoctiluca pelagica	
Prorocentrum cordatum	
Pyramimonas longicauda	
Sargassum muticum	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=117117
Smittoidea prolifica	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=140363#
Styela clava	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138836
Ulva australis	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=178829
Acartia tonsa	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/literature2/reference.php?id=2589
Amathia gracilis	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=140426
Amphibalanus amphitrite	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=144201
Balanus balanus	
Blackfordia virginica	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=174678
Bugulina stolonifera	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=140392
Conticribra guillardii	
Corbicula fluminea	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=137187
Dreissena rostriformis	
Ensis directus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=137237
Gammarus tigrinus	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143428
Hydroides elegans	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=138017
Melita nitida	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143397
Musculista senhousia	
Mya arenaria	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=137301
Neomysis americana	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=172398
Petricolaria pholadiformis	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=137159
Rangia cuneata	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=175454
Rhithropanopeus harrisii	http://www.nederlandsesoorten.nl/linnaeus_ng/app/views/species/nsr_taxon.php?id=143685

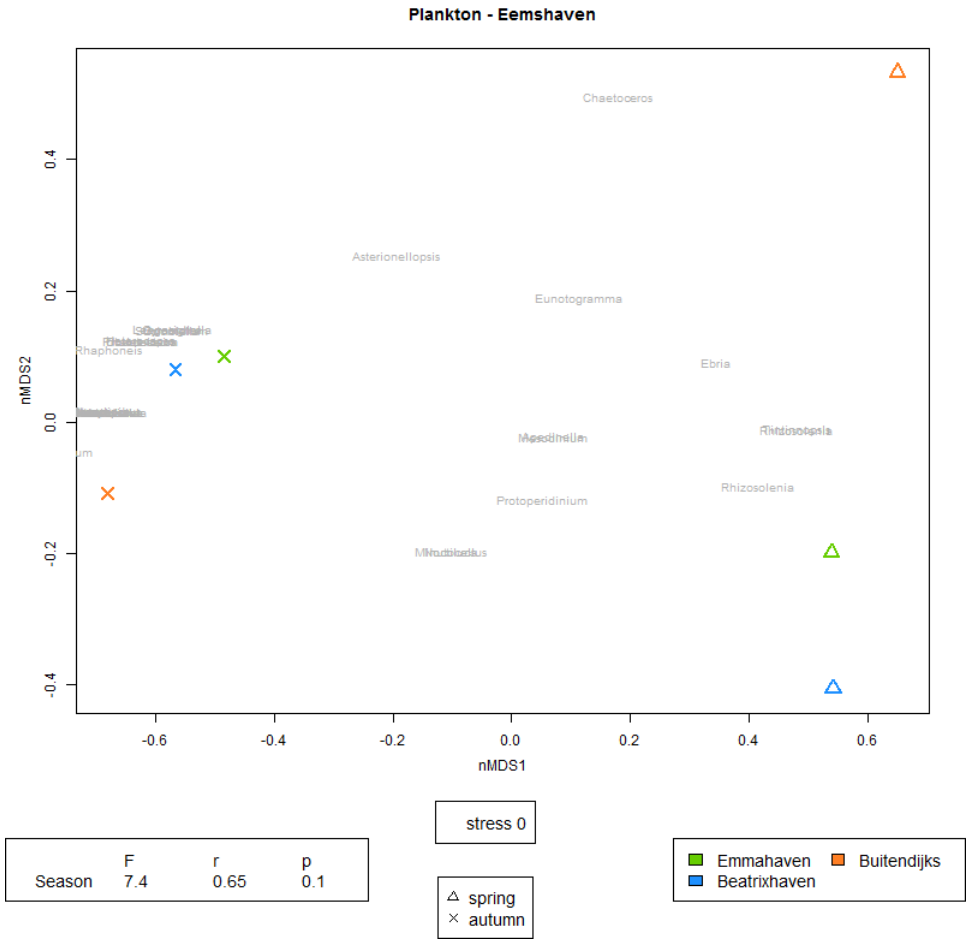
Table 13. *Overview of NIS species found in this study, compared to Gittenberger (2015).*

