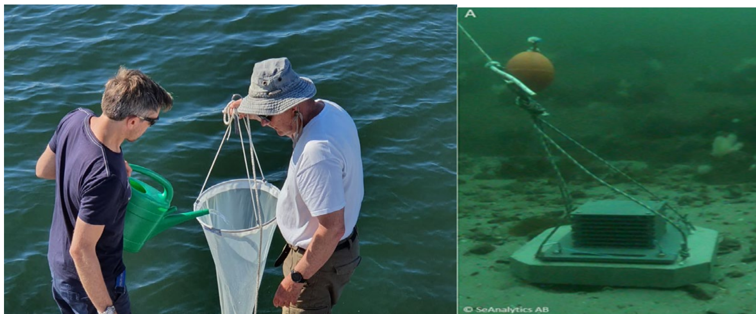
Genetic methods in environmental monitoring

Early detection and monitoring of non-indigenous species based on DNA



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Havs och Vatten myndigheten

Genetic methods in environmental monitoring

Early detection and monitoring of non-indigenous species based on DNA

PER SUNDBERG, ALIZZ AXBERG, NAURAS DARAGMEH, MARINA PANOVA and MATTHIAS OBST

SeAnalytics AB Samsmarka 14, 475 37 Bohus-Björkö, Sweden and University of Gothenburg, Department of Marine Sciences P.O. Box 461, 405 30 Gothenburg, Sweden

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Preface

The methodology for detecting of marine invasive alien species always needs to be evaluated and improved for a more efficient surveillance system for early warning. Novel methods have been tested in the Swedish part of the North Sea.

Sweden has a relatively new monitoring on marine non-indigenous in place which is based on principles from the EU Marine Strategy Framework Directive and the Regional Sea Conventions to implement the international Ballast Water Management Convention. The criteria is to have a monitoring system for early detection of introduction of invasive alien species and their spread to new areas. The methods for monitoring are designed to operate in high risk areas where it is most likely to detect new introductions or follow the spread of the species. The sampling and analysis methodology always needs to be tested and verified to be able to implement the best available methods that are sensitive and adopted for occurrence of non-indigenous species. This will ensure that the objectives are met in an efficient way and. Novel methods like autonomous sampling of species and traces of species by environmental DNA as well as analysis by photographic imaging, machine leering carry a great potential to improve monitoring of non-indigenous species and biodiversity.

The fact that the number of non-indigenous species dominate by settling animal species with a planktonic life stage makes it clear that methods on collecting these species are fundamental. The current report presents how genetic methods from autonomous could improve the lag time and sensitivity for an effective early warning system on new invaders. The results show that methods and techniques which are effective and consistent for collection are available. The project was conducted off the Swedish west coast and involves a collaboration within an international network for biodiversity where open data helps to disseminate the results in an open and transparent manner. The results provide with an experience on how monitoring of changes in biodiversity including detection of invasive alien species could improve a surveillance system which enables improvement of a knowledge base for well-informed decision making.

The report has been commissioned by the Swedish Agency for Marine and Water Management as part of our development of long-term monitoring on non-indigenous species in the marine environment. This report should be of interest both to environmental monitors at national, regional and municipal level, but also to researchers and developers of new methods for this monitoring.

The report has been reviewed by the analysts Niclas Engene and Andrea Ljung. Erland Lettevall has been responsible for the assignment of the project as well as reviewing and editing of the report.

This report is published in Swedish as well (same report number).

Summary

Collecting marine hard bottom organism with autonomous techniques in combination with DNA analyses for species identification has proven to be efficient monitoring of biodiversity and detection of non-indigenous species. This methodology provides well to early warning surveillance of invasiv alien species.

The University of Gothenburg has, on behalf of the Swedish Agency for Marine and Water Management and in collaboration with SeAnalytics AB, carried out two pilot studies to investigate whether plankton samples and settling panels in combination with DNA-based species identification is an effective method for early detection and continuous monitoring of non-indigenous species (NIS). The surveys were conducted during the winter, spring and summer of 2020 at various locations along the Swedish west coast, from the fjord Brofjorden in the north to Helsingborg in the south. The sites were chosen based on previous modelling (Bergkvist et al. 2020a) hotspots for introduction of alien species.

The settling panel study followed the protocols for monitoring of hard bottom organisms as set up in the international ARMS (Autonomous Reef Monitoring Structures) project (Leray & Knowlton, 2015; Obst et al. 2020). A total of 16 ARMS settling panels were deployed during the winter and spring months and were submerged between 3–4 months before taken up for further processing and analysis. Plankton samples were taken from six of these sites, on three different occasions, and two samples from each site or occasion.

DNA was extracted from the settling panels and plankton and metabarcoding libraries prepared for three molecular markers (COI, 18S, ITS), together with positive and negative controls, altogether 284 libraries. Thirty-four NIS were recorded, of which fourteen are classified as invasive alien species (IAS) by the Swedish Species Information Center and on the list of Invasive Alien Species of Union concern. The remaining 20 NIS are listed on the Aqua-NIS list and need to be further investigated for their alien and invasive status in the country.

The tested methods perform well both for early detection of unknown NIS as well as for regular monitoring of already known NIS. The chosen markers COI and 18S performed well, while ITS did not produce sufficient species observations and can be omitted as a marker gene.

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1 Introduction

A species (and subspecies) is defined as non-indigenous species (NIS) if it has been introduced (with or without human help) outside its historical or contemporary distribution. Species that are spread and introduced to new environments with the help of humans can affect the environment and ecosystems in negative ways. They can have major economic consequences for society, pose a threat to biodiversity, and also have direct health effects on both humans, animals and plants. These species are defined and categorized as invasive alien species (IAS). Ships are a significant vector for the introduction and spreading of IAS in the aquatic environment, via fouling on the hull or the ballast water used to control ship stability during operation. Therefore, ports and shipping lanes are particularly interesting in terms of control and monitoring of IAS since they are likely places where introductions take place. There may also be a secondary spread from commercial ports by recreational boats via fouling or gear (e.g. anchors) and it is therefore also of interest to monitor recreational boat marinas, although they are probably not the primary site for the introduction of IAS.

The need to monitor and assess the prevalence and distribution of NIS has increased in recent years. For the aquatic environment, this is governed by the Water Framework Directive (WFD), the Marine Strategy Framework Directive (MSFD), the Ballast Water Management Convention, the European Regional Sea Conventions Helcom and Ospar, and the EU Regulation on Invasive Alien Species (IAS Regulation). Effective monitoring requires effective and efficient methods, and this is especially important in order to be able to take actions as quickly as possible to prevent establishment and further spread. While other biodiversity monitoring programs may allow reporting of individuals at higher taxonomic levels such as genus, family, phylum, monitoring of NIS requires that organisms are identified to species level.

The ability to quickly, accurately and cost-effectively identify species is therefore an essential and crucial component of any marine monitoring program on NIS. This requires efficient tools that can quickly and with high reliability identify these species, not just the adults but also (and maybe more importantly) their larvae, eggs and juvenile stages. Traditionally, species are identified based on habitus and detailed morphological characters which requires good knowledge and experience of the taxa to be determined. Given the vast species richness and general diversity of the marine environment, it is not possible for an individual expert to identify everything with certainty. In addition, it may be difficult to identify juvenile forms of species that look completely different as adults, or to be able to determine larvae and eggs. Given that rapid detection is important for NIS/IAS, methods need to be developed that can reliable and rapidly identify all life stages of a species.

Another crucial part of an NIS monitoring program is the sampling strategy. Sweden has since 2019 a surveillance system for assessing trends of marine NIS, under MSFD. One program involves simple quantitative and semi-quantitative so-called rapid assessment survey methods on NIS in hotspots for introduction of marine and brackish NIS[1]. The other is citizen science based on aquatic NIS[2]. In addition, directed monitoring programs on NIS due to thermal water pollution

[1] The Swedish monitoring program of marine NIS by rapid assessment survey, under the Marine Strategy Framework Directive: https://www.havochvatten.se/overvakning-och-uppfoljning/miljoovervakning/marin-miljoovervakning/frammande-arter.htm.

[2] The Swedish monitoring program of marine NIS by citizen science, under the Marine Strategy Framework Directive: https://www.havochvatten.se/overvakning-och-uppfoljning/miljoovervakning/marin-miljoovervakning/medborgarforskning-gallande-frammande-arter.html.

from nuclear power plants have been running since the end of 1960's[3]. These include phyto- and zooplankton, growth organisms, scrapings of hard bottom fauna, soft-bottomed macro fauna and flora, as well as traps for mobile epifauna.

The present report evaluates how DNA-based species identification can be used in NIS and IAS monitoring programs. The pilot study focuses on plankton samples (water column) and settling panels (hard bottom). The study was carried out during the winter–spring–early summer period in 2020, at commercial ports along the west coast assumed to be gateways for NIS introductions via ballast water and biofouling on ship hulls. The commercial ports included in the study are the Preemraff Lysekil in the fjord of Brofjorden and the Port of Gothenburg, Port of Varberg and Port of Helsingborg. The study also includes the Marstrand fjord area – a place where shipping traffic to Uddevalla and Stenungsund passes. It also included places just north of the Port of Gothenburg, which were chosen because of the dominant water currents out of the port area (Green 2013). In addition, two marinas (Getterön and Marstrand) were included in this study because they are popular entry points for recreational boats from abroad, and due to their vicinity to commercial ports and the potential of secondary spread from these ports.

1.1 Plankton

Many marine species have pelagic larval and fry stages in their life cycles. Given the ecology and high mortality rate for this way of life, a larva, egg or fry of an invasive species is much more likely to be encountered than an adult individual. High mortality rates are also expected for planktonic species with entirely pelagic forms. It is therefore reasonable to assume that NIS are more readily detected among pelagic samples, while larvae may not settle and develop further. Plankton samples are also relatively cheap and easy to take and can therefore cover a larger geographical area and time span.

Identification of larvae and juveniles is in general problematic since many lack identifying characters. This will lead to relatively more time for species identification when using traditional methods, compared to when identifying adult forms. Even with experienced and competent plankton taxonomists, there can be problems in determining plankton by species. See, for example, Table 2 in Sundberg et al. (2018) which shows that only nine (15%) individuals could be identified as a species of a total of 61 identified unique zooplankton taxa. For comparison, DNA-based metabarcoding was able to identify 143 taxa, of which 95 could be determined to species (66%), in a parallel sample taken at the same time and at the same site. Manual traditional identification is thus not only difficult but also time-consuming and thus costly.

DNA-based identification of plankton samples will get a more accurate picture of the species composition and can also be faster depending on the approach. DNA metabarcoding will give an idea of the entire species composition in the plankton sample, but this involves sequencing and bioinformatics analysis. An alternative that is both faster and cheaper is to focus on a number of species, i.e., a target species list, to be monitored. Depending on the purpose and which species to be monitored, there may be a manageable number of species that can be analysed in close connection with the sampling.

[3] Swedish monitoring program on NIS and thermal water pollution in the sea, under the Marine Strategy Framework Directive https://www.havochvatten.se/overvakning-och-uppfoljning/miljoovervakning/marin-miljoovervakning/effekter-av-kylvatten.html.

1.2 Settling by using ARMS panels

Unlike soft-bottom benthos, which can be surveyed by dredging and grabs, it is technically difficult to sample hard bottom fauna. These organisms can instead be monitored and detected by placing settling panels in the area to be monitored. The technology for capturing hard bottom fauna has been developed in recent years, but there is yet no national or regional standard for the design, size, and number of settlement panels. In the HELCOM guidelines (2020), it is stated that there must be three (grey) qualdratic panels with each side of 15 centimetres (or 14 centimetres) made of PVC. They should be submerged at slightly different depths (suggested 1, 3 and 7metres) according to this protocol, while the international ARMS program (Leray & Knowlton 2015) has developed a system of Autonomous Reef Monitoring Structures (ARMS) with a stack of so called ARMS panels, which creates crevices and microhabitats.

