UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries, and Economics

Evaluation of blue mussel *(Mytilus edulis)* as a natural sampler of environmental DNA and pathogens in aquaculture

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Abstract

In recent years analysis of environmental DNA has shown promising results for biodiversity monitoring. The area of applicability for eDNA-based methods is extending and has been proposed as an approach to detect and monitor pathogens in aquaculture. Filter feeding organisms have been advocated as ideal candidates as natural samplers of eDNA due to their ability to efficiently filter water. Several studies have been performed to evaluate the potential of natural samplers, but mainly their ability to detect eDNA from fish species in comparison to visual or traditional surveys. In this study, eDNA from blue mussel samples and water samples collected near an aquaculture site for 15 months were amplified and sequenced with the Leray XT primer set for COI to compare the eDNA diversity obtained from the two sampling methods and their ability to detect pathogens and harmful species. In the blue mussel samples, 718 species were detected which was lower than half the number of species detected in the water samples (2250), and a significant difference in the eDNA community retrieved with the two sampling methods was observed. Seasonal signals between samples collected in the "summer months" (May-October) and "winter months" (November-April) were detected in both blue mussel samples and water samples, but the three main species that contributed most to these signals were different for the two sampling methods. The water samples had a higher species richness and abundance in all pathogens detected except for one species, *Paramoeba sp*. Even though the species richness and abundance were greater in the water samples than in the blue mussel samples, the blue mussels have shown the ability to detect eDNA in seawater and that they can be used as natural samplers. The blue mussels present an opportunity to easily avoid the processing and filtration that aquatic eDNA samples require, but further investigation of which species the blue mussels are able to detect will provide information on where the use of blue mussels as natural samplers could be most suitable.

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Acknowledgement

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1 Introduction

Farming of Atlantic Salmon has become an important industry in Norway and is one of the most exported goods from Norway. The aquaculture industry in Norway began in the 1970s, and since then Norway has grown to become the world's largest exporter of Atlantic Salmon. Between the 1970s and today, the industry has experienced a massive increase in production, from 640 tons in 1971 (Berge, 2002) to 1.28 million tons in 2021 (Figure 1).

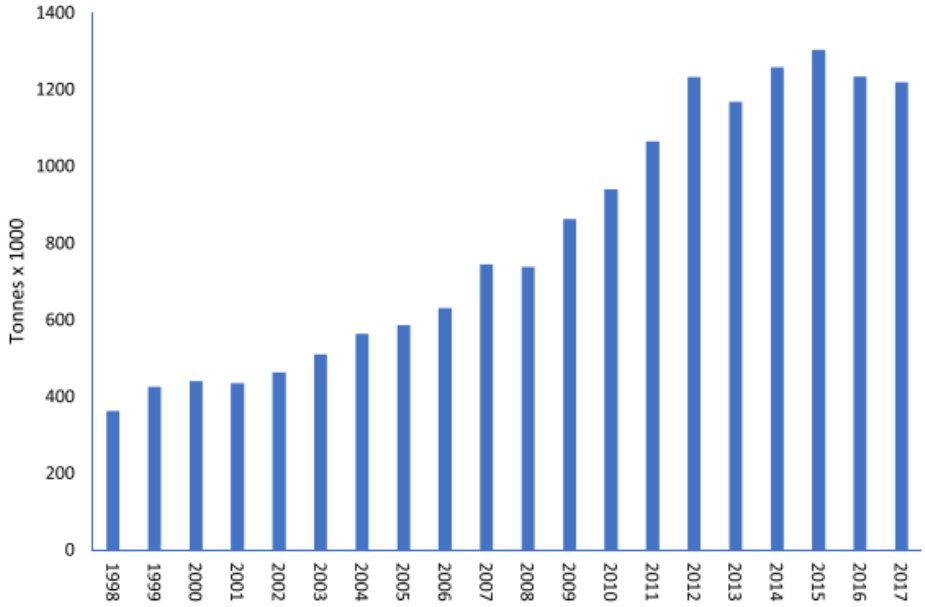

Figure 1. The annual sale of Norwegian farmed Atlantic Salmon from 1998 to 2017. Y-axis is in thousand tons. Source: (Aas et al., 2019).

