UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

MONITORING MARINE ALIEN AND INVASIVE SPECIES

A comparison between the traditional method (eRAS) and DNA-based identification of species.

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Degree project for Master of Science (120 hec) with a major in biology.

BIO795, Degree project biology (master), 30 hec.

Semester/year:	Spring 2023
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Abstract

There is a major concern with the uncontrolled spreading of alien and invasive species, caused by an increase in travelling by boat globally. These are dispersed via ballast water, by fouling on ships and in fishing gear. If an alien or invasive species is introduced into a foreign environment, it can have negative effects on the native species composition on the site. The aim of this study was to investigate which method, DNA-based or traditional, that is more suitable when tracking and identifying alien and invasive species in harbours. Detecting alien or invasive species in an early stage is crucial to prevent spreading, and for this, sensitive methods that can detect species in all their life stages are needed. The research question was "Which method is best to track and determine potentially alien – or invasive species in harbours?". The DNA-based method, water samples, plankton samples and arms settling panels (Autonomous Reef Monitoring Structures), collected genetic material and metabarcoding was used to identify and species determine any findings. For the traditional methods; panels, scrapings and artificial habitats, only visual morphological identification was used for identification and determination of the findings. The results showed that the DNA-based method found ten alien and invasive species in their samples, were two, round goby and the red algae *Bonnemaisonia hamifera*, are classified as invasive. The traditional method recovered zero alien or invasive species in their samples. The traditional method are today included in the Swedish monitoring program and with the results of this study, it is proposed that these are replaced by the DNA-based method for future enhanced monitoring of alien and invasive species.

Sammanfattning

Ett stort problem med ökningen av globala båtresor är spridningen av främmande och invasiva arter. Arterna sprids via barlastvatten, som påväxt på skepp eller i fiskeutrustning. Då en främmande art introduceras i en främmande miljö finns det risk att de inhemska arterna påverkas negativt. Att upptäcka främmande och invasiva arter i ett tidigt skede är avgörande, därför behövs metoder som kan upptäcka arterna i alla dess olika livsstadier. Studiens syfte var att undersöka vilken typ av metod av DNA-baserad alternativt traditionell metod (eRAS) som är lämpligast när det kommer till att spåra och artbestämma främmande och invasiva arter i hamnar. Detta genom frågeställningen ”vilken metod är bäst lämpad när det kommer till att spåra och artbestämma främmande eller invasiva arter i hamnar?”. Den DNA-baserade

metoden; vattenprover, planktonprover och arms settling-paneler, samlade in genetiskt material och metabarcoding användes därefter för att artbestämma och identifiera eventuella fynd. Den traditionella metoden; paneler, skrapningar och artificiella habitat, identifierade och artbestämde sina fynd med enbart visuell morfologisk identifiering. Resultaten visade att den DNA-baserade metoden återfann tio främmande arter, varav två ,svartmunnad smörbult och japantofs, är klassade som invasiva. Den traditionella metoden (eRAS) registrerade inga främmande eller invasiva arter i sina prover. Resultaten visar att den DNA-baserade metoden är överlägsen den traditionella metoden (eRAS) vid identifiering och artbestämning. Den traditionella metoden (eRAS) är idag inkluderad i det svenska marina övervakningsprogrammet och med resultaten i denna studie föreslås att den byts ut mot DNA-baserade metoder för framtida effektiviserad övervakning av främmande och invasiva arter

Introduction

Marine species have been introduced to new habitats and ecosystems since humans first started their global boat traveling, however, you can see a significant increase in introduced species over the last 150 years. This is mainly due to the fact that technology has developed, which has thus increased global travel both in number and in length (Geburzi & McCarthy, 2018). The introduced species are mainly spread: via ship ballast as hitch hikers or fouling on ships, by expanding their range due to climate change and by fishing gear or instruments on small recreational boats (Van Berkel, 2022; Kelly et al., 2013). Through these human activities, the species are spread to areas that are beyond their natural range and distribution limits, which can cause large problems for the local flora and fauna. Today it is common to see hot spots of exotic species right next to harbours and marinas (Geburzi & McCarthy, 2018). Of the large number of introduced species, only a few are able to survive permanently in their new environment. (Molnar et al., 2018). The species that survive and become established are called alien species, since they don't belong to the native flora and fauna. If any of these alien species start to spread heavily and cause damage the native species composition, the ecosystem, economy or have impacts on health, they are called invasive (SVAM, n.d.).The spreading of alien and invasive species poses major threats to the biodiversity of our oceans (Molnar et al., 2018). One major risk with invasive species are the infections and parasites that the species can carry with them and thereby infect the native species. An example of this is, the signal crayfish (*Pacifastacus leniusculus)*, which carries a crayfish plaque that is fatally dangerous to our native river crayfish (*Astacus astacus)* (SVAM, 2015).

Since Sweden is a member of the European Union, we are obligated to follow the marine environmental regulation (The Swedish parliament, 2018). To do this, Sweden has developed a monitoring program

regarding alien and invasive species in Swedish waters (SWAM, 2019). In order to prevent the spread of invasive species, it is important that the species are identified correctly before they establish. Determining and identifying a species requires a great deal of specialist knowledge. With the increasing number of exotic species there is an imminent risk of knowledge gaps arising regarding these. This means that the species cannot be correctly identified (Sundberg et al., 2018). There are also many difficulties regarding identification based on morphology as it may require that there are large parts of an individual intact, that the species is relatively easy to find or that the species is in a special stage of its life cycle (Ibrahimovic, 2016).

Species identification based on DNA information can be very effective when detecting the presence of different organisms without actually having to capture or observe it (Gelis et al., 2021). The method neither damages nor affects habitat, species or ecosystems during sampling, which is advantageous when working with endangered species or ecosystems (Sahu et al., 2022). All organisms release some type of genetic material into their environment. It can be through faeces, germ cells, mucus, blood, hair, skin cells etc. This is called environmental DNA (eDNA) (LeBlanc et al., 2020). This genetic material can easily be collected through various methods such as water samples, plankton samples or soil samples etc. and analysed using DNA techniques such as metabarcoding (Gelis et al., 2021). A study from South Korea, used eDNA collection to study the presence of the invasive species *Bugula neritina* in 35 different harbours. By carrying out water samples containing genetic material with subsequent DNA analyses, the result showed that *Bugula neritina* was found in 27 of the 35 ports (Kim et al., 2018).The DNA-based methods are commonly used in marine environments, but can also be used in terrestrial environments (Davis, J. n.d).

Using DNA analyses for species identification was first introduced by Arnot et al., (1993), but became more standardized by Herbert et al., (2003) who also introduced the concept of DNA barcoding. When first introduced, it was only possible to only identify one species at the time. However, with technological advances, barcoding evolved into metabarcoding which enabled the identification of several different species from different taxonomic groups at the same time (Cristescu et al., 2014).

