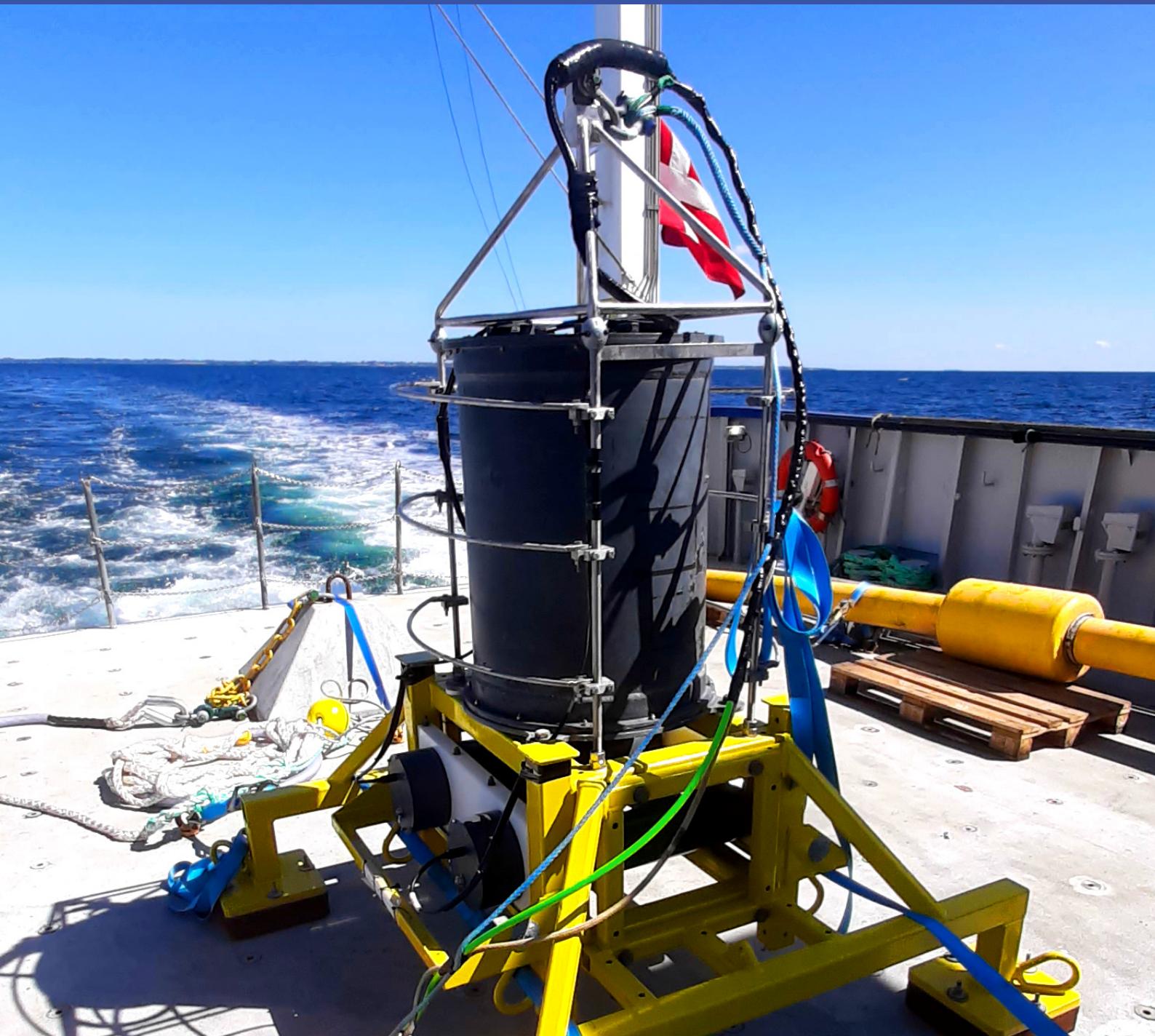


Monitoring offshore biodiversity through the collection of eDNA using an autonomous ecogenomic sensor

Manuel Arcieri

Master's thesis



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Approval

This thesis was prepared over six months in collaboration with the National Institute of Aquatic Resources, at the Technical University of Denmark, in partial fulfilment of the MSc Eng. in Bioinformatics and Systems Biology.

It is assumed that the reader has a basic knowledge of statistics and fish ecology.

Abstract

Marine biodiversity is undergoing rapid changes due to global warming and human activities, which are leading to unprecedented extinction rates among fish. Despite the seriousness of these changes, there is limited research documenting them, making it challenging to assess marine ecosystems accurately. The impact of human activities on marine life is extensive and has significant consequences. Global factors, such as rising temperatures and increased ocean acidity, worsen the situation.

Monitoring marine ecosystems is often costly and labour-intensive, and it typically focuses more on larger species. Traditional methods, such as trawling and video capture, can be invasive or require skilled personnel. Therefore, there is a crucial need for non-destructive and efficient biomonitoring techniques to support effective conservation efforts.

To address some of these challenges, the National Institute of Aquatic Resources developed a second-generation Environmental Sample Processor. This portable DNA laboratory can be deployed at sea to automatically collect and analyse marine environmental DNA samples. By combining these two techniques, researchers can assess local biodiversity in a cost-effective and non-invasive way. The instrument operates without human intervention, allowing for the investigation of variations over entire weeks or months. DTU Aqua conducted three campaigns to test this machine in Lysekil, Horsens Fjord, and North Sea.

Valuable data was collected on the presence and composition of bony fishes, and it was also possible to detect traces of elusive marine mammals like the Harbour porpoise. The horizontal, vertical, and temporal components of sampling significantly impact the outcomes of experiments. Additionally, sea currents were found to be an important factor influencing eDNA concentration. However, it was not possible to detect traces of Elasmobranchs during any of the expeditions, which hints at the potential drawbacks of this technology.

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May this be the first step toward a bright and peaceful future.

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1 Introduction

Marine biodiversity is undergoing significant changes due to various factors, including global warming and human activities [1]. These transformations are affecting the overall composition and abundance of species within marine ecosystems. Continuous monitoring and documentation of these shifts in biodiversity are essential for addressing the challenges and evaluating the effectiveness of initiatives aimed at preserving or restoring marine environments.

Current extinction rates for marine species are higher than they have ever been [2], and yet most of these changes are still undocumented due to limited research attention compared to terrestrial species and technical difficulties to assess the condition of marine ecosystems, especially in oceans.

The intensification of human activities is one of the factors driving this decline in biodiversity. As an example, less than 1% of the entire Mediterranean Sea is estimated to be unaffected by human impact, and over 20% of its surface is highly impacted by these changes [3]. Global effects include the rise of temperature and acidity.

Monitoring offshore marine ecosystems is challenging. The cost efficiency and labour efforts play a key role in implementing biomonitoring methods [4]. Ideally, they should provide a comprehensive assessment of local biodiversity in a non-destructive, minimally invasive manner. Traditional techniques may involve diver-operated video, bottom trawling, fishing, acoustic methods, or baited remote underwater video capture [5, 6, 7]. These methods are expensive, labour-intensive, and may detect a limited number of species compared to the whole ecosystem, with a bias for larger and more common animals. Experienced personnel are also needed to visually identify species.

1.1 Environmental DNA

Sampling and analysis of environmental DNA (eDNA) is a novel and increasingly popular monitoring technique to assess the biodiversity of an ecosystem [8]. Here, eDNA is defined as the collection of genetic material that is shed into the environment by animals and plants. It can be extracted from faeces, urine, mucous, skin cells, hair, sperm, eggs, and degraded organisms, providing insights into the composition of local communities. Common sources of eDNA are soil, cave sediments, permafrost, and sea, river, or lake water. Aquatic eDNA samples are normally collected by passing water through a micro-pore filter that captures cells, particles with bound DNA, and free DNA. The filter is then brought into a laboratory where the captured DNA is extracted and examined using different methods that allow species designation based on DNA-based analysis.

One such method is quantitative polymerase chain reaction (qPCR): it generates billions of copies of DNA segments that are targeted and amplified as needed using a pair of primers, a probe, a DNA polymerase enzyme, and nucleotides [9]. Heat cycles are repeated until the product reaches a predetermined DNA concentration. The choice of primers will determine which genetic regions are amplified. Species-specific primers target a single species, while universal primers amplify regions belonging to a broader taxonomic group [8]. Primers are picked based on the scope of the study. Compared to the traditional method, quantitative PCR can also estimate the precise number of DNA copies in real-time. This is accomplished thanks to the binding of fluorescent molecules (probes) to the amplified DNA segments. A standard curve is also required to calibrate the analysis,

enabling the estimation of DNA copies and allowing the calculation of the eDNA concentration in the original water samples.

When using universal primers, qPCR can be paired with so-called metabarcoding. This technique uses taxon-specific PCR targeting DNA from broader taxonomic groups (e.g., bony fishes, elasmobranchs, marine mammals) to concentrate DNA from those species. After PCR, the amplicons (sequences) are sequenced using next-generation sequencing (NGS) platforms to generate thousands or even millions of reads per samples. Sequence targets can be compared to a reference database, enabling the taxonomic identification of species, genera, or families. The species abundance can be used to derive statistics on the biodiversity of an ecosystem.

eDNA is a powerful tool for the monitoring and surveillance of oceans, lakes, and other environments. It is often more efficient than alternative methods, less error-prone, non-invasive, non-destructive, and easier to standardise [10]. Thanks to the constantly reducing cost of DNA sequencing, it is also becoming cheaper and more accessible [4].

Metabarcoding of eDNA samples offers a higher-resolution snapshot of the biodiversity of an ecosystem, including smaller or more elusive species that are hard to observe using traditional visual methods. However, eDNA assays may sometimes be challenging to design due to the need to find a good compromise between the sequence length and its number of polymorphic sites [11]. The targeting of short primers (< 200 nucleotides) may be insufficient to distinguish between the DNA sequences of two closely related species, resulting in ambiguous taxonomic assignments [12]. Building a good reference dataset for taxonomic assignment may also be challenging. Using longer amplicons may increase the overall accuracy. In the best-case scenario, whole genome sequencing data could be used to identify species with a negligible error rate. However, environmental data usually contains such a small amount of biological content that it would be close to impossible to reassemble the genome of an individual, as bacterial and algae DNA would dominate the results.

Additionally, degradation imposes hard limits on the lifespan of free DNA particles. The spatial and vertical dimensions of sample extraction also play a key role in interpreting eDNA results. Environmental factors like sea currents, salinity, and temperature may influence data interpretation. For example, it has been shown that stratification can prevent the mixing of the water column and lead to differences in eDNA signals between surface and bottom water in lakes and marine waters [13, 14]. In general, marine eDNA particles are expected to degrade within days, allowing eDNA transport across seascapes. However, eDNA is diluted from its source, which likely explains why several recent marine eDNA studies have detected biodiversity differences even at small geographical scales. Environmental DNA also has several other challenges, and some are particularly relevant for offshore marine biodiversity monitoring. Here, one of the main challenges of using eDNA for offshore marine monitoring is the use of dedicated vessel time, which increases operational costs and reduces the number of samples which can be collected and analysed. However, this problem may be solved using autonomous eDNA samplers that can be deployed at sea and collect tens or even hundreds of samples over several weeks or months [15]. Still, few studies have investigated the general potential for using such devices to collect and analyse complex marine biodiversity data.

1.2 Metabarcoding

Species-specific primers may not be available for all taxa, or their specificity might be insufficient to distinguish closely related species. Moreover, it requires *a priori* knowl-

edge of our target group, but sometimes we lack this information or prefer not to set strict taxonomic bounds for the analysis.

The metabarcoding of environmental DNA is a novel technique that allows us to perform taxonomic inference of arbitrary genetic samples. Less specific primers are used to amplify DNA regions common to all organisms belonging to a certain class, order, or phylum. Short strings of nucleotides, called barcodes, are added at both strands of the amplified DNA segments to uniquely tag them and identify their collection area and other information during downstream analysis. The samples are then sequenced and can be studied with generic or task-specific bioinformatic tools for genomic data.

This pipeline has the advantage of being able to identify hundreds or even thousands of different species all from the same data [8]. DNA sequencing and analysis are progressively cheaper to perform and more standardised, reducing the cost and effort to assess the biodiversity of an ecosystem.

For eDNA analysis, we are looking for short genomic regions that maximise the chances of being assigned to the right species. 2nd generation shotgun sequencing machines may also impose hard limits on the length of these regions: most of them can read strands up to 300 nucleotide-long, but the quality may degrade starting from around 150-200 base pairs.

Among the most common targeted loci, we can find the 12S, 16S, and 18S ribosomal DNA regions, and the Cytochrome b and c mitochondrial genes (especially COX1). These regions are highly conserved within species yet show high variation between species, making them a great choice for metabarcoding.

1.3 Environmental Sample Processor

To tackle some of the issues linked with eDNA collection, the National Institute of Aquatic Resources designed and implemented a second-generation Environmental Sample Processor (2-G ESP) [15]. It is an unmanned electromechanical fluidic system acting as a mobile DNA laboratory.

The ESP is powered by batteries and can operate independently for up to two months, automatically collecting environmental samples. The most prominent feature is its ability to collect and store eDNA samples (or analyse previous samples in real-time) using species-specific quantitative PCR (qPCR) [15]. In total, the ESP can extract and hold up to 132 filters (archived samples) or collect and analyse up to 66 filters (in-situ samples). Mixing these two types of filter processing is also possible. After deployment, the sampled DNA material can be retrieved and analysed using qPCR or metabarcoding methods. During deployment, the ESP can be connected to a tele-buoy using either the GSM or satellite network, allowing scientists to control and operate the instrument from land, as well as to download and analyse qPCR results in real time. The instrument can be deployed in several ways: i) at the docking site using an external pump; ii) directly at the seabed using a bottom lander module; or iii) pelagically using a buoyant foam module surrounding the instrument. Standard moorings allow for deployment at a depth of 10-20 metres underwater. However, deep-water deployments can also be conducted using specialized deep-sea moorings [16]. In addition to eDNA sampling, the ESP can also be equipped with advanced sensors and analytical tools. As such, the ESP can measure parameters such as temperature, salinity, pH, chlorophyll concentration, and nutrient levels [16].

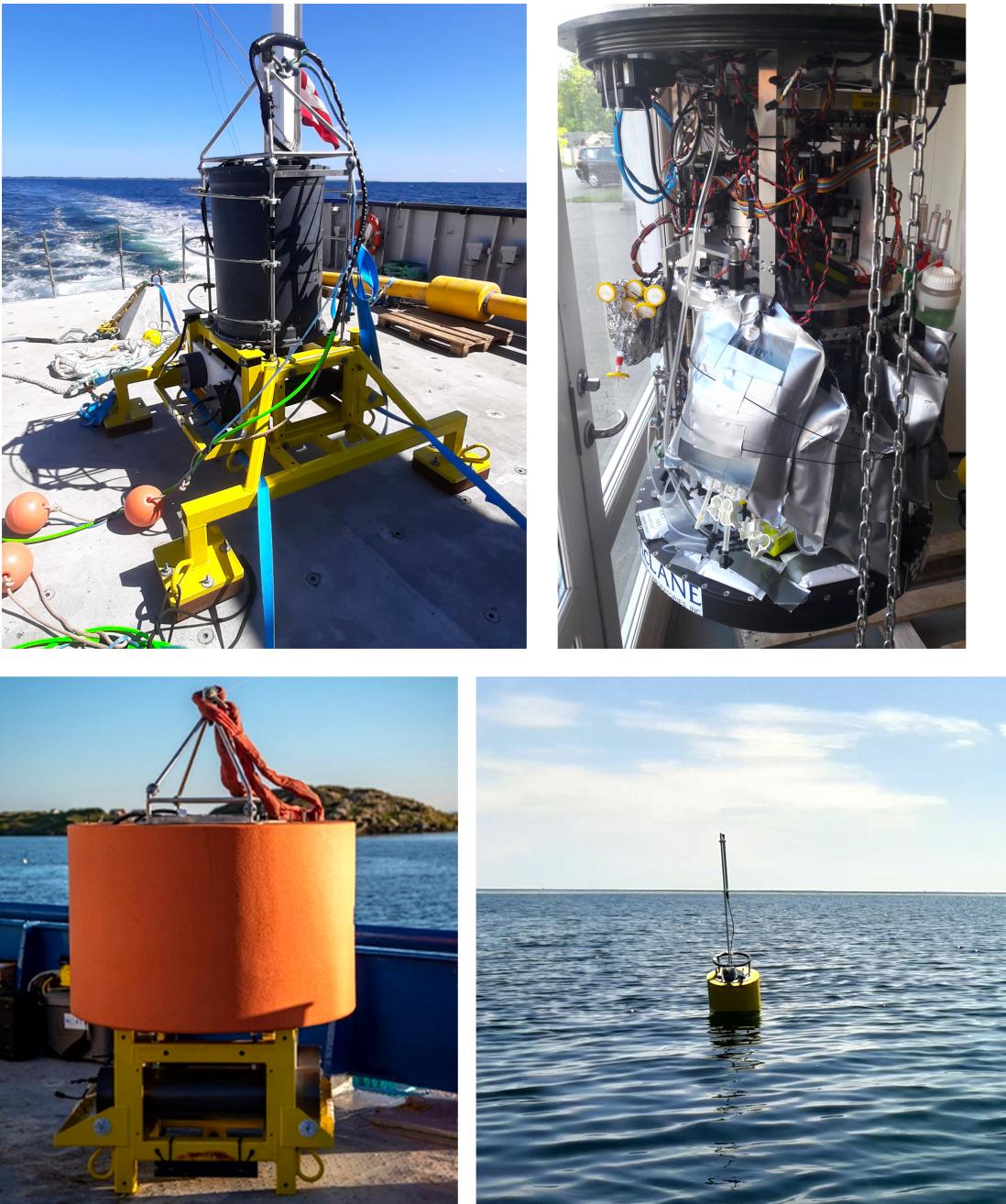


Figure 1.1: A collection of pictures of the ESP taken during separate campaigns, both in the lab and at sea.

1.4 Research questions for this project

This study aimed to investigate the potential for using the Environmental Sample Processor (ESP) to collect offshore eDNA data for biodiversity monitoring. The project focused on data collected from three sampling campaigns conducted in 2018, 2019, and 2020 in Skagerrak, the North Sea and Kattegat. The collected eDNA samples were analysed using both qPCR and metabarcoding methods, which allowed testing different questions about future deployment strategies. In this regard, qPCR targeted a few species of high relevance for the individual deployments, while metabarcoding enabled analyses for fish, but also rarer species of elasmobranchs and marine mammals.