The salmon production in Norway is mostly produced in open net pens in the marine environment. This makes the farmed fish susceptible to diseases, toxic algae, and other harmful substances (Tveteras, 2002). Sea lice (Caligideae) and regional outbreaks of serious diseases, like Infectious Salmon Anemia (ISA) and Pancreas Disease (PD), are ongoing problems (Bergheim, 2012), in addition to the threat of harmful algal blooms like the event in 2019 that was estimated to cause the death of 8 million salmon (Davidson et al., 2020). Globally, diseases account for approximately 40% of lost aquaculture production (Gomes et al., 2017). To continue the growth in the aquaculture industry the loss caused by diseases and parasites is one of the major obstacles that need to be tackled (Olesen et al., 2010).

1.1 Environmental DNA

Analysis of environmental DNA has shown promising results in biodiversity monitoring and has recently been introduced as a possible tool for the detection and monitoring of pathogens in aquaculture (Peters et al., 2018; Shea et al., 2020; Krolicka et al., 2021). Environmental DNA (eDNA) is genetic material obtained directly from environmental samples without any obvious signs of biological source material (Thomsen & Willerslev, 2015). The DNA is secreted or shed by organisms into the surrounding environment in the form of cellular debris, excretion, tissue, and blood from wounds (Hansen et al., 2018). This DNA is derived from a mixture of genetic materials, ranging from chromosomes and plasmids within intact cells and cellular remains to extracellular DNA fragments freely floating in the environment that can be captured as eDNA (Barnes et al., 2014). Capturing and analyzing eDNA makes it possible to detect species' presence even at low densities, without direct observation or invasive methods (Ficetola et al., 2008). The traditional biodiversity survey techniques are invasive, costly, and often destructive methods. Equipment such as bottom trawl and gill nets have limitations, not only in terms of the conditions and areas they can be used but also because they have a certain species and size selection (Thomsen et al., 2012a; Valentini et al., 2016). Habitat destruction is also a concern with bottom trawls and other mobile fishing gear as they crush, bury, and expose marine animals and structures on and in the seabed (Watling & Norse, 1998). Resource management of marine species relies on biotic data to draw recommendations and conclusions. Biotic data is conventionally retrieved through traditional survey methods. Many European countries base their resource management upon recommendations from The International Council for the Exploration of the Sea (ICES) and their database. The accuracy of the resource management is therefore dependent on robust survey methods that capture the actual diversity and abundance in the sea. There have been reports about the ICES database containing inaccurate information on species (Daan, 2001). Traditional survey methods have several possible sources of error, such as incorrect identification of cryptic species or juvenile life stages (Thomsen & Willerslev, 2015) and variation in detectability during different developmental stages and periods (Ficetola et al., 2008). Using eDNA analysis for surveying biodiversity can be useful when the traditional survey techniques give low-quality results or require a large effort. Metabarcoding can usually circumvent improper taxonomic assignments derived from morphology as it relies on genetic information (Bohmann et al., 2014). Collection of environmental samples is possible almost everywhere and together with eDNA-based methods it provides an opportunity to easily assess the biodiversity (Aylagas et al., 2016; Lacoursière-Roussel et al., 2018), especially for monitoring rare or threatened species (Thomsen et al., 2012b). The applications of eDNA as a

biodiversity assessment tool also have some disadvantages. Although eDNA can detect species that are difficult to find using the traditional methods, the traditional surveys can often provide more detailed information about the organisms like size, sex, age, or health status (Valentini et al., 2016). Another limitation of eDNA analysis for biodiversity surveys is the difficulty to provide quantitative estimates of the species abundance (Bista et al., 2018; Hansen et al., 2018; Peters et al., 2018). Possible explanations for this are the variable shedding of eDNA from species to the environment (Sassoubre et al., 2016; Allan et al., 2021), and DNA degradation in water. DNA can exist in different lengths, sequences, and conformations, and each of these characteristics influences how DNA interacts with its environment and degrades over time (Barnes et al., 2014). There are large variations in the environmental conditions in the ocean, both in different areas of the ocean and within the same areas but at different depths. In these habitats, there are contrasted physiochemical characteristics, material transport, biomass, and biological activity that will determine the persistence of eDNA (Taberlet et al., 2018). There are uncertainties about the physical processes that influence eDNA persistence and its fate within the environment (Harrison et al., 2019), but a review by Barnes et al. (2014) organized possible influences on eDNA degradation into three main categories: characteristics of the DNA molecule, abiotic environmental characteristics, and biotic environmental characteristics (Figure 2).