Metabarcoding, which is commonly used in DNA-based identification methods, is a tool that has the ability to identify large amounts of species by using standardized DNA sequences that are matched against reference sequences in various databases (Rodrigues et al., 2017). The tool differs from previous conventional methods, which compared species morphology, in that it uses and compares the species' genome instead (Ibrahimovic, 2016). A genome is the complete genetic material of a species and must

contain all the information needed for the organism to function. The genetic material is stored in long molecules of DNA called chromosomes (Nature education, 2014). Since a genome of a multicellular organism is enormous, it would not be time-efficient to constantly compare the entire genome against the genome of other individuals. To make the work more efficient, a standardized gene sequence was developed. This gene sequence can be found in all the genomes of all individuals belonging to the animal kingdom. The gene region that were chosen for the identification can be found in the mitochondria within every animal species and is called Cytochrome c oxidase 1 (COI) I. COI has a length of 648 base pairs (Valentini et al., 2009).

COI is used primarily for three reasons. Firstly, it has universal primers. A universal primer is a primer that have the ability to amplify the targeted region of DNA over a wide range of species. Even if the species are completely different, they still got some regions in their sequences where their nucleotides are exactly the same. This is why you can use universal primers. You can, for example, use a universal primer for fish and thus gain access to all fish species in your sample, instead of using one primer per species (Haider, 2011). Secondly, COI has a rapid rate of evolution, which means that there are small differences in each region of COI in each species. This makes it possible to distinguish between species, which you want to do during metabarcoding (Pentinsaari et al., 2016).Thirdly, there are a huge number of mitochondria's in an individual's body mass, which means that there are large amounts of mitochondrial DNA in circulation. This makes it relatively easy to get access to mitochondrial DNA (Chial & Craig, 2008).

An example of when metabarcoding is at its finest is a study conducted by Rodgers et al, 2017 on Borro Colorado Island, Panama. The study wanted to find out what kind of mammals that were present on the island by using DNA-based techniques. They did this by trapping a number of carnivorous flies, Carrion flies (*Calliphoridae*). The flies eat mammal meat, which caused them to ingest the DNA of mammals into their bodies. Rodgers et al, 2017 extracted the DNA from the stomachs of the flies and then used universal primers for mammals and vertebrates only, this so that the flies own DNA would not be included in the process. They performed metabarcoding and the results showed 20 different mammals, 4 birds and 1 lizard species from the flies' stomach contents. This shows how effective this DNA technology is in comparison to e.g. capturing, cameras etc. (Rodgers et al., 2017).

Swedish Agency for Marine and Water Management has developed a monitoring program of alien and invasive species in Swedish waters. Today the traditional method (eRAS - extended rapid assessment survey) is mainly used in this program (SWAM, 2017).The eRAS method mainly consists of taking samples such as scrapings, panels, divers and artificial habitats and then determining and identifying the

organisms in them by visual morphological identification executed by a taxonomist (Hoppe et al., 2016). The monitoring program is currently under evaluation regarding which method to use when monitoring the species and this is why SWAM assign this study to SeAnalytics AB.

The aim of this study was to compare the traditional method (eRAS) to the DNA-based method, to see which method were more suited to track and identify alien and invasive species in Wallhamn harbour, Tjörn. The study and material collection was conducted in Wallhamn harbour and marina during summer 2022, and the analyses during spring 2023. To detect alien or invasive species in an early stage is crucial to prevent spreading, for why, sensitive methods that can detect species in all their life stages are needed. The main research question to be answered was "Which method is best to track and determine potentially alien – or invasive species in harbours?". The hypothesis was that the DNA-based method would find and determine more species than the traditional ones (eRAS). The study also included the research question "What is the most common method used to collect genetic material in studies of invasive species?", which was answered by a literature study. The study was assigned by Swedish Agency for Marine and Water Management to SeAnalytics AB, and is based on a previous project that was carried out by Sundberg et al., (2018). A new study is motivated by the fact that the technology regarding DNA analyses has advanced a lot since the previous study.

Materials and methods

Collecting of material

All sampling and data collection was conducted by SeAnalytics AB during summer 2022 in Wallhamn harbour, Tjörn.

DNA-based method

Water samples

Water samples were collected with a water sampler (ruttner) at different depths and was then pooled in 1 litre containers (figure 1). The water was then filtered with a Sterivex 0,45µm filter and fixed with 95% ethanol. It was then kept in a freezer (-20 °C) until extraction. The procedure was done three times during June, July and August 2022, which resulted in 9 water samples in total.

Figure 1. Water sampler which were used to collect 3x3 water samples in June, July and August 2022. Illustrator: Cecilia Casper.

Plankton samples

To collect the plankton samples a Hydro-bios Apstein 90-micromilimetre net with an opening diameter of 40 centimetres was used (figure 2). The net gives a sampling volume of 1.25 cubic meter per sample. By vertically drag the net from the bottom (5-10 meters) and up to the surface, three samples were collected. This procedure was performed three times in June, July and August 2022, resulting in 9 plankton samples in total. Every sample was fixed with 95% ethanol and back at the laboratory the samples was decanted and new ethanol was added. The samples were kept in a freezer (-20 °C) until extraction.

Figure 2. Hydro-bios Apstein 90-micromilimetre net which were used to collect 3x3 plankton samples in June, July and August 2022. Illustrator: Cecilia Casper.

Autonomous Reef Monitoring Structures (ARMS settling panels)

Three Autonomous Reef Monitoring Structures (ARMS) that consisted of nine PVC-plates (22.5 cm x 22.5 cm, plus a bottom plate 45 cm x 35 cm) were placed in three different locations on the pier on 2 meters depth in 23/6-2022 and where brought up in 9/9-2022 (figure 3). They were photographed and all the organisms were scraped off, divided into three size fractions by sieving, decomposed with a mixer rod, fixed in a buffer and then followed DNA extraction.

Figure 3. ARMS settling panels which were placed under the water surface from June to September 2022 to gather genetic material. Illustrator: Cecilia Casper.

Traditional method (eRAS)

Panels

The panels consisted of three PVC-plates (15 cm x 15 cm, 2 mm thick), which were sandpapered each three times vertically and three times horizontally to increase growth. The panels were then attached to a rope with 1, 3 and 7 meters apart, resulting in three panels per rope (figure 4). The ropes were then placed under water on the piers between 23/6 - 9/9 - 2022. There were three ropes in total. When brought out of the water, the panels were cut off and placed in individual plastic bags. The degree of coverage of organisms were analysed and species determined on both sides of the panel.

Figure 4. Illustration of the panels that were used in the traditional method to gather species. Illustrator: Cecilia Casper.

Scrapings

Scrapings from three different substrates on piers were collected and then fixed with 95% ethanol (figure 5). The scrapings samples were filtered through a sieve and were then placed in treys for taxonomical identification.