Overall, this study aimed at: i) investigating the general use of the ESP to monitor biodiversity, focused on fish, as well as rare species like elasmobranchs and marine mammals. Specifically, this was done through both qPCR and metabarcoding approaches; ii) explore the importance of sampling depth for eDNA observations in Danish waters by comparing observations from different deployments and manual samples collected at the surface vs. few meters over the seabed; iii) test the importance of temporal samples to cover full biodiversity at a fixed location. This was done by testing eDNA observations through time to understand day-to-day variation; iv) investigating the importance of external factors like current velocity and water salinity on eDNA observations.

1.5 Deployment campaigns

The ESP was deployed between 2018 and 2020 at three separate locations to collect eDNA data on the local biodiversity. Filters (25 mm, 0.22 µm) were collected at a regular rate during a period ranging from 4 to 21 days.

Location	Period	# of days	Species	# of samples
Lysekil	October 2018	21	Atlantic mackerel, Atlantic tuna, Garfish	16
North Sea	September 2019	4	Atlantic mackerel, Harbour porpoise, Whiting	4
Horsens Fjord	May 2020	15	Comb jellies, Pacific oyster, Rainbow trout	21 + 6 (manual)

Table 1.1: Summary of the three expeditions covered by this project.

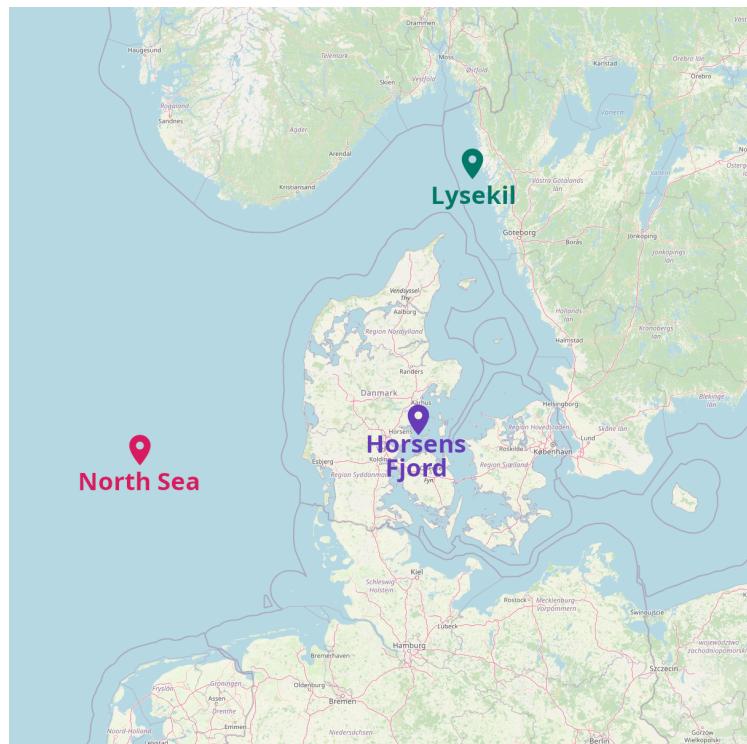


Figure 1.2: Overview of the location of the three campaigns.

The first ESP deployment originally focused on tracking migratory and prey species in East Skagerrak, 15 km west of the town of Lysekil. The deployment focused on important migratory species with special focus on the Atlantic bluefin tuna (*Thunnus thynnus*), and prey species of Atlantic mackerel (*Scomber scombrus*) and garfish (*Belone belone*). The deployment was conducted between the 1st and 21st of October 2018 using a bottom lander deployed at a depth of around 13 metres on an underwater plateau surrounded by 40-50 metres-deep water.

The second expedition focused on the monitoring of rare marine species in the North Sea. The device was deployed 200 meters from TotalEnergies's Dan F oil platform, around 200 km west of Esbjerg, as Harbour porpoises gather around this area in summer and autumn. In addition to Harbour porpoise, the deployment focused on its potential prey species of Atlantic mackerel (*Scomber scombrus*) and Whiting (*Merlangius merlangus*). As the depth at the site was too deep for seabed deployment (43 meters), the ESP was deployed pelagically at approximately 15 metres of water. Due to technical problems, the ESP stopped working after four days.

The last deployment took place south of Horsens Fjord, Kattegat, in the As Vig bay, and it targeted invasive species. There were several Rainbow trout farms in the area. The deployment was carried out between the 5th and 28th of May. The ESP was deployed directly on the seabed at a depth of 13 metres. The site was chosen based on the presence of several Rainbow trout (*Oncorhynchus mykiss*) fish farms in the area, as well as possible presence of Pacific oyster (*Magallana gigas*) and the Warty Comb jelly (*Mnemiopsis leidyi*), which are also considered invasive species in Denmark. The deployment site was located roughly 700 meters south of a rainbow trout fish farm (Hundshage, see Figure 1.3).

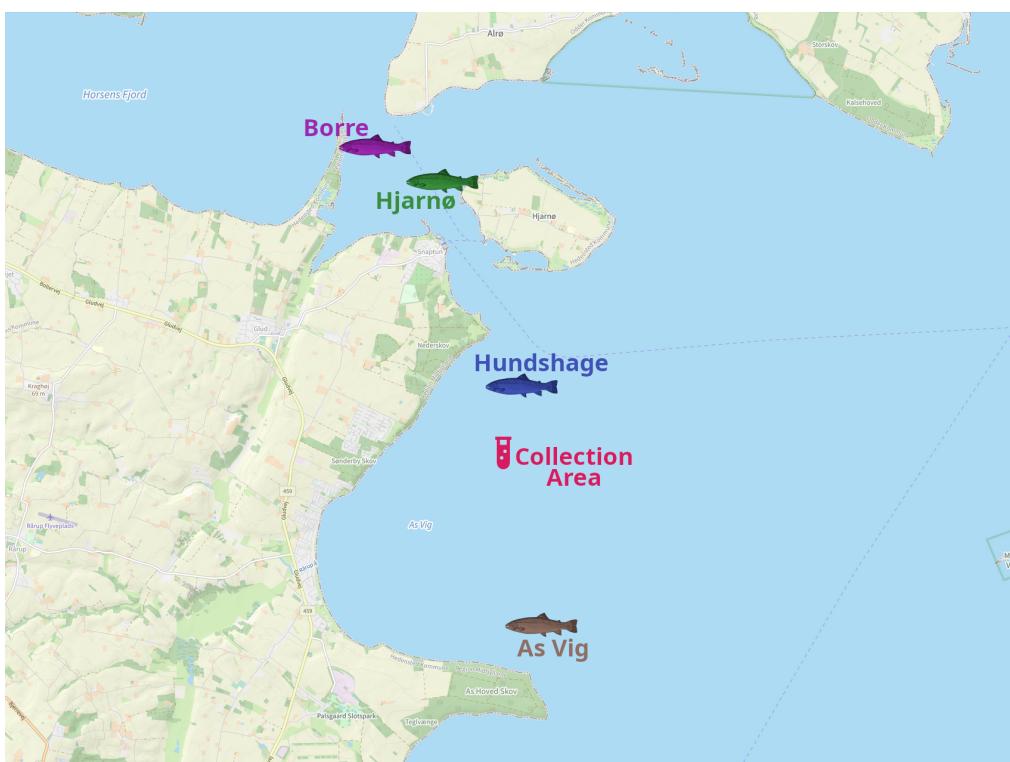


Figure 1.3: Geographical distribution of the Rainbow trout farms in Horsens Fjord. The deployment site is marked, too.

Additional manual samples were collected from two deployment sites at Dan F and Horsens Fjord. Water was collected using a weighted 4L Nisking water sampler. It was poured into a sterile 10L bucket and filtered on-site using Sterivex 0.22 µm filters (SVGPL10RC, Sigma-Aldrich, St. Louis, MO, USA), a sterile 60 mL disposable syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and wearing disposable nitrile gloves. After sampling, filters were stored in a freezer (-20°C) until extraction. For Horsens Fjord, triplicate 1L samples were collected from the surface (2 m below the surface) and bottom water (2 m above the seabed). Given the greater depth at the ESP deployment site (Dan F platform) in the North Sea, triplicate 3L water samples were collected from both surface water (2 m below the surface), mid-water (at the same depth as the ESP), and bottom water (2 m above the seabed). Sampling and filtering procedures were the same as for Horsens Fjord.

In addition to the samples collected at the ESP deployment site, seven other locations were sampled on the same day. The sites were chosen based on earlier acoustic studies [7] on the presence of Harbour porpoises in the North Sea, showing higher abundances of harbour porpoises around Dan F and the oil well (Regnar) compared to the other stations. In total, samples were collected in transects away from the Dan F oil and gas platform to Esbjerg harbour. Overall, the manual samples were used to investigate the possibility of vertical eDNA variation.

Site	Date	Depth	# of samples
Dan F	18 May 2019	Surface, middle, bottom	3 per depth
1600 m East	24 May 2019	Surface	3
6400 m East	24 May 2019	Surface	3
Regnar	15/18 May 2019	Surface, bottom	3 per depth
Ref South	15/18 May 2019	Surface, bottom	3 per depth
Ref East	15 May 2019	Surface, bottom	3 per depth
Norvana shipwreck	24 May 2019	Surface	3
Esbjerg harbour	15 May 2019	Surface	3

Table 1.2: Collection sites for the manual samples from the North Sea.

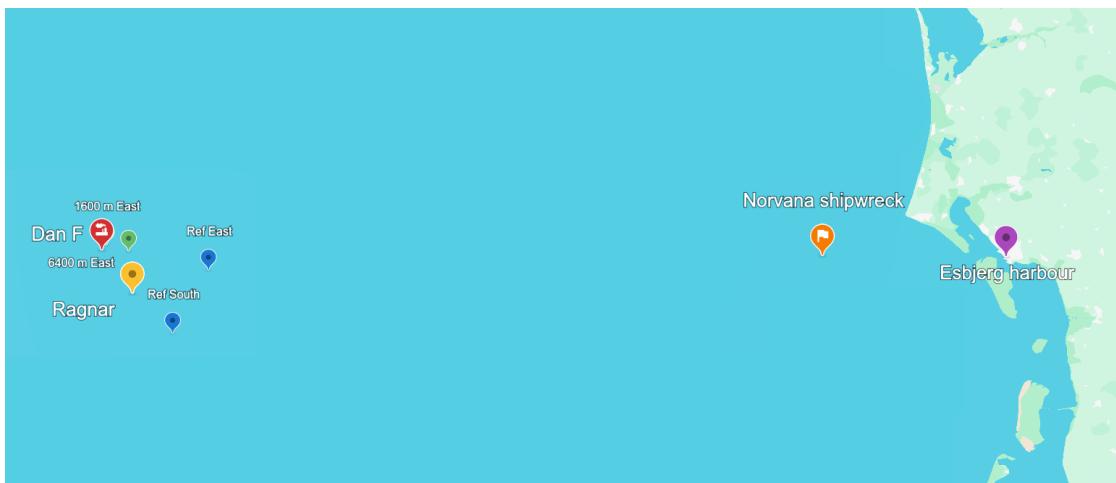


Figure 1.4: Geographical distribution of the collection sites from the North Sea.

2 Materials and methods

2.1 Sampling regimes and manual samples

All deployments originally included a mixture of in situ and archival sampling. An overview of all samples can be found in Appendix V. Quantitative PCR assays of specific target species were performed in real-time directly on the ESP. Archival samples were also analysed by the ESP after deployment targeting the same species. Overall, the in-situ analysis did not perform well, with only two positive detections from all three test cases (one for the Rainbow trout and another for the Warty comb jelly). Hence, this study focuses only on the subsequent qPCR and metabarcoding analyses performed on the archival samples.

For all sampling events, the instrument followed a cleaning protocol that involved using bleach, followed by rinsing with nuclease-free water to clean all tubes inside the instrument. Additionally, each ESP expedition included samplings of sterile water, referred to as ESP-negatives, both before and after deployment. This was done to analyse for potential DNA contamination, which could arise from DNA residues left over from previous sampling events.

2.2 DNA extraction

All filters were collected after retrieving the instrument. The samples from Sweden were extracted using a protocol exactly mimicking the ESP extraction protocol (Hansen et al. 2020), while samples from the two Danish deployments were extracted using the protocol by Spens et al. [17] using the Qiagen blood and tissue kit. All extractions were performed in a clean room. Procedures included wiping all surfaces with 5% bleach before and after use, UV-light exposure after use, and wearing sterile full-body suits. All extractions included the collection of blanks to control potentially exogenous DNA contamination originating from the laboratory or sample cross-contamination. DNA concentration was measured for all samples using a Qubit fluorometer and the Qubit dsDNA High-sensitivity kit.

2.3 Analysis and archival samples: Quantitative PCR (qPCR)

Samples were analysed for eDNA from pre-defined target species using qPCR. Specifically, we looked for Atlantic bluefin tuna (*Thunnus thynnus*), Atlantic mackerel (*Scomber scombrus*), and Garfish (*Belone belone*) during the Swedish deployment. For the North Sea, we analysed Harbor porpoise (*Phocoena phocoena*), Atlantic mackerel (*Scomber scombrus*), and Whiting (*Merlangius merlangus*). Finally, for the deployment close to Horsens Fjord, we targeted Rainbow trout (*Oncorhynchus mykiss*), Pacific oyster (*Magallana gigas*), and the Warty comb jelly (*Mnemiopsis leidyi*).

All used qPCR assays consisted of already developed and published species-specific assays (Appendix IV). Reactions were run on a StepOnePlus Real-time PCR instrument (Life Technologies, Carlsbad, CA, USA), using 10 min initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s. Tests for potential PCR inhibitors were conducted by running a positive control (IPC, Applied Biosystems, Foster City, CA, USA) on all extracts using the same volume as in the species tests. All samples and extraction blanks were run in triplicates and three negative controls were run per 96-plate. Estimation of number of copies per reaction was performed using a dilution series of 1-105 copies of synthetic amplicons (gBlocks). The average copy number estimates across triplicates

were used to calculate the average eDNA copy number per litre (copies/L) of water in the samples, which was used for downstream analyses. This data is the basis for the qPCR analyses in the report.

2.4 qPCR: statistical analysis

The data from the qPCR was calculated in eDNA concentrations in the original samples (copies/L) and studied using the R programming language. The DNA concentration of the selected species was associated with the time of collection and sample volume. The Pearson correlation coefficient was used to calculate the correlation matrix between the following variables: target DNA concentration, meridional and zonal velocity of the sea currents, water salinity, and temperature. The same method was used to estimate the *p*-value for the variables showing the highest correlation values (i.e., meridional velocity and salinity).

Information about the temperature, salinity, and velocity of sea currents was made available by the Danish Defence Center for Operational Oceanography (FCOO). These variables were tested and recorded every hour for the whole duration of the expedition. This dataset was joined with the sample table by extracting the weather records with the smallest delay compared to the extraction day and time.

2.5 Analysis and archival samples: Metabarcoding

To investigate the broader biodiversity, all eDNA samples were further analysed using metabarcoding. Depending on the collection area, we targeted up to three taxonomic groups: bony fishes (MiFish-U), elasmobranchs (Elas02), and marine mammals (Mamm02). Analyses of marine mammals were performed for eDNA samples collected from the North Sea region. We amplified the 12S rRNA region of the mitochondrial DNA for the first two groups and the 16S rRNA subunit for the latter. The final PCR reactions were performed in 20 µL reaction volumes containing 10 µL of AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA, USA), 0.16 µL BSA (20 µg/ µL), 10 µM of each primer, and 2 µL of DNA. Reactions were run on a standard PCR machine, using 10 minutes at 95°C, followed by 40 cycles including a 1 min DNA denaturation step at 94°C, a 1 min annealing step at 45°C, and a 1 min extension step at 72°C for 1 min. A final 5 min extension step at 72°C was performed after all 40 cycles.

A total of three 20 µL reactions were run per sample. Negative controls (NTCs) were included to test for DNA contamination during PCR setup. Since all NTCs contained no measurable DNA, we used 12 µL of these samples to ensure maximal representation of any amplified DNA. To discriminate between samples, PCR reactions were performed using different combinations of uniquely barcoded primers. Each barcode was 7 bp long and had a minimum of 3 mismatches to other barcodes. Barcodes were applied on both forward and reverse primers to ensure a low risk of misidentification. Following PCR, positive amplification of target DNA was confirmed by gel electrophoresis. We pooled the triplicate PCR reactions and measured DNA concentrations on a Qubit fluorometer. The measured concentrations were used as a reference for the subsequent pooling of all the samples in equimolar concentrations.

After pooling the samples, DNA libraries were prepared following the KAPA PCR-free library kit using dual-indexed barcodes. Sequencing was conducted using either High-output or Mid-output flow cells (150 PE) and run on a MiniSeq sequencing instrument (Illumina, San Diego, California, United States). To simplify DNA sequencing, all the samples were split into three libraries, summarised in the table below.

Library	Campaigns	Target	Taxa	# of samples
Lib01	North Sea (2019), Horsens Fjord (2020)	12S, 16S	Bony fish, Elasmobranch, Mammals	87
Lib02	Lysekil (2018), North Sea (2019)	12S	Bony fish, Elasmobranch	71
Lib04	North Sea (2019)	12S	Bony fish, Elasmobranch, Mammals	44

Table 2.1: Number of samples and targeted taxonomic groups for each deployment.

2.6 Metabarcoding: statistical analysis

Given the short length of our targeted primers and the high similarity with closely related taxonomic groups, we decided to restrict the OTU labelling to a subset of species living in the North Sea. First, we downloaded a taxa list of Danish sea animals from the SealifeBase database [18]. We also added the species used as positive controls (i.e., Sand tiger shark, Roach), the 25 most frequent marine mammals found in the North Sea, and common sources of natural contamination (i.e., Human, Chicken, Horse, Pig, Domestic cattle, and Sheep). We ended up with a total of 294 species: 234 fishes, 25 marine mammals, 25 birds, 6 contaminants, and 4 positive controls. The list can be found in Appendix III.

Reference DNA sequences for the 12S and 16S ribosomal RNA were obtained from the MIDORI2 database [19] (GenBank 261), filtering out taxa not belonging to our list of pre-defined species.

The paired-end (PE) DNA sequencing data extracted from the samples was merged and trimmed using PEAR [20] with its default parameters. Unassembled and low-quality reads ($p\text{-value} > 0.01$) were discarded. FastQC [21] was used to qualitatively assess the original and trimmed FASTQ files.