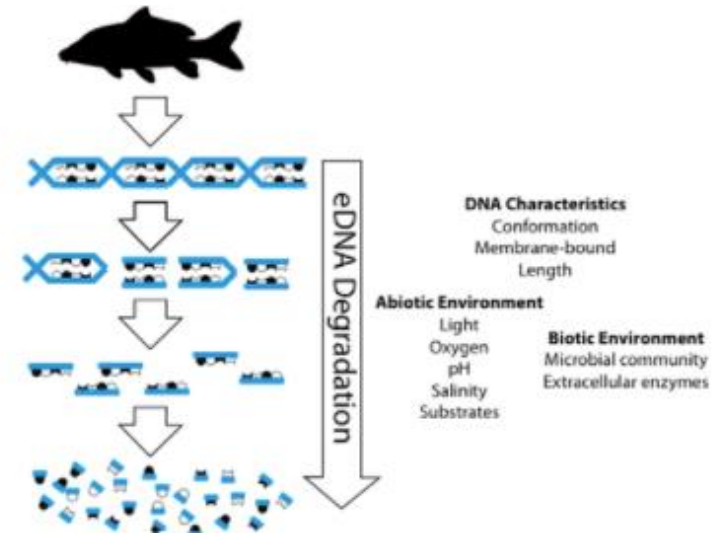

Figure 2. Three categories of underlying factors that affect the degradation of eDNA in aquatic environments. Source: (Barnes et al., 2014).

The difference in the decay rate of eDNA in water is a result of both the characteristics of the shredded DNA and the conditions in the surrounding water. The accuracy of species diversity based on analysis of eDNA compared to traditional survey methods has been thoroughly investigated for different habitats (Taberlet, 2018; Antich et al., 2020; Fraija-Fernández et al., 2020; Ershova et al., 2021;). However, the concentration of eDNA in water can vary (Lacoursière-Roussel et al., 2016) and the possibility of getting a false negative (species that is present but not detected) or a false positive (species that are absent but is detected) should be minimized. The species' mobility may also affect the amount and area where eDNA from the species can be found. Species with low mobility and abundance may have a smaller area where their eDNA can be detected, and the opposite for species with high abundance and mobility. The environmental conditions where the eDNA samples are collected and the target species biology should be taken into consideration when the experimental setup is decided, and the sampling effort should be high to capture the entire taxonomic complexity. An intensified sampling of water causes increased effort of water filtration that can be time-consuming and dependent on expensive equipment. Extended sampling time and sampling in remote areas can also affect the processing and storage of the eDNA samples, which is important for optimal preservation of eDNA samples (Curtis et al., 2020). Combined with traditional survey methods, eDNA may provide complementary information and better data for biodiversity assessment and resource management.

Although there are several questions about eDNA and metabarcoding as a method for detection of species in the aquatic environment that needs to be further investigated, the eDNA-based survey approach has the potential to become a useful tool in biodiversity monitoring in a wide range of aquatic ecosystems including disease detection in aquaculture. The current way that most diseases are discovered in aquaculture fish today is when the fish show symptoms of the disease through monthly routine visits from veterinarians. In many cases when a disease is discovered, it is too late to stop the disease from spreading. In the everyday production of farmed salmon, the producer strives to impose as little handling stress on the fish as possible. eDNA monitoring of pathogens presents a possibility to continuously evaluate the pathogen situation in the nearby water in a non-invasive way without causing unnecessary stress to the farmed salmon. Additionally, continuous monitoring of pathogens can make it possible to act pro-actively against pathogen outbreaks. Several challenges need to be solved before eDNA-based monitoring approaches can have a commercial application in aquaculture, such as developing a low-cost sequencing method that can enable bulk processing of field samples

(Peters et al., 2018) and efficient filtration of water samples. If eDNA is to become a useful tool in aquaculture, an easy-to-use, efficient, and low-cost method needs to be established.