Figure 5. A scraper which were used to scrape piers and structures in the area to gather species. Illustrator: Cecilia Casper.

Artificial habitats

There were in total two artificial habitats which consisted of two plastic trays filled with potsherds (this to keep the trays down) and covered by a net to keep the potsherds in place (figure 6). The holes were cut open to allow fish and other species to move around freely in the habitats. The artificial habitats were placed on two different locations, one on a depth of 10 meters and one on 3 meters depth between 23/6 – 4/8 - 2022. When brought up they were examined for fish, crustaceans, and sessile organisms/egg collections.

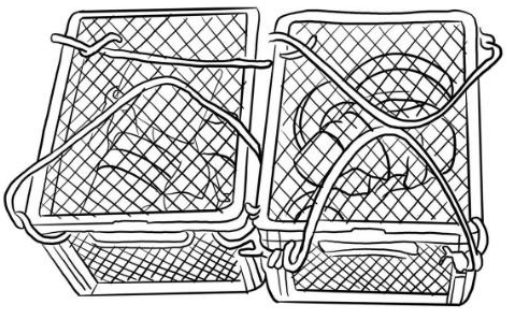

Figure 6. Two artificial habitats were used to gather species for the traditional method. Illustrator: Cecilia Casper.

Bioinformatic analyses, Metabarcoding.

In order to analyse the genetic data regarding water samples, plankton samples and arms settling panels, the dada2 pipeline was used in the software Rstudio version 4.2.2. Before analyses, the data had to be cleared from all unwanted primers. The specific primers used for the COI gene were "*GGWACWGGWTGAACWGTWTAYCCYCC*" for forward reads and *TANACYTCNGGRTGNCCRAARAAYCA*" for reverse reads. The primers presence and location were determined by using the function "*allOrients"*, then counted by the function "*primerHits"*. To remove the located primers in the data, Cutadapt (version 4.2) via Python (3.9.13) was used. Cutadapts function was to find and remove unwanted primers from the sequences. With Cutadapt, the data was "washed" of unwanted parts. When the primers were removed, both forward and reverse reads were plotted separately trough the commando *"plotQualityProfile*". This to be able to determine where the quality of the reads started to drop off in every sample. This information was necessary when deciding where the data should be filtered and trimmed. The filterAndTrim-function contained four steps, *trimLeft*=10 which removes 10 nucleotides per read, *truncLen=c(200, 140)* which truncate forward reads at 200, *maxN=0* filteres out all reads with more *maxN=0* indecent nucleotides. Lastly it filtered out all the reads that contained more than two expected errors. By using the *learnErrors* function, it calculated the parametric error model in both forward and reverse reads. Until this step, the forward and reverse reads had been treated separately, but where through the next step combined into complete sequences.

The function "*mergepairs"* was used to merge forward and reverse reads into full denoised sequences. Pairs which did not overlap or contained too many mismatches were rejected. The commando "*hist"* was used to make a histogram over the distribution of sequence length. When regarding the COI gene, sequences of 312-314 base pairs are desirable, why only sequences with this length were kept. Once the sequences have been merged together it was important to remove chimeras from the data. Chimeras are created during PCR amplification and form sequences where half the read consists of half a sample sequence and half from another sample. The removal of chimeras was done by the function "*removeBimeraDenovo".* The last step in dada2's pipeline produces a table where each row shows the sample names and each column shows "input", "filtered", "denoisedF", "denoisedR", "merged", "nonchim".

Assign taxonomy

Regarding COI, there is no reference database included in dada2, so a database must be created via BOLDigger software via Python first. This was then applied in dada2 in Rstudio. The table produced in

the step above was formatted into a file, which were then placed in the BOLDigger software. BOLDigger provided an excel file with all the taxonomy of the species found in each sample. A text file was then created with all amplicon sequence variant (ASV) counts. By converting the fasta file , created above, into a tabular file, a DNA-table was created. The ASV-counts, DNA-table and the species list from BOLDigger were then merged together. The end result created a submission with sequences, asv-counts, phylum, class, order, family, genus, species, sub species, similarity, status, process ID and sample name. The data in the submission file was then sorted so only the ASV-counts with higher similarity than 98% was kept. Irrelevant species were left out.

To pick out the native species, each submission was run against a list of native species from Artdatabanken. This was carried out in the software Rstudio. To extract the invasive and alien species, all submissions were run against one list from SWAM and another that included Artdatabanken, AquaNIS, SWAM and helOSP. This was done on the submissions from the water samples, plankton samples and ARMS settling panels. The procedure above was not performed on any of the traditional methods (panels, artificial habitats and scrapings) but was carried out by hand since the samples contained very few or no species.

Literature study

To answer the research question “What is the most common method used to collect genetic material in studies of invasive and alien species?” a literature study was conducted. The search strings used were “DNA AND invasive species AND marine AND sampling methods” which generated 156 articles in total on the search portal Web Of science.The 156 abstracts were read and irrelevant articles were sorted out. The articles listed as relevant contained studies that generally searched for invasive and alien species. Studies which focused only on one or a few species were sorted out. This was motivated whit that if a study look for one type of species, there is often a specific method to use. After sorting out irrelevant articles 20 remained. The articles were thoroughly read and by their used method. The different categories were “Water samples”, “Plankton samples”, “ARMS settling panels”, “Mixed methods” and “Other”.

Statistical analyses

Kruskal Wallis test

As the study contained only one replicate, no statistical testing was possible. Therefore, test data was created, to be enable statistical analyses, which is a criteria in the Master thesis course. The test data consisted of 10 replicates of each method: water samples, plankton samples, arms settlings panels, panels, artificial habitat and scrapings. The number of findings was estimated and created based on the numbers that replicate one contained. The methods ability to discover total number of findings were tested and the data was plotted in a histogram using Rstudio (version 4.2.2) to see if the data was normally distributed. The histogram showed that the data were non-parametric which led to the decision to conduct a non-parametric Kruskal Wallis test with post hoc in Rstudio (version 4.2.2).

Results

Invasive and alien species

The results regarding invasive and alien species concluded that there are big differences between the DNA-based method (water samples, plankton samples and ARMS settling panels) and the traditional method (eRAS) (panels, artificial habitats and scrapings) (figure 7). A total of ten invasive or alien species were found with the DNA-based method, while the traditional method (ERAS) found none (table 1). There were also an overlap of the findings between the different methods where water samples (four species) overlap plankton samples (five species) with one species, *Penilia avirostris* (figure 8).