For the present study we chose the ARMS protocol (ARMS handbook 2020), both because it is a published standard but also because there is already an existing monitoring program which allows for comparisons between surveys in other countries and geographic areas (Obst el al 2020). The ARMS panels (Figure 1) consist of nine PVC plates (22.5 cm x 22.5 cm) plus a bottom plate of 45 cm x 35 cm. These nine plates are mounted on top of each other at a distance of a few centimetres.

According to ARMS, the first step after the panels have been retrieved from the water is to document the fouling by photography. The settling organisms are in the second step removed by scraping the panels, and divided into three size fractions by sieving. The organisms are decomposed with a mixer rod, fixed in a buffer, followed by DNA extraction and sequencing for three molecular markers COI, 18S and ITS. Sequences are then matched to existing reference libraries to determine species composition. Thereafter species observations have to be matched against checklists for IAS and NIS.

Figure 1. Left: Assembled ARMS panels before deployment at sampling site, with a concrete slab attached to the bottom plate. Right: ARMS photographed on the seafloor 12 months after deployment. Photo: Per Sundberg (left), Matthias Obst (right)

2 Sampling and analysis

2.1 Sampling

Sampling sites (Figure 2) were selected based on assumed “hotspots” for the introduction of alien invasive species (Bergkvist et al. 2020a). With regard to the sites around Gothenburg, these were chosen based on prevailing current patterns in, and around the port. There is an outgoing current from the inner harbour, which meets the (most common) northbound current and panels were accordingly placed in the mouth and north of the port. ARMS panels have previously been placed in the Kosterfjord area in connection with another research project, and these were included in the final analysis as a reference site less affected by ship traffic.

Figure 2. Sampling sites along the Swedish west coast. Blue dots: settling panels; grey dots: both settling panels and plankton samples (map from Lantmäteriet). See Table 2 below for exact positions.

2.1.1 Settling and biofouling organisms by using ARMS panels

ARMS panels were deployed in five different geographical areas: Gothenburg and surroundings, Marstrand and Marstrand fjord, Varberg and Getterön, Preemraff Lysekil/Brofjorden, and the Port of Helsingborg between 2020-01-31 and 2020-04-01 and stayed in the water during three to four months (for details, see chapter 5 below). At four sites around Gothenburg (1–4) and two in the Marstrand fjord (Marstrand 2–3) the panels were placed on the bottom (depth 7–10 meters) with a rope and an attached buoy to the surface. In the Port of Helsingborg (Helsingborg 1–2), the panels were placed on the bottom (about 2 metres deep) with rope to the quay. In other sites, the panels hung vertically from jetties to a depth of 1.5 to 2 meters.

Panels have previously been placed in the Koster area as part of an ongoing international ARMS project (Obst et al. 2020). The results from these are included here as a comparison with long-

term deployments (12 months) and in an area relatively unaffected by commercial shipping and nearby major ports.

Directly after the panels were taken out of the water, each individual plate (both sides) was photographed for documentation and identification of species based on habitus (Figure 3). In some situations, the panels were placed in barrels with filtered water for transportation to places where the photographic documentation could take place. It is important not to let the panels dry out and they were kept in trays with water during photography. The next step was to scrape of all organisms from the plates and sieve them into three different size fractions (< 40 µm, 100–500 µm, > 500 µm). Larger organisms (like tunicates and mussels) were removed before sieving. Organisms were fixed in DSMO (Obst et al. 2020) and stored in a freezer at -20 °C until DNA extraction.

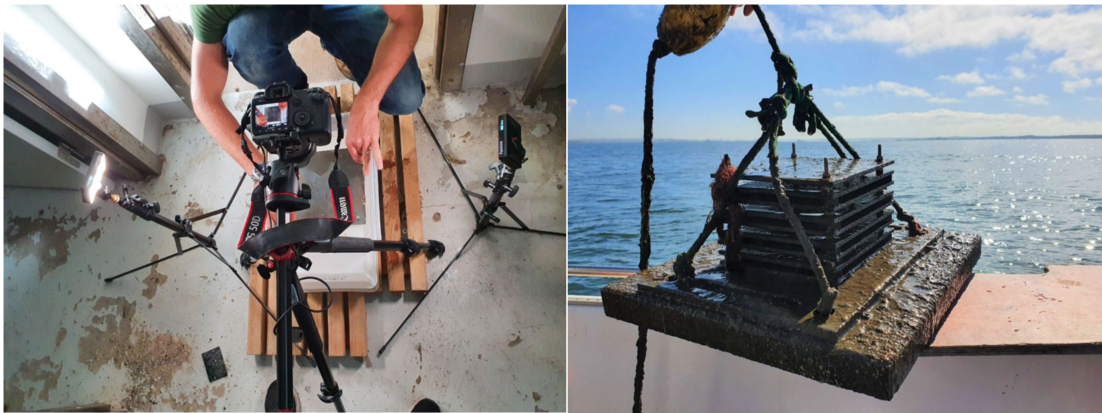

Figure 3. Photographic set-up for documentation of the panels and an example of growth on a panel from the Port of Gothenburg. Photo: Alizz Axberg

2.1.2 Plankton

Plankton samples were taken at six sites: four around Gothenburg (labelled: Gothenburg 1, Gothenburg 2, Hjuvik, Bessekroken); two in the Marstrand area (labelled: Marstrand, Marstrand fjord). Exact positions are listed in Chapter 5. The locations correspond to the placement of ARMS panels. Sampling took place during the period 2020-05-20 to 2020-07-13. Each site was sampled on three occasions during the period. On each occasion, two vertical plankton tows from a depth of about 10 meters were taken per site. The depth on the sites varied between 10 and 15 meters. Plankton was collected with a Hydro-Bios Apstein 90-micromilimetre net, with an opening diameter of 40 centimetres, which resulted in a sampling volume of around 1.25 cubic metre per sample.

The samples were fixed on board in sterile disposable jars with an amount of ethanol that was estimated to have a concentration of at least 60–70 per cent. After field sampling, the samples were filtered with 80-micromililmetre sieve on return and fixed again with a higher ethanol content (95%). The samples were stored at -20 °C until extraction.

2.2 Analysis

2.2.1 DNA extraction ARMS and plankton samples

Appendices I and II.

2.2.2 Negative and positive controls for possible contamination

Plankton: Two negative controls (blank samples) were created by the same extraction procedure as described in Appendix II but without the plankton. No DNA could be detected in these samples, with neither Nanodrop nor Qubit. The blank samples were included in the preparation of libraries (below) as a further control for contamination during the extraction of DNA. Another negative control was performed during PCR.

ARMS: Three negative controls were created in the same way for control of contamination.

A positive control, co-called mock community, was included as a quality control for PCR and sequencing. This sample consists of DNA from nine local invertebrate species and one fish in the equal amounts.

Appendix III describes the procedures and results in more detail.

2.2.3 DNA library preparation for sequencing

Three molecular markers (18S, COI and ITS) were amplified for each sample, using primers from Obst el al. (2020). Same PCR protocols were used for metabarcoding of the ARMS and plankton samples, see Appendix IV.

Library preparation and sequencing of the ARMS samples were carried out by the Hellenic Centre for Marine Research[4] using the ARMS MBON protocols (Obst et al 2020). Library preparation of the plankton samples were performed by SeAnalytics and sequencing done by Eurofins Genomics. Altogether 120 libraries were formed for the plankton samples (36 field samples + 3 blank samples + 1 mock x 3 molecular markers), and 164 libraries for the ARMS samples (18 ARMS panels x 3 fractions x 3 markers = 162 samples + 2 blanks).

More details can be found in Appendix IV.

The plankton DNA libraries were sequenced with Illumina MiSeq, 2 x 300 bp with an average of 16 million reads, which means about 133 thousand readings per sample. The ARMS libraries were also sequenced with Illumina MiSeq 2 x 300 bp. More details can be found on the Molecular Standard Operating Procedures (ARMS MSOP 2020) as published by Obst et al (2020). Amount of sequenced data per sample was compatible between the ARMS and plankton samples.

2.2.4 Bioinformatics

The analysis was performed in the R-environment with a DADA2 package (Callahan et al. 2016). After initial quality control, the sequences were filtered for low quality, as well as adapters and primers were removed. Sequencing errors were corrected by calculating an error model (DADA2 error model); and after that singletons and chimeras were removed using the “pseudo-pooling” function in DADA2. The filtered and quality-controlled COI sequences were matched against the BOLD database[5] with the python package BOLDigger (Buchner & Leese 2020). The 18S sequences were matched against the PR2 and SILVA databases and the ITS sequences against the UNITE database. All subsequent analysis was performed with customized R scripts where identified species were matched against different NIS and IAS species reference lists. Identified

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[4] The Hellenic Centre for Marine Research: https://www.hcmr.gr/en/.

[5] Barcode of Life Data System, BOLD: http://www.boldsystems.org/.

species (at the >98% similarity) that were new records from the area were examined individually to exclude bioinformatics or taxonomic sources of error.

Species matching was carried out against four partially overlapping checklists for NIS. The lists from the SLU Swedish Species Information Centre (152 species) (Strand et al. 2018), from the Swedish Agency for Marine and Water Management (including the list of IAS of Union concern) (171 species), and OSPAR/HELCOM (64 species) includes species which are risk-classified and identified as invasive. In addition, species were also matched against the AquaNIS list with 1906 species, but some of them have not been risk evaluated (Table 1). Information on the genetic diversity in the samples, as well as geographic distribution and abundance in the sampled area were compiled for each NIS discovered in the genetic analysis. Finally, we conducted a manual review for the species that were unexpected in the sampling area with databases such as WoRMS[6], BOLD[5], GBIF[7], and the Swedish Analysis portal (that will be replaced in 2022 by Artfakta at the SLU Swedish Species Information Centre).

2.2.5 Data management

Data were managed according to the plan developed by the European ARMS project[8] to ensure good documentation processing, as well as archiving and access according to the FAIR[9] principles.

The study generated various kinds of data, including metadata, field measurements, photographic images, raw and processed sequence data (fastq and fasta format), as well as inferred species observations. All raw sequences data are published in the European Nucleotide Archive (https://www.ebi.ac.uk/) under the following accession numbers: ERR4914146-ERR4914160 (COI), ERR4914204-ERR4914218 (ITS), ERR4914088-ERR4914102 (18S). All metadata, field measurements, photographic images as well as derived fasta sequences are published under the PlutoF project[10]. Sequence data will also become available through the Swedish Biodiversity Data Infrastructure[11]. All data collected during this project are available free of charge under the CC BY 4.0 license.

Protocols for the field and laboratory procedures and techniques can be downloaded from ARMS MBON website[12].

[6] World Register of Marine Species, WoRMS. https://www.marinespecies.org/index.php.

[7] Global Biodiversity Information facility, GBIF: https://www.gbif.org/.

[8] The European ARMS program (ARMS-MBON) at the Marine Biodiversity Observation Network for genetic monitoring of hard-bottom communities: http://www.arms-mbon.eu/.

[9] The FAIR Guiding Principles for scientific data management and stewardship, GO FAIR: https://www.go-fair.org/fair-principles/.

[10] Pluto F, Data management and Publishing Platform: https://plutof.ut.ee/#/study/view/81139.

[11] Open access to Sweden´s biodiversity data at the Swedish Biodiversity Data Infrastructure, SBDI: https://biodiversitydata.se/.

[12] The European ARMS program (ARMS-MBON) at the Marine Biodiversity Observation Network for genetic monitoring of hard-bottom communities: http://www.arms-mbon.eu/.