1.2 Natural samplers

In recent years, the use of species as natural samplers, such as filter feeders (Mariani et al., 2019; Turon et al., 2020; Jeunen et al., 2021; Weber et al., 2021) or scavenging shrimps (Siegenthaler et al., 2018; Urban, 2019) has been explored as an alternative to water samples of eDNA. Filter feeders can filtrate water at a higher rate than artificial devices (Mariani et al., 2019), and for instance, sponges can process up to 10 000 liters of water per day (Kahn et al., 2015). Although the filtration rate of filter feeders can contribute to avoiding the problems associated with water filtration, their performance as eDNA samplers needs to be further evaluated. The potential that sponges have as natural sampler was first presented in Mariani et al. (2019) where DNA extracted from sponges from two different locations (Mediterranean and Antarctic) gave distinguishable taxon assemblage from the studied regions. Turon et al. (2020) obtained eDNA from sponges as natural samplers, collected at different eutrophication levels, and detected DNA from fish species known to inhabit the investigated habitat. They concluded that the sponges reasonably captured the fish diversity considering the limiting effort that was required compared to traditional survey techniques. These two studies suggest that sponges do behave like biological eDNA filters that retain eDNA particles from the surrounding environment and can be used as a tool in biodiversity assessments. Studying gut content has also been suggested as a source for retrieving eDNA and information about biodiversity. During metabarcoding of shrimp stomach content Sigenthaler et al. (2018) recovered twice as many species than with traditional survey methods, while Urban (2019) obtained higher fish richness from metabarcoding of stomach content from *Pandalus borealis* than obtained by traditional bottom trawl surveys. In addition, the study revealed that gelatinous zooplankton was the most important component in the diet of *P. borealis*, in contrast to previously published stomach content analysis (Urban et al., 2022). This illustrates the potential of the molecular approach to provide more insight into trophic relations when used for diet analysis. Only two of the previous natural sampler studies contrasted the taxonomical diversity obtained by metabarcoding DNA from natural samplers and water (Jeunen et al., 2021; Weber et al., 2021), and it remains to be investigated whether the increased filtering volume of water with natural samplers also provides higher or more realistic diversity estimates.

1.3 Blue mussels as a natural sampler

Following the promising results of using sponges as natural samplers of eDNA the idea of other filter feeders as possible natural samplers have gained focus. Mussels are one of the targeted filter feeders that only recently have been tested as a potential eDNA sampler (Jeunen et al., 2021; Weber et al., 2021).

Mussels are efficient water filters (Møhlenberg & Riisgård, 1979). There have been several attempts to determine the pumping rate of mussels, with two main categories of experimental design, a direct and indirect method (more details on these methods in (Morton, 1983; Famme et al., 1986)). The results from these methods differ both within and across the categories and because of this, there is doubt as to which of the pumping rates are most applicable (Jones et al., 1992). The rate that the mussel pumps water is related to the capacity for feeding and filtration rate (Møhlenberg & Riisgård, 1979). Jørgensen et al. (1988) found that pump pressure and flow rate in mussels varied with valve gape and extension of both mantle edges and siphons and that the rate of water pumping is created through the spatial geometry of the interfilament canals and the mantle cavity, rather than a physiologically regulated process. The reduction of the valve gape does not seem to be a mechanism to control water processing and therefore feeding, but rather a secondary effect of suboptimal environmental conditions. Generally, bivalves are known to be very sensitive to mechanical or chemical disturbances (Møhlenberg & Riisgård, 1979). As a consequence, the environmental conditions can impact how much water the blue mussels filtrate and thus the amount of eDNA they collect.

Mussels are active suspension feeders. Typically, the blue mussel (*Mytilus edulis*) feeds on bacteria, phytoplankton, detritus, and dissolved organic matter that is suspended in the water column. They actively sweep, pump, or in the case of blue mussels create a localized current to ingest the feed (The Marine Biological Association, n.d.). The main component of the diet is phytoplankton, which can vary significantly in size and structural features (Rouillon & Navarro, 2003). The cilia on the gills pump water, remove, transport, and sort food particles from the water. The gills of blue mussels retain most particles that are larger than 3 to 4 μm (Vahl, 1972; Tuttle-Raycraft & Ackerman, 2018). The maximum retention efficiency of particles has been found to be 30 to 35 μm, while particles smaller than 4 μm had a variable retention efficiency which indicates that smaller particles occasionally are important components in the blue mussel diet (Strohmeier et al., 2012). The particle size of eDNA is most likely highly variable depending on species, type of DNA, and extent of degradation. In freshwater, eDNA from Carp