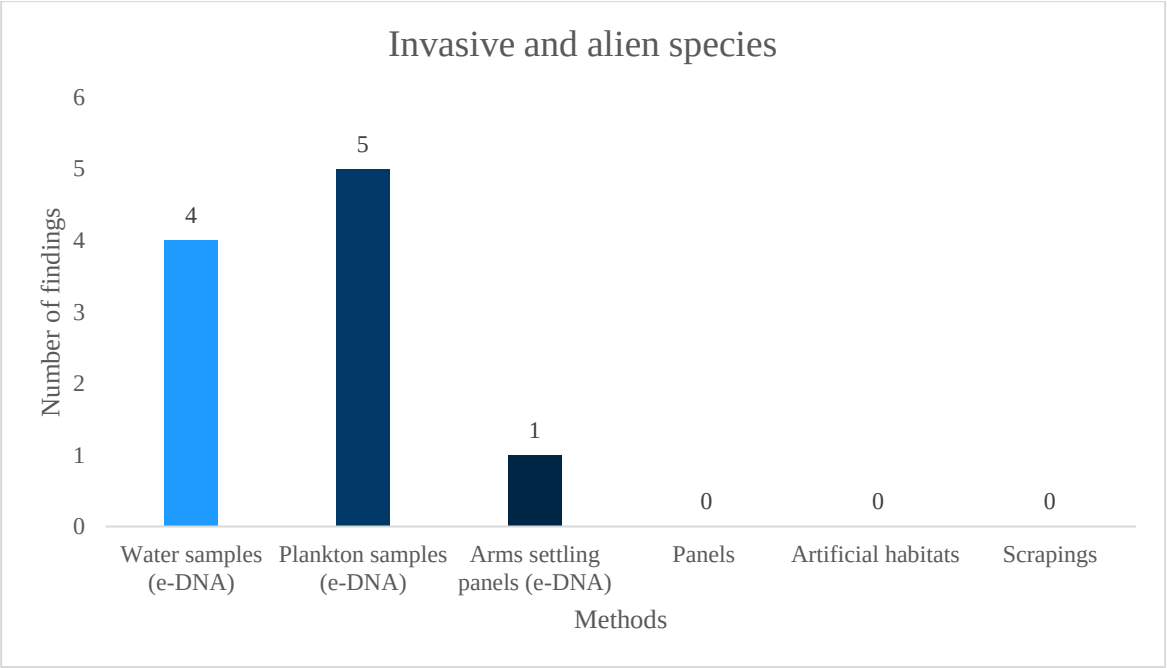

Figure 7. Number of findings of invasive and alien species in the DNA-method: water samples (4), plankton samples (5), Arms settling panels (1) and the traditional method (eRAS): panels (0), artificial habitats (0) and scrapings (0).

Species	Invasive or alien	Method	Risk of being invasive (SWAM)
Penilia avirostris (crustacean)	Alien	Water samples (e-DNA), Plankton samples	0 (not risk classified)
Fibrocapsa japonica (Brown algae)	Alien	Water samples (e-DNA)	5 (very high risk)
Alexandrium minutum (dinoflagellat)	Alien	Water samples (e-DNA)	4 (high risk)
Round goby, Neogobius melanostomus (fish)	Invasive	Water samples (e-DNA)	Invasive
Crepidula fornicate (sea snail)	Alien	Plankton samples (e-DNA)	4 (high risk)
Bonnemaisonia hamifera (red algae)	Invasive	Plankton samples (e-DNA)	Invasive

Neosiphonia harveyi (red algae)	Alien	Plankton samples (e-DNA)	5 (very high risk)
Mnemiopsis leidyi (cnidaria)	Alien	Plankton samples (e-DNA)	5 (very high risk)
Amphibalanus improvisus (Crustacean)	Alien	ARMS settling panels (e-DNA)	2 (low risk)

Table 1. The total amount of invasive and alien species found in the studied methods water samples, plankton samples and ARMS settling panels, (here sorted by method). Also included is the risk classification done by Swedish Agency for Marine and Water Management and Artdatabanken. The classification scale spans from 0 – not classified to 5 – very high risk of being invasive in Sweden.

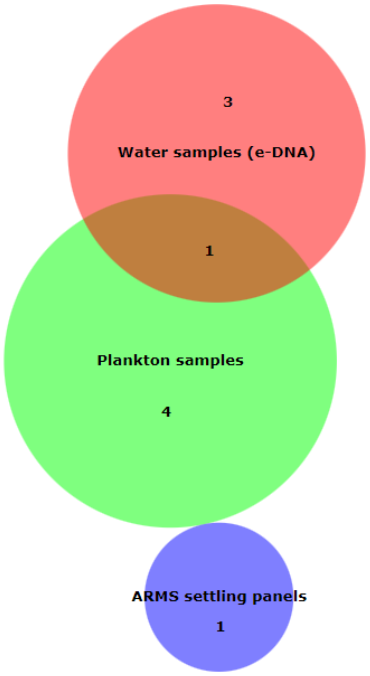

Figure 8. The results found ten invasive and alien species in total in the DNA-based method water samples, plankton samples and ARMS settling panels. The findings overlap between the different methods water samples (4), plankton samples (5) and ARMS settling panels (1). Water samples and plankton samples overlap with one species, Penilia avirostris.

Native species

Regarding findings of native species, it can be stated that the DNA-based method are superior to the traditional method (ERAS). The water samples (e-DNA) found 20 species, the plankton samples (e-DNA) found 52 species and the arms settling panels (e-DNA) found 32 species (figure 9). This compared to the

traditional method (ERAS) were panels found nine species, artificial habitat found five species and scrapings found three species. The results also show the total findings of unique species per method were water samples e-DNA has six unique species, plankton samples has 32 unique species, ARMS settling panels has 18 unique species and artificial habitats has two unique species (figure 9).

The results show that the different methods overlap in species findings and that all of the methods overlap another method in some way (figure 10).