3 Results and discussion

3.1 Results

3.1.1 NIS and IAS identified from ARMS panels and plankton samples

No alien or invasive species could be detected during the initial inspection of the photographs (Obst et al. 2020). However, after the sequence analysis indicated the presence of NIS some of these species could be confirmed on the images (Martaeng & Obst pers. comm.).

The analysis based on the ITS marker did not detect any NIS and in general provided very few species-level identifications, therefore we suggest that this marker is excluded in future surveys.

Altogether 34 either NIS were identified based on the two molecular markers 18S and COI. Out of these, 13 are reported on different risk lists and identified as IAS (Table 1). The similarity between the sequences in the samples and the databases is equal or higher than 98 per cent for both markers, and in most cases 100 per cent, in all matches against different reference databases. Species that have previously been reported as rare as well as new in Sweden are noticeable in Table 1. Overall, the results show that there are several new species in Sweden that should be risk assessed with regard to the effect on biodiversity, economy and health in the Swedish environment.

Table 1 (continued on next page). Compilation of non-indigenous species (NIS) and risk-classified invasive alien species (IAS) found in the plankton samples (P), ARMS settling panels (A), or both (AP). To find IAS, the sequences were matched against the following reference lists: HELCOM and OSPAR (HEL & OSP), the Swedish Agency for Marine and Water Management (SwAM) and Artfakta at the SLU Swedish Species Information Centre Species Data Bank (SLU), and generally NIS against the AquaNIS database. Max sim (%) shows the maximum percentage similarity between the recovered sequence and the reference sequence for COI (in the reference libraries BOLD and MIDORI) or 18S (in the reference libraries SILVA and PR2). Species that have previously been reported as rare in Sweden are marked with an asterisk*. New records of NIS are marked with two asterisks**.

Scientific name	Phylum	Class	SwAM	HEL & OSP	SLU	Aqua-NIS	Max sim (%)	Collec-ting method (A, P, AP)
Acartia clausi	Arthropoda	Copepoda				x	100	AP
Acartia tonsa	Arthropoda	Copepoda		x		x	100	P
Acrochaetium moniliforme	Rhodophyta	Florideophyceae				x	100	A
Aglaothamnion halliae	Rhodophyta	Florideophyceae	x		x	x	100	A
*Amathia imbricata**	Bryozoa	Gymnolaemata				x	99.67	AP
Amphibalanus amphitrite	Arthropoda	Thecostraca				x	100	AP
Amphibalanus eburneus	Arthropoda	Thecostraca				x	100	AP
Amphibalanus improvisus	Arthropoda	Thecostraca	x		x	x	100	AP
Antithamnionella spirographidis	Rhodophyta	Florideophyceae				x	98.71	A
Bonnemaisonia hamifera	Rhodophyta	Florideophyceae	x		x	x	100	AP
Calanus euxinus	Arthropoda	Copepoda				x	100	P
Caprella mutica	Arthropoda	Malacostraca	x	x	x	x	100	A
Crepidula fornicata	Mollusca	Gastropoda	x	x	x	x	100	A
*Dasya baillouviana***	Rhodophyta	Florideophyceae	x		x	x	98.00	P
Dasysiphonia japonica	Rhodophyta	Florideophyceae	x		x	x	100	AP

Scientific name	Phylum	Class	SwAM	HEL & OSP	SLU	Aqua-NIS	Max sim (%)	Collec-ting method (A, P, AP)
*Ercolania viridis***	Mollusca	Gastropoda				x	99.35	P
*Gonionemus vertens**	Cnidaria	Hydrozoa				x	100	A
*Haminoea solitaria***	Mollusca	Gastropoda				x	99.68	P
*Hydroides elegans***	Annelida	Polychaeta		x		x	98.00	A
*Hymeniacidon sinapium***	Mollusca	Gastropoda				x	99.00	AP
Jassa marmorata	Arthropoda	Malacostraca				x	100	A
*Lyrodus pedicellatus***	Mollusca	Bivalvia				x	99.00	AP
Mnemiopsis leidyi	Ctenophora	Tentaculata	x	x	x	x	99.80	P
Monocorophium acherusicum	Arthropoda	Malacostraca				x	100	A
Monocorophium sextonae	Arthropoda	Malacostraca				x	100	A
Mya arenaria[1]	Mollusca	Bivalvia				x	100	AP
Mytilus trossulus	Mollusca	Bivalvia				x	100	A
Penilia avirostris	Arthropoda	Branchiopoda	x		x	x	100	P
Petricolaria pholadiformis	Arthropoda	Copepoda			x	x	99.00	P
*Pileolaria militaris***	Annelida	Polychaeta				x	100	A
Proceraea cornuta	Annelida	Polychaeta				x	100	A
*Pseudochattonella verruculosa***	Ochrophyta	Dictyochophyceae	x	x	x	x	98	P
*Pseudodiaptomus marinus***	Arthropoda	Copepoda				x	99.68	P
Sparus aurata	Chordata	Actinopteri				x	100	A

[1] *Mya arenaria* is not defined as NIS in Sweden according to definition in Strand et al. (2018) but is included here because it is considered NIS in Denmark.

More species were found in the settling panels (23) than among the plankton samples (20 species), 10 species are common to the two collection methods (Figure 4). The COI marker also identified more species than the marker 18S (26 compared to 12, three of which are in both databases), which is an effect of the COI reference libraries for macroorganisms that include more taxa. The marker 18S is furthermore not a marker that works as well as COI when it comes to distinguishing between species, since the SILVA database contains only genus level information and the species-level PR2 database is smaller.

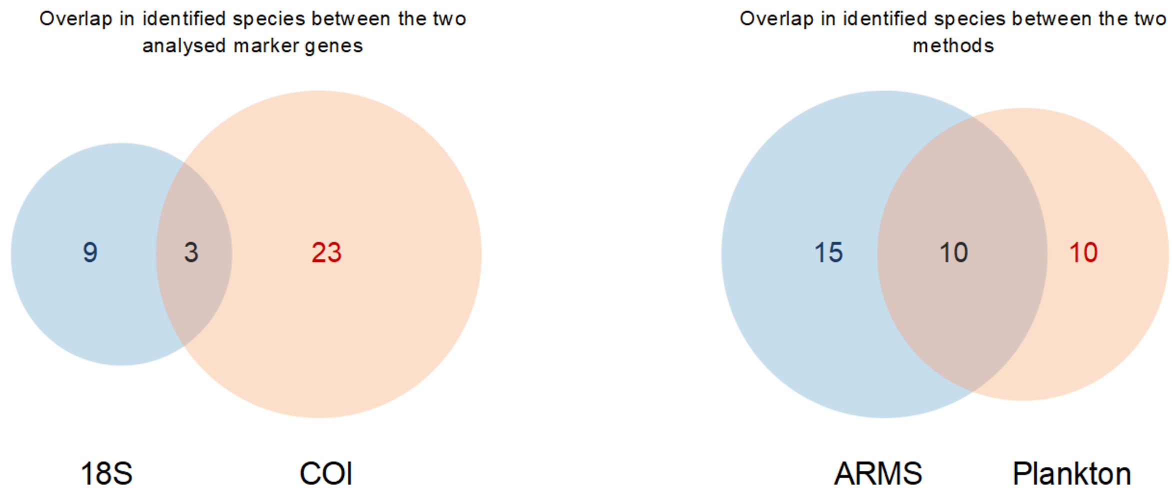


Figure 4. The figure shows the distribution of NIS found between the markers COI and 18S, and how many species have been found by both markers. The figure also shows graphically the distribution of alien species between the ARMS panels and the plankton sample, and the number of species that overlapped between the two different collecting methods.

The number of identified NIS is higher in the ARMS panels than the plankton samples (Figure 4), but the abundance is higher in the plankton samples (Figure 5).

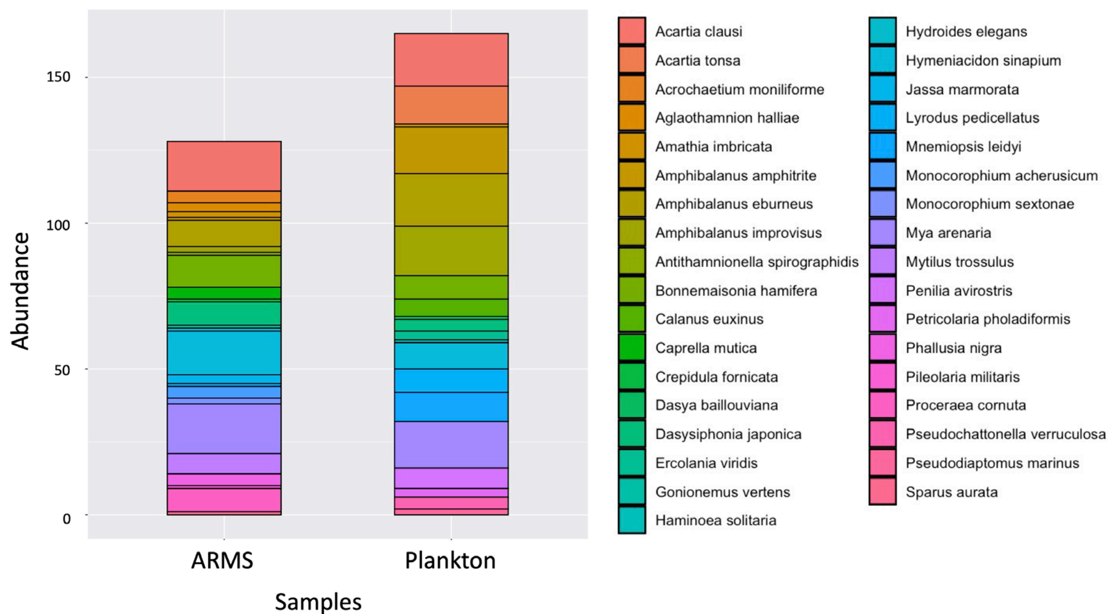


Figure 5. A graph showing the difference in species abundance between ARMS panels and plankton samples. Abundance is calculated as the sum of samples where a particular species is present.

3.1.2 NIS on settling panels – comparing presence between sampling sites

The amount of NIS found on the settling panels varies between sampling localities, with the highest number in Brofjorden (the Preem refinery) (Figure 6). This is probably a combination of the fact that these plates were in the water later in the spring and at a site with higher salinity, two factors favouring biological activity. Another site with a somewhat relatively higher number of detected NIS is the Marstrand marina. This can be explained by good conditions for marine life – high turnover saline water due to prevailing currents going through the strait where the marina is placed. It is also a very popular marina with a large number of visiting boats, also from abroad. This can thus be a gateway for alien species both directly from boats from abroad, but also secondary spread from, for example, the neighbouring Gothenburg area with its large number of marinas and recreational boats.

The panels in the Koster area had been in the water for a year, but have on average fewer NIS species than panels placed in ports assessed as possible introduction places. The results show the importance of choosing the right time and place to maximize the probability of finding invasive species.