and Brook Trout has been determined to be most abundant between 1 and 10 μm (Turner et al., 2014; Wilcox et al., 2015), while total eDNA was most abundant below 0.2 μm (Turner et al., 2014). In marine water, eDNA from fish is mostly associated with particles larger than 1 μm (Sassoubre et al., 2016), whereas also eDNA from Japanese Jack Mackerels (*Trachurus japonicus*) was most abundant in the size fraction between 0.4 and 10 μm (Jo et al., 2019). However, fish is only a small part of the marine ecosystems and hardly anything is therefore known about particle size in the total marine natural environment. This is especially because eDNA is "sticky" (Barnes et al., 2021) and is thought to adhere to sinking organic matter that has considerable particle size (Alldredge, 2001; Turner, 2015). The size selection in particle retention of blue mussels indicates that a proportion of eDNA in water may have a particle size too small to be retained. Blue mussels use several hours (10-15 hours) to digest dietary particles (Hawkins et al., 1990). In contrast to water samples that give a snapshot of the DNA that is present only when the samples are taken, DNA retrieved from blue mussel intestines and stomach may represent extracellular DNA fragments, DNA bound to particles, as well as bacteria, phytoplankton, organismal DNA and living organisms integrated over a wider period.

Mussels play a fundamental role in the marine ecosystem (Suárez-Ulloa et al., 2013), and the widespread distribution of mussels makes them available in different environmental conditions with variations in the food supply. Their importance and distributions make mussels a natural sampler that is easy to access in many coastal areas (Figure 3). Five species of mussels are occurring in the Northern hemisphere: *Mytilus trossulus*, *Mytilus edulis*, *Mytilus galloprovinvialis*, *Mytilus californianus*, and *Mytilus coruscus*, and three in the Southern Hemisphere: *Mytilus chilensis*, *Mytilus galloprovincialis*, and *Mytilus platensis* (Gaitán-Espitia et al., 2016). The taxonomy in the Mytilus genus is not fully clarified, and because of the overlapping distribution and ability to interbreed the different populations are often mixed with hybrids (Gosling et al., 2008). Whether the morphological differences between mussels from different locations represent important measures of taxonomical differentiation is not clear (McDonald et al., 1991).

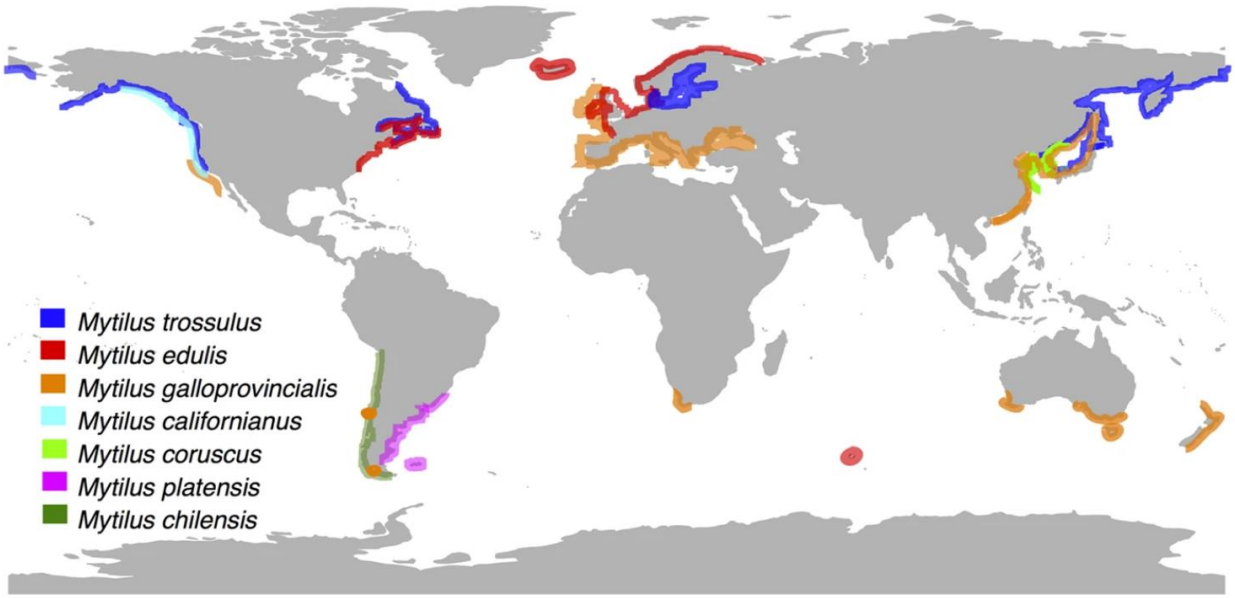

Figure 3. Distribution of different marine mussel species. With permission from Gaitán-Espitia et al. (2016).