Next, Cutadapt [22] was utilised to remove the sequencing primers from the reads and to demultiplex them depending on their barcode sequences. We set a maximum allowed error rate of 10%, a minimum overlap of 7 nucleotides, and a minimum and maximum read lengths according to the targeted sequences. After this, we obtained an annotated FASTQ file for each library. All reads are associated with a specific sample ID to trace them back to the original collection site and target group.

Operational Taxonomic Units (OTU) were assembled following the OBITools pipeline [23] for metabarcoding:

1. obiuniq: clustering of identical reads;
2. obigrep: removal of sequence records with a single occurrence in the whole library;
3. obiclean: removal of reads that may have been caused by PCR or sequencing errors (using a threshold ratio between rare/abundant counts of 0.5);
4. obiannotate: trimming of unused metadata from sequence record;

5. `obitab`: metadata extraction and conversion to a tabular file.

Negative controls were checked, confirming the absence of unwanted contaminations. OTUs with less than 2 reads were removed.

The taxonomic assignment of OTUs was based on the algorithm created by Joseph Sevigny [24]. Briefly, a consensus assignment is used to classify OTUs by comparing the top 5 hits from a BLASTN query. We set a minimum identity of 97%, minimum coverage of 97%, and a maximum E-value of 10^{-3} .

Finally, the metadata tables exported with `obitab` and the results from BLAST were merged and plotted using the R programming language. Rarefaction analysis was performed using the `vegan` [25] package, while the `phyloseq` [26] package was used for dissimilarity analysis and ordination. Dendrogram clustering analysis was performed by building a sample-taxon matrix and estimating the similarity between samples using the Jaccard index. The resulting Newick tree was then plotted using TreeViewer [27].

3 Results

3.1 qPCR data

Quantitative PCR detected the presence of eDNA from mackerel and garfish at Lysekil and mackerel and whiting at the Dan F platform in the North Sea. Atlantic bluefin tuna and Harbour porpoise were not detected and left out of subsequent analyses. Analysis of samples from Horsens Fjord showed the presence of the Warty comb jelly and rainbow trout, but not pacific oyster. All NTCs, extraction blanks, and ESP-negatives were negative, excluding the risk of DNA contamination affecting the results. IPCs showed no sign of sample inhibition.

3.1.1 Daily variations in observed eDNA concentrations

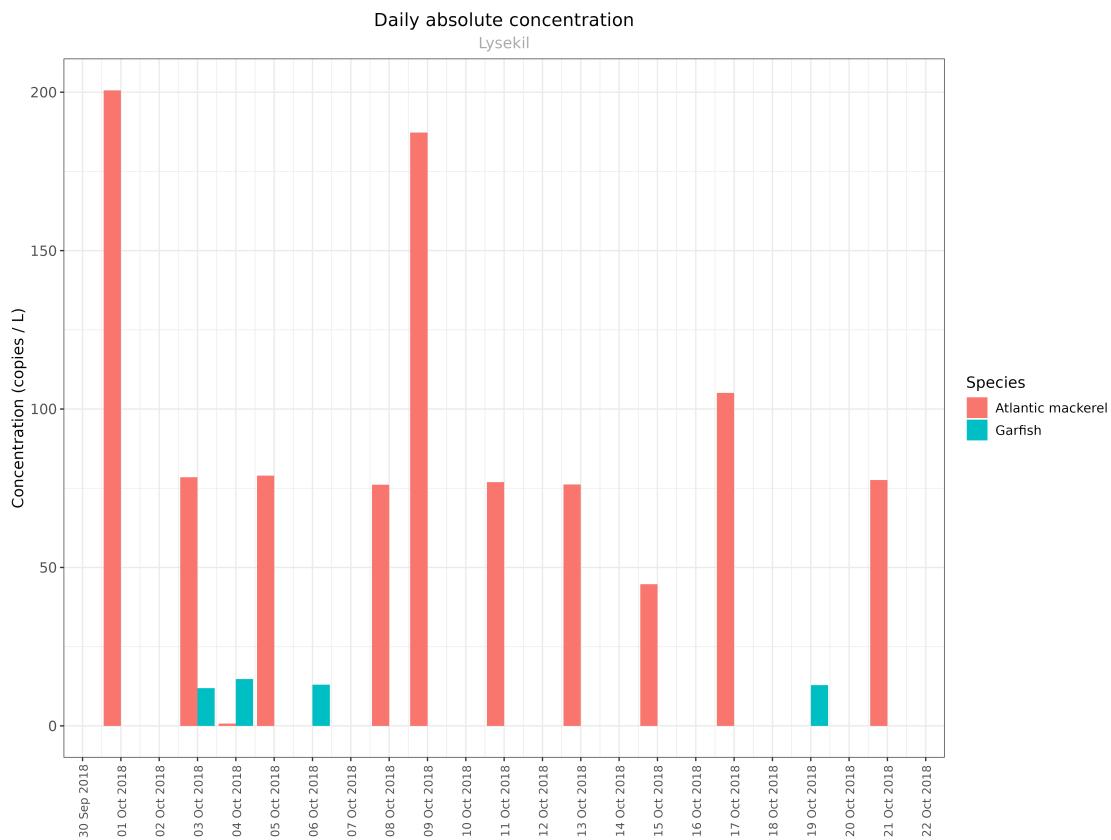


Figure 3.1: Daily eDNA concentrations for the targeted species from Lysekil.

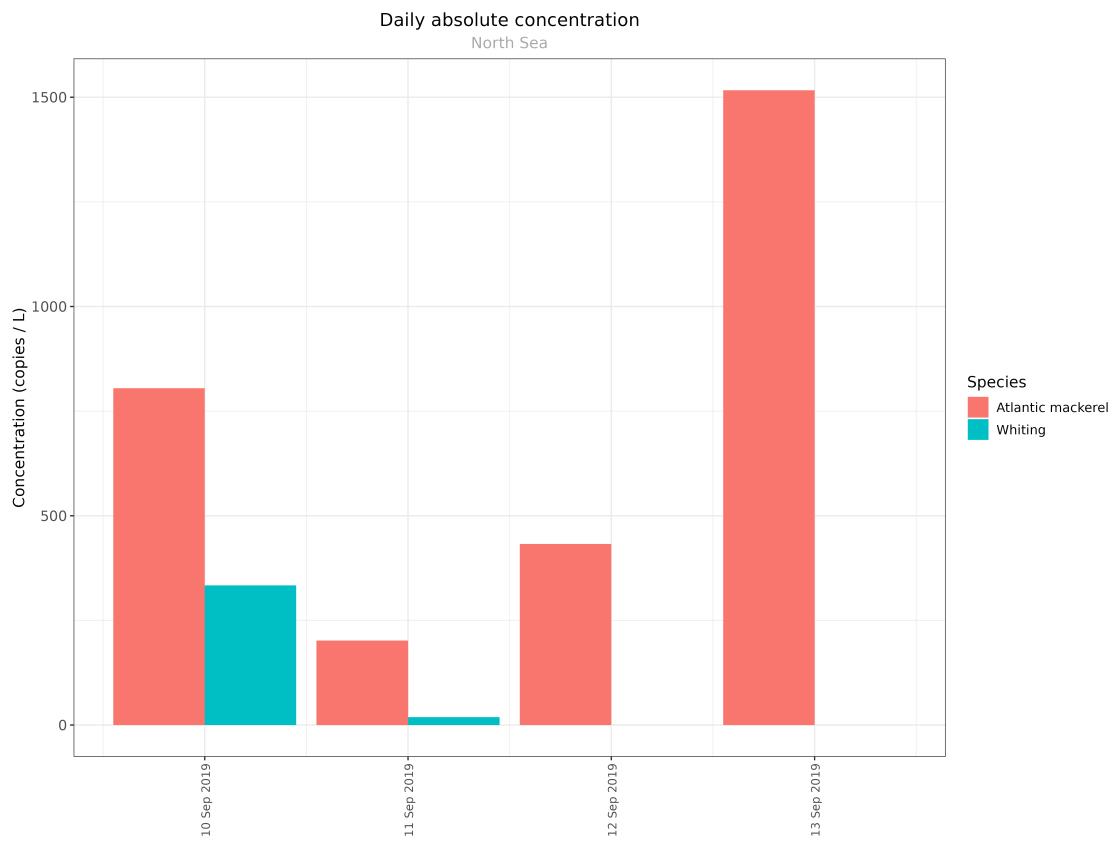


Figure 3.2: Daily eDNA concentrations for the targeted species from the North Sea.

The eDNA concentrations of target species measured by qPCR varied considerably from day to day at all deployment sites. In Lysekil and the North Sea regions, we detected the highest concentrations of Atlantic mackerel while DNA concentrations from garfish and whiting were much lower. At Lysekil, eDNA concentrations from mackerel varied from 0 to around 200 copies/L, while mackerel DNA concentrations at the Dan F platform in the North Sea varied from approximately 200 to 1,500 copies/L. Whiting eDNA concentration varied from 0 to about 300 copies/L, and garfish showed very low concentrations. In Lysekil, mackerel was detected in 47% (10/21) of the collected samples and garfish in 19% of the samples. In the North Sea samples, mackerel was detected in all four collected samples, while whiting was detected in 50% (2/4) of them.

The campaign at Horsens Fjord provided more data and higher eDNA concentrations, allowing us to perform a more exhaustive analysis. Overall, the ESP collected a total of 21 samples over 15 days, while another 6 were collected through manual sampling.

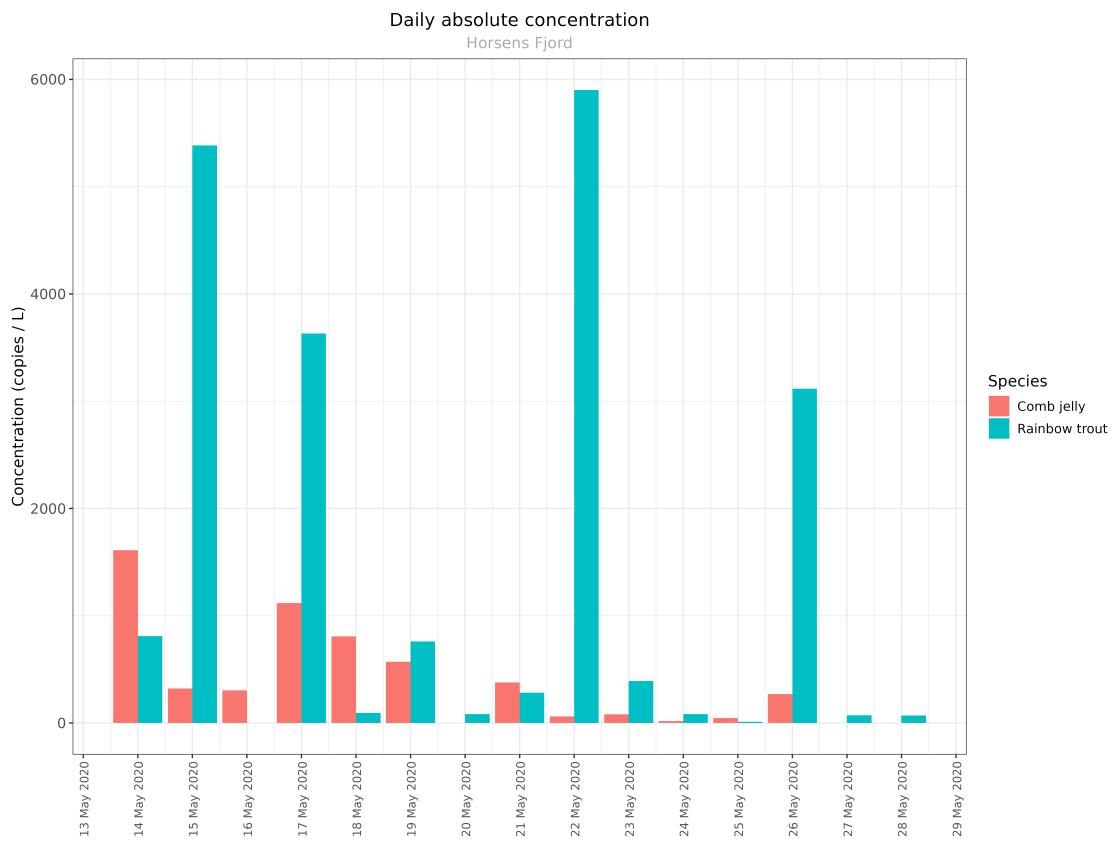


Figure 3.3: Daily eDNA concentrations for the targeted species from Horsens Fjord.

Here the qPCR focussed on the Rainbow trout and Comb jelly, two species considered invasive in Denmark. As for the samples collected at the other sites, we saw a high variation in eDNA concentration between consecutive days. DNA concentrations for the Rainbow trout varied from 0 to around 6,000 copies/L, while DNA concentrations from the Warty comb jelly varied from 0 to approximately 1,800 copies/L. Days with observed high concentrations from one species did not correspond with similar high concentrations for the other. This fact supports that the observed differences were not due to technical artefacts, like sample extraction efficiencies, as such bias should lead to similar trends for both species. Further, while DNA concentrations and sampling volume showed a significant correlation, the same was not observed between the overall DNA concentration and species-specific estimates, ensuring the absence of a potential sampling bias.

3.1.2 Correlation with abiotic conditions

We observed sudden spikes in DNA concentration for the Rainbow trout. Given the presence of several farms in the area, it is plausible to think that the two elements are related.

Hence, we calculated a correlation matrix per species using the Pearson correlation coefficient to highlight possible relationships between variables. We included the ocean data collected by FCOO's buoy. Sea currents were split into their zonal (east to west) and meridional (south to north) components.

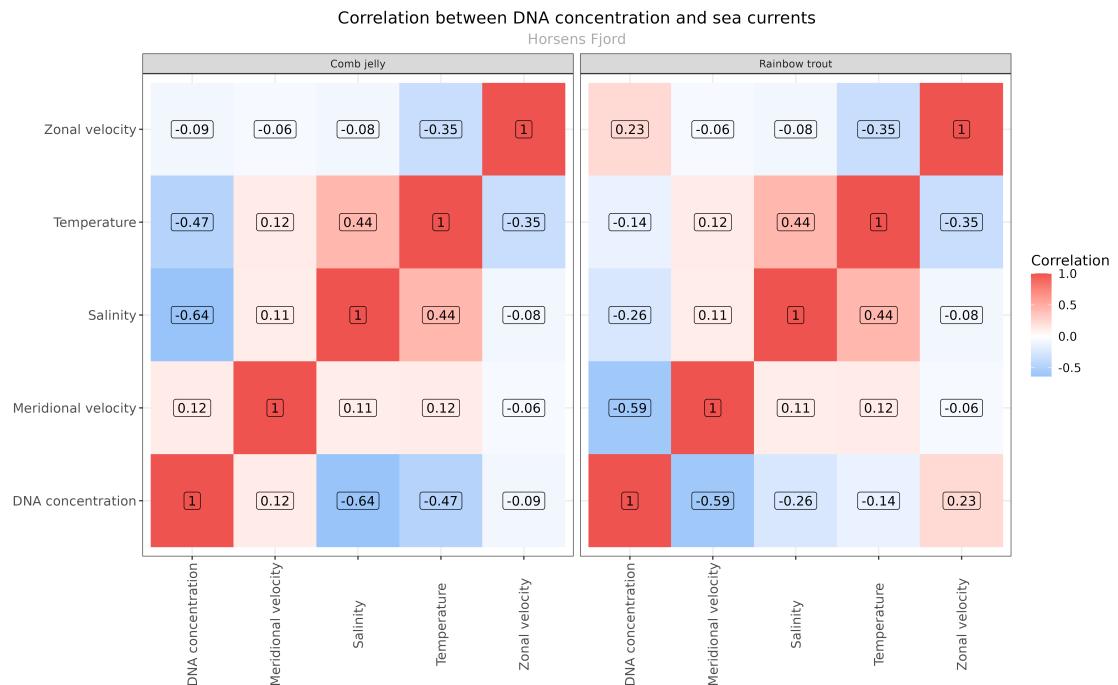


Figure 3.4: The Pearson correlation matrix was used to find statistically valid associations between DNA concentration and abiotic factors.

The analysis showed two particularly strong factors influencing DNA concentration: the meridional velocity of the water currents, for the Rainbow trout, and the salinity, for the Comb jelly. We can plot the relative variation of these factors against the DNA concentration to better visualise their relations.

Rainbow trout and sea currents

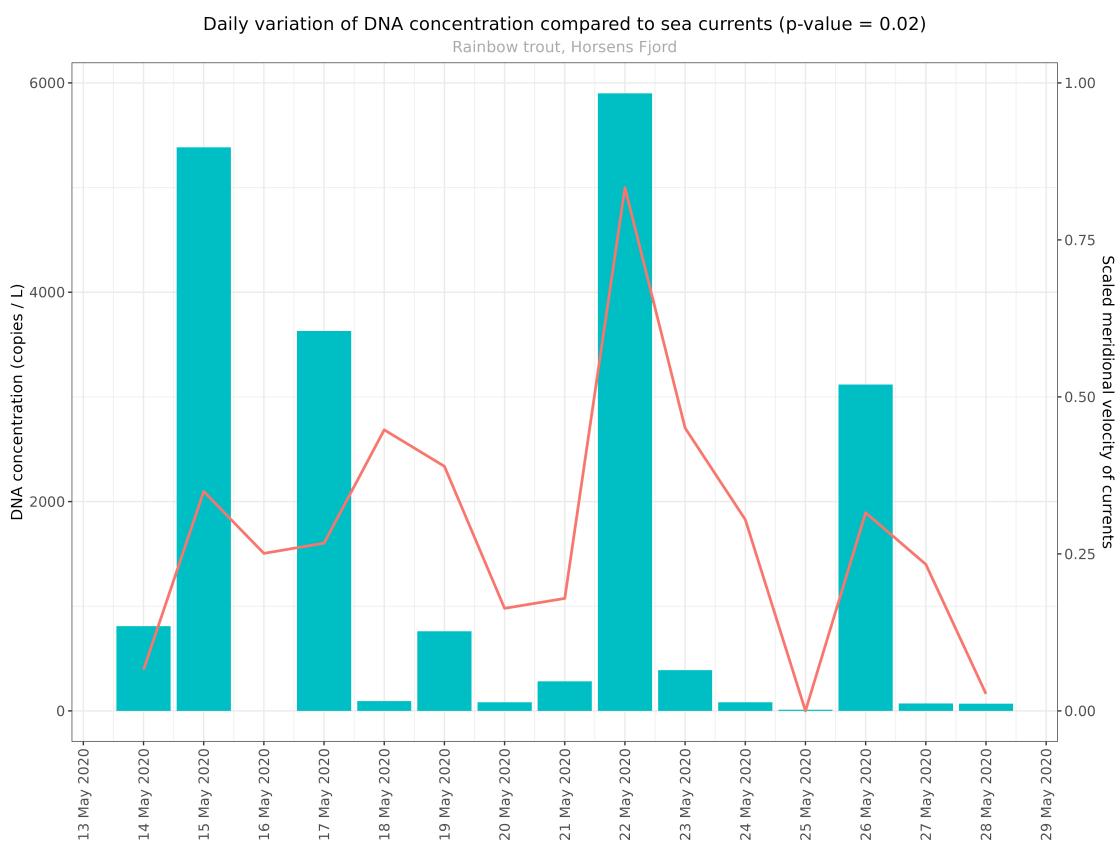


Figure 3.5: Direct comparison between the daily eDNA concentration for the Rainbow trout (blue columns) and the scaled meridional speed of the sea currents (red line). Higher values of the line indicate stronger currents from north to south.