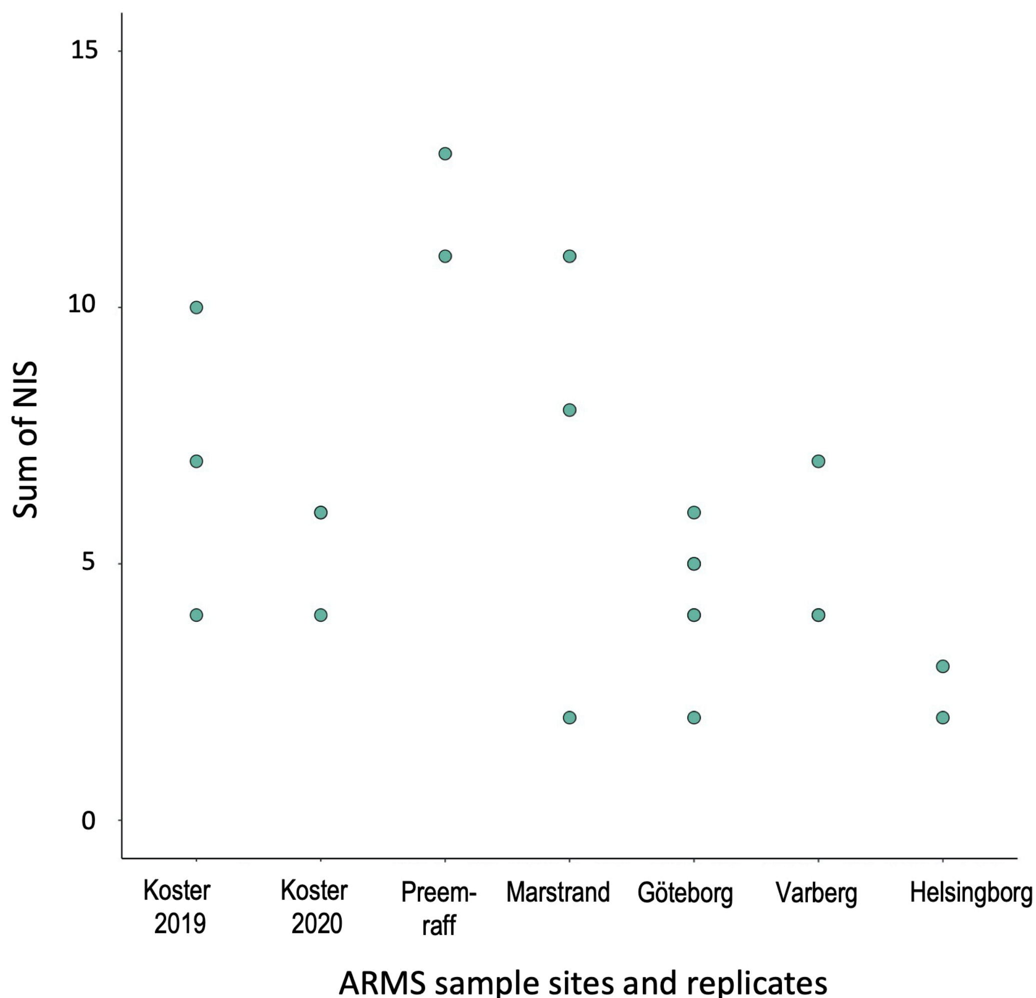


Figure 6. Number of non-indigenous species (NIS) per settling panel and sampling site.

3.1.3 NIS in plankton samples – comparing presence between sampling dates and sites

The number of NIS is relatively evenly distributed over the different sites among the plankton samples (Figure 7). The variation between the sampling dates (Figure 8) is greater, which may be due to a seasonal change in larval supply and abiotic factors (e.g. ocean currents). This can be compensated with a larger number of replicates per site and time in the future.

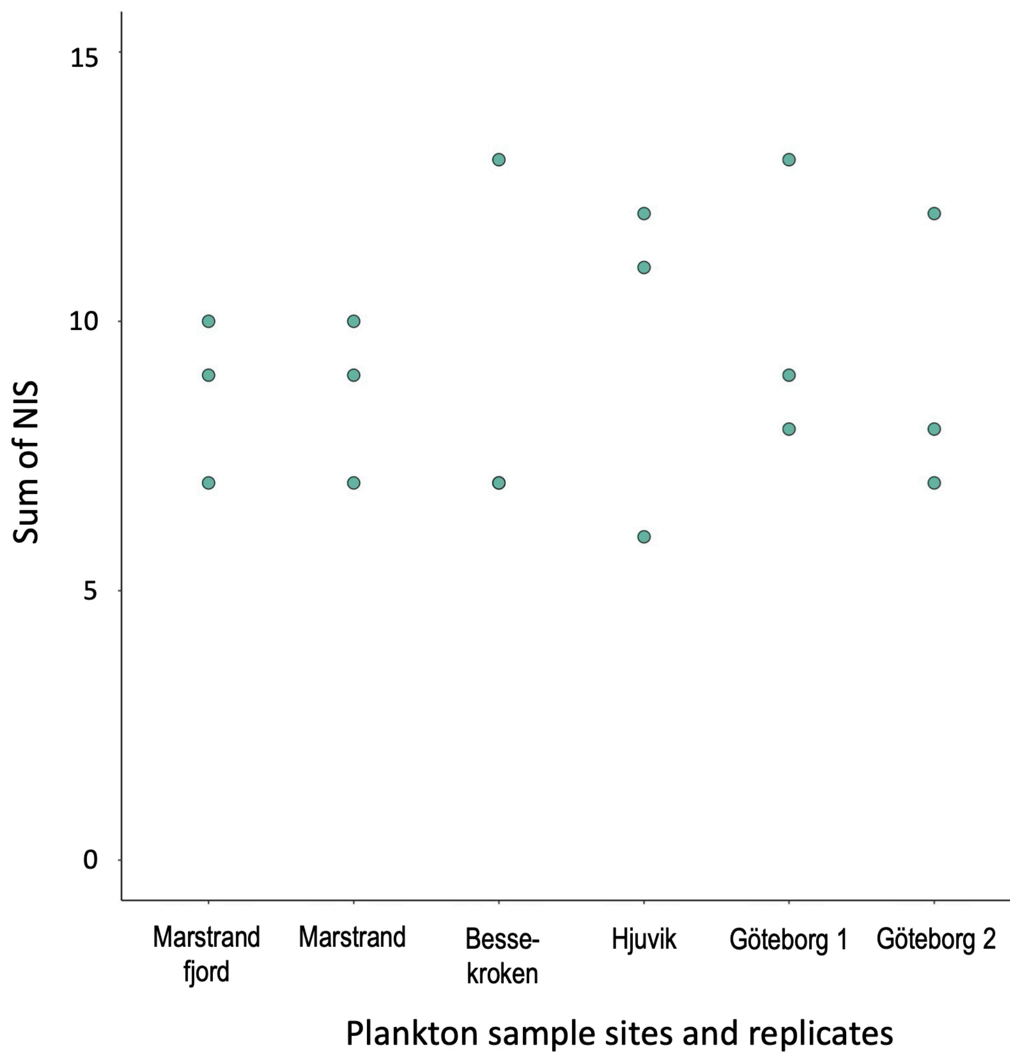


Figure 7. Number of non-indigenous species (NIS) in relation to date of sampling and site.

Sampling over time (Figure 8) shows a slight decrease in number of alien species found in the samples taken in late May. It is however difficult to draw any conclusions without knowing in detail the settlement periods of different species and other (abiotic) factors that can have influenced this.

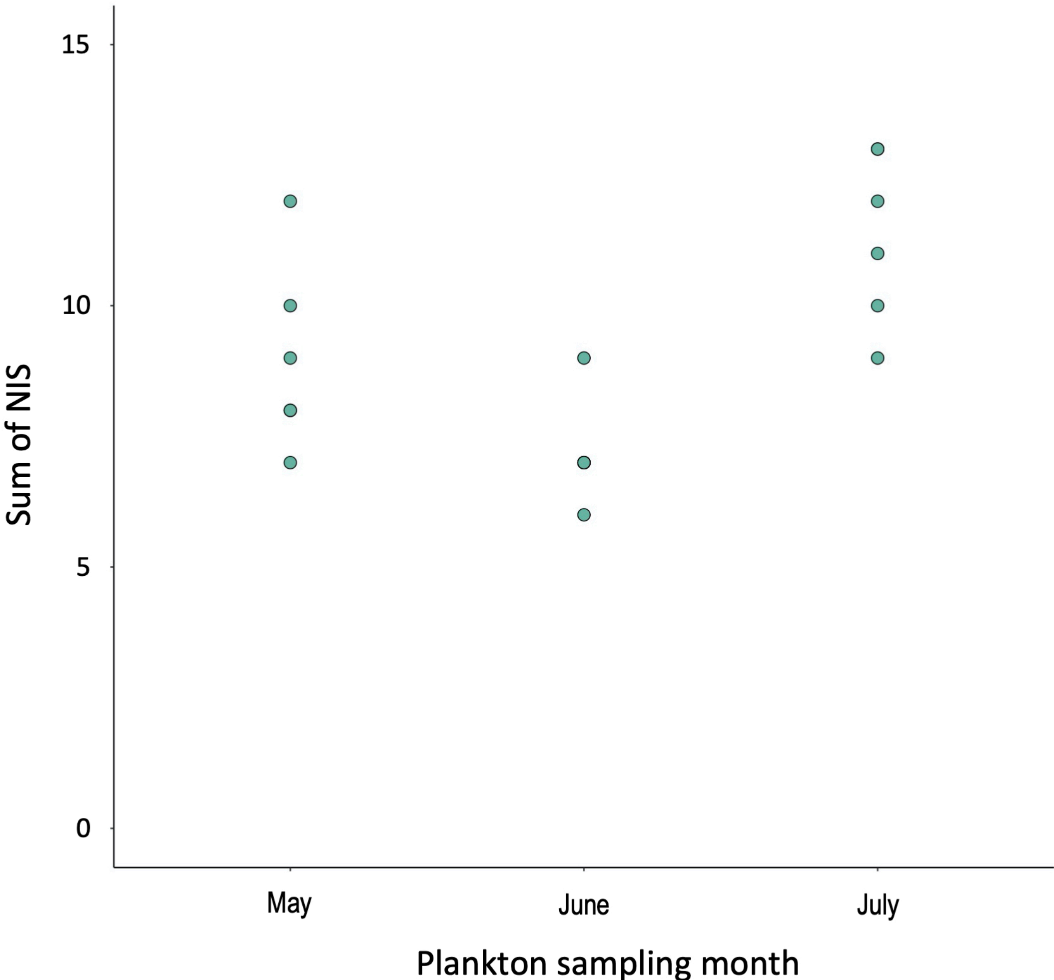
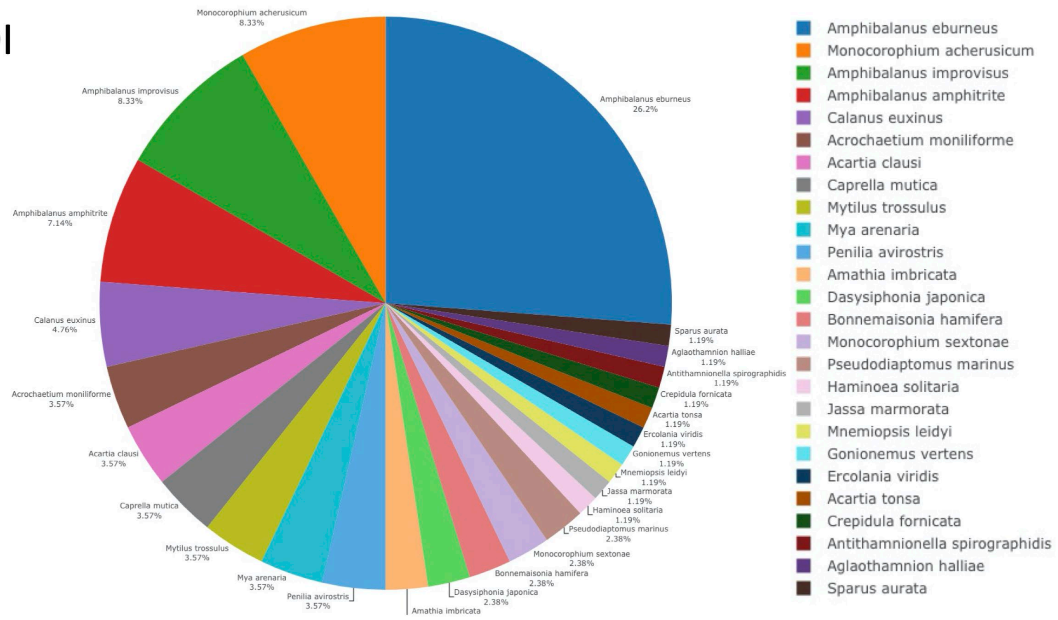


Figure 8. Number of NIS and IAS in plankton samples, and collecting period.