The protocol for how to extract eDNA from blue mussels is far from established, and in previous studies, different approaches have been used. Some studies used gill tissue or the digestive system, while others finely grounded the entire tissue of the mussel (Strohmeier et al., 2012; Turner et al., 2014; Weber et al., 2021). Using filter feeders as natural samplers for retrieving eDNA has the potential to become a useful tool in eDNA survey methodology. Most natural sampler studies have compared fish diversity obtained from eDNA retrieved in a natural sampler with either visual detection of fish species or traditional survey techniques such as trawling. This study is the first to use COI to investigate blue mussels as a natural sampler over a long period compared to eDNA found in water samples.

1.4 Metabarcoding

Morphological based identification technologies can be time-consuming and dependent on specific expertise. Metabarcoding can potentially detect all species in a community, regardless of developmental stage or preservation of distinguishing features (Ershova et al., 2021). DNA metabarcoding can be used on bulk samples with whole genomic DNA and environmental samples containing little and often degraded DNA (eDNA metabarcoding) (Deiner et al., 2017). Samples containing stomach content are somewhere in between these two, as they contain whole genomic DNA, but it can be highly degraded or digested (Van der Loos & Nijland, 2021), and DNA retrieved from the stomach of natural samplers such as blue mussels can contain both whole genomic DNA and eDNA. DNA metabarcoding is a promising approach to rapid identification and biodiversity surveying. Furthermore, the use of the cytochrome c oxidase

subunit I (COI) gene, found in the mitochondrial genome, has been proposed as the core of a global bio identification system for animals (Herbert et al., 2003), although the quantitative value of metabarcoding itself is still disputed (Bucklin et al., 2016; Van der Loos & Nijland, 2020).

Most members of the animal kingdom have a primary barcode sequence consisting of 658 base pairs (bp) of the mitochondrial COI gene (Herbert et al., 2003). One or more signature barcode region, such as COI, from a DNA sequence can be used to make high-throughput taxonomic identification. However, this gene region is rather long for eDNA metabarcoding, thus smaller fragments of this gene region such as the Leray fragment, with a length of 313 bp (Leray et al., 2013) have gained more interest. Additionally, Wangensteen et al. (2018) developed a new primer Leray-XT which ensures high affinity against several phyla of Animalia kingdom and other eukaryotes. The pool of barcode sequences obtained from environmental samples can be assigned to species using a DNA reference sequence database. The quality of the results from metabarcoding is therefore dependent on a reference database with high-quality reference sequences to provide accurate and species-level resolution. Databases such as The Barcode of Life Data System (BOLD) and National Center for Biotechnology Information (NCBI) provide free-to-use databases where you can run your sequences against billions of annotated sequences from more than 2 million taxa. However, improvement and curation of databases are still important for better taxonomic resolution (Sinniger et al., 2016; Weigand et al., 2019).

Although metabarcoding studies are subject to several choices and considerations such as sampling design, DNA extraction, primer of choice, PCR biases, and sequencing errors (Van der Loos & Nijland, 2020), studies have shown reliable results for inferring community composition and measurement of biodiversity. However, interpretation of eDNA metabarcoding results needs to be conducted cautiously.

1.5 Objectives

The overall objective of the study was to investigate blue mussels as a natural sampler of eDNA in seawater. To achieve this main objective, the secondary objectives were to; i) compare differences in diversity obtained from metabarcoding of eDNA isolated from blue mussel samples and water samples throughout 15 months of sampling, and ii) investigate how the blue mussels perform as a sampler of pathogens and harmful species in aquaculture compared to water samples.

I hypothesize that

i) Analysis of eDNA from blue mussels will provide higher species richness, compared to eDNA derived from water samples.
ii) Blue mussels will detect a higher richness of pathogens at higher absolute abundances compared to what can be detected in water samples.

because blue mussels, as a natural sampler, should integrate the eDNA signal.