For the Rainbow trout, there is a negative correlation between the meridional velocity and the DNA concentration. This means we register stronger biological signals when the currents flow from north to south. To more easily visualise this relationship, the red line on the plot represents the inverse of the velocity. The observations match with a primary eDNA source from the nearest farm in the area, the Hundshage fish farm, situated around 700 metres north of the ESP deployment site. Thus, we can hypothesise that the spikes in DNA concentration are due to the material carried southwards from the fish farm by the sea currents.

Concentration and meridional velocity are correlated by a p -value = 0.02. We can reject the null hypothesis and confirm that this correlation is statistically sound.

Comb jelly and water salinity

The story changes when we look at the data for the Comb jelly and the water salinity.

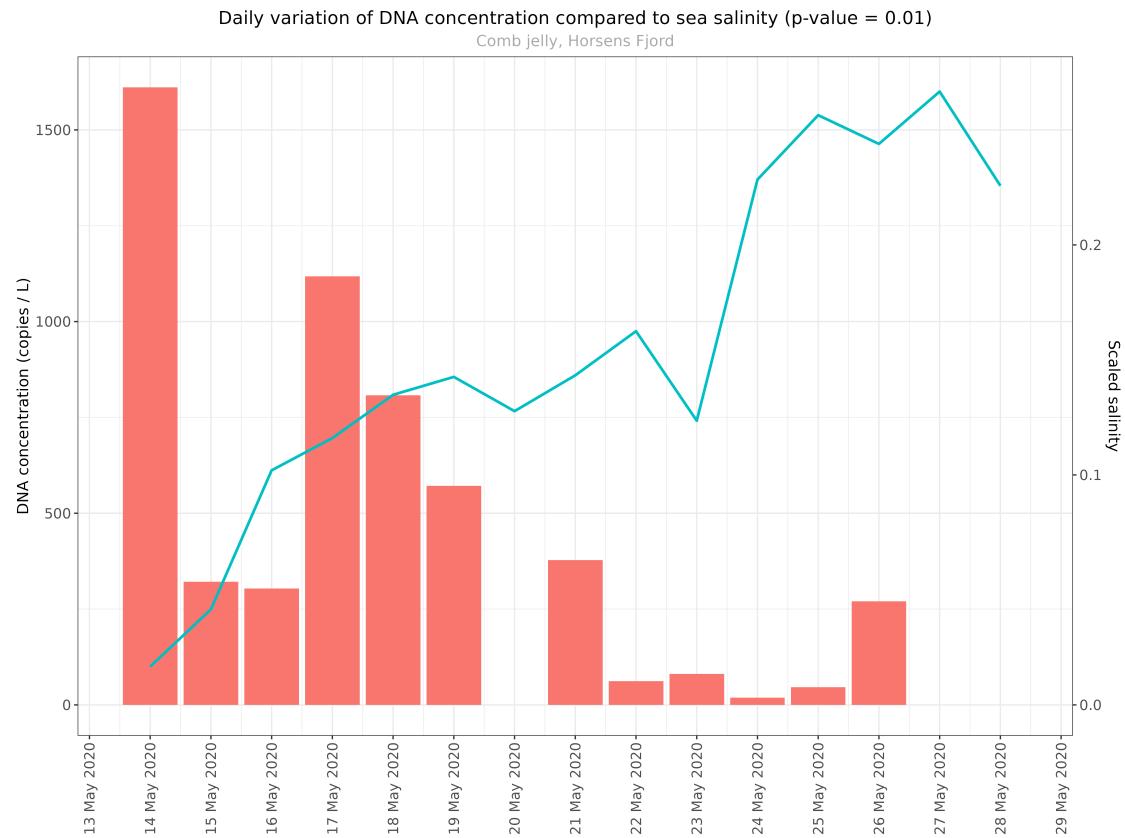


Figure 3.6: Direct comparison between the daily eDNA concentration for the Warty comb jelly (red columns) and the scaled salinity of seawater (blue line). Higher values of the line indicate higher concentrations of salt in the water.

At first glance, we might say that the concentration of Comb jelly goes down when the salinity increases. This is also supported by a small p -value = 0.01.

3.1.3 Sampling variation

The unique sampling design at Horsens Fjord allowed us to explore the sampling variation through analysis of the relative deviation from the average concentration per sample. We divided the data into three categories: i) temporal samples containing all the ESP observations for the whole period; ii) daily samples, a special cycle of the ESP collecting multiple samples within a 24-hour window; iii) six samples that were manually extracted without the use of the ESP, all on the same day. Half were collected from surface water, while the other half were collected 2 metres above the seabed.

Overall, there was a clear difference between the data for the Comb jelly and the Rainbow trout. Jellies were harder to detect, and we have seen that their concentration decreases over time. This is reflected in the deviation from the mean, with values that fluctuate a lot between sampling, even when they are collected a few hours apart.

The Rainbow trout was easier to detect and yielded much higher DNA concentrations. This helped us to get more reproducible results, with a deviation from the mean that resembles what we would expect. The values obtained from the same-day data collection stick close together and experience minor variations compared to the total temporal data. A remarkable distinction can be seen with the manual extraction, where surface and bottom samples clearly follow different trends. Bottom samples show the highest eDNA concentration (1,340 copies/L), while values from the surface have around one-third of the concentration (390 copies/L).

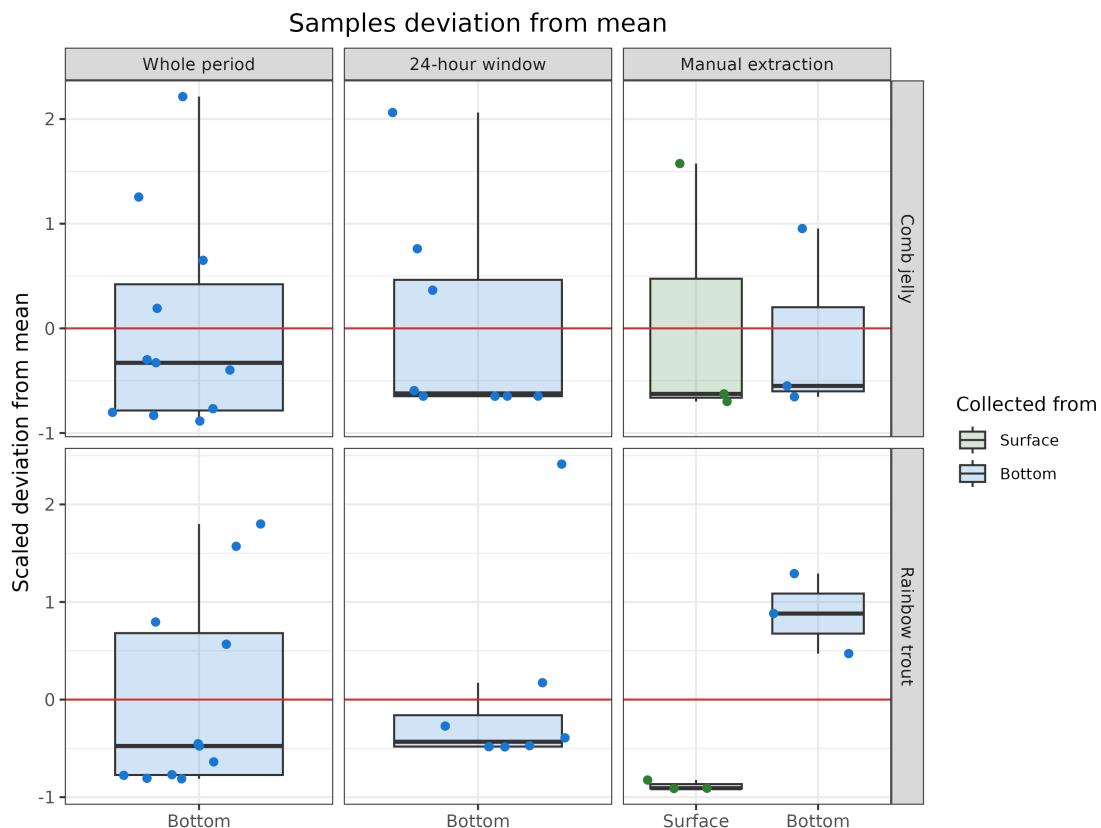


Figure 3.7: Box plot showing the deviation from the mean ($Y = 0$) of individual samples, categorised by species and collection strategy. Samples (points) above the mean (red line) have higher DNA concentrations than the average for that category. The opposite is valid for the samples below the mean.

3.2 Metabarcoding data

Primer selection is the factor that mostly influenced the performance of our libraries. MiFish-U produced the best results, capturing DNA sequences from a large variety of bony fish. Among the 50 most common taxa across all libraries, five of them could only be labelled at a family level (*Clupeidae*, *Pleuronectidae*, *Gobiidae*, *Triglidae*, *Gadidae*) and one at the genus level (*Pomatoschistus*). Two possible causes for this are the absence of the correct species from our reference database or excessive similarities between genomic regions.

Mamm02 captured some faint traces of marine mammals, while Elas02 failed to detect the presence of any member of the Elasmobranch subclass except for the positive controls.

The following table summarises the average and maximum depths of the OTUs found for each primer. The data before filtering contains all reads obtained from DNA sequencing, including non-targeted species and positive controls. Negative controls were manually assessed for potential contamination, detecting only non-marine species (almost entirely humans and pigs). Since this category of organisms was already excluded, we did not have to perform other forms of calibrations.

Library	Target	Primer	Avg. depth - Before filtering	Max. depth - Before filtering	Avg. depth - After filtering	Max. depth - After filtering
Lib01	Bony fish	MiFish-U	337	1,403,466	12,075	298,341
	Elasmobranch	Elas02	337	1,403,466	0	0
	Mammals	Mamm02	1,952	1,284,167	3	6
Lib02	Bony fish	MiFish-U	397	624,243	7,329	56,165
	Elasmobranch	Elas02	397	624,243	0	0
Lib04	Bony fish	MiFish-U	52	490,412	1,017	22,583
	Elasmobranch	Elas02	74	1,185,623	0	0
	Mammals	Mamm02	578	5,495,450	40	302

Table 3.1: List of primers used for each targeted taxonomic unit along with the average and maximum depths before and after filtering.

The following analyses focus on the bony fish data, as it is the only class that produces larger biodiversity data.

3.2.1 Elasmobranchs, marine mammals, and natural contaminants

During the analysis, virtually all OTUs were assigned to Bony fish. Nonetheless, we managed to detect some faint traces of marine mammals, mostly Harbor porpoises (*Phocoena phocoena*), concentrated in the North Sea (1,025 copies at Dan F, 334 in Regnar, 78 at the Norvana shipwreck, 32 in 6400 m East), and Harbor seals (*Phoca vitulina*; 218 copies at Esbjerg harbour, 123 at 6400 m East). No elasmobranchs were found in our data. Positive controls gave a strong signal, including DNA extracted from Sand tiger shark, used as positive control, but also water collected from the Nordsøen Oceanarium, which contains several species of sharks and rays.

Finally, it is worth to spend a few words on contaminants. When collecting environmental samples like seawater, we should always expect to encounter some OTUs belonging to non-marine species. Human DNA had the highest abundance in all libraries, probably due to all the manual labour required to deploy and retrieve the ESP. Pig, chicken, domestic cattle, and sheep DNA was also present in various amounts. They may stem from sewage wastewater and human activities close to the collection areas, like offshore oil platforms

or coastal towns. We also included 25 marine birds in our taxonomic database, but we did not find any trace of them.

3.2.2 Rarefaction

To get a better understanding of whether the libraries were sequenced deep enough, we conducted a rarefaction analysis. In short, it can be used to visualise the species distribution over a random subset of observations. Despite large variations in sequencing depth, all rarefaction curves for fish biodiversity (MiFish) reached a plateau for the number of species, showing that the sequencing depth was sufficient to find all the species in the samples. All rarefaction plots can be found in Appendix I.

3.2.3 Biodiversity clustering between ESP deployment sites

Dendrogram clustering analysis by species compositions (Jaccard beta-diversity index) showed a clear separation of samples by deployment site. Overall, we see that there is a strong relationship between samples and locations. There are a few spurious elements in the tree (e.g., ESP 11, ESP 13, ESP 14) caused by atypical concentrations of one or more rarer species compared to the other samples collected from the same area.

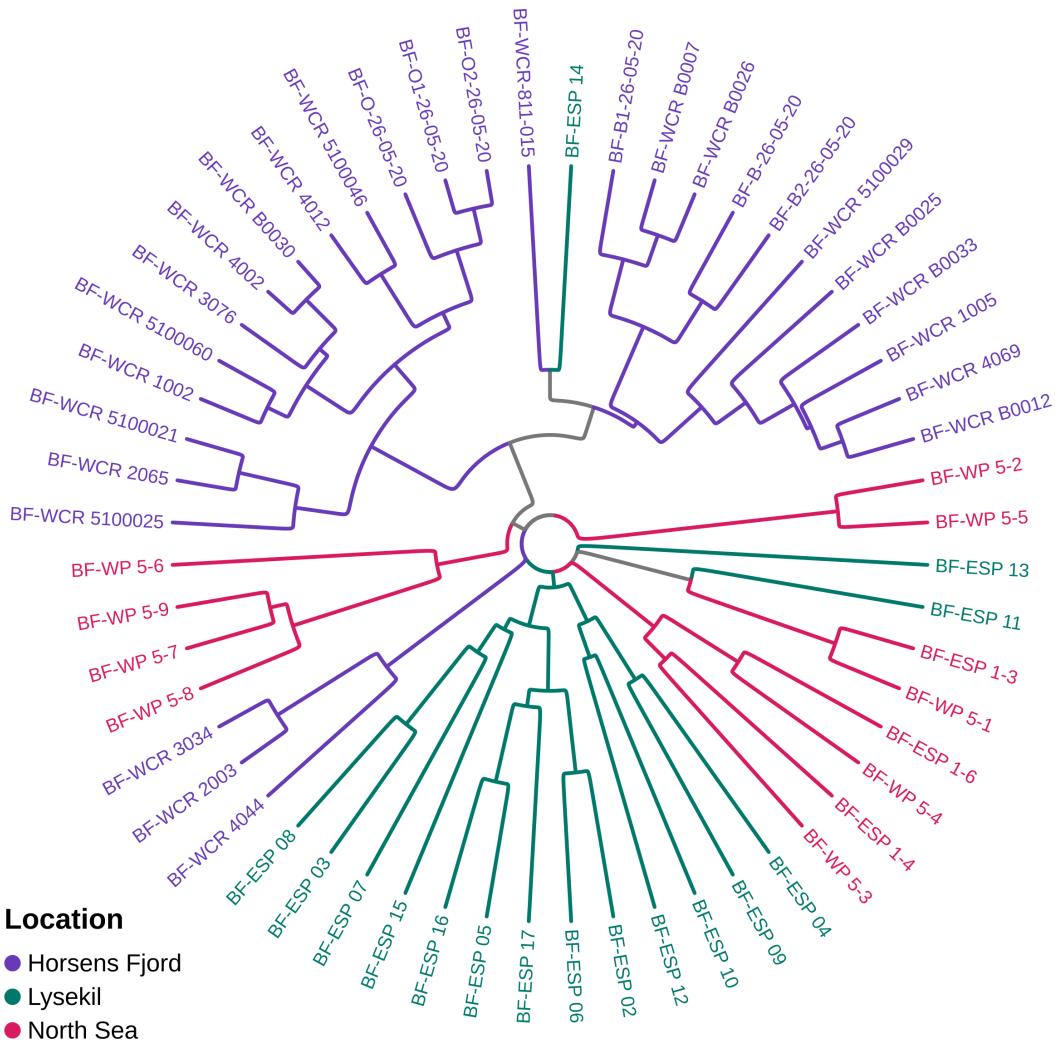


Figure 3.8: Dendrogram built on top of the Jaccard dissimilarity matrix showing clustering between samples collected from the same deployment sites.

A dendrogram specific to the spatial data can be found in Appendix II.