3.1.4 Intra-specific genetic variation

DNA-based methods also yield information on intra-specific genetic variation. Figure 9 shows intraspecific haplotype variation (ASV diversity) for the detected species, based on the COI marker gene (Figure 9A) and based on the 18S rRNA marker gene (Figure 9B).



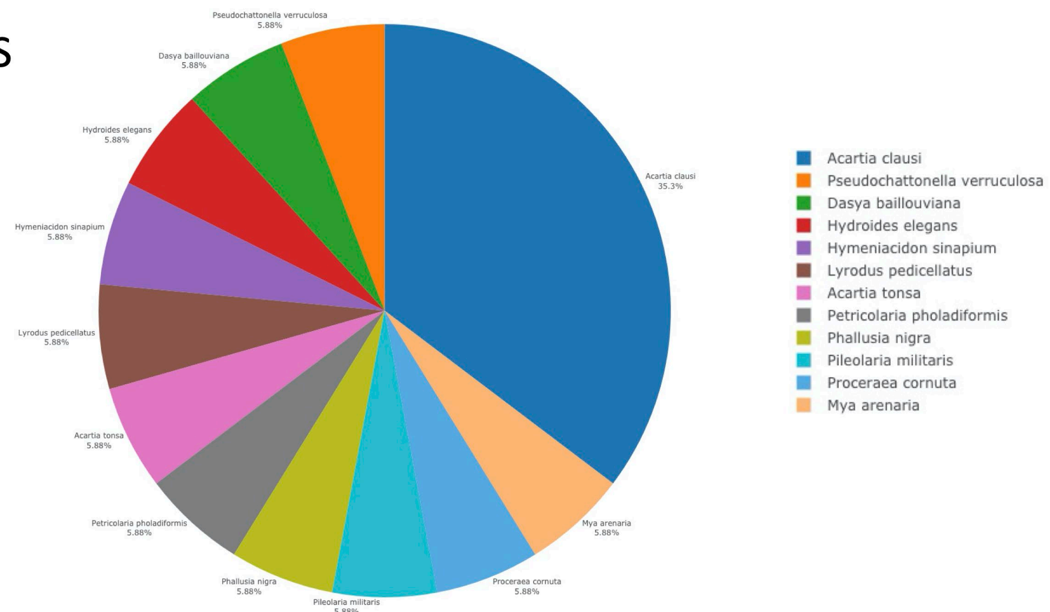


Figure 9. Haplotype diversity for detected species and for the molecular markers in A. COI and in B. 18S.

The genetic variation and the number of haplotypes (Figure 9) allows for different interpretations. A low number of haplotypes may indicate that either the species has been introduced recently or by limited number of individuals (founder effect). A larger genetic variation, and several haplotypes, in a sampled species may indicate that this species in fact has been in the area for long time, or that there were several more or less simultaneously introductions from genetically distinct populations to the area. For example, a large genetic variation among NIS in a port area may indicate continuous and repeated introduction though ships. However, there are only few studies of genetic variation in relation to ports and ship traffic and hence do not permit wider conclusions. One should also be aware of that the level of intraspecific genetic variation varies substantially among marine invertebrates and among marker genes.

Another reason for, a seemingly, high haplotype variation could be cryptic species complexes, i.e., several species are hidden under the same name. This is further discussed and elaborated in section 3.3 below.

3.2 Discussion

The traditional method for species identification based on habitus and morphology requires that there is a specimen (preferably in undamaged condition) to observe and maybe to take measurements from. Organisms are often damaged or distorted during sampling in such a way that it becomes impossible to identify them. For some taxa (for example ribbonworms, phylum *Nemertea*) it is furthermore necessary in many cases to observe the living animals for a proper identification, which is basically impossible in monitoring programs since it would require various experts to be at site during sampling. Furthermore, juveniles of many species do not resemble the adult individual and taxonomic keys are normally based on adult characters and features. Zooplankton organisms are especially difficult to determine, both holopelagic species, but perhaps especially mesoplankton which consist of larvae, eggs, and juveniles that can have a strongly different appearance compared to the adult specimen.

Another obstacle for correct species identification is the lack of the taxonomic competence, an expertise emphasized as essential by Granhag (2016) in “Methods for monitoring of alien species – Protocol for sampling in ports and shipping lanes” (authors´ translation). All phyla are represented in the marine realm, and it is thus impossible for any single person to be able to identify all species in a phylum diverse sample. Although biologists involved in monitoring programs are usually able to identify most of the common and easy identifiable species, they do not have the knowledge to cover the entire diversity sampled in marine ecosystems. Especially not species that come from afar, species that are rare, and species that are difficult to identify - all of typical characteristics for NIS. Hence, it is very likely that early observations of NIS are ignored by in monitoring programs.

DNA sequencing technology has developed enormously in recent years, and it is today possible to conduct the analysis starting from very small amounts of DNA. It is also not required that individuals are sorted before analysis, unsorted bulk samples can be sequenced (metabarcoding) and data matched to reference libraries with DNA sequences for different species. Another overwhelming advantage, especially in a monitoring program, is that damaged organisms or parts of organisms and all life forms of a species can be identified. A potential problem is that it assumes that there are sequence reference libraries that include the species of interest. This is not always the case and it means that there is a risk of missing invasive species, but we consider that risk small in comparison with traditional species identification, especially in

the case of plankton. There are furthermore many international initiatives to fill these gaps in the libraries and we foresee this to be a lesser problem in the future.

Metabarcoding is therefore potentially a well-functioning method for providing an overview of alien invasive species present in a plankton sample, or on a settling panel. There are many studies showing DNA metabarcoding to be a reliable and effective method of finding alien species, for example by Duarte et al. (2020, and references therein) and Couton et al. (2019). However, more studies are needed where DNA metabarcoding is compared with traditional methods. Such a comparison is found in Sundberg et al. (2018) but further studies would be valuable and with detailed cost and time comparisons for different methods considered. It is already well known that plankton species are difficult, and time consuming, to identify by traditional means. Andersen et al. (in press) mention, for example, how difficult copepods in the genus *Acartia* are to determine and how dissection and microscope preparations may be required for a correct identification. *Acartia tonsa* is a NIS on the list of target species for the North East Atlantic (OSPAR area)[13] to monitor, and while Andersen et al. (in press) had problems separating different species in the genus, the genetic identification reported here succeeded.

Settling panels were used by Bergqvist et al. (2020a) to test and evaluate the Swedish monitoring program on NIS. Among nine panels that submerged during a year in the Port of Nynäsham and the nearby marina, as well as Bullandö Marina; nine species were found and identified, of which one was alien (*Amphibalanus improvisus*). Although the comparison is not entirely relevant as these are ports in the Baltic Sea, it is still a much smaller number of species than in the present study. Besides geographic area, another explanation may be that the panels used by Bergkvist et al. (2020b) were of a different type and submerged individually at different depths and they also used habitat collectors. The construction of the ARMS panels creates space between the tiles in a way that we believe offer better conditions for settling organisms to find good living space.

Another advantage of using DNA-based methods is that they provide information on genetic variation that can be used to understand the dynamics of introductions and geographic distribution. Although the interpretations above about the link between low genetic variation and number of introduction events must be considered preliminary, it stills shows the possibilities. Increased sampling efforts would improve this type of analysis and it is relatively easy to increase the number of samples, both in terms of time and geographical coverage.

It is important to carefully consider time and place when planning the sampling part of an NIS monitoring program. The plankton sampling was carried out during spring and early summer when the biological activity is high. It is also in these samples that we have found a relatively higher number of IAS compared to the settling panels (Table 1) in view of the lesser number of sampling sites. The result from the settling panels also shows the importance of placing these during a period of higher biological activity. The panels in Brofjorden were those submerged and taken out of water later (spring time) than the others and these panels show most identified NIS. However, it may also be because the Brofjorden is probably a more favourable environment, from a diversity perspective, than for example the port of Gothenburg. When it comes to the settling panels, we also see that depth can be a factor that affects the result. The ARMS that were hung from jetties (1.5–2 meters) were affected by light favouring algae growth and it might had been better to put them at a greater depth. In cases where they were hung from floating jetties, it would

[13] “Target species” for ballast water exemptions are available at HELCOM and OSPAR Ballast Water Exemptions Decision Support Tool. https://maps.helcom.fi/website/RA_tool/.

have been possible to place them in the shade under the bridge. There are also other differences between premises that can affect the number of species on the panels. The sites in the mouth of the Port of Gothenburg are affected by the water running from the Göta älv river (Green 2013), which means a generally lower biodiversity than, for example, the Koster fjord area. The plates that had been placed in the Kosterfjord also showed a generally greater diversity (pers. obs.) which is not only explained by the fact that they were in longer, but also generally more favourable conditions for hard-bottomed organisms. A more general biodiversity analysis of plates and plankton can create a basis for planning the best time to lay plates, and take plankton samples.

We followed the ARMS protocol (Obst et al. 2020) that also includes photographic documentation of the plates (top and bottom). This is an important step when using these structures for more general studies of hard bottom fauna, but if they are to be used for early detection of NIS, this step may not be necessary based on the experience of this study. Our results show that the molecular analysis finds far more species than the visual inspection. Many species are difficult to identify and determine with certainty, even for an experienced and competent biologist. In addition, species are not fully developed at an early stage and may only be present on the plate as a larva, or embryo – something that the DNA determination can detect but not the visual assessment. Considering the time it takes to take the photos, handle the data, and analyse the images we recommend to skip this part in view of the outcome in relation to cost.

3.3 Sources of errors in genetic species identification

There are several things that can go wrong with a manual, traditional identification of species. For example, lack of experience and knowledge of the species, incorrect identification literature, species that require living organisms to be identified, species that require special techniques for which there is no time for. Some of these elements disappear when genetic identification is used. No special taxonomic knowledge is needed given that the species name that is linked to DNA sequences in reference libraries is accurate and has been examined by taxonomic expertise. However, there are other issues to consider.

The taxonomy within a group is not always correct and clarified which could lead to that a sequence in a reference library is incorrectly coupled to a species name. There are also cases where Linnean name in fact covers many species, cryptic species are such a case. Nor can a molecular marker always distinguish between species – which do not even have to be closely related. So for example, it is difficult to unambiguously distinguish between different *Amphibalanus* and *Mytilus* species based on COI. The risk of false positives must also be taken into account – DNA can fall from land (like insect contamination), or come from a ship that has passed.

Nor can it be sufficiently pointed out that many public databases with DNA sequences (for example GenBank) are not quality assured. This means that there are errors where the published sequence does not belong to the species specified. This may of course lead to wrong decisions with consequences. It is therefore important to:

- Have a critical mind when interpreting the results of the bioinformatic analysis; the choice of pipeline, database, parameters and filters may affect the list of identified species and potentially cause erroneous species reports.

- Double-check all observations of a species new to the area through a special “species report” where the 10 best matches against databases are examined followed by a tree-based species determination. Appendix V gives an example of this procedure.
- Confirm/evaluate an observation if the species is not reported before or for some reason unexpected in the area (in view of the habitat for example).

3.3.1 Example of manual control of species identification

Three species (*Ercolania viridis*, *Haminoea solitaria* and *Pseudodiaptomus marinus*), not previously recorded from Sweden were discovered in the plankton samples. An extended analysis was carried out to ensure that the identification of these species is correct. This analysis included:

- Library searches to check for the possibility that it is a matter of other closely related species. Especially, this is important to consider when the match between sequence and species is below 100%.
- A phylogenetic analysis to quantify the relationships with closely related sequences and species.
- Check for contamination. If the species is present in both the blank negative control and in the "sharp" samples it is a reason to be suspicious.
- Make a literature search and check, as well as correspond with the relevant taxonomic expertise.