NIS this study	Gittenberger (2015)
Mysta picta	x
Ficopomatus enigmaticus	x
Austrominius modestus	x
Amphibalanus improvisus	x
Caprella mutica	x
Monocorophium acherusicum	x
Hemigrapsus sanguineus	x
Hemigrapsus takanoi	x
Crepidula fornicata	x
Magallana gigas	x
Smittoidea prolifica	x
Diplosoma listerianum	x
Molgula manhattensis	
Botrylloides violaceus	x
Antithamnionella spirographidis	x
Ceramium tenuicorne	x
Gracilaria vermiculophylla	x
Sargassum muticum	x
Ulva australis	x
Polydora ciliata	x
Aplidium glabrum	x
Styela clava	x
Cordylophora caspia	x
Mnemiopsis leidyi	x
Pronoctiluca pelagica	
Pauliella taeniata	
Apedinella radians	
Prorocentrum cordatum	
Fibrocapsa japonica	
Pyramimonas longicauda	
Heterosigma akashiwo	
Mediopyxis helysia	
Odontella sinensis	
Acartia tonsa	
Amathia gracilis	
Amphibalanus amphitrite	
Balanus balanus	
Blackfordia virginica	
Bugulina stolonifera	x
Conticribra guillardii	
Corbicula fluminea	
Dreissena rostriformis	
Ensis directus (Ensis leei)	x
Gammarus tigrinus	
Hydroides elegans	
Melita nitida	x
Musculista senhousia	
Mya arenaria	x
Neomysis americana	
Petricolaria pholadiformis	
Rangia cuneata	
Rhithropanopeus harrisii	

Table 14. *Overview of NIS species found in Gittenberger (2015) compared to this study.*

Species_GIMARIS(2015)	This study
Antithamnionella spirographidis	*x*
Ceramium botryocarpum	
Ceramium tenuicorne	*x*
Codium fragile subsp. atlanticum	
Codium fragile subsp. fragile	
Colpomenia peregrina	
Dasysiphonia japonica	
Gracilaria vermiculophylla	*x*
Neosiphonia harveyi	
Sargassum muticum	*x*
Ulva pertusa	*x*
Undaria pinnatifida	
Alitta virens	
Ficopomatus enigmaticus	*x*
Marenzelleria viridis	
Neodexiospira brasiliensis	
Streblospio benedicti	
Botrylloides violaceus	*x*
Didemnum vexillum	
Styela clava	*x*
Bugula stolonifera	
Smittoidea prolifica	*x*
Cordylophora caspia	*x*
Diadumene cincta	
Diadumene lineata	
Austrominius modestus	*x*
Caprella mutica	*x*
Eriocheir sinensis	
Hemigrapsus sanguineus	*x*
Hemigrapsus takanoi	*x*
Jassa marmorata	
Leptomysis lingvura	
Melita nitida	
Palaemon macrodactylus	
Mnemiopsis leidyi	*x*
Crassostrea gigas	*x*
Crepidula fornicata	*x*
Ensis directus	
Mya arenaria	
Hymeniacidon perlevis	