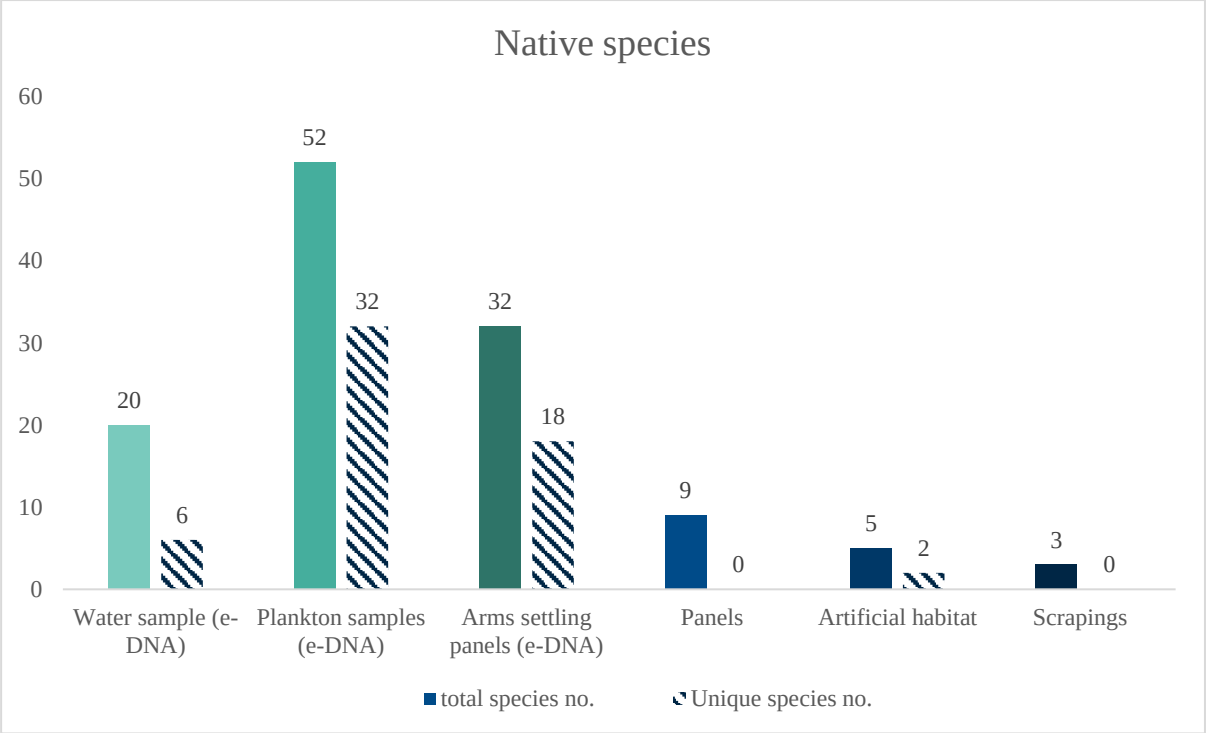

Figure 9. The green bars represent the findings in the DNA-based method: water samples (20), plankton samples (52) and ARMS settling panels (32). The blue bars represent the traditional method (eRAS) with panels (9), artificial habitat (5) and scrapings (3). The number over each bar represent the number of findings per method. The striped bars contain total findings of unique species per method, water samples (6 unique), plankton samples (32 unique), ARMS settling panels (18 unique) and Artificial habitats (2 unique).

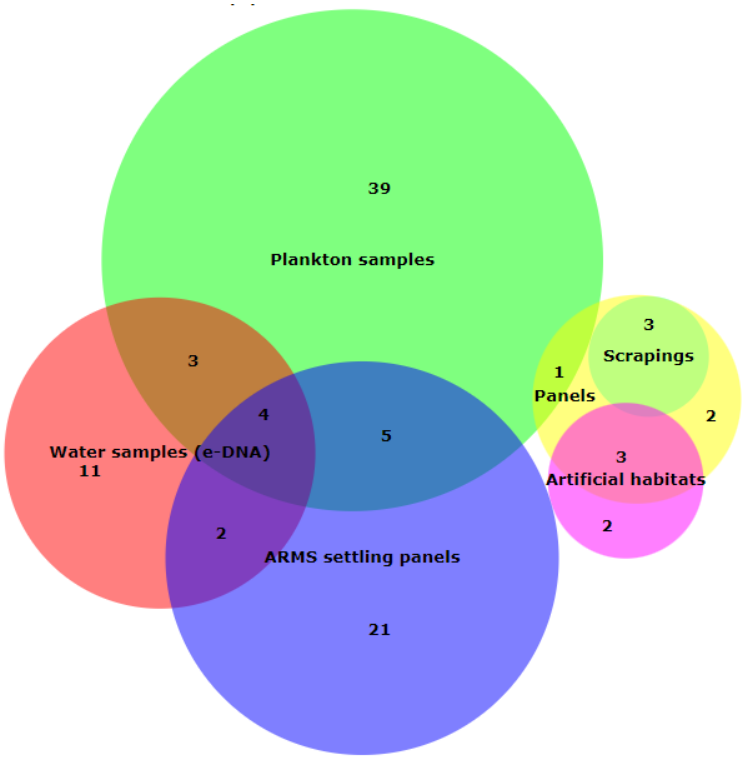

Figure 10. The overlap regarding total amount of species between the methods water samples, plankton samples, ARMS settling panels, panels, artificial habitats and scrapings.

Species in total

Regarding the total amount of species, the results shows that the DNA-based method (water samples, plankton samples and ARMS settling panels) are superior to the traditional method (panels, artificial habitats and scrapings) (figure 11).

Water samples found a total of 37 species, plankton samples found 81 species and arms settling panels found 46 species. While the traditional method (eRAS) panels only found 12, artificial habitats five and scrapings found three.

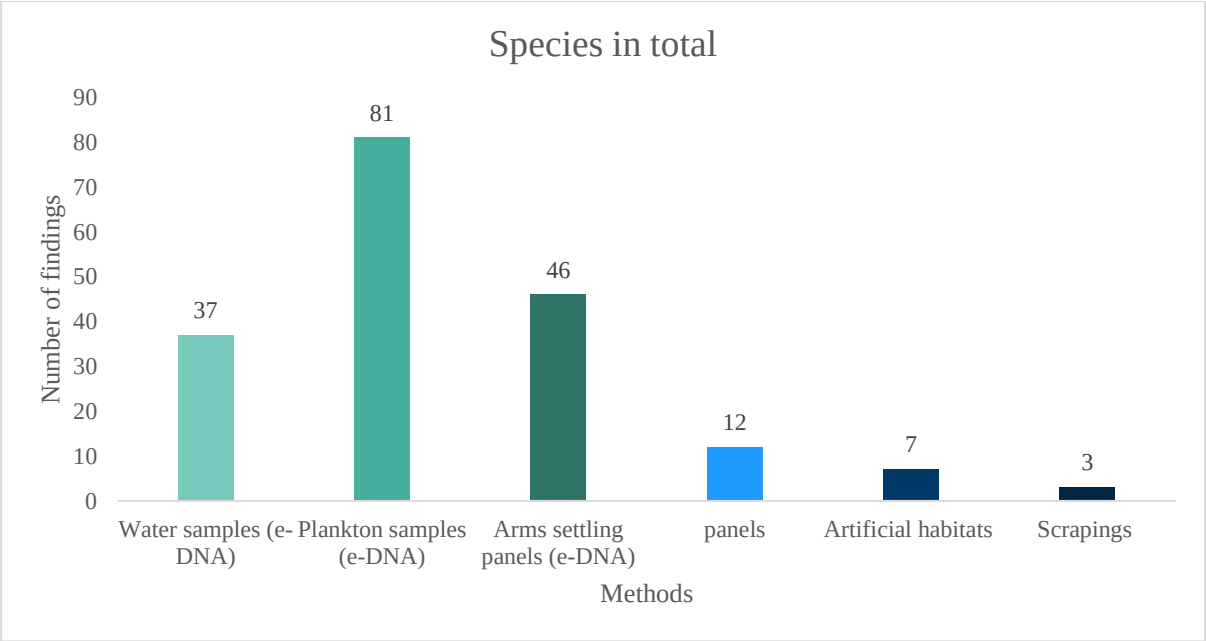

Figure 11. The green bars represent the total amount of species found in the DNA-based method: water samples (37), plankton samples (81) and ARMS settling panels (46). The blue bars contains the total amount of species found in the traditional method (eRAS): panel (12), artificial habitats (7) and scrapings (3). The number beside each bar represent the total amount of species for each method.