4 Conclusions

Plankton sampling is a relatively simple, cost-effective, and rapid method for monitoring of NIS. At the same time zooplankton is difficult to identify with traditional methods and this may mean that species are not detected (Sundberg et al. 2018). This report shows that a modest repeated sampling from six sites with a sampling volume of 1.25 cubic metre per sample is sufficient to both identify known NIS, as well as discover new NIS that can potentially be judged as invasive.

The study also shows that settling panels work excellent to detect alien and invasive species. This sampling is also cheap and easy. We perceive the ARMS system with plates at a height of a few centimetres at intervals are better than the panels specified by e.g. Granhag (2016) and HELCOM (2013). However, we advise against the photographic analysis found in the ARMS protocol because it is difficult to correctly determine species from images and it is relatively time consuming.

The collected sequence data also enables a deeper analysis that can provide knowledge about the introduction, spread, distribution and abundance of alien species. This is a clear advantage over traditional, manual nature identification and allows the technology to be a truly "early warning" system.

The data produced in the DNA-based monitoring must be stored in a secure and accessible manner. Analytical tools and methods are constantly developing so that new information may be obtainable from stored data, but it is also important that there are time series of the genetic information. It is therefore crucial to include a clear data management plan in DNA-based monitoring reports.

5 Sampling sites and dates

Table 2. Sites for ARMS settling panels and reference panels (2020) with positions and dates for deployment and uptake.

Site	Latitude (N)	Longitude (E)	Deployment dates (MMDD)	Uptake dates (MMDD)
Hjuvik	57.7032	11.7113	0131	0518
Björkö Bessekroken	57.7179	11.6799	0131	0518
Gothenburg 1	57.6648	11.7147	0206	0520
Gothenburg 2	57.6646	11.7328	0206	0520
Gothenburg 3	57.6805	11.7405	0206	0520
Gothenburg 4	57.6808	11.7283	0206	0520
Varberg Getterön 1	57.1133	12.2299	0213	0603
Varberg Getterön 2	57.1126	12.2302	0213	0603
Varberg	57.1107	12.2439	0213	0603
Marstrand 1	57.9144	11.5941	0228	0527
Marstrand 2	57.9035	11.5815	0228	0527
Marstrand 3	57.8892	11.5857	0228	0527
Helsingborg 1	56.0262	12.7005	0305	0604
Helsingborg 2	56.0180	12.7005	0305	0604
Brofjorden Preem 1	58.3533	11.4348	0401	0715
Brofjorden Preem 2	58.3540	11.4339	0401	0715
Reference panels Koster VH1	58.8751	11.1031	190527	200716
Reference panels Koster VH2	58.8763	11.1118	190527	200716
Reference panels Koster VH3	58.8598	11.0804	190527	200716

Tabell 3. Sampling sites for plankton (2020) with positions and dates. Two plankton tows were taken at each site, and each site was sampled three times during a period from May 24 to July 13.

Site	Latitude (N)	Longitude (E)	Date (MMDD)
Gothenburg 1	57.6699	11.7188	0520
Gothenburg 1	57.6720	11.7401	0626
Gothenburg 1	57.6712	11.6773	0713*)
Gothenburg 2	57.6826	11.7351	0529
Gothenburg 2	57.6842	11.7372	0626
Gothenburg 2	57.6811	11.7311	0713
Hjuvik	57.7026	11.7082	0529
Hjuvik	57.7020	11.7088	0626
Hjuvik	57.7016	11.7111	0713
Björkö	57.7194	11.6702	0529
Björkö	57.7191	11.6761	0626
Björkö	57.7156	11.6772	0713
Marstrand	57.8843	11.5884	0527
Marstrand	57.8825	11.5918	0626
Marstrand	57.8830	11.5870	0712
Marstrandsfjorden	57.9154	11.5964	0527
Marstrandsfjorden	57.9102	11.6021	0626
Marstrandsfjorden	57.9045	11.5945	0712

*) The sample at Gothenburg 1, on July 13, 2020, was taken slightly westwards from previous samples due to rough weather and sea conditions.

6 Acknowledgements

We are grateful to the staff in the port of Helsingborg, port of Preemraff Lysekil, and Getterön Marina for their help and support in connection with deployment of ARMS panels.

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Appendix I. DNA extraction ARMS panels

Detailed protocols for the various parts in the ARMS-MBON project, including DNA extraction and sequencing information, can be downloaded on the projects website http://www.arms-mbon.eu) under "Documentation".

Appendix II. DNA extraction plankton

Plankton samples were fixed in 95% ethanol in 1 L jars and kept at -20 °C until DNA extractions. Preliminary tests showed that it is essential to remove all ethanol before DNA extractions. The following procedure was developed to remove ethanol in several steps.

During the storage, the material precipitated on the bottom of the jar (Figure 1 left) and the first step was to decant excess of ethanol from jars leaving 100–150 mL of liquid. The samples were mixed by shaking, and 50 mL per sample was transferred to 50 mL Falcon tubes. The Falcon tubes were centrifuged at 4 000 rpm and 10°C for 10 min to precipitate the material (Figure 1 middle) and the ethanol was again decanted. Approx. 0.5 g of wet material per sample (as in the ARMS protocol) was transferred to Eppendorf tubes. The Eppendorf tubes were centrifuged at 1 000 rpm and 10 °C for 10 min to pellet the material (Figure 1 right) and the ethanol was removed with a pipette. Finally, the samples were left to air-dry for 45 min to remove all ethanol residuals.

Figure 1. Different steps of removing ethanol from the plankton samples. See text. (photo Marina Panova)

DNA extraction was performed using Qiagen DNeasy Power Soil kit. The dry samples were re-suspended in the lysis buffer (60 uL of C1 buffer plus the liquid from the power beads tubes), transferred back to the power beads tubes and processed according to the manufacturer's protocol.

DNA extractions quantity and quality was checked using Nanodrop spectrophotometer and Qubit fluorometer. The ratio 260/280 was equal 1.8–1.9 and the ratio 260/230 ≥2 for most of the samples, indicating a good DNA purity. The DNA concentration varied 10–400 ng/uL, with the most concentrations between 100 and 200 ng/uL. For the library preparation all DNA samples were diluted to 10 ng/uL.

Two negative controls (blanks) were produced during the DNA extractions following the protocol without adding any sample at the start. No DNA was detected in those samples, neither by Nanodrop, nor by Qubit. These blank samples were included in the library preparation as a control for contamination at the DNA extraction step.

Appendix III. Negative and positive controls during sequencing

In connection to the genetic analysis of the settlement panels, three negative controls were taken, one for each PCR reaction. Three negative controls were also performed in connection with the plankton samples: two during DNA extraction (blank extractions without plankton samples) and another during PCR (blank PCR with water instead of DNA template). These negative controls were sequenced and analysed along with the samples.

Negative controls contained overall few sequences (0n1,160 after filtering) compared to the samples (average 20,000 sequences per sample after filtering). The COI analysis showed that the most common contamination was from human DNA (Table 1). There were some sequences from marine species. Of these, two barnacle species (*Amphibalanus improvisus* and *A. eburneus*) are also on the NIS list. However, these species were found only in the plankton controls and with low occurrence at the same time as they were found in both the settling panel and plankton samples with much higher occurrence. Therefore, it is unlikely that findings of *Amphibalanus* sp. DNA in samples would have come from lab contamination, and the observations in the samples are considered accurate and reliable observations.

Analysis of the control samples for the marker 18S again showed low occurrences of marine species (Table 2 in Appendix VI). There was no overlap with species detected with COI, which illustrates that the two different markers complement each other. One alien species, the sea brush worm (*Hydroides elegans*) was found in only one ARMS blank and in a much lower occurrence than in the settlement panels (12 vs 303 sequences). Therefore, it is unlikely that the species' findings on the settlement panels were the result of contamination.

Positive control (mock community) in the form of DNA mixture from nine known invertebrate species plus one fish was analysed together with the plankton samples (Table 3). The control generated approximately 55 000 sequences after filtering. With the COI marker and the BOLD database we found 9 out of 10 species, while with the MIDORI database 6 out of 10 species. The number of reads per species varied from 40% to 0.1%, which may be due to the fact that universal primers are not as effective for different groups of organisms. In addition to the control species, two fish species (rainbow and black butterbur) were found with very low occurrences (0.04–0.007%).

Table 1. Analysis of negative controls showing number of sequences, for marker COI in the BOLD database.

Class	Species	TOTAL blanks	ARMS .Blank .2018	CP.BLANK. 2019.S332	Blank. 2020	Blank extr1	Blank extr2	Blank PCR
Mammalia	Homo sapiens	686	630	0	56	0	0	0
Mammalia	Homo denisova	530	530	0	0	0	0	0
Elasmobranchii	Scyliorhinus canicula	236	0	0	236	0	0	0
Thecostraca	Amphibalanus eburneus	103	0	0	0	33	70	0
Elasmobranchii	Scyliorhinus canicula	86	0	0	86	0	0	0
Copepoda	Pseudocalanus acuspes	66	0	0	0	0	66	0
Thecostraca	Amphibalanus eburneus	50	0	0	0	0	50	0
Actinopterygii	Cyprinus carpio	32	0	0	32	0	0	0
Elasmobranchii	Squalus blainville	28	0	0	28	0	0	0
Gastropoda	Nassarius nitidus	24	0	0	24	0	0	0
Thecostraca	Amphibalanus improvisus	20	0	0	0	20	0	0
Polychaeta	Alitta virens	5	0	0	5	0	0	0
Copepoda	Amphiascopsis cinctus	2	0	2	0	0	0	0
Malacostraca	Monocorophium insidiosum	1	0	1	0	0	0	0

Table 1b. Analysis of negative controls showing number of sequences, for marker COI in the MDORI database.

Class	Species	TOTAL blanks	ARMS .Blank .2018	CP.BLANK. 2019.S332	Blank. 2020	Blank extr1	Blank extr2	Blank PCR
Mammalia	Homo sapiens	686	630	0	56	0	0	0
Chondrichthyes	Scyliorhinus canicula	236	0	0	236	0	0	0
Maxillopoda	Amphibalanus improvisus	103	0	0	0	33	70	0
Chondrichthyes	Scyliorhinus canicula	86	0	0	86	0	0	0
Maxillopoda	Pseudocalanus acuspes	66	0	0	0	0	66	0
Maxillopoda	Amphibalanus improvisus	50	0	0	0	0	50	0
Actinopteri	Cyprinus carpio	32	0	0	32	0	0	0
Maxillopoda	Amphibalanus improvisus	20	0	0	0	20	0	0
Polychaeta	Alitta virens	5	0	0	5	0	0	0
Malacostraca	Monocorophium insidiosum	1	0	1	0	0	0	0

Table 2a. Analysis of negative controls showing number of sequences, for marker 18S in the SILVA database.