3.2.4 Overall biodiversity

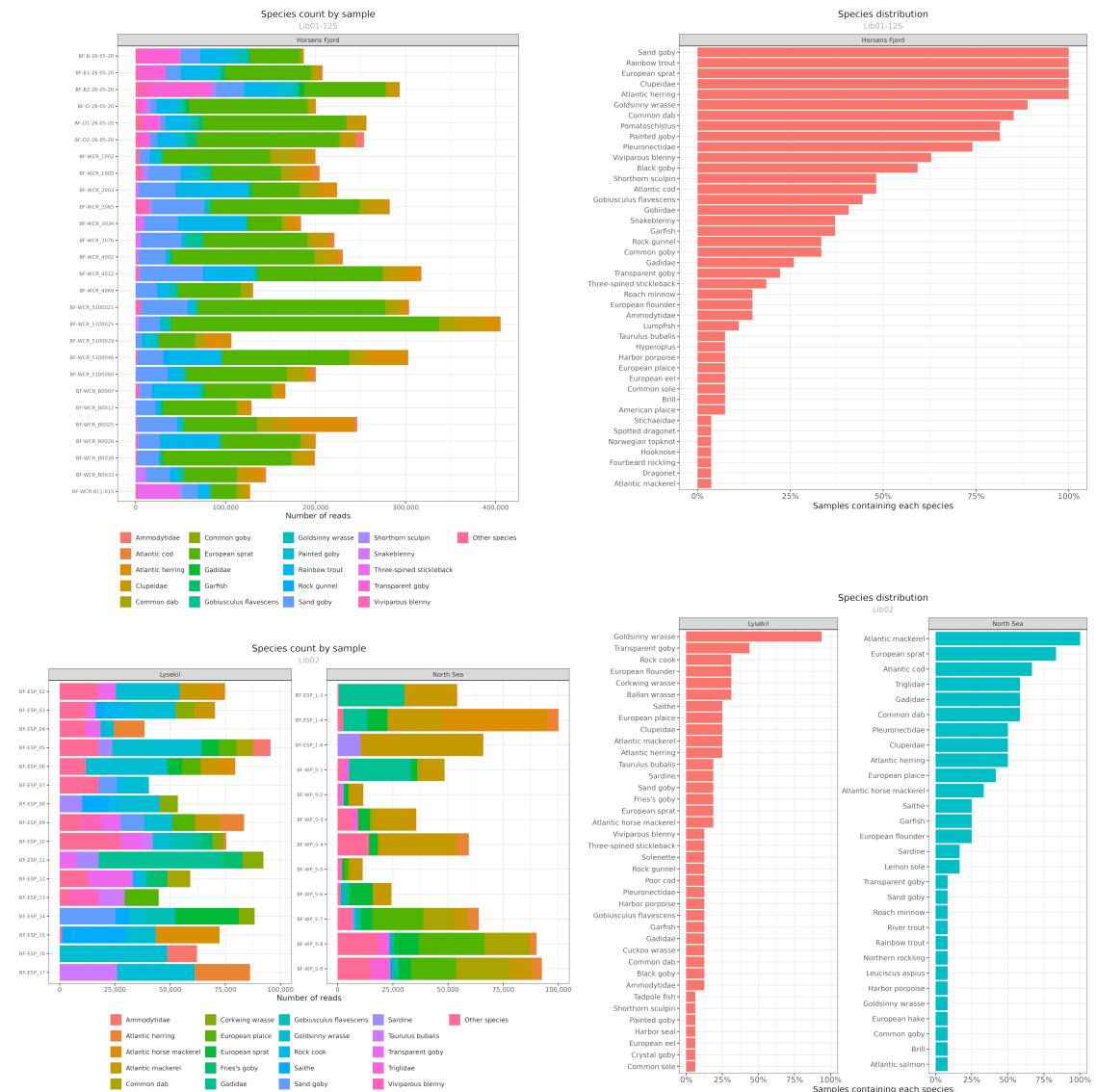


Figure 3.9: On the left: distribution of read counts from all samples, coloured by species and split by collection site. On the right: percentage of samples where we detected at least one copy for each species, split by collection site.

The taxonomic composition of the fishes found in Horsens Fjord showed generally similar results in terms of the relative species proportions of ray-finned fishes among most samples. Overall, there was a strong presence of European sprat, Goldsinny wrasse, and Sand goby, which were found in all samples. The results from Lysekil and the Dan F oil platform in the North Sea also showed distinct patterns. In Lysekil, results indicated a high presence of benthic fishes like wrasses (*Labridae* family), gobies (*Gobiidae* family), and European flounders. In the North Sea, we mostly detected pelagic fishes like the Atlantic mackerel and European sprat, but also fish like Atlantic cod, and other species belonging to the *Triglidae* and *Gadidae* families. Interestingly, the patterns matched well with the sampling depth. As such, we observed a higher presence of benthic fish at Lysekil and Horsens Fjord, where the ESP was deployed directly on the seabed, while we found that the most abundant species at Dan F belonged to pelagic species. Just like for the

qPCR data, the Rainbow trout dominates the results from the metabarcoding analysis. The results from qPCR and metabarcoding analysis matched. As an example, samples WCR_2003 (May 22nd) and WCR_1005 (May 19th) had, respectively, a high and low concentration of Rainbow trout in the qPCR data. The same trend can be observed for the other samples.

3.2.5 Biodiversity: spatial data

The spatial data gathered samples extracted from multiple locations along the route from the Dan F oil platform to Esbjerg harbour. For many of those sites, samples were collected from both the bottom and surface of the water, providing us with a vertical component to analyse.

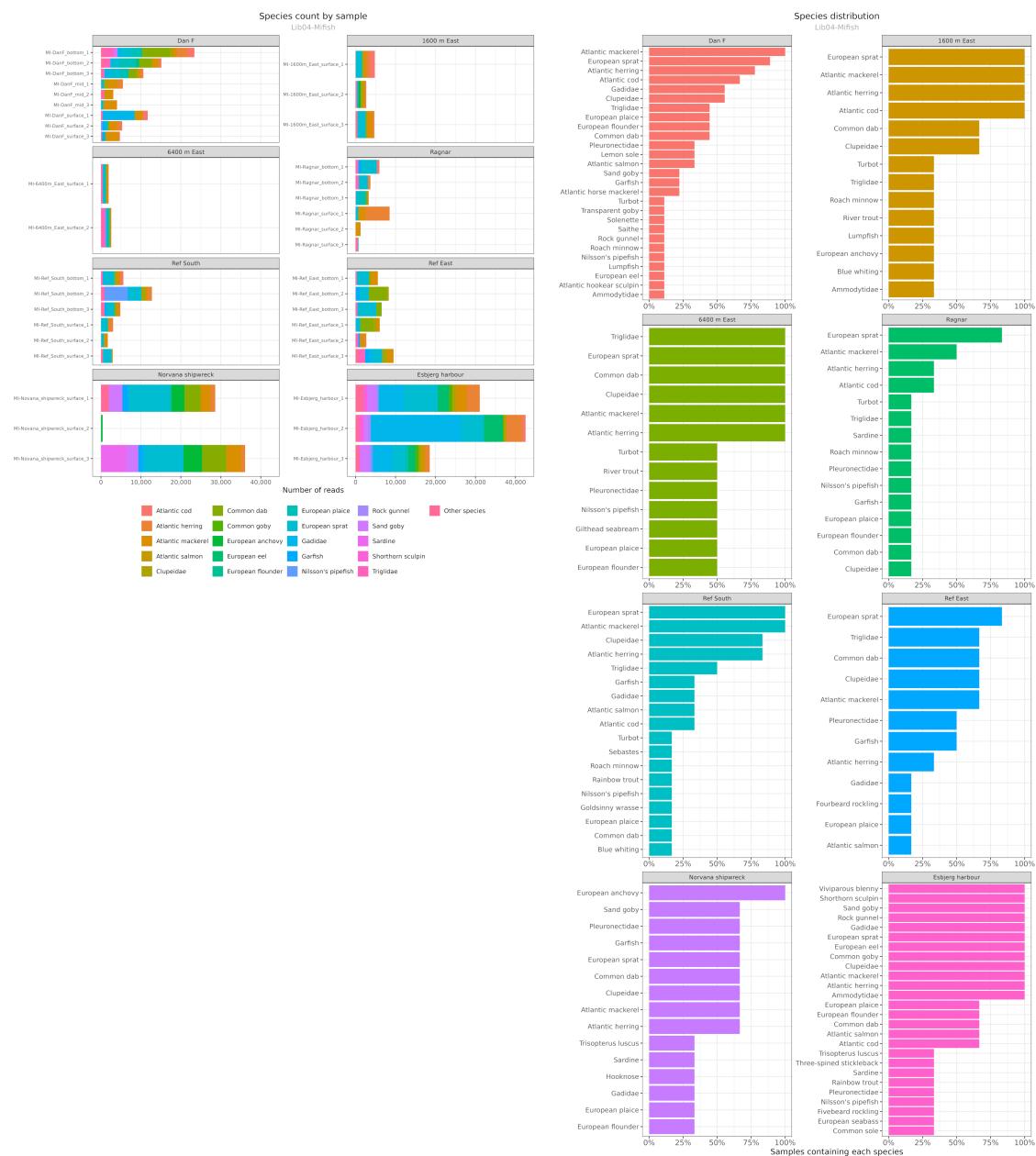


Figure 3.10: On the left: distribution of read counts from all samples, coloured by species and split by collection site. On the right: percentage of samples where we detected at least one copy for each species, split by collection site.

We can observe different patterns for each location. Species-wise, the locations can be split into two categories: oceanic and coastal sites. The former is composed of all sites close to Dan F, showing similar trends for the North Sea as we saw for Lib02. The latter includes Norvana shipwreck and Esbjerg harbour, with a higher concentration of coastal and pelagic fish, like Gobies and Shorthorn sculpins.

3.2.6 Species clustering

Non-metric multidimensional scaling (NMDS) was used to visualise the difference between surface and bottom samples. It showed a clear difference between beta-diversity and sampling depth, especially for the ESP site and Ragnar samples.

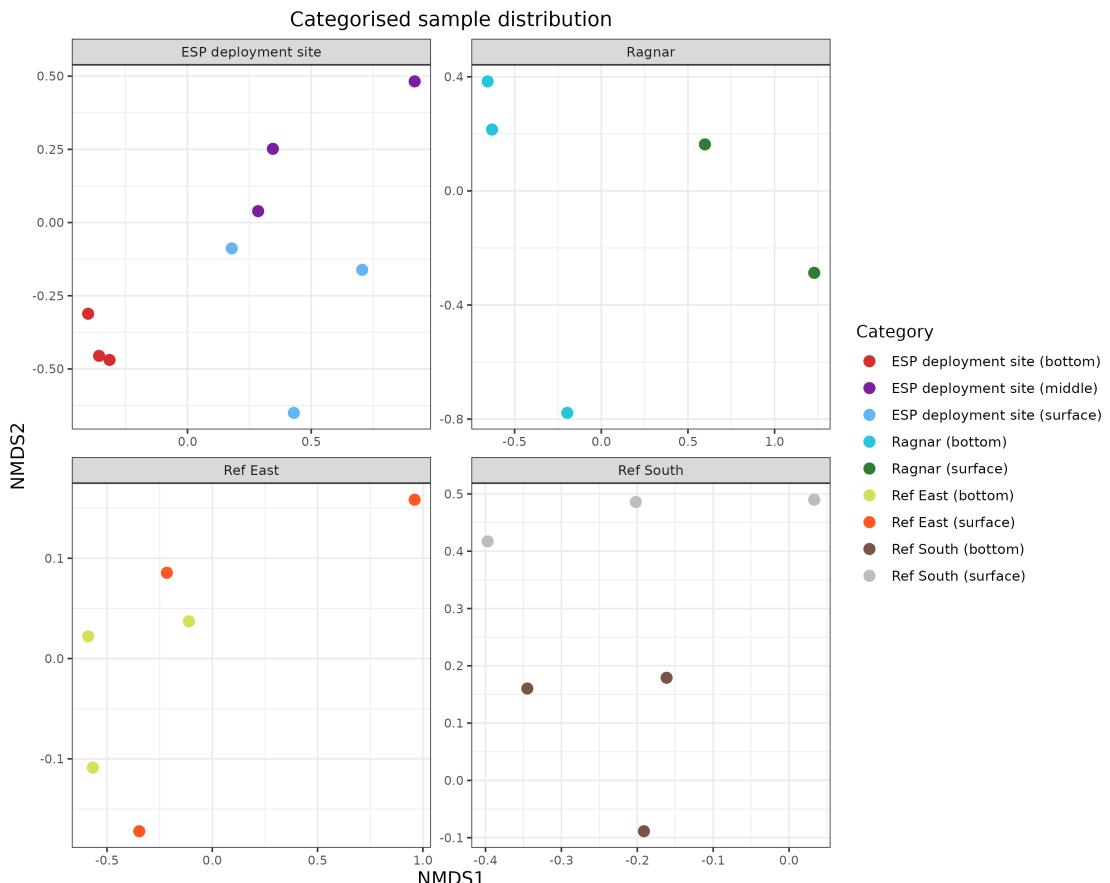


Figure 3.11: Non-metric multidimensional scaling plot for the North Sea's collection sites where we extracted both surface and bottom samples.

3.2.7 Temporal dimension

We performed an analysis to estimate the minimum number of days one should sample at each location to cover the whole detected local biodiversity. It represents a best-case scenario where daily samples were ordered by abundance and kept being extracted until we found at least one copy for all species.

Overall, the ESP performed differently at each location, probably reflecting sample volume and species diversity. In Horsens Fjord, it could identify 65% of all species in two days, but it required 13 more days to cover the whole spectrum. In Lysekil, 60% of all species were detected in four days, requiring 9 more days of sampling for full coverage. The ESP data from the North Sea is too limited for a good comparison.

For the spatial data, multiple samples were collected on the same day for most locations, so we cannot talk in terms of days, but extractions needed to cover the whole detected biodiversity.



Figure 3.12: The histograms show how many days (on the left) or sampling activities (on the right) we need to cover the whole detected biodiversity. Individual plots are split by collection site.

4 Discussion

Throughout this study, we investigated the use of a novel method for marine biodiversity assessment involving the use of an Environmental Sample Processor to collect eDNA samples, which were subsequently analysed using both quantitative PCR and metabarcoding methods. This combination of technologies enables users to monitor aquatic species in a non-invasive and low-effort manner. Automatic samplers like the ESP can be deployed offshore to autonomously collect and analyse samples at regular intervals. Filters can be retrieved weeks or even months later for DNA sequencing and data processing. This facilitates time series studies, which might better capture biodiversity than traditional “snapshots” collected using manual sampling.

However, like traditional eDNA methods, the use of automatic samplers also has pitfalls that may affect the quality of results. Technical problems, such as component malfunctions, and external factors, like deployment depth and environmental conditions, should be carefully considered before and after an expedition. Below we discuss the results of extensive analyses conducted in this report and discuss key considerations for future deployment strategies in Danish waters.

4.1 Comparing different primers

Due to the limited DNA concentration in seawater, adequate PCR primers should be selected to target relevant taxonomic groups. In our study, we tested the use of MiFish-U, Mamm02, and Elas02 primers to analyse eDNA from bony fish, marine mammals, and elasmobranchs. We also performed species-specific quantitative PCR targeting Atlantic bluefin tuna, Harbour porpoise, Atlantic mackerel, Garfish, Whiting, Rainbow trout, and the Warty comb jelly.

As expected, the MiFish-U primer proved to be a good choice for detection and taxonomic assignment, identifying more than 50 species of bony fish, distributed among all deployment sites.

The remaining two metabarcoding primers performed worse and mostly captured eDNA from non-target species, like fish, and terrestrial contamination from pigs, cattle, and humans. Elas02 did not detect any elasmobranch from the ESP expeditions. Nevertheless, we successfully verified its capacity to capture elasmobranch eDNA from positive controls collected from Nordsøen Oceanarium in Hirtshals, supporting that the used primers worked as intended.

We barely detected any trace of marine mammals from our samples. One exception was the Harbor porpoise, with just a few hundred copies from the North Sea (around 0.1% of the total). However, Harbor porpoises are known to shed little eDNA [28]. Combined with a relative low abundance of individuals in nature, eDNA observations are expected to be difficult. Hence, identifying even tiny portions of their DNA is already a remarkable accomplishment. Interestingly, the Regnar and Dan F sites showed the highest number of positive replicates with respectively 100% (3/3 replicates) and 55% (5/9) of the samples positive for Harbor porpoise eDNA. This result matches well with knowledge of acoustic studies on Harbor porpoise abundance in the North Sea, showing higher specimen abundance around these two sites [29].

Interestingly, Harbour porpoise concentration from the North Sea’s ESP samples collected at Dan F was much lower (13 copies over four samples). This finding is not surprising

given the limited number of samples from this deployment and the likely low concentration of eDNA from Harbour porpoises. Analysing additional ESP data would be beneficial to determine whether the long storage of filters in the ESP may have contributed to DNA degradation, potentially increasing the risk of false negatives. However, Harbour porpoises were also detected in eDNA samples collected from Lysekil (1,013 copies) and Horsens Fjord (36 copies) using MiFish primers, which are expected to be less effective for amplifying this species. This finding tentatively suggests that longer storage times of samples do not negatively affect the detection of rare species in eDNA analysis.

4.2 Horizontal and vertical diversity

In this study, we observed distinct patterns from different deployment sites and noted a strong clustering between these locations when comparing the observed beta diversity. This finding was expected due to the significant separation of the sites both spatially and temporally. Additionally, the results from the spatial samples collected in the North Sea indicated consistent changes in biodiversity as we moved farther away from the coast.

The deployment depth and closeness to the seabed is another important factor to account for. Samples collected by the ESP represent snapshots of the local biodiversity. The concentration of eDNA from different species might, however, not uniformly spread throughout the seawater. For example, water stratification is known to lead to differences in eDNA signals between surface and bottom water in freshwater lakes [13, 14]. However, published marine eDNA studies show opposite results on the vertical stratification of eDNA concentration, suggesting that this segregation may be more accentuated in certain regions than others. Dukan et al. [30] showed that eDNA samples collected at different depths do not exhibit distinct vertical concentration patterns, comparing their finding with more traditional trawl catch data. In contrast, Jeunen et al. [31] collected eDNA samples at three depths next to a rock wall, finding distinct concentration patterns along the water column, and hinting at the presence of vertically localised communities.

Our study further supports the concept that different biodiversity is observed at various depths. In Lysekil and Horsens Fjord, the ESP was placed close to the sea bottom, either on an underwater plateau or directly on the seabed. Here, we detected more benthic fish and bottom feeders like gobies, wrasses, flounders, and plaice compared to the deployment in the North Sea.

In the North Sea, the ESP was deployed mid-water at around 15 metres from the surface and identified mostly pelagic species like mackerels, sprats, herrings, and sardines. The observed differences between deployment sites suggest that water depth influences eDNA results. This is further supported by our analyses of surface and bottom samples from the other stations in the North Sea. In this case, sample clustering using the Jaccard index and non-metric multidimensional scaling revealed clear dissimilarities between collection sites and deployment depths. Rainbow trout eDNA samples collected as part of the Horsens Fjord deployment also showed clear differences, with a much higher concentration in bottom samples compared to the surface. This finding further reinforces the general impact of sampling depth across various sites. Other studies [32, 31] also found that bottom samples, including sediment samples, tend to have higher concentrations of species, with different patterns detected at separate depths. Thus, factors like deployment depth might influence biodiversity observations using automated samplers like the ESP, heavily influencing downstream data interpretation.

4.3 Temporal changes in eDNA observations

Seasonal changes and shifts in biodiversity are known to be reflected in eDNA observations [33, 34]. However, so far little is known about the potential effects of short-term variations. Jensen et al. [35] suggest considering marine communities as dynamic entities, with a strong correlation to the sampling time, especially for daylight vs. dark hours. Daily patterns can provide insights into the behaviour of certain species. This information can be used to refine future experiments by collecting samples at different hours of the day and night. Our study offered a unique opportunity to investigate the importance of short-term temporal sampling and how it can be used to better cover an area's real biodiversity.