Literature study

The results show that water samples dominate with 12 articles and is the most commonly used method when gathering genetic material in studies looking for invasive and alien species (figure 12). Second is mixed methods, thirdly plankton samples and lastly ARMS settling panels, structures and other.

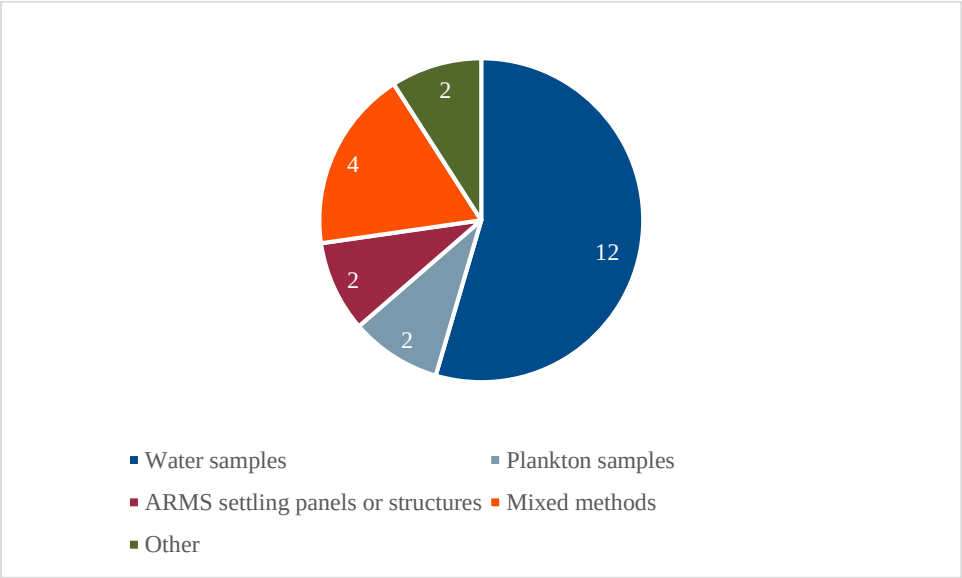

Figure 12. Water samples (12) is the most used method when gathering genetic material, followed by mixed methods (4), plankton samples (2), Arms settling panels and structures (2) and other (2).

Stastistical analyses

Kruskal Wallis test with post hoc

Kruskal Wallis test with post hoc showed that there was a significant difference ($p < 0.001$) between the DNA-based method and traditional method (eRAS).The largest differences were between water samples – scrapings ($p < 0.001$), arms settling panels – scrapings ($p < 0.001$), artificial habitats – plankton samples ($p < 0.001$), plankton samples – scrapings ($p < 0.001$), arms settling panels – artificial habitats ($p < 0.001$) and panels – plankton samples ($p < 0.001$) (figure 13).

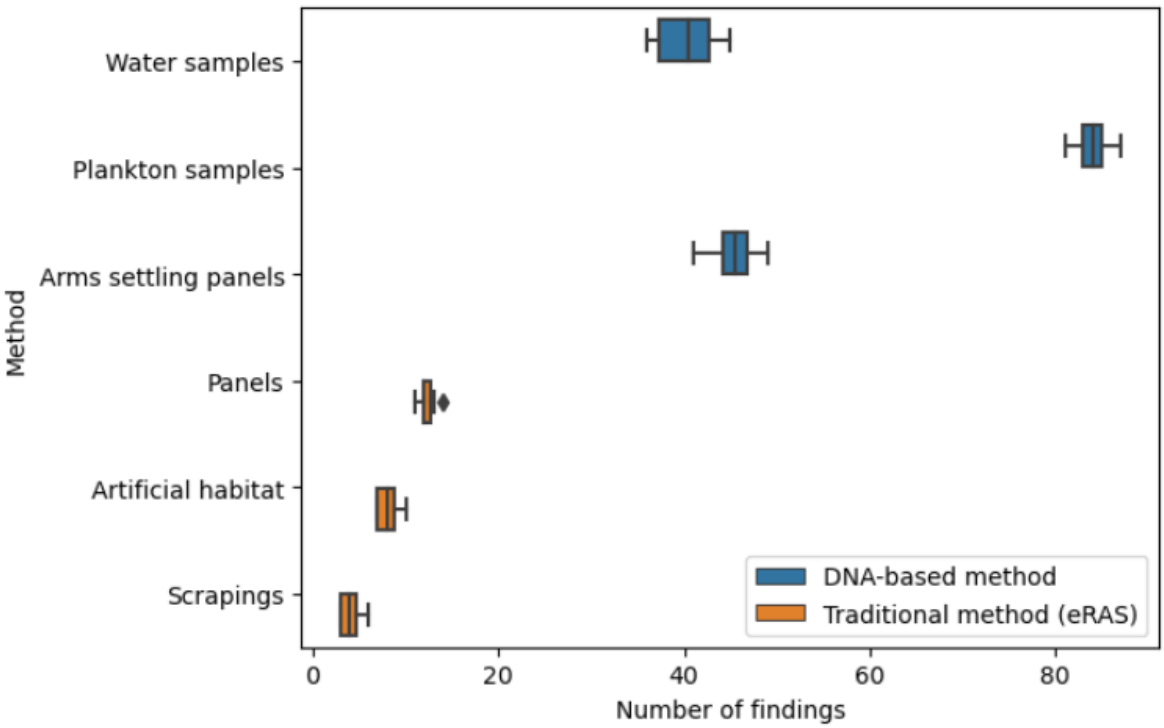

Figure 13. The different sampling methods and the findings of species for each sampling method, were the three highest median was found within the DNA-based method. Water samples, MD=40.50), plankton samples, MD=84.00), Arms settling panels, MD=45.50), panels, MD=12.00), artificial habitats, MD=8.00) and scrapings, MD=4.00).