Class	Species	Blanks_ total	ARMS .Blank .2018	CP.BLANK. 2019	Blank. 2020	Blank extr1	Blank extr2	Blank PCR
Malasseziaceae	uncultured fungus	297	105	88	104	0	0	0
Malasseziaceae	uncultured fungus	255	118	35	102	0	0	0
Heteroconchia	Parvicardium exiguum	205	205	0	0	0	0	0
Copepoda	Acartia clausii	129	111	0	0	0	18	0
Neopterygii	Merluccius merluccius (European hake)	76	76	0	0	0	0	0
Podocopa	Propontocypris pirifera	54	54	0	0	0	0	0
Rhodymeniophy cidae	Spermothamnion repens	44	44	0	0	0	0	0
Neoptera	Sericoderus sp. 1 JAR-2007	41	0	41	0	0	0	0
Euplotia	Gastrocirrhus monilifer	38	38	0	0	0	0	0
Eumalacostraca	Leptochelia sp. WW-2002	35	35	0	0	0	0	0
Neopterygii	Merluccius merluccius (European hake)	23	0	23	0	0	0	0
Mediophyceae	Cymatosira belgica	16	16	0	0	0	0	0
Copepoda	Acartia clausii	15	15	0	0	0	0	0
Neoptera	Sericoderus sp. 1 JAR-2007	13	0	13	0	0	0	0
Eumalacostraca	Thoralus cranchii	10	10	0	0	0	0	0
Cladophorales	Rhizoclonium lubricum	8	8	0	0	0	0	0
Scolecida	Notomastus latericeus	7	7	0	0	0	0	0
Copepoda	Acartia hudsonica	6	6	0	0	0	0	0
Demospongiae	Halisarca dujardini	3	0	0	3	0	0	0

Table 2b (continued on next page). Analysis of negative controls showing number of sequences, for marker 18S in the PR2 database.

Class	Species	Total_blanks	ARMS.Blank.2018	CP.BLANK.2019	Blank.2020	Blank_extr1	Blank_extr2	Blank_PCR
Annelida	Scoloplos armiger	881	881	0	0	0	0	0
Basidiomycota	Malassezia globosa	255	118	35	102	0	0	0
Mollusca	Parvicardium exiguum	205	205	0	0	0	0	0
Arthropoda	Acartia clausii	129	111	0	0	0	18	0
Urochordata	Phallusia nigra	109	109	0	0	0	0	0
Embryophyceae	Pogostemon cablin	60	0	0	60	0	0	0
Embryophyceae	Musa basjoo	57	0	57	0	0	0	0
Arthropoda	Propontocypris pirifera	54	54	0	0	0	0	0
Florideophyceae	Spermothamnion repens	44	44	0	0	0	0	0
Arthropoda	Sericoderus sp.	41	0	41	0	0	0	0
Annelida	Spio sp.	41	41	0	0	0	0	0
Arthropoda	Leucothoe sp.	38	38	0	0	0	0	0
Arthropoda	Leptochelia sp.	35	35	0	0	0	0	0
Cnidaria	Obelia geniculata	35	35	0	0	0	0	0
Bryozoa	Cryptosula pallasiana	35	35	0	0	0	0	0
Bryozoa	Tubulipora liliacea	32	32	0	0	0	0	0
Litostomatea	Didiniidae X sp.	28	0	28	0	0	0	0
Cnidaria	Halecium pusillum	25	25	0	0	0	0	0
Dinophyceae	Prorocentrum lima	23	23	0	0	0	0	0
Endomyxa-Ascetosporea	Paradinidae X sp.	22	22	0	0	0	0	0
Syndiniales	Dino-Group-II-Clade-56 X sp.	21	0	21	0	0	0	0
Arthropoda	Acartia clausii	15	15	0	0	0	0	0
Mollusca	Dendrodoris nigra	14	14	0	0	0	0	0
Arthropoda	Temora longicornis	13	0	0	0	10	3	0
Telonemia_X	Telonemia-Group-2 X sp.	13	13	0	0	0	0	0
Arthropoda	Sericoderus sp.	13	0	13	0	0	0	0
Platyhelminthes	Philactinoposthia ischiae	12	12	0	0	0	0	0
Arthropoda	Centropages hamatus	12	0	1	0	4	7	0
Annelida	Hydroides elegans	12	12	0	0	0	0	0
Bryozoa	Callopora lineata	11	11	0	0	0	0	0
Endomyxa-Phytomyxea	Phagomyxa sp.	11	11	0	0	0	0	0
Ichthyosporea	Abeoformidae Group MAIP 1 X sp.	11	11	0	0	0	0	0
Platyhelminthes	Haplopharynx rostratus	10	10	0	0	0	0	0
Labyrinthulomycetes	Oblongichytrium sp.	9	9	0	0	0	0	0
Ichthyosporea	Abeoformidae Group MAIP 1 X sp.	8	8	0	0	0	0	0
Annelida	Spirorbis spirorbis	8	8	0	0	0	0	0

Class	Species	Total_blanks	ARMS.Blank.2018	CP.BLANK.2019	Blank.2020	Blank_extr1	Blank_extr2	Blank_PCR
Gregarinomorphea	Eugregarinorida XX sp.	8	8	0	0	0	0	0
Annelida	Notomastus latericeus	7	7	0	0	0	0	0
Arthropoda	Crustacea XX sp.	6	6	0	0	0	0	0
Arthropoda	Stenothoe brevicornis	6	6	0	0	0	0	0
Monothalamids	Allogromia sp.	6	6	0	0	0	0	0
Filosa-Granofilosea	Massisteria marina	6	6	0	0	0	0	0
Arthropoda	Harpacticus sp.	5	3	0	0	0	2	0
Urochordata	Oikopleura sp.	5	0	0	0	0	5	0
Bacillariophyta	Chaetoceros socialis	5	5	0	0	0	0	0
Bacillariophyta	Cerataulina pelagica	5	0	0	0	0	5	0
Nematoda	Pareurystomina sp.	5	5	0	0	0	0	0
Syndiniales	Dino-Group-I-Clade-4 X sp.	4	4	0	0	0	0	0
Syndiniales	Dino-Group-I-Clade-1 X sp.	4	4	0	0	0	0	0
Arthropoda	Centropages typicus	3	0	0	0	0	3	0
Arthropoda	Eurytemora affinis	3	3	0	0	0	0	0
Arthropoda	Mysidopsis gibbosa	3	3	0	0	0	0	0
Porifera	Halisarca dujardini	3	0	0	3	0	0	0
Arthropoda	Corycaeus speciosus	2	0	0	0	0	2	0
MAST-12	MAST-12A XX sp.	2	2	0	0	0	0	0
Bacillariophyta	Minutocellus sp.	2	2	0	0	0	0	0
Syndiniales	Dino-Group-I-Clade-4 X sp.	1	1	0	0	0	0	0

Table 3. Analysis of positive controls for marker COI in the BOLD and MIDORI databases.

Class	Species	BOLD	MIDORI
Actinopterygii	Sander lucioperca	20984	20984
Malacostraca	Liocarcinus depurator	16281	16281
Polychaeta	Alitta virens	7347	7360
Asteroidea	Marthasterias glacialis	4998	4998
Gastropoda	Littorina littorea	2835	0
Anthozoa	Alcyonium digitatum	1484	0
Gymnolaemata	Flustra foliacea	1193	1193
Anthozoa	Virgularia mirabilis	664	0
Asteroidea	Astropecten irregularis	195	0
Ascidiacea	Phallusia ingeria	0	2192
Actinopterygii	Oncorhynchus mykiss	21	21
Actinopterygii	Gobius niger	4	4

Appendix IV. DNA library preparation for sequencing

PCR protocols for each marker 18S, ITS and COI in 30µl reaction volume.

18S	uL
KaPa Hifi Ready PCR mix, 2x	15
Forward primer, 5 mM	1.8
Reverse primer, 5 mM	1.8
BSA, 20 mg/mL	0.6
PCR-grade H2O	8.8
DNA template, 10 ng/uL	2

ITS	uL
KaPa Hifi Ready PCR mix, 2x	15
Forward primer, 10 mM	1.5
Reverse primer, 10 mM	1.5
BSA, 20 mg/mL	0.6
PCR-grade H2O	9.4
DNA template, 10 ng/uL	2

COI	uL
KaPa Buffer A, 10x	3
MgCl2, 25 mM	0.6
dNTPs, 10 mM each	0.8
KaPa Taq polymerase, 5U/uL	0.9
Forward primer, 10 mM	1.8
Reverse primer, 10 mM	1.8
BSA, 20 mg/mL	0.6
PCR-grade H2O	18.5
DNA template, 10 ng/uL	2

PCR cycling

18S: 95 °C for 3 min; 30 cycles at 98 °C for 20 s, 58 °C for 15 s, 72 °C for 15 s; 72 °C for 3 min.

ITS: 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min.

COI (touchdown PCR): 95 °C for 5 min; 16 cycles at 95 °C for 10 s, 62 °C (-1 °C/cycle) for 30 s, 72 °C for 1 min; 24 cycles at 95 °C for 10 s, 46 °C for 30 s, 72 °C for 1 min; 72 °C for 7 min.

A blank sample with PCR-grade H_2O was included together with template samples in the different PCR steps as control for contamination. A mock sample with 9 known invertebrate species and one fish species was included as positive control during the different PCR steps

PCR-products were cleaned with AMPure XP beads, in accordance with the "Illumina amplicon library preparation" protocol (see the link: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf)

Index PCR carried out in 50 uL reaction volume with Eurofins Genomics indexes UDI_96_set1 and UDI_24_set1 as follows:

Reactions	uL
KaPa Hifi Ready PCR mix 2x	25
Cleaned up first PCR	15
Eurofins Indexes	10

PCR cycling: 95 °C for 3 min; 10 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min.

The PCR products were cleaned up with AMPure XP beads, following the Illumina amplicon library preparation protocol (see the link: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf)

The concentrations of the cleaned PCR were assessed by Qubit and were 40-400 ng/uL. Libraries for each marker were pooled in the equimolar concentrations. Fragment size and purity were confirmed at the gel electrophoresis (Figure 2).

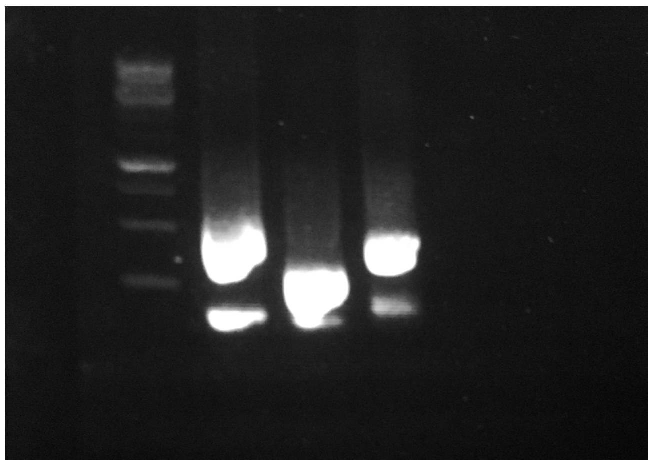


Figure 2. Gel electrophoresis of the library pools: ITS, 18S, COI.

Altogether, there were 120 libraries: (36 samples + 3 blanks + 1 mock) x 3 markers.

Appendix V.

Below are examples of how observations of new species in a region can be manually double-checked to exclude false identifications. Important elements that should be included in such a control are a brief description of the hitherto known biogeographical distribution, as well as an examination of taxonomic identity (i.e. are there any possible synonyms or taxonomic ambiguities). Such information can be obtained from relevant databases, including WoRMS (http://www.marinespecies.org/), GBIF (https://www.gbif.org/), or the Swedish Biodiversity Data Infrastructure (https://biodiversitydata.se/). In addition, the at least 10 of the best individual sequence matches (e.g. from BOLD) investigated and a tree-based identification should be carried out. Below are examples of a manual double check for some of the new species observed in this report.