2 Materials and Methods

2.1 Description of aquaculture site

This project used an aquaculture production site, Skogshamn, located on the north side of Dyrøya in Solbergsfjorden, northern Norway (Figure 4) to obtain water and blue mussel samples The permits for salmon production in Skogshamn are co-owned by The Arctic University of Norway in Tromsø (UiT) and NOFIMA and is managed by Havbruksstasjonen Tromsø A/S. Daily operations and commercial practices are performed by Salmar Farming AS. This aquaculture site has a cleared capacity of 5280 tons of Atlantic Salmon per production cycle. During this production cycle, six net pens with a circumference of 157 meters were used.

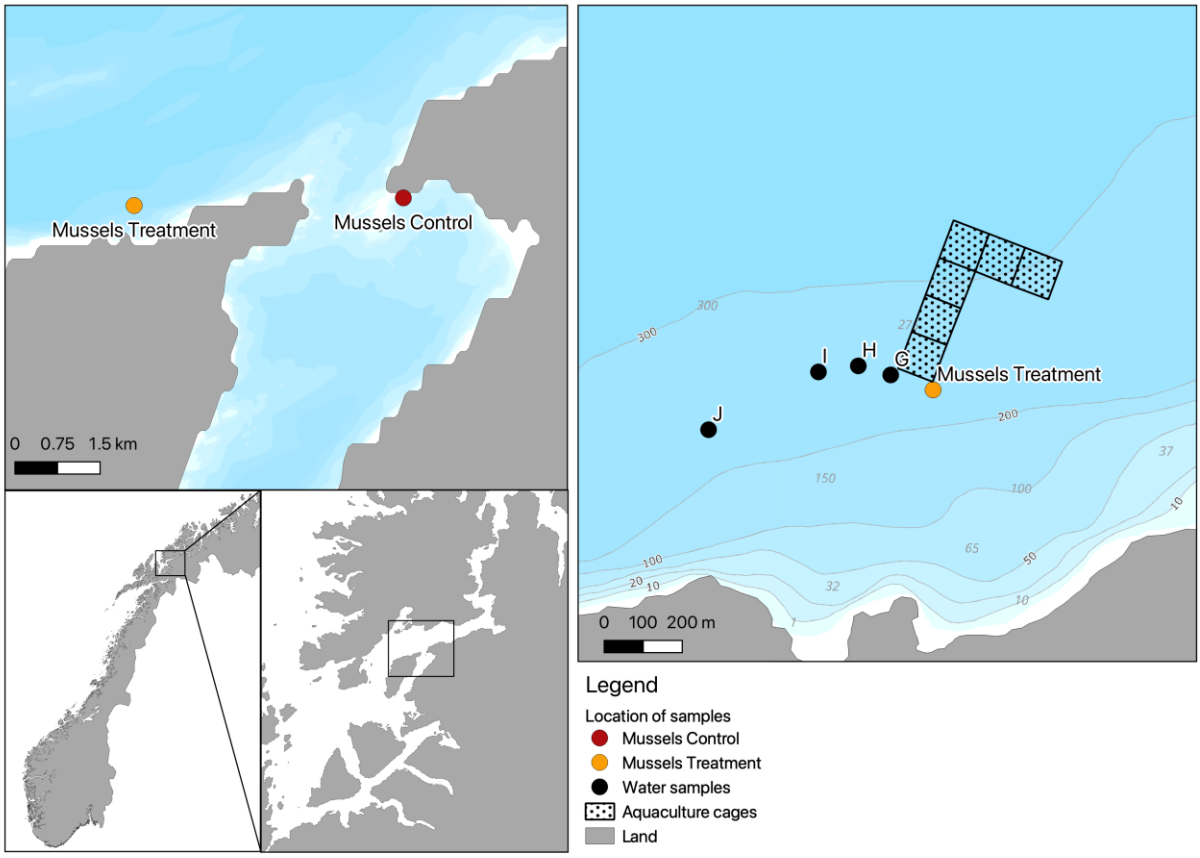

Figure 4. Location of Skogshamn aquaculture site and sampling points for both water and blue mussels. The water samples are taken at a distance ranging from 50m to 500m. Treatment mussels are located approximately 50m from the aquaculture site. Produced by Gledis Guri.