Discussion

The main results in this study shows that the DNA-based method are superior to the traditional method (eRAS), when it comes to tracking and identifying alien and invasive species in harbours. The DNA-based method found ten invasive and alien species all together while the traditional method (eRAS) found none. With this result the study could answer the research question "Which method is best to track and determine potentially alien or invasive species in harbours?" by conducting that the DNA-based method are the best choice when looking for alien or invasive species in harbours. This result corresponds well with the result of the pilot study from Sundberg et al., (2018) on which this study is based. The result from the pilot study showed that the DNA-based method which they used, were better at tracking and identifying alien and invasive species, than the traditional method (eRAS). Since the results showed DNA-based method to be very superior to eRAS the study decided to conduct a literature study to see which DNA-based method sampling method that was the most commonly used when gathering genetic material. The result showed that water samples are the most common method when gathering genetic material in studies that look for alien and invasive species in general. Out of 20 articles, 12 studies used water samples.

The DNA-based method is better suited when tracking and identifying alien and invasive species. The different sampling methods yield different amounts of species, but also different types of species, which allows a conclusion to be drawn that the DNA-based sampling methods should be used together to complement each other. The method that generated the greatest number of species, both regarding alien and invasive species, total number of species but also native species, is plankton samples. Normally each arms settling panel is run separately, but in this study, all of the arms settlings panels were combined due to cost zeal.This could be the reason why arms settling panels in this study had such a low number of invasive and alien species. When the arms settling panels are combined there is a great risk that larger specimens overshadow smaller pieces of DNA making the smaller pieces less visible and is therefore missed. If the arms settling panels were run separately, more species could been expected to be found, using this method. With this in mind, it is not certain that arms settling panels would bring in more finds than plankton samples did. This study might go as far as to say that plankton samples would be the method of choice if only one had to be used. The method is easy to execute, it brings up large volumes of water and can capture both larvae and fry, which makes it possible to detect alien or invasive species in their young life stages.

The results in the literature study showed that water samples is the most commonly used method when gathering genetic material in these types of studies. If this result is compared to the result in this study, it's easy to see that if this study had only used water sample, four alien and invasive species would have been missed. This also clarifies that the methods should be used together as a complement.

The results regarding the statistical analyses are not relevant for this discussion since they were executed on test data which were produced solely so that the study could carry out some form of statistical analysis. As the study lacked sufficient replicates to be able to perform statistical analyses, a decision was made to use test data only to achieve the criteria on statistical analyses included in the Masters course in which this study is included.

An Kruskal Wallis with post hoc were performed and the results showed that there was a significant difference between groups, which can conclude that the methods differ between each other in total number of findings. When looking at the results from the post hoc test it can be observed that within the DNA-based method there were no difference, nor were there differences between the traditional method (eRAS). However when comparing the DNA-based methods and the traditional methods they differ in mean findings, which can conclude that DNA-based and the traditional methods (eRAS) differ in total number of findings. This support the rest of the results in this study, which states that the DNA-based methods are better suited.

The national monitoring program requires that native species should be reported with these types of studies, why native species are included in the result. A total of 58 native species were found within all of the methods (DNA-based and traditional methods combined). The largest amount was recovered in the plankton samples with 32 unique species, then 18 unique species on the arms settling panels, 6 in water samples and 2 in the artificial habitats.These results can also strengthen the other results and the conclusion that the DNA-based method recover more species than the traditional one (eRAS) with plankton samples having the most findings of species. The results are also pleasing in that sense that so many native species was found in one of Sweden's largest industrial harbours which is trafficked with heavy boat traffic daily and where there is major disturbances in form of noise pollution, pollution and other disturbances (Ahmed, 2022) (Erbe et a., 2022). A great diversity of native species is beneficial since it creates higher resilience against disturbances like invasive species. If the ecosystem has a high resilience it is more likely that it can withstand or recover from attacks from invasive species (SSNC, 2023). Therefore it's reasonable to want to keep track of how the native species composition looks and which method that covers it best.

To discover species that occurs in only a few individuals is important since this can help to detect alien and invasive species in time. The traditional method (eRAS) has difficulties discovering species in small numbers, partly as it requires actual findings, but also requires knowledge of the species` different life stages for identification. Most of the taxonomists are specialized in a couple of species or one group of organisms, which makes it hard to cover everything that show up in the samples (Brown et al., 2016). Since the DNA-based method are more sensitive and only need a part of the species genome, it increases the probability that the method will encounter unusual or rare species that occur in low numbers (Jerde et al., 2011).

The DNA-based method found ten alien species, were two are classified as invasive, Round Goby(*Neogobius melanostomus)* and the red algae *Bonnemaisonia hamifera.* Round goby (*Neogobius melanostomus*) originates from the Caspian Sea and the Black sea. It is known for being very aggressive and territorial which out-compete many native species. The fish feeds on fish roe, which causes harm to other fish species, it also eats mussels, which negatively affect birds e.g. eiders (Artdatabanken, n.d).That the species are found in our Swedish waters is of great concern, since the species affects so many other species, both in marine and terrestrial environments. The other invasive species found in the study was *Bonnemaisonia hamifera,* a red algae that spreads through tiny bits of fragment and originates from Japan. The species survive and establish quickly because it lacks natural enemies. The species has a chemical composition which makes it uneatable for herbivores (Enge et al., 2012). In this case it is also great that our methods detects the species, so that possible action work can be started. The eight other species that were found in the methods are not assessed as invasive yet, but according to the classification by Swedish Agency for Marine and Water Management and Artdatabanken, three of the species are still judged to be at the top of the classification with a very high risk (5) of becoming invasive in Swedish waters. Two of the species are assessed to have a high risk (4) of spreading and becoming invasive. The different species found in the DNA-based method belong to crustacean, brown algae's, red algae's, sea snails, fish and dinoflagellates. This variation shows how important it is to use a combination of the DNA-based sampling methods since all of these species were found in different methods, with plankton and water samples accounting for the most findings.

The DNA-based method and metabarcoding are not perfect but has some disadvantages such as false positives and false negatives. False positives can for example be DNA from terrestrial animals that somehow ends up in the water and show up in the results. Or it can be DNA that were brought to the site by ships or other, which causes a positive hit in the results, but actually the species does not appear in the water (Ficetola et al., 2016). This can cause really big problems since they show something that isn't

present in the sampling area. To deal with this problem, some studies remove detections that have low read counts. This however, could lead to the removal of perfectly usable data, which also affects the outcome of the sampling negatively. To remove low reads can affect findings of rare species in the samples, which is not preferred when looking for new invasive or alien species (Drake et al., 2020). How to best deal with false positives are still under discussion and should be decided from case to case. False negatives are when a taxon is not identified even though it is present in the sampling area or in the sample. To avoid this, it is advocated to perform multiple replicates of sampling in the same area over a period of time but also to do the amplification process of the same sample multiple times (Ficetola et al., 2015). Another problem is that many Linnaean names covers many different species, i.e. cryptic species. This means that many different species can be hidden under the same Linnaean names, which can cause problems when identifying (Wei et al., 2022).