Overall, we saw large fluctuations in DNA concentration throughout the 15 days of sampling in Horsens Fjord. This is especially true for the Rainbow trout, which went from near-zero values to over 6,000 copies/L in just 24 hours, highlighting the importance of distributing sampling activities over time. The concentration of Warty comb jelly DNA also showed significant daily variations, going from 0 to 1,600 copies/L, with an average of 380 copies/L.

The deviation from the mean of the observed eDNA concentrations in the samples gives us some further insights into its variability. The DNA concentration in samples collected within a 24-hour window showed little deviation from the average the Rainbow trout. The same did not apply to the Comb jelly, presenting much higher fluctuations, possibly due to the smaller amount of captured eDNA or because of the outliers from larger cell aggregates in the water. Variation in eDNA concentration is larger over time, supporting the need for a higher temporal resolution during sample collection.

The major distinction, however, involves the extraction depth. Surface samples revealed a much lower eDNA concentration for Rainbow trout compared to bottom samples. This further suggests that the vertical component of eDNA analyses can influence findings and downstream results. This clustering was not detected for the Comb jelly.

We conducted a time analysis to investigate the minimum days required to cover the full detected biodiversity. This is a best-case estimate that does not take into account the collection order of individual samples.

Multiple days of data extraction are required to capture the largest possible amount of species. Spending more time sampling from the same location leads to increasingly accurate information about the local biodiversity.

While this finding is not surprising, it is challenging to determine the exact number of days needed to obtain a comprehensive snapshot of the current situation. In Horsens Fjord, the ESP identified 65% of all species in two days, but it required 13 more days to cover the whole spectrum. In Lysekil, 60% of all species were detected within four days, requiring 9 more days of sampling for full coverage.

It is important to remember that these estimates are based on a best-case scenario. Real collection campaigns may require more days of sampling to achieve the same results. As a general guideline, we should aim for at least 10 samples per location during future deployments, preferably collected on different days.

Another limitation is that we currently do not know if the rarer species that were observed inhabit the area close to the deployment site, or if some genetic material has been transported there by sea currents or as part of the excretions of predators. More data is needed

to refine this metric, but there are limits to what can be inferred from the environment using eDNA methods alone.

Similarly, even though we can detect a very strong signal for Rainbow trout in Horsens Fjord, we may not be able to capture it from other nearby locations if the ESP is deployed upstream.

Using automatic sampling stations can be significantly cheaper compared to other methods when assessing data over extended periods. This approach eliminates the need for deploying vessels or having stationary personnel supervising the device, as all the work is done autonomously. The device can be retrieved weeks after deployment, which will save costs and at the same time enable the collection of extensive time series data.

4.4 Impact of environmental factors

After being deployed, the ESP remains stationary until its retrieval. Therefore, variations in observed eDNA concentrations are mostly dictated by changes in water flow, dilution, and animal activities in the surrounding area.

We found an especially strong correlation with sea currents in Horsens Fjord. The Hundshage Rainbow trout farm was located around 700 metres north of the deployment site. The eDNA concentration for this species is in an inverse relationship with the meridional component of the water currents, recording much higher values when they flow from north to south (p -value = 0.02).

This observation has several important implications for this study, but also in a more general eDNA context. DNA transportation is believed to be a key factor in understanding eDNA results [36]. Here, the focus has been on the potential risk of eDNA being transported over vast areas, hence leading to potential false positives [8, 36]. However, transport might also impact observed eDNA concentrations on a local scale, impacting the interpretations of species abundance and biodiversity.

Although many research groups have studied the relationship between biomass or abundance and eDNA, a clear connection has yet to be established (Hansen et al. 2018). So far, controlled experimental studies from aquaria or ponds have reported significant associations between biomass and eDNA concentration, with other abiotic factors like temperature, fish diet, population size, and DNA extraction methods also being important [37, 38]. However, studies in natural habitats are much less consistent and often report weak or no relationship between eDNA and biomass or species abundance [39, 40, 41, 42].

The results from this study, which demonstrates a clear impact of current velocity on eDNA concentration despite a large stationary biomass, highlight the importance of incorporating current data into marine eDNA monitoring when investigating biomass-eDNA relationships. In our case, the results emphasize the same need for information on currents when interpreting results from stationary sampling. While this might lead to increased complexity when analysing eDNA data, it may also improve deployment planning and facilitate the capture of target-specific eDNA when current patterns are known. In such scenarios, eDNA samplers, like the ESP, can be deployed down-current to better capture eDNA from specific areas. Potential areas of interest include oil and gas platforms, wind parks, and aquaculture sites, as all may benefit from monitoring biodiversity [43] in the case of aquaculture, pathogens [44].

Finally, we also detected a negative correlation (p -value = 0.01) between the concentration of Comb jelly and water salinity, but we could not establish if it is a spurious relation-

ship, a confounder effect linked to seasonality, or a proper association between these two variables.

4.5 Advantages and pitfalls of environmental DNA

eDNA and metabarcoding proved to be a powerful tool for biodiversity assessment, but it has also some potential issues compared with other monitoring methods like underwater video capture, netting, or acoustic analyses. The advantages include the possibility of capturing a broader taxonomic spectrum, including rarer and more elusive species. Autonomous sampling devices are non-invasive and potentially cheaper than alternative methods, especially for longer offshore monitoring campaigns, and do not require taxonomic expertise to interpret the data. The temporal dimension of the analysis lets us trace the variation in eDNA biodiversity observations over days or weeks, and this data can be further enhanced with sea current information.

However, eDNA detection may not directly translate into demographic information, especially if we want to determine the size of local fish communities. False negatives are also a problem for species shedding little eDNA. Negative eDNA results do not exclude the presence of a species since it may have not been active at the time of sequencing, or it might shed such a small amount of genetic material that cannot be detected [45]. Reference databases used for taxonomic assignment may incorrectly label species with genetically close relatives, but this effect can be attenuated by compiling a list of acceptable taxa based on the specific location [46, 12]. Lastly, abiotic factors may also influence eDNA concentration in ways that are only partially understood, with fluctuations from day to day [35].

4.6 Conclusion

Research worldwide has seen a recent surge in studies on eDNA and metabarcoding, highlighting the power of this technique and the many uses and benefits that may derive from it. Quantitative PCR and metabarcoding can be powerful tools for marine biodiversity assessment from environmental DNA in the hands of ecologists and other researchers. However, eDNA also has several fundamental challenges, of which some are particularly relevant for offshore marine biodiversity monitoring.

One of the main challenges of using eDNA for offshore marine monitoring is the use of dedicated vessel time, which increases operational costs and reduces the number of samples which can be collected and analysed. In the study, we investigated the use of an autonomous eDNA sampler, the ESP, to collect offshore eDNA data. By deploying such samplers and subsequently analysing eDNA data, we can cut down operating costs, streamlining the process of marine fauna surveillance and assessment.

To get the best results possible, current data should be matched to the collection areas so that the ESP can be deployed in pivotal locations. By placing it downstream, we can use the water currents to our advantage, letting them carry genetic material from surrounding areas directly to our sampler.

The deployment depth is another discriminant when it comes to biodiversity assessment, and we observed different biological signals depending on whether the ESP was placed closer to the seabed or mid-water. This matched with vertically collected manual samples, showing distinct differences in biodiversity between surface and bottom water.

Significant variations in eDNA concentrations can also be recorded from day to day, or even from daytime to nighttime. Longer sampling periods should be preferred to get a better overview of the local biodiversity.

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A Appendix I - Rarefaction data

Rarefaction curves are a way to represent the species distributions of samples in a probabilistic manner. By choosing X observations from a randomised dataset, we will likely find Y unique species among them.

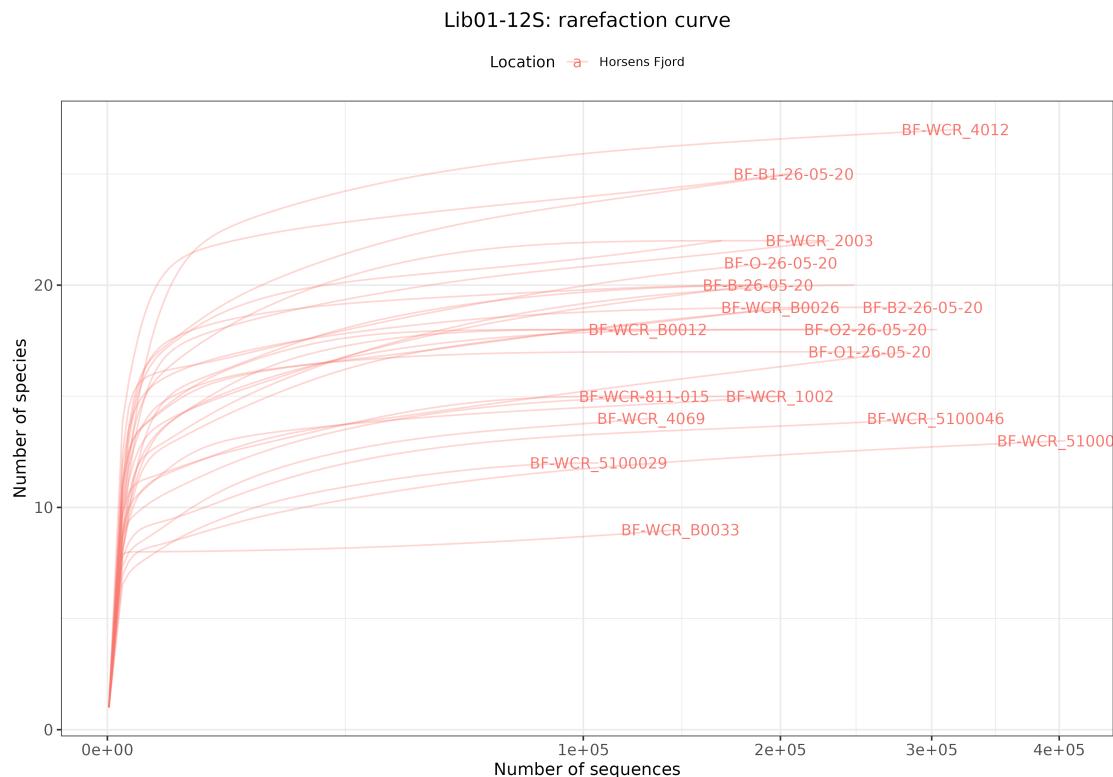


Figure A.1: Rarefaction curve for Lib01.

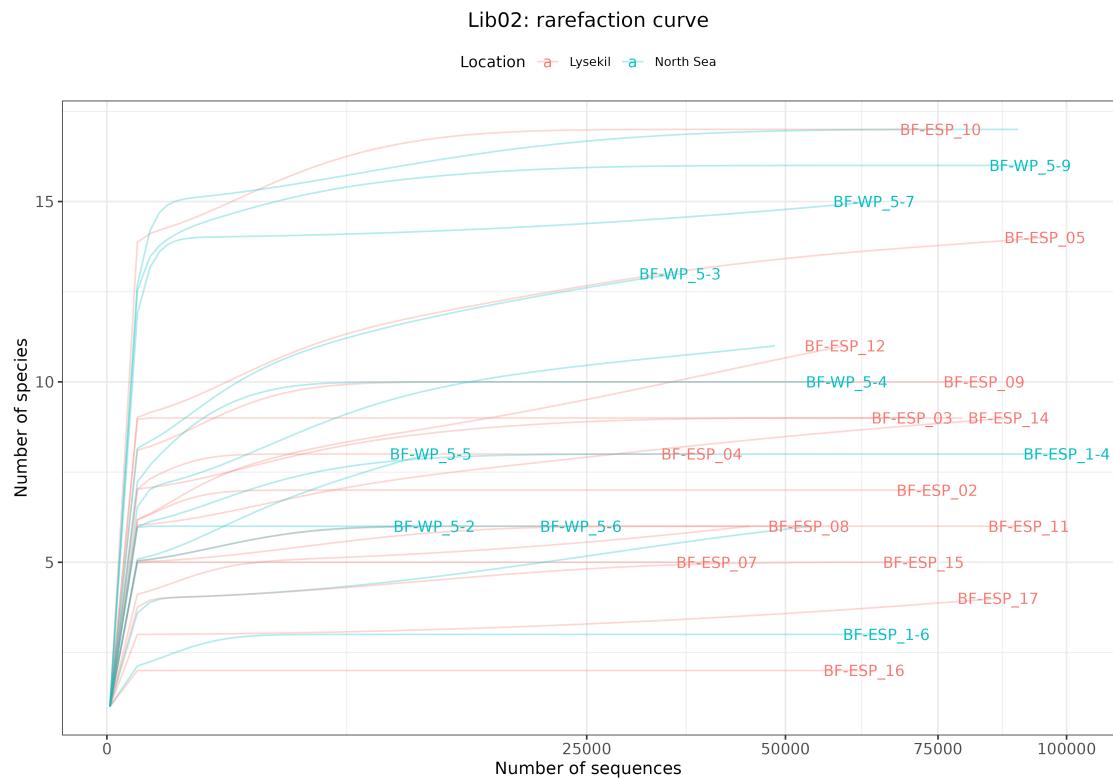


Figure A.2: Rarefaction curve for Lib02.

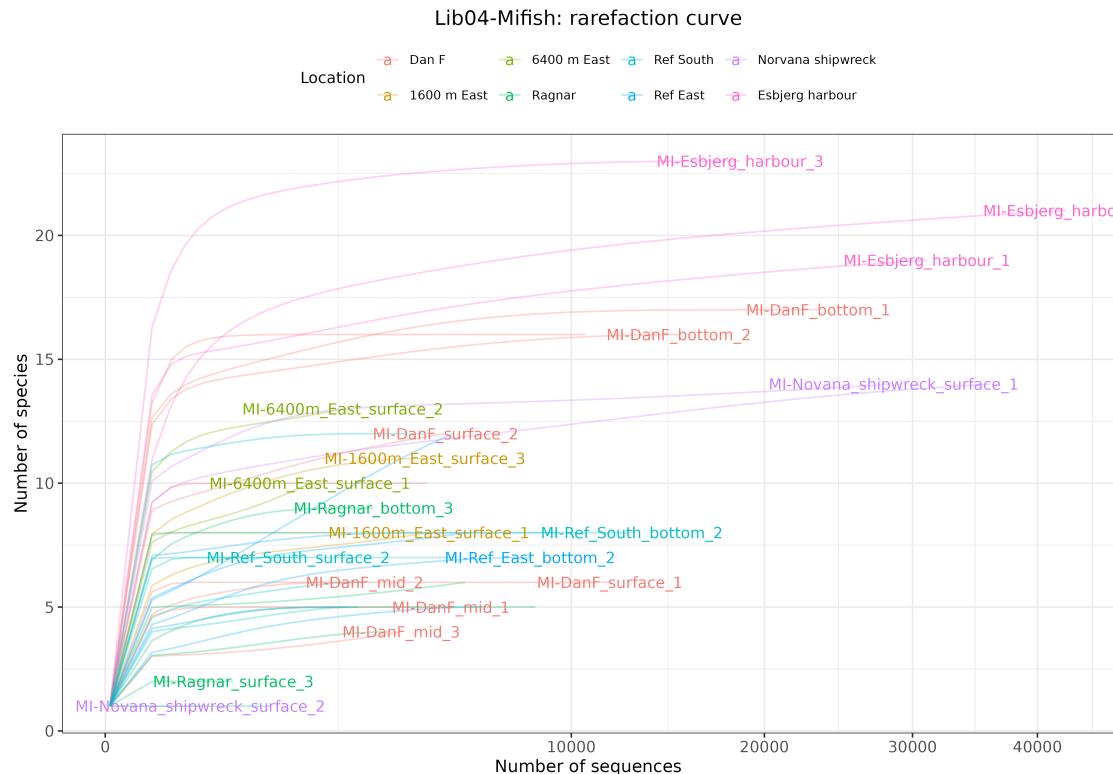


Figure A.3: Rarefaction curve for Lib04.

B Appendix II - Clustering of spatial data

This is an overview of the spatial data collected in Lib04. Here, it is tougher to define clear clusters. Many deployment sites in the North Sea were close to each other, showing little variation.

Three sub-clusters are well-defined: Esbjerg harbour, Norvana shipwreck, and the bottom samples from Dan F. These areas are distant from one another and have unique environmental compositions.

All the other samples represent similar ecosystems, making it challenging to separate them using the Jaccard index. In addition, surface and bottom samples are known to have different species concentrations.

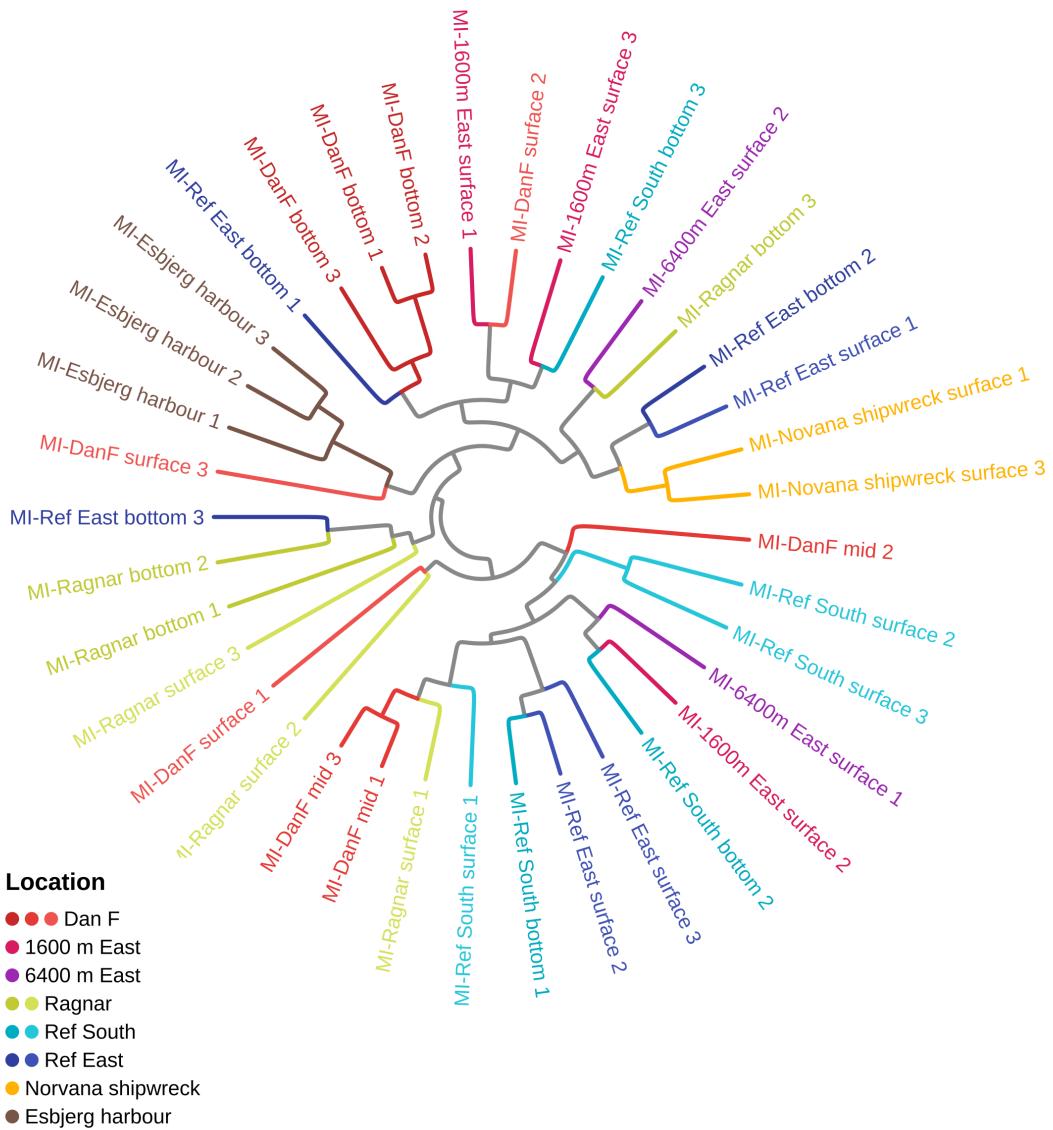


Figure B.1: Dendrogram based on the Jaccard dissimilarities for the spatial data from the North Sea (Lib04).