Annex 2 Background tables and figures



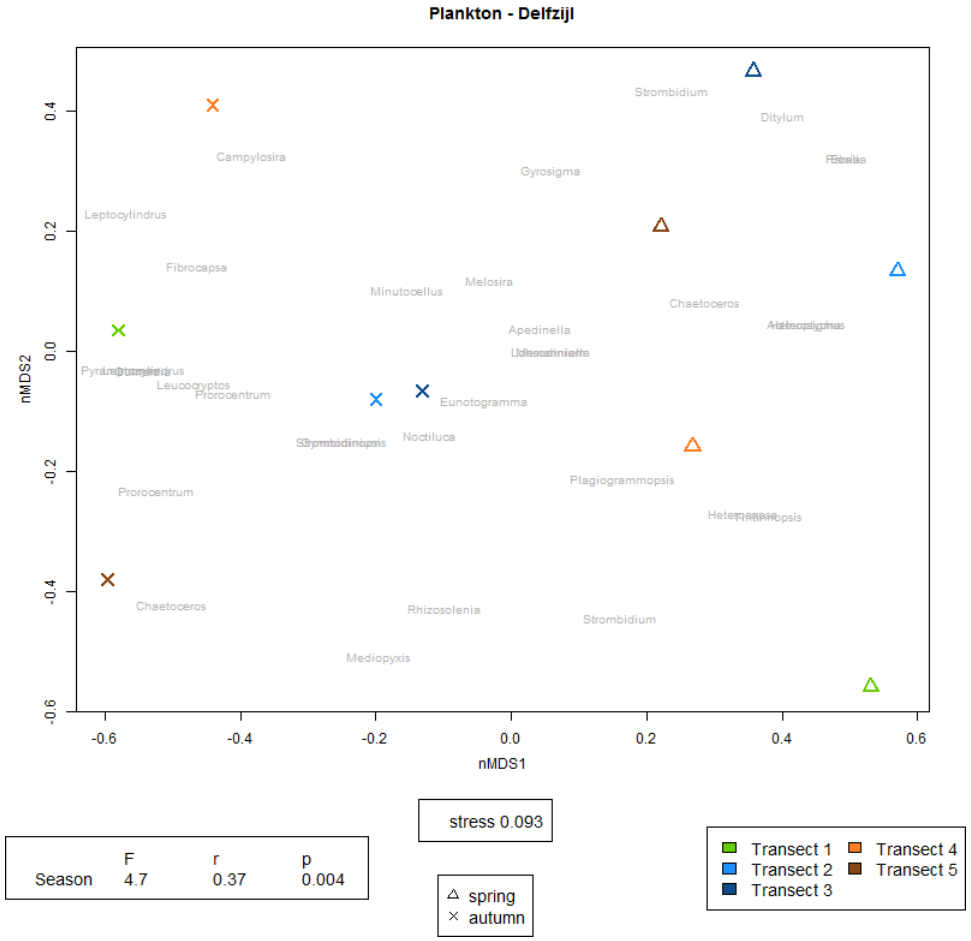


***Figure 29**. nMDS plots representing water samples at various locations and seasons.*

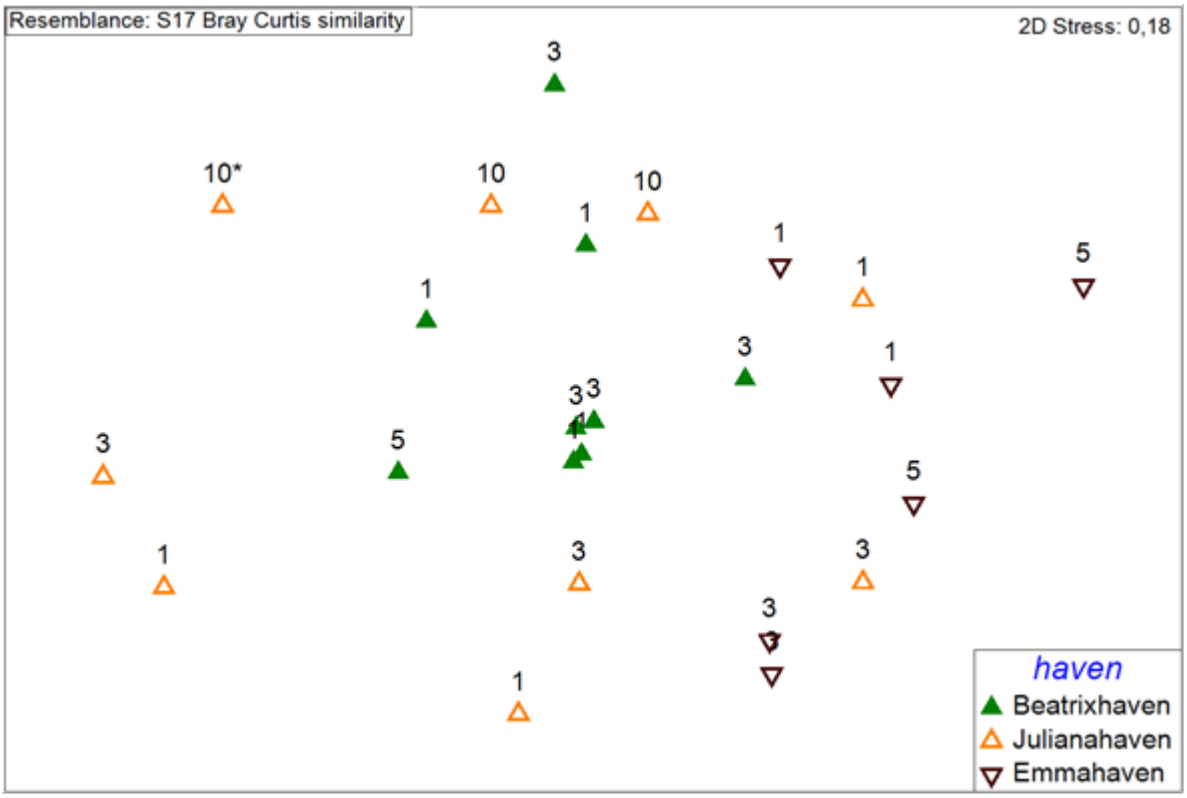


***Figure 30**. MDS plot of all SETL plates (deployed in June 2016, retreived in September 2016) in the three basins of Eemshaven. Depths (1, 3, 5 en 10 m) per sample are oted woth a corresponding number.*

***Table 15**. Planktonic NIS. Expressed is the number of samples (#) taken in total during the survey and the % of samples in which the species is observed.*

Species	Eemshaven		Delfzijl	
	#	%	#	%
Apedinella radians	6	100	10	80
Prorocentrum cordatum	6	33.3	10	30
Fibrocapsa japonica	6	50	10	40
Pyramimonas longicauda			10	30
Heterosigma akashiwo			10	20
Mediopyxis helysia			10	20
Odontella sinensis	6	16.7	10	10

***Table 16**. Percentage of NIS observed within the total of hard substrate samples per applied technique technique (FD= floating dock, QP= Quay/pillar scraping.*

		Eemshaven					Delfzijl		
Phylum	Species	SETL	FD dive	FD scrape	Dike	QP	SETL	Dike	QP
Annelida	*Mysta picta*					22.2			
Annelida	*Ficopomatus enigmaticus*						86.7		58.3
Annelida	*Polydora ciliata*	27.7	41.7						