Species identification report
Ercolania viridis

Known distribution: Mediterranean

Query: ASV_3189
Top Hit: Mollusca Gastropoda - *Ercolania viridis* (99.35%)

Search Result:

The submitted sequence has been matched to *Ercolania viridis*. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

A species page is available for this taxon: SPECIES PAGE

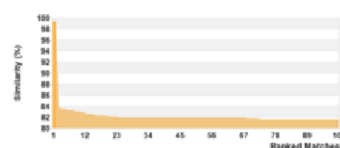
Closest matching BIN (within 3%): BIN PAGE

For a hierarchical placement - a neighbor-joining tree is provided: TREE BASED IDENTIFICATION

Identification Summary

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Mollusca	100
Class	Gastropoda	100
Order		100
Family	Limapontiidae	100
Genus	*Ercolania*	100
Species	*Ercolania viridis*	99.4

Similarity Scores of Top 100 Matches



Top 10 Matches

Display: Top 10

Phylum	Class	Order	Family	Genus	Species	Subspecies	Similarity (%)	Status
Mollusca	Gastropoda		Limapontiidae	*Ercolania*	*viridis*		99.35	Published
Mollusca	Gastropoda		Limapontiidae	*Ercolania*	*viridis*		99.35	Published
Mollusca	Gastropoda		Limapontiidae	*Ercolania*	*sp.*		83.86	Published
Mollusca	Gastropoda		Hermaeidae	*Cyerce*	*nigra*		83.5	Published
Mollusca	Gastropoda		Hermaeidae	*Cyerce*	*nigra*		83.5	Published
Mollusca	Gastropoda		Plakobranchidae	*Elysia*	*nigrocapitata*		83.17	Published
Mollusca	Gastropoda		Platyhedylidae	*Gascoignella*	*nukuli*		83.17	Published
Mollusca	Gastropoda		Hermaeidae	*Cyerce*	*nigricans*		83.17	Published
Mollusca	Gastropoda		Limapontiidae	*Ercolania*	*fuscata*		82.96	Published
Mollusca	Gastropoda	Stylommatophora	Ariophantidae	*Everettia*	*sp. 5 TSL-2008*		82.95	Published

Tree-based identification (*Ercolania viridis*)

Bulinus globosus|[27]|Angola|Planorbidae|'
Bulinus globosus|[26]|Angola|Planorbidae|'
Unknown Specimen||'
Ercolania viridis|[16]|France|Limapontiidae|'
Ercolania viridis|[15]||Limapontiidae|'
Placida brookae|[14]|United States|Limapontiidae|'
Placida brookae|[13]|Ecuador|Limapontiidae|'
Placida brookae|[12]|United States|Limapontiidae|'
Placida brookae|[11]|United States|Limapontiidae|'
Plakobranchus sp. blue JV2013|[10]|Guam|Plakobranchidae|'
Plakobranchus aff. 1 JV2013|[9]|Papua New Guinea|Plakobranchidae|'
Cyerce nigricans|[8]|Guam|Hermaeidae|'
Cyerce nigra|[7]|United States|Hermaeidae|'
Cyerce nigra|[6]|United States|Hermaeidae|'
Ercolania fuscata|[5]|Bermuda|Limapontiidae|'
Ercolania fuscata|[4]|United States.Florida|Limapontiidae|'
Ercolania sp.|[3]|United States.Florida|Limapontiidae|'
Volvatella viridis|[2]|Volvatellidae|'
Ercolania sp. 5 JEV-2014|[1]|Japan|Limapontiidae|'
Elysia cf. tomentosa 5 JV2013|[25]|Thailand.Krabi|Plakobranchidae|'
Elysia cf. tomentosa sp. 5 AAN-2018|[24]|Iran|Plakobranchidae|'
Elysia marginata|[23]||Plakobranchidae|'
Elysia cf. japonica ALB-2006|[22]||Plakobranchidae|'
Elysia bangtawaensis|[21]|Thailand|Plakobranchidae|'
Thuridilla bayeri|[20]||Plakobranchidae|'
Elysia nigrocapitata|[19]|South Korea|Plakobranchidae|'
Elysia singaporensis|[18]|Singapore|Plakobranchidae|'
Gascoignella nukuli|[17]|Thailand|Platyhedylidae|'
Everettia sp. 5 TSL-2008|[83]||Ariophantidae|'
Quantula striata|[82]|Malaysia.Sabah|Dyakiidae|
Quantula striata|[81]|Malaysia.Sabah|Dyakiidae|
Quantula striata|[80]|Malaysia.Sabah|Dyakiidae|
Quantula striata|[79]|Malaysia.Sabah|Dyakiidae|
Haplotrema concavum|[78]|United States.Alabama|Haplotrematidae|
Lymnaea taurica|[77]||Lymnaeidae|'
Lymnaea taurica|[76]||Lymnaeidae|'
Acroloxus lacustris|[75]|Italy|Acroloxidae|'
Acroloxus lacustris|[74]|Austria.Vorarlberg|Acroloxidae|
Acroloxus lacustris|[73]|Italy|Acroloxidae|'
Acroloxus lacustris|[72]|Italy|Acroloxidae|'
Indoplanorbis exustus|[71]|Bangladesh|Planorbidae|'
Burnupia sp.|[70]|Brazil.Rio de Janeiro|Burnupiidae|
Burnupia sp.|[69]|Brazil.Rio de Janeiro|Burnupiidae|
Burnupia sp.|[68]|Brazil.Rio de Janeiro|Burnupiidae|
Bulinus camerunensis|[67]|Cameroon|Planorbidae|'
Bulinus camerunensis|[66]|Cameroon|Planorbidae|'
Bulinus camerunensis|[65]|Cameroon|Planorbidae|'
Bulinus forskalii|[64]|Burkina Faso|Planorbidae|'
Bulinus forskalii|[63]|Senegal.Dakar|Planorbidae|'
Bulinus globosus|[56]|Angola|Planorbidae|'
Bulinus globosus|[53]|Angola|Planorbidae|'
Bulinus globosus|[52]|Angola|Planorbidae|'
Bulinus globosus|[45]|Angola|Planorbidae|'
Bulinus globosus|[44]|Angola|Planorbidae|'
Bulinus globosus|[43]|Angola|Planorbidae|'
Bulinus globosus|[42]|Angola|Planorbidae|'
Bulinus globosus|[41]|Angola|Planorbidae|'
Bulinus globosus|[40]|Angola|Planorbidae|'
Bulinus globosus|[39]|Angola|Planorbidae|'
Bulinus globosus|[38]|Angola|Planorbidae|'
Bulinus globosus|[37]|Angola|Planorbidae|'
Bulinus globosus|[36]|Angola|Planorbidae|'
Bulinus globosus|[35]|Angola|Planorbidae|'
Bulinus globosus|[34]|Angola|Planorbidae|'
Bulinus globosus|[33]|Angola|Planorbidae|'
Bulinus globosus|[32]|Angola|Planorbidae|'
Bulinus globosus|[31]|Angola|Planorbidae|'
Bulinus globosus|[30]|Angola|Planorbidae|'
Bulinus globosus|[29]|Angola|Planorbidae|'
Bulinus globosus|[28]|Angola|Planorbidae|'

0.03

Species identification report
Haminoea solitaria

Known distribution: Origin East coast USA, recently observed in the Baltic

Query: ASV_7455
Top Hit: Mollusca Gastropoda - Cephalaspidea - *Haminoea solitaria* (99.68%)

Search Result:

The submitted sequence has been matched to *Haminoea solitaria*. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

A species page is available for this taxon: SPECIES PAGE

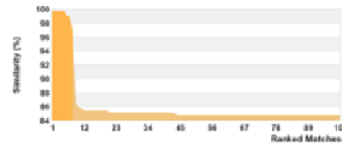
Closest matching BIN (within 3%): BIN PAGE

For a hierarchical placement - a neighbor-joining tree is provided: TREE BASED IDENTIFICATION

Identification Summary

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Mollusca	100
Class	Gastropoda	100
Order	Cephalaspidea	100
Family	Haminoeidae	100
Genus	*Haminoea*	100
Species	*Haminoea solitaria*	99.7

Similarity Scores of Top 100 Matches

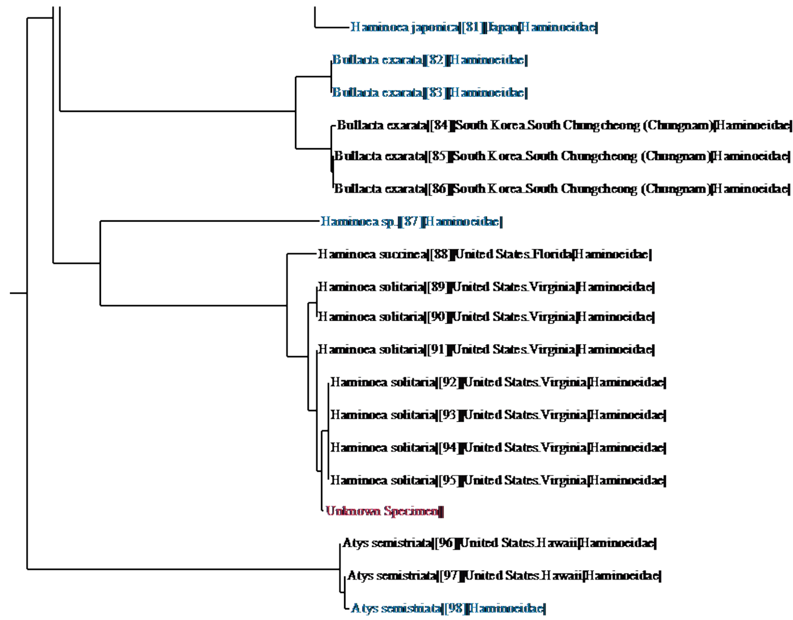


Top 10 Matches

Display: Top 10

Phylum	Class	Order	Family	Genus	Species	Subspecies	Similarity (%)	Status
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.68	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.68	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.68	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.68	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.68	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.03	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*solitaria*		99.03	Private
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	*succinea*		96.76	Private
Arthropoda	Insecta	Lepidoptera	Geometridae	*Chiroclystis*	*semiscripta*		86.6	Published
Mollusca	Gastropoda	Cephalaspidea	Haminoeidae	*Haminoea*	sp.		85.84	Published

Tree-based identification (*Haminoea solitaria*)



Species identification report
Pseudodiaptomus marinus

Known distribution: known from the North Sea, but unclear about Sweden

Query: ASV_5580
Top Hit: Arthropoda Copepoda - Calanoida - *Pseudodiaptomus marinus* (99.68%)

Search Result:

The submitted sequence has been matched to *Pseudodiaptomus marinus*. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

A species page is available for this taxon: SPECIES PAGE

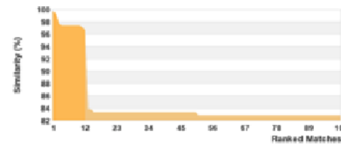
Closest matching BIN (within 3%): BIN PAGE

For a hierarchical placement - a neighbor-joining tree is provided: TREE BASED IDENTIFICATION

Identification Summary

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Arthropoda	100
Class	Copepoda	100
Order	Calanoida	100
Family	Pseudodiaptomidae	100
Genus	*Pseudodiaptomus*	100
[illegible]	[illegible]	[illegible]

Similarity Scores of Top 100 Matches



Display: Top 10

Top 10 Matches

Phylum	Class	Order	Family	Genus	Species	Subspecies	Similarity (%)	Status
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		99.68	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		99.26	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.78	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published
Arthropoda	Copepoda	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus*	*marinus*		97.41	Published

Tree-based identification (*Pseudodiaptomus marinus*)

Genetic methods in environmental monitoring

Early detection and monitoring of non-indigenous species based on DNA

Collecting marine hard bottom organism with autonomous techniques in combination with DNA analyses for species identification has proven to be efficient monitoring of biodiversity and detection of non-indigenous species. Along the Swedish west coast, it as investigate whether plankton samples and settling panels in combination with DNA-based species identification are effective methods for early detection and continuous monitoring of non-indigenous species. This report presents how these methodologies provides well to early warning surveillance of invasiv alien species.

Vi arbetar för levande hav och vatten

Havs- och vattenmyndigheten, HaV, är en statlig förvaltningsmyndighet inom miljöområdet. Vi arbetar på regeringens uppdrag för bevarande, restaurering och hållbart nyttjande av sjöar, vattendrag, hav och fiskresurserna