C Appendix III - Selected species for the analysis

Table C.1: List of acceptable taxa that were included as part of the reference database for the metabarcoding analysis.

TaxId	Scientific name	Common name	Category
7748	<i>Lampetra fluviatilis</i>	European river lamprey	Fishes
7757	<i>Petromyzon marinus</i>	Sea lamprey	Fishes
7769	<i>Myxine glutinosa</i>	Atlantic hagfish	Fishes
7781	<i>Raja clavata</i>	Thornback ray	Fishes
7788	<i>Torpedo marmorata</i>	Marbled electric ray	Fishes
7797	<i>Squalus acanthias</i>	Spiny dogfish	Fishes
7815	<i>Prionace glauca</i>	Blue shark	Fishes
7830	<i>Scyliorhinus canicula</i>	Smaller spotted catshark	Fishes
7849	<i>Lamna nasus</i>	NA	Fishes
7852	<i>Alopias vulpinus</i>	Thresher shark	Fishes
7871	<i>Chimaera monstrosa</i>	Rabbit fish	Fishes
7902	<i>Acipenser gueldenstaedtii</i>	Russian sturgeon	Fishes
7903	<i>Acipenser stellatus</i>	Stellate sturgeon	Fishes
7936	<i>Anguilla anguilla</i>	European eel	Fishes
7950	<i>Clupea harengus</i>	Atlantic herring	Fishes
8017	<i>Oncorhynchus gorbuscha</i>	Pink salmon	Fishes
8019	<i>Oncorhynchus kisutch</i>	Coho salmon	Fishes
8022	<i>Oncorhynchus mykiss</i>	Rainbow trout	Fishes
8030	<i>Salmo salar</i>	Atlantic salmon	Fishes
8032	<i>Salmo trutta</i>	River trout	Fishes
8036	<i>Salvelinus alpinus</i>	Arctic char	Fishes
8049	<i>Gadus morhua</i>	Atlantic cod	Fishes
8056	<i>Melanogrammus aeglefinus</i>	Haddock	Fishes
8058	<i>Merlangius merlangus</i>	Whiting	Fishes
8060	<i>Pollachius virens</i>	Saithe	Fishes
8063	<i>Merluccius merluccius</i>	European hake	Fishes
8074	<i>Lophius piscatorius</i>	Angler	Fishes
8097	<i>Myoxocephalus scorpius</i>	Shorthorn sculpin	Fishes
8103	<i>Cyclopterus lumpus</i>	Lumpfish	Fishes
8175	<i>Sparus aurata</i>	Gilthead seabream	Fishes
8204	<i>Anarhichas lupus</i>	Atlantic wolffish	Fishes
8226	<i>Katsuwonus pelamis</i>	Skipjack tuna	Fishes
8228	<i>Euthynnus alletteratus</i>	Little tunny	Fishes
8232	<i>Sarda sarda</i>	Atlantic bonito	Fishes
8237	<i>Thunnus thynnus</i>	Atlantic bluefin tuna	Fishes
8245	<i>Xiphias gladius</i>	Swordfish	Fishes
8260	<i>Platichthys flesus</i>	European flounder	Fishes
8262	<i>Pleuronectes platessa</i>	European plaice	Fishes
8267	<i>Hippoglossus hippoglossus</i>	Atlantic halibut	Fishes

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Table C.1 – Continued from previous page

TaxId	Scientific name	Common name	Category
13225	<i>Pomatoschistus minutus</i>	Sand goby	Fishes
13489	<i>Dicentrarchus labrax</i>	European seabass	Fishes
13677	<i>Scomber scombrus</i>	Atlantic mackerel	Fishes
27588	<i>Raja montagui</i>	Spotted ray	Fishes
27590	<i>Tetronarce nobiliana</i>	Atlantic torpedo	Fishes
27689	<i>Acipenser baerii</i>	Siberian sturgeon	Fishes
27697	<i>Sardina pilchardus</i>	Sardine	Fishes
27722	<i>Trisopterus luscus</i>	NA	Fishes
27771	<i>Limanda limanda</i>	Common dab	Fishes
29151	<i>Osmerus eperlanus</i>	European smelt	Fishes
29154	<i>Micropogonias undulatus</i>	Atlantic croaker	Fishes
30804	<i>Chelon ramada</i>	Thin-lipped mullet	Fishes
34785	<i>Callionymus lyra</i>	Dragonet	Fishes
34817	<i>Hippoglossoides platessoides</i>	American plaice	Fishes
34819	<i>Glyptocephalus cynoglossus</i>	Witch	Fishes
36177	<i>Acipenser oxyrinchus</i>	Atlantic sturgeon	Fishes
36212	<i>Trachurus trachurus</i>	Atlantic horse mackerel	Fishes
36219	<i>Boops boops</i>	NA	Fishes
37007	<i>Mullus barbatus</i>	Red mullet	Fishes
38941	<i>Diplodus sargus</i>	White seabream	Fishes
47308	<i>Neogobius melanostomus</i>	Round goby	Fishes
48171	<i>Chelon labrosus</i>	Thicklip grey mullet	Fishes
48191	<i>Chelon auratus</i>	Golden grey mullet	Fishes
48416	<i>Zoarces viviparus</i>	Viviparous blenny	Fishes
50593	<i>Pagellus erythrinus</i>	Common pandora	Fishes
50595	<i>Spondyliosoma cantharus</i>	Black seabream	Fishes
52904	<i>Scophthalmus maximus</i>	Turbot	Fishes
56720	<i>Lipophrys pholis</i>	Shanny	Fishes
56723	<i>Labrus bergylta</i>	Ballan wrasse	Fishes
56726	<i>Pholis gunnellus</i>	Rock gunnel	Fishes
56737	<i>Trachinus draco</i>	Greater weever	Fishes
57982	<i>Cetorhinus maximus</i>	NA	Fishes
61643	<i>Taurulus bubalis</i>	NA	Fishes
61674	<i>Acipenser sturio</i>	Sturgeon	Fishes
61971	<i>Huso huso</i>	Beluga	Fishes
64108	<i>Zeus faber</i>	John dory	Fishes
65125	<i>Scophthalmus rhombus</i>	Brill	Fishes
68454	<i>Scyliorhinus stellaris</i>	Nursehound	Fishes
68502	<i>Maurolicus muelleri</i>	Pearlsides	Fishes
69293	<i>Gasterosteus aculeatus</i>	Three-spined stickleback	Fishes
77914	<i>Galeus melastomus</i>	Blackmouth catshark	Fishes
80722	<i>Trisopterus minutus</i>	Poor cod	Fishes
80723	<i>Trisopterus esmarkii</i>	Norway pout	Fishes
81370	<i>Lampris guttatus</i>	North Atlantic opah	Fishes
81389	<i>Regalecus glesne</i>	King of herrings	Fishes
81636	<i>Micromesistius poutassou</i>	Blue whiting	Fishes
81638	<i>Brosme brosme</i>	NA	Fishes

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Table C.1 – Continued from previous page

TaxId	Scientific name	Common name	Category
81640	<i>Enchelyopus cimbrius</i>	Fourbeard rockling	Fishes
82655	<i>Conger conger</i>	European conger	Fishes
84621	<i>Ammodytes tobianus</i>	Small sandeel	Fishes
85417	<i>Gobius niger</i>	Black goby	Fishes
86063	<i>Galeorhinus galeus</i>	Tope shark	Fishes
87757	<i>Mullus surmuletus</i>	Striped red mullet	Fishes
87759	<i>Sarpa salpa</i>	NA	Fishes
90068	<i>Pegusa lascaris</i>	Sand sole	Fishes
90069	<i>Solea solea</i>	Common sole	Fishes
90071	<i>Buglossidium luteum</i>	Solenette	Fishes
90074	<i>Microchirus variegatus</i>	Thickback sole	Fishes
94237	<i>Mola mola</i>	Ocean sunfish	Fishes
94984	<i>Echiichthys vipera</i>	Lesser weever	Fishes
94987	<i>Hexanchus griseus</i>	NA	Fishes
98815	<i>Dentex maroccanus</i>	Morocco dentex	Fishes
98817	<i>Pagellus acarne</i>	Axillary seabream	Fishes
98819	<i>Pagellus bogaraveo</i>	Blackspot seabream	Fishes
98928	<i>Leuciscus aspius</i>	NA	Fishes
106175	<i>Microstomus kitt</i>	Lemon sole	Fishes
107241	<i>Beryx decadactylus</i>	Alfonsino	Fishes
109282	<i>Hippocampus guttulatus</i>	Long-snouted seahorse	Fishes
109283	<i>Hippocampus hippocampus</i>	Short-snouted seahorse	Fishes
112230	<i>Mustelus mustelus</i>	Smooth-hound	Fishes
112232	<i>Mustelus asterias</i>	Starry smooth-hound	Fishes
112728	<i>Polyprion americanus</i>	Wreckfish	Fishes
114866	<i>Myctophum punctatum</i>	Spotted lanternfish	Fishes
118170	<i>Nemichthys scolopaceus</i>	Slender snipe eel	Fishes
125806	<i>Notoscopelus elongatus kroyeri</i>	NA	Fishes
126330	<i>Coris julis</i>	Mediterranean rainbow wrasse	Fishes
129037	<i>Belone belone</i>	NA	Fishes
129061	<i>Scomberesox saurus</i>	NA	Fishes
134920	<i>Pungitius pungitius</i>	Ninespine stickleback	Fishes
146480	<i>Ammodytes marinus</i>	Lesser sand-eel	Fishes
154550	<i>Lepidorhombus whiffiagonis</i>	Megrim	Fishes
159077	<i>Nerophis ophidion</i>	Straightnose pipefish	Fishes
159850	<i>Pomatoschistus lozanoi</i>	Lozano's goby	Fishes
161455	<i>Entelurus aequoreus</i>	Snake pipefish	Fishes
161584	<i>Syngnathus acus</i>	Greater pipefish	Fishes
161589	<i>Syngnathus rostellatus</i>	Nilsson's pipefish	Fishes
161592	<i>Syngnathus typhle</i>	Broad-nosed pipefish	Fishes
163112	<i>Molva molva</i>	Ling	Fishes
163115	<i>Phycis blennoides</i>	NA	Fishes
163117	<i>Raniceps raninus</i>	Tadpole fish	Fishes
163118	<i>Coryphaenoides rupestris</i>	Roundnose grenadier	Fishes
170817	<i>Dalatias licha</i>	Kitefin shark	Fishes
171732	<i>Centrolabrus exoletus</i>	Rock cook	Fishes
171735	<i>Ctenolabrus rupestris</i>	Goldsinny wrasse	Fishes

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Table C.1 – Continued from previous page

TaxId	Scientific name	Common name	Category
171736	<i>Syphodus melops</i>	Corkwing wrasse	Fishes
172269	<i>Argyrosomus regius</i>	Meagre	Fishes
173339	<i>Trachinotus ovatus</i>	Derbio	Fishes
182854	<i>Dipturus oxyrinchus</i>	Longnosed skate	Fishes
184585	<i>Engraulis encrasicolus</i>	European anchovy	Fishes
185738	<i>Molva dypterygia</i>	Blue ling	Fishes
185739	<i>Pollachius pollachius</i>	Pollack	Fishes
191678	<i>Lophius budegassa</i>	Black-bellied angler	Fishes
191813	<i>Somniosus microcephalus</i>	Greenland sleeper shark	Fishes
196075	<i>Sprattus sprattus</i>	European sprat	Fishes
202727	<i>Helicolenus dactylopterus</i>	NA	Fishes
206100	<i>Capros aper</i>	Boarfish	Fishes
206124	<i>Macroramphosus scolopax</i>	Longspine snipefish	Fishes
206126	<i>Spinachia spinachia</i>	Sea stickleback	Fishes
208529	<i>Arnoglossus laterna</i>	Scaldfish	Fishes
213384	<i>Leucoraja circularis</i>	Sandy ray	Fishes
215414	<i>Lycenchelys sarsi</i>	Sar's wolf eel	Fishes
215424	<i>Lycodes vahlii</i>	Vahl's eelpout	Fishes
217026	<i>Auxis rochei</i>	NA	Fishes
223862	<i>Raja brachyura</i>	Blonde ray	Fishes
223888	<i>Leucoraja naevus</i>	Cuckoo ray	Fishes
223889	<i>Leucoraja fullonica</i>	Shagreen ray	Fishes
225753	<i>Pomatoschistus microps</i>	Common goby	Fishes
257537	<i>Pomatoschistus pictus</i>	Painted goby	Fishes
257538	<i>Pomatoschistus norvegicus</i>	Norway goby	Fishes
257540	<i>Gobiusculus flavescens</i>	NA	Fishes
263718	<i>Squatina squatina</i>	Angelshark	Fishes
268865	<i>Aphanopus carbo</i>	Black scabbardfish	Fishes
278164	<i>Alosa alosa</i>	Allis shad	Fishes
278165	<i>Alosa fallax</i>	Twaite shad	Fishes
303685	<i>Balistes capriscus</i>	Grey triggerfish	Fishes
303921	<i>Atherina presbyter</i>	Sand smelt	Fishes
305631	<i>Remora remora</i>	Common remora	Fishes
316130	<i>Centrolophus niger</i>	Blackfish	Fishes
319769	<i>Magnisudis atlantica</i>	Duckbill barracudina	Fishes
335025	<i>Deania calcea</i>	Birdbeak dogfish	Fishes
335027	<i>Centrophorus squamosus</i>	Leafscale gulper shark	Fishes
335028	<i>Centroscymnus crepidater</i>	Longnose velvet dogfish	Fishes
348057	<i>Gaidropsarus vulgaris</i>	Three-bearded rockling	Fishes
348443	<i>Ciliata mustela</i>	Fivebeard rockling	Fishes
349651	<i>Brama brama</i>	Atlantic pomfret	Fishes
372798	<i>Nesiarchus nasutus</i>	Black gemfish	Fishes
386614	<i>Amblyraja radiata</i>	Thorny skate	Fishes
394699	<i>Sebastes norvegicus</i>	Golden redfish	Fishes
394708	<i>Sebastes viviparus</i>	Norway redfish	Fishes
414887	<i>Trachipterus arcticus</i>	Dealfish	Fishes
417956	<i>Rajella fyllae</i>	Round ray	Fishes

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Table C.1 – Continued from previous page

TaxId	Scientific name	Common name	Category
420460	<i>Dipturus batis</i>	Blue skate	Fishes
425520	<i>Anarhichas denticulatus</i>	Northern wolffish	Fishes
426094	<i>Chelidonichthys cuculus</i>	Red gurnard	Fishes
426098	<i>Eutrigla gurnardus</i>	Grey gurnard	Fishes
426100	<i>Gaidropsarus mediterraneus</i>	Shore rockling	Fishes
430084	<i>Trigla lyra</i>	NA	Fishes
441899	<i>Aphia minuta</i>	Transparent goby	Fishes
441903	<i>Crystallogobius linearis</i>	Crystal goby	Fishes
441905	<i>Lesueurigobius friesii</i>	Fries's goby	Fishes
442169	<i>Argentina sphyraena</i>	European argentine	Fishes
446415	<i>Argentina silus</i>	Greater argentine	Fishes
446792	<i>Liparis liparis</i>	Striped seasnail	Fishes
446793	<i>Liparis montagui</i>	Montagu's sea snail	Fishes
455296	<i>Centroscymnus coelolepis</i>	Portuguese dogfish	Fishes
473298	<i>Argyropelecus olfersii</i>	NA	Fishes
473364	<i>Etmopterus princeps</i>	Great lanternshark	Fishes
508551	<i>Callionymus maculatus</i>	Spotted dragonet	Fishes
508552	<i>Callionymus reticulatus</i>	Reticulated dragonet	Fishes
508553	<i>Ciliata septentrionalis</i>	Northern rockling	Fishes
508554	<i>Labrus mixtus</i>	Cuckoo wrasse	Fishes
508607	<i>Agonus cataphractus</i>	Hooknose	Fishes
508657	<i>Hyperoplus lanceolatus</i>	Great sand-eel	Fishes
508659	<i>Zeugopterus norvegicus</i>	Norwegian topknot	Fishes
509777	<i>Paralepis coregonoides</i>	Sharpchin barracudina	Fishes
526623	<i>Zeugopterus punctatus</i>	Topknot	Fishes
546515	<i>Cheilopogon heterurus</i>	Mediterranean flyingfish	Fishes
546530	<i>Nerophis lumbriciformis</i>	Worm pipefish	Fishes
546535	<i>Schedophilus medusophagus</i>	Cornish blackfish	Fishes
557337	<i>Bathyraja spinicauda</i>	Spinetail ray	Fishes
630738	<i>Malacocephalus laevis</i>	Softhead grenadier	Fishes
648166	<i>Etmopterus spinax</i>	Velvet belly lantern shark	Fishes
651723	<i>Pteroplatytrygon violacea</i>	Pelagic stingray	Fishes
673854	<i>Dasyatis pastinaca</i>	Common stingray	Fishes
674131	<i>Coregonus maraena</i>	Maraena whitefish	Fishes
862826	<i>Echinorhinus brucus</i>	Bramble shark	Fishes
980323	<i>Artediellus atlanticus</i>	Atlantic hookear sculpin	Fishes
980417	<i>Leptoclinus maculatus</i>	NA	Fishes
990611	<i>Myliobatis aquila</i>	Common eagle ray	Fishes
1016941	<i>Oxynotus centrina</i>	Angular roughshark	Fishes
1266874	<i>Lumpenus lampretaeformis</i>	Snakeblenny	Fishes
1266884	<i>Triglops murrayi</i>	Moustache sculpin	Fishes
1306753	<i>Gymnammodytes semisquamatus</i>	Smooth sandeel	Fishes
1365585	<i>Thorogobius ephippiatus</i>	Leopard-spotted goby	Fishes
1365694	<i>Lebetus guilleti</i>	Guillet's goby	Fishes
1482869	<i>Gadiculus argenteus thori</i>	Silvery pout	Fishes
1484357	<i>Hyperoplus immaculatus</i>	Greater sand-eel	Fishes
1552233	<i>Buenia jeffreysii</i>	Jeffrey's goby	Fishes