Arthropoda	*Austrominius modestus*	46.8	58.3	100	80	77.8	13.3	75	25
Arthropoda	*Amphibalanus improvisus*	12.8	25	33.3			73.3	25	75
Arthropoda	*Caprella mutica*	12.8	41.7	100					
Arthropoda	*Monocorophium acherusicum*					66.7			25
Arthropoda	*Hemigrapsus sanguineus*			33.3	40	11.1			8.3
Arthropoda	*Hemigrapsus takanoi*	4.3	41.7		30			50	8.3
Arthropoda	*Hemigrapsus hemigrapsus*			100		66.7			8.3
Chordata	*Diplosoma listerianum*					11.1			
Chordata	*Molgula manhattensis*	44.7	25	33.3		11.1	80		33.3
Chordata	*Botrylloides violaceus*	74.5	100	100		55.6			
Chordata	*Aplidium glabrum*	2.1							
Chordata	*Styela clava*	17	83.3						
Mollusca	*Crepidula fornicata*				10	22.2			
Mollusca	*Crassostrea gigas*	17	83.3	83.3	70	88.9		100	75
Bryozoa	*Smittoidea prolifica*	2.1				11.1	20		
Ochrophyta	*Sargassum muticum*		8.3						
Rhodophyta	*Ceramium sungminbooi*					22.2			
Rhodophyta	*Antithamnionella spirographidis*		58.3	33.3					
Rhodophyta	*Ceramium sungminbooi*		91.7						
Rhodophyta	*Ceramium tenuicorne*		25						
Rhodophyta	*Gracilaria vermiculophylla*				10				
Chlorophyta	*Ulva australis*		75						
Ctenophora	*Mnemiopsis leidyi*		100						
Cnidaria	*Cordylophora caspia*						26.7		

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