The main idea with metabarcoding is that the DNA sequences are matched to a database with lists of species. These lists could contain alien and invasive species for example. There are a handful of databases used today, but one major problem with these databases is that they are not complete. The species in the databases are known and identified, which means that species that are unknown and unidentified does not appear in the database. If you have an unknown species in your sample, this will not appear in the results which is problematic, especially if the species tends to become invasive. Even though the DNA- based method comes with some disadvantages it is still a powerful technique that never harms habitats, ecosystems or species. It can detect really rare or endangered species without having to capture them, it can be used both in terrestrial or aquatic environments.The technique is fairly easy to perform and can be thought to almost anyone, why we can conclude that the technique can be a great tool for future conservation work (Sahu et al., 2020).

When considering the strengths of the study, one of the major ones is that it is relatively easy to implement and the instructions regarding the bioinformatical steps (metabarcoding) could be thought to anyone with some computer knowledge. As well as that, despite its small size, the study still contributes with valuable knowledge base that is of great importance for future management work. To place the study in a harbour increases the knowledge about alien and invasive species, since the harbours are often hot spots and starting point for alien and invasive species (Bergkvist et al., 2017). Furthermore, the relevance of comparing the different methods can be questioned. To compare advanced DNA techniques with methods that use only visual identification may be perceived as unfair. The methods differ greatly, water samples for example contributes with a snapshot of what is present in the water right when the sample is taken. This means, for example, that a large load of alien species or DNA may have come in from a ship that is

then captured in the water sample. The alien species may not establish but they still end up in the samples and results, and therefore contributes with inaccurate information. ARMS settling panels can provide a fairer picture of the species composition since they hang out longer during the year which gives many different species a chance colonize them.While the traditional method (eRAS) completely depends on what is found in the samples and which taxonomist that identify and species them.

A future study would therefore exclude the traditional method (eRAS), and scale up a bigger study regarding the DNA-based method, were they were tested on many different places with multiple replicates. It would be interesting to see how, and if the results would differ from this study. A future study could also include sediment samples, since sediment can hold a great deal of DNA:s. A conclusion can state that even though DNA-based and traditional methods should not be compared, it still gives a general view of what happens if you use these methods in the same place and for the same purpose.

This study is especially important since Sweden is obligated to monitor alien and invasive species in our waters. The knowledge from this study can bring clarity in how to best do this. The earlier alien or invasive species are discovered, the faster measures can be taken against them. The knowledge from this study can therefore show which methods that will detect the species best, and thereby which ones that should be chosen when working with monitoring of these species in the future. The results established in this report also show that the traditional methods (eRAS) used today do not meet the standards when it comes to monitoring alien and invasive species. If this study only would have used the traditional methods (eRAS) in Wallhamn, it would have missed ten alien and invasive species that are present in the harbour. The Swedish Agency for marine and Water management asked SeAnalytics AB to carry out this study, so that the result and knowledge could help in making a decision regarding with identification method to use in the future.

To summarize the report and the study, the results show that the DNA-based technique are superior to the traditional method (eRAS). Today, the traditional method (eRAS) are used in the Swedish monitoring program and this study suggests that these should be replaced with a DNA-based method in future monitoring of alien and invasive species in Swedish waters.

Acknowledgements

Firstly I want to thank my supervisors Mattias Obst and Per Sundberg for great guidance during this thesis. I have learn so much and this project has opened up a whole new field for me, so thank you for having me on this great project. To my examiner Karin Hårding for input and feedback. Fanny Berglund and Justine Pagnier for making me look like a professional regarding the bioinformatical steps in Rstudio. I wouldn't have gotten past the first line of code without you guys. My husband Nils Odebo Länk for putting up with my bad mood, stress and sometimes stupid questions. Lastly, Malin Jonsson and Nathalie Edwards for your unconditional support throughout this thesis, but also over the last two years as we have struggled and laughed our way through our masters. I would never have been able to do this without you and I am sure I have found friends for life.

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Appendix 1

How do we best find and determine uninvited guests in our marine environments?

It's great to go out with the boat, preferably to some tropical waters were the marine wildlife is teeming. Boat life in all its glory, but did you know that it's very easy to bring uninvited guests home with you? In the last 150 years, peoples global boat travels have increased drastically and with this means that unwelcomed species are spread with these vehicles. The uninvited guests can catch a ride in the ballast water of large ships, as growth of the hull or why not among your fishing tools?

Alien vs invasive species

If the uninvited guests end up in the wrong environments, this can cause very big consequences to the species that already exists on the site. You may have heard of alien and invasive species? But what exactly is the difference? An alien species is a species that have been brought to an unknown environment by humans. If the species establish but doesn't cause any harm to the surrounding species, the species is called alien. If the species should establish and then start to spread heavily and cause damage to other species, the ecosystem or cause economical or health-threatening damage, it is called invasive.

In Sweden we have an obligation that we have to monitor alien and invasive species in our waters and we have different methods to do this. With this study, we wanted to know which one of these methods are more suited when tracking and determine alien and invasive species in the harbour Wallhamn at Tjörn.

Which methods are used today?

In the monitoring program today, they use traditional methods called eRAS to determine alien and invasive species. The eRAS methods contain for example different substrates that are lowered in the water and then species are allowed to grow on them. They can also contain divers or artificial habitats that are placed at different spots in the area. When the substrates or artificial habitats are brought up a taxonomist can visually identify and determine which species that are present.

In the opposite ring corner we have the DNA-based methods which have become a great help in the identifying – and determining process. The DNA technique is based on collecting genetic material, DNA, from for example water samples, plankton samples or settling panels. The DNA that are found in the samples are the sequenced and can then be fed into a computer program that generates a list of the species found in each method. Very science fiction, isn't it?
So what was the research question then, I promise that you are wondering now? Yes of course, the research question was stated "Which method is more suited when tracking and determine alien and invasive species in harbours?"
To test this we placed panels, artificial habitats and did some scrapings on piers for the traditional methods (eRAS). For the DNA-based methods we took water samples, plankton samples and lowered settling panels into the water.

What did we find?

The tension was unbearable and the results showed that the DNA-based methods found eight alien species and two invasive species, while the traditional methods (eRAS) found none. One of the alien species that were found was the Round goby (*Neogobius melanostomus*) which is a fish species that out-compete the native flora and fauna, by becoming very aggressive and territorial. It lives of fish roes and mussels which causes massive problems for native fish species, but also for mussel-eating birds. In

other words, this little rascal causes troubles on many fronts with its presence and is most unwelcome.

Round goby was one of the invasive species that the DNA- methods found in our samples.

The results in this study is hereby crystal clear, the method that is most suited to track and determine alien and invasive species in harbours is the DNA-based methods. With these clear results we can draw the conclusion that the traditional methods (eRAS) should be replaced in the monitoring program with the DNA-based methods in order to have a shoot against the alien and invasive species in the future.

Source: https://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=713

Appendix 2

Articles from literature study

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