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Table C.1 – Continued from previous page

TaxId	Scientific name	Common name	Category
1819258	<i>Echiodon drummondii</i>	NA	Fishes
2014788	<i>Lebetus scorpioides</i>	NA	Fishes
2576622	<i>Chelidonichthys lucerna</i>	Tub gurnard	Fishes
2719052	<i>Chirolophis ascanii</i>	NA	Fishes
2719090	<i>Rajella lineata</i>	Sailray	Fishes
2814718	<i>Dipturus intermedius</i>	NA	Fishes
2821466	<i>Micrenophrys lilljeborgii</i>	NA	Fishes
8879	<i>Mergus serrator</i>	NA	Birds
13746	<i>Uria aalge</i>	Common murre	Birds
28689	<i>Alca torda</i>	Razorbill	Birds
28691	<i>Alle alle</i>	NA	Birds
28701	<i>Fratercula arctica</i>	Atlantic puffin	Birds
30455	<i>Fulmarus glacialis</i>	Northern fulmar	Birds
37050	<i>Podiceps auritus</i>	NA	Birds
37051	<i>Puffinus gravis</i>	Great shearwater	Birds
37052	<i>Puffinus griseus</i>	Sooty shearwater	Birds
48670	<i>Puffinus assimilis</i>	NA	Birds
48681	<i>Puffinus mauretanicus</i>	NA	Birds
48688	<i>Puffinus puffinus</i>	NA	Birds
52122	<i>Calonectris diomedea</i>	Scopoli's shearwater	Birds
52124	<i>Oceanodroma leucorhoa</i>	Leach's storm-petrel	Birds
57069	<i>Gavia arctica</i>	NA	Birds
76058	<i>Somateria mollissima</i>	Common eider	Birds
79651	<i>Hydrobates pelagicus</i>	European storm-petrel	Birds
79653	<i>Oceanites oceanicus</i>	Wilson's storm-petrel	Birds
126723	<i>Sterna sandvicensis</i>	Sandwich tern	Birds
126865	<i>Gelochelidon nilotica</i>	NA	Birds
197941	<i>Clangula hyemalis</i>	Long-tailed duck	Birds
198027	<i>Melanitta nigra</i>	European common scoter	Birds
345573	<i>Podiceps cristatus</i>	Great crested grebe	Birds
371864	<i>Melanitta fusca</i>	Velvet scoter	Birds
1323832	<i>Calonectris borealis</i>	Cory's shearwater	Birds
9707	<i>Odobenus rosmarus</i>	Walrus	Marine mammals
9711	<i>Halichoerus grypus</i>	Gray seal	Marine mammals
9718	<i>Pusa hispida</i>	Ringed seal	Marine mammals
9720	<i>Phoca vitulina</i>	Harbor seal	Marine mammals
9728	<i>Delphinus delphis</i>	Saddleback dolphin	Marine mammals
9731	<i>Globicephala melas</i>	Long-finned pilot whale	Marine mammals
9733	<i>Orcinus orca</i>	Killer whale	Marine mammals
9737	<i>Stenella coeruleoalba</i>	Striped dolphin	Marine mammals
9739	<i>Tursiops truncatus</i>	Common bottlenose dolphin	Marine mammals
9742	<i>Phocoena phocoena</i>	Harbor porpoise	Marine mammals
9749	<i>Delphinapterus leucas</i>	Beluga whale	Marine mammals
9755	<i>Physeter macrocephalus</i>	Sperm whale	Marine mammals
9760	<i>Ziphius cavirostris</i>	Cuvier's beaked whale	Marine mammals
9767	<i>Balaenoptera acutorostrata</i>	Minke whale	Marine mammals
9768	<i>Balaenoptera borealis</i>	Sei whale	Marine mammals

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Table C.1 – Continued from previous page

TaxId	Scientific name	Common name	Category
9770	<i>Balaenoptera physalus</i>	Fin whale	Marine mammals
9771	<i>Balaenoptera musculus</i>	Blue whale	Marine mammals
9773	<i>Megaptera novaeangliae</i>	Humpback whale	Marine mammals
27606	<i>Eubalaena glacialis</i>	North Atlantic right whale	Marine mammals
27610	<i>Lagenorhynchus albirostris</i>	White-beaked dolphin	Marine mammals
27615	<i>Kogia breviceps</i>	Pygmy sperm whale	Marine mammals
39293	<i>Cystophora cristata</i>	Hooded seal	Marine mammals
48744	<i>Hyperoodon ampullatus</i>	Northern bottlenose whale	Marine mammals
48745	<i>Mesoplodon bidens</i>	Sowerby's beaked whale	Marine mammals
90246	<i>Lagenorhynchus acutus</i>	Atlantic white-sided dolphin	Marine mammals
9031	<i>Gallus gallus</i>	Chicken	Contamination
9606	<i>Homo sapiens</i>	Human	Contamination
9796	<i>Equus caballus</i>	Horse	Contamination
9823	<i>Sus scrofa</i>	Pig	Contamination
9913	<i>Bos taurus</i>	Domestic cattle	Contamination
9940	<i>Ovis aries</i>	Sheep	Contamination
30501	<i>Carcharias taurus</i>	Sand tiger shark	Positive control
54563	<i>Rutilus frisii</i>	Common roach	Positive control
563643	<i>Rutilus pigus</i>	Danube roach	Positive control
48668	<i>Rutilus rutilus</i>	Roach minnow	Positive control

D Appendix IV - Assay table

Figure D.1: Information on all qPCR assays used for eDNA analysis. Concentrations of primers and probes can be found in the relevant papers or obtained by requesting them from the supervisors.

Species english name	Species latin name	Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')	Reference
Atlantic tuna	<i>Thunnus thynnus</i>	cytB	GAATTCAGGCATTAACGAG	TGGACTGGATGGTAGGCTCT	CCAAAGTCTCTCATCTCTGACATCTCGT	DTU Aqua, unpublished
Antlantic mackerel	<i>Scomber scombrus</i>	cytB	TTCCTCTGCTTGCTCTGT	GCGGACTGAGTTGATGCTG	TTC CAAATCCTCACAGGACTATTC	Knudsen et al. 2018
Garfish	<i>Belone belone</i>	cytB	GCAGCCCTACTAACTCAGCT	AACTAATGGTTGGCTGGGG	TTCGCCAAATCTCTTAAAGTGACCCGG	DTU Aqua, unpublished
Harbor porpoise	<i>Phocoena phocoena</i>	CytB	GTTCCTCTATTGCTTTATATCCATATTG	GCACCTCTAAAATGATTTGTC	CTATGACTTTAGTAGTAAGAGCACCC	DTU Aqua, unpublished
Antlantic mackerel	<i>Scomber scombrus</i>	cytB	TTCCTCTGCTTGCTCTGT	GCGGACTGAGTTGATGCTG	TTC CAAATCCTCACAGGACTATTC	Knudsen et al. 2018
Whiting	<i>Merlangius merlangus</i>	COI	CTCTACTTGGTTTCCTCGTA	TCTCCCTAGTAGATTAGGGCA	ACTCTTAGGCTTAAC TGCTCTGGCCCT	Hansen et al. 2020
Pacific Oyster	<i>Magallana gigas</i>	COI	TTGAGTTTGGCAGGGCTC	ACCAGCAAGGTGAGGCTTA	AACATTGTAGAAACGGAGTTGGGC	Knudsen et al. 2022
Warty comb jelly	<i>Mnemiopsis leidyi</i>	COI	ACGGTCCCTTGAGTAGAGC	TCTGAGAAAGGCTCGGACAT	GTGCTCTCTGGTGTGAGCAATATCT	Knudsen et al. 2022
Rainbow trout	<i>Oncorhynchus mykiss</i>	ITS2	ACCTCCAGCCATCTCTCAGT	AGGACGGGGAGGGAAAGTAA	TGAGCCGTCTAGTTACTTGCTGTCTT	Knudsen et al. 2022

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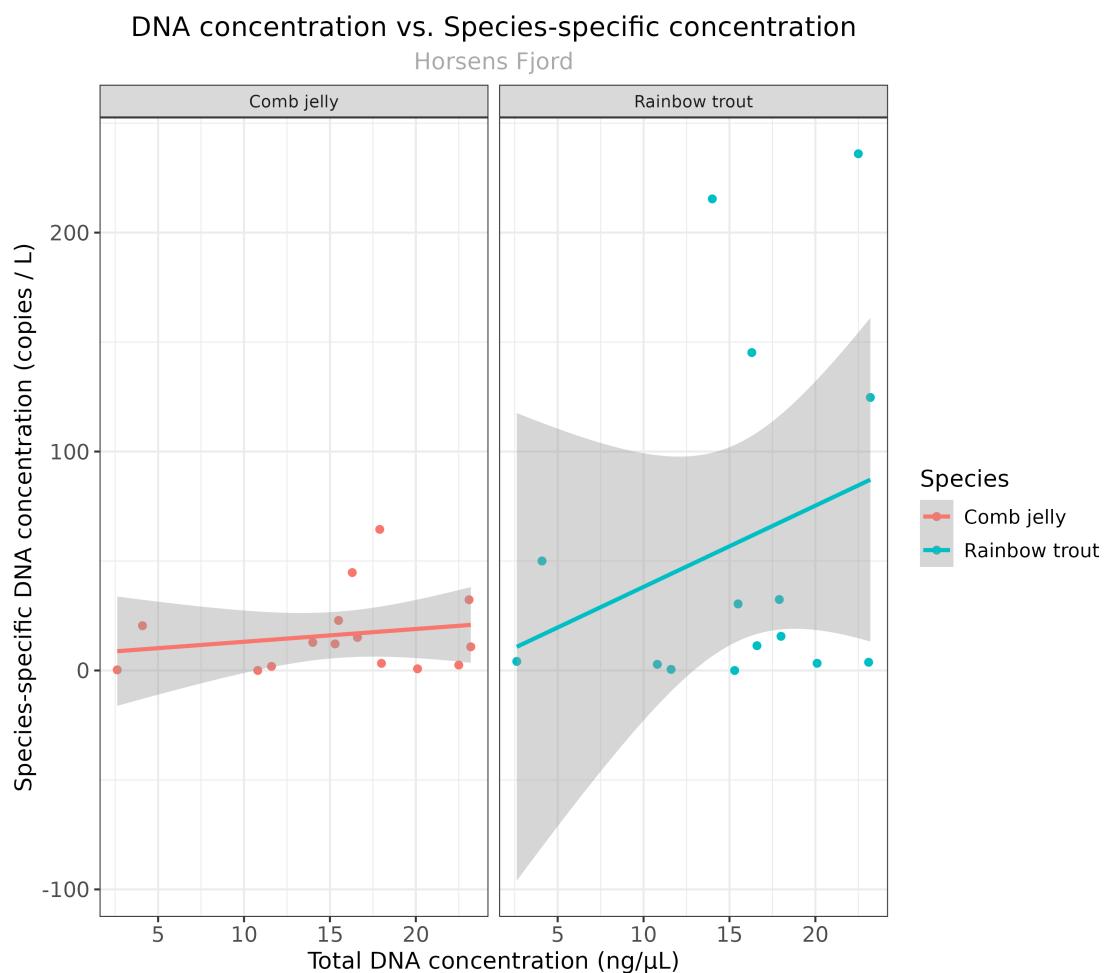
E Appendix V - ESP samples table

Figure E.1: List of all ESP samples collected from the various deployment sites.

Sample name	Sample type ESP	Sample type	Date	Start Time	Sample volume (ml)	DNA conc (ng/ml)	qPCR analyses	Metabarcoding
ESP01	Premissioning control	Premissioning control	15/09/2018	06:00	300	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP02	Stidphase	On-site sample, ESP, Lyskull, Sweden	01/10/2018	20:00	1425	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP03	Coreleg	On-site sample, ESP, Lyskull, Sweden	02/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP04	Stidphase	On-site sample, ESP, Lyskull, Sweden	03/10/2018	16:00	1225	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP05	Stidphase	On-site sample, ESP, Lyskull, Sweden	04/10/2018	17:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP06	Stidphase	On-site sample, ESP, Lyskull, Sweden	05/10/2018	16:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP07	Stidphase	On-site sample, ESP, Lyskull, Sweden	06/10/2018	16:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP08	Stidphase	On-site sample, ESP, Lyskull, Sweden	07/10/2018	17:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP09	Stidphase	On-site sample, ESP, Lyskull, Sweden	08/10/2018	16:00	1150	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP10	Stidphase	On-site sample, ESP, Lyskull, Sweden	09/10/2018	16:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP11	Stidphase	On-site sample, ESP, Lyskull, Sweden	11/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP12	Stidphase	On-site sample, ESP, Lyskull, Sweden	13/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP13	Stidphase	On-site sample, ESP, Lyskull, Sweden	15/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP14	Stidphase	On-site sample, ESP, Lyskull, Sweden	17/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP15	Coreleg	On-site sample, ESP, Lyskull, Sweden	18/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP16	Stidphase	On-site sample, ESP, Lyskull, Sweden	19/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP17	Stidphase	On-site sample, ESP, Lyskull, Sweden	21/10/2018	15:00	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP18	Coreleg	Post-mission negative control	20/11/2018	NA	500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP19	Coreleg	Post-mission negative control	24/11/2018	NA	500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP20	Stidphase	post-mission positive control, Hirshals aquarium	NA	NA	1500	N/A	Atlantic tunc, Garfish, Atlantic mackerel, Atlantic bonito	Mifish (bony fishes), Elas02 (Easmobranchs)
ESP-1	Coreleg	Premission negative control	17/05/2019	NA	1000	Out of range	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
ESP-2	Coreleg	Premission negative control	05/09/2019	NA	100	Out of range	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
ESP-1.3	StidPhase	On-site sample, ESP, North Sea, Darrf	10/09/2019	22:46	1900	112	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
ESP-1.4	StidPhase	On-site sample, ESP, North Sea, Darrf	11/09/2019	22:22	2000	92.1	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
ESP-1.5	archive	On-site sample, ESP, North Sea, Darrf	12/09/2019	20:12	1700	124	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
ESP-1.6	archive	On-site sample, ESP, North Sea, Darrf	13/09/2019	00:12	1550	107	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
ESP-1.7	Coreleg	Postmission negative control	05/09/2019	NA	1000	Out of range	Harbor porpoise, Whiting, Atlantic mackerel	Mifish (bony fishes), Elas02 (Easmobranchs), Mamm02 (Marine mammals)
WCR4944	General activation	Premission control	NA	NA	NA	Out of range	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR8012	Stidphase	On-site sample, ESP, South of Horsensford	14/05/2020	14:54	425	17.9	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR8026	Stidphase	On-site sample, ESP, South of Horsensford	15/05/2020	10:00	475	14	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR8030	Stidphase	On-site sample, ESP, South of Horsensford	16/05/2020	10:00	600	15.3	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR8032	Stidphase	On-site sample, ESP, South of Horsensford	17/05/2020	10:00	575	16.3	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR8025	Stidphase	On-site sample, ESP, South of Horsensford	18/05/2020	10:00	625	23.1	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR1005	Stidphase	On-site sample, ESP, South of Horsensford	19/05/2020	10:00	475	15.5	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR510029	Stidphase	On-site sample, ESP, South of Horsensford	20/05/2020	10:00	150	4.09	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR111015	Stidphase	On-site sample, ESP, South of Horsensford	20/05/2020	17:34	325	10.8	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR3024	Stidphase	On-site sample, ESP, South of Horsensford	20/05/2020	22:00	300	12.1	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR510021	Stidphase	On-site sample, ESP, South of Horsensford	21/05/2020	04:00	300	16.6	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR8003	Coreleg	On-site sample, ESP, South of Horsensford	21/05/2020	10:00	300	16.8	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR203	Stidphase	On-site sample, ESP, South of Horsensford	22/05/2020	10:00	375	22.5	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR2076	Stidphase	On-site sample, ESP, South of Horsensford	23/05/2020	10:00	350	18	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR1002	Stidphase	On-site sample, ESP, South of Horsensford	24/05/2020	10:00	450	20.1	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR4002	Stidphase	On-site sample, ESP, South of Horsensford	25/05/2020	10:00	450	11.6	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCRB0007	Stidphase	On-site sample, ESP, South of Horsensford	26/05/2020	10:00	400	23.2	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCRB0033	Stidphase	On-site sample, ESP, South of Horsensford	27/05/2020	10:00	75	2.62	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR510006	Coreleg	On-site sample, ESP, South of Horsensford	27/05/2020	22:00	300.00	8.38	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR4059	Stidphase	On-site sample, ESP, South of Horsensford	27/05/2020	04:00	225.00	10.8	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR2065	Stidphase	On-site sample, ESP, South of Horsensford	28/05/2020	10:00	225.00	10.8	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR5-03	Coreleg	On-site sample, ESP, South of Horsensford	28/05/2020	10:00	9.62	1.11	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR10006	Coreleg post-can	Premission negative control	NA	NA	NA	Out of range	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)
WCR10068	Coreleg	Premission negative control	NA	NA	NA	Out of range	Pacific Oyster, Warty comb jelly, rainbow trout	Mifish (bony fishes), Elas02 (Easmobranchs)

F Appendix VI - Correlation between species-specific DNA concentration and total DNA mass

Figure F.1: Correlation between the total DNA concentration per sample (X) and the copy number for each species (Y). No direct link between the two variables was detected.



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