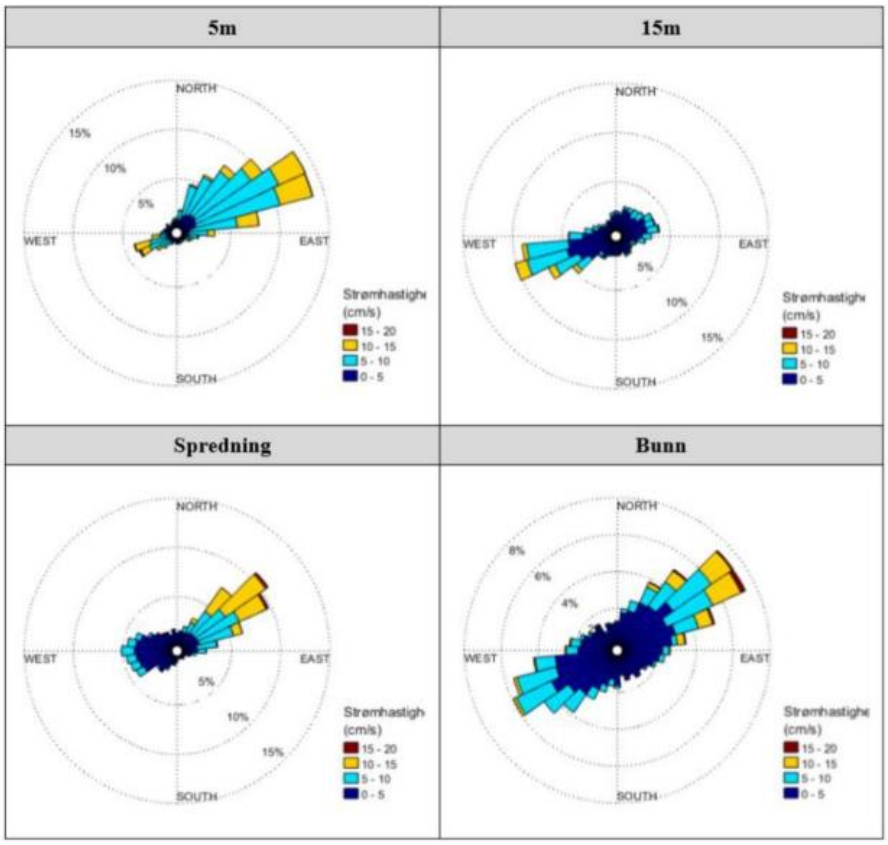

Figure 5. Circular barplot of current velocities and directions at aquaculture site Skogshamn by depth. Current velocities (cm/s) are represented by color, and their % occurrence in each direction at those velocities is indicated by each bar length extending from the focal point. Four plots are displayed for four depths where this data was collected at the farm (5m, 15m, net spreading, and fjord bottom)

The seabed under the production site is steep down towards the deepest areas of the Solbergsfjord. The depth where the net pens are located varies from between 160 to 340 meters. The main direction of the current is northeast (Figure 5).

2.2 Sampling

2.2.1 Sampling design

To investigate blue mussels as a natural sampler of eDNA, seawater and blue mussel samples were collected for 15 months at the same location.

2.2.2 Using blue mussels as natural sampler of eDNA

Blue mussels were collected manually from a harbor nearby the aquaculture site and placed in a holding net attached to a buoy at the fish farm. At the time of collection in the harbor, 10-12 blue mussels were preserved in 96 % ethanol as a reference (control) for the biodiversity the blue mussels may bring to the aquaculture site. The holding net was restocked with blue mussels

three times during the experiment and reference samples were collected on each occasion (04.09.20, 28.09.20, and 23.12.20). At the aquaculture site, the holding net was placed approximately 50 meters from the nearest net pen located at a depth of around 2 meters. The blue mussels were collected approximately every month, from February 2020 to April 2021, with a total collection of 166 blue mussels distributed over 15 sampling dates in that period (Table 1).

Table 1. Blue mussel and water samples collected at Skogshamn from February 2020 to April 2021 including information about number of samples and where the samples were collected. Control mussels are the ones that were collected in the harbor and treatment mussels are the ones collected close to the aquaculture site. G, H, I and J are the different sampling points for water. G= 25 meter from net pen. H=100 meters from net pen. I=250 meter from net pen. J=500 meters from net pen.

Sampling date	**Blue mussels**	**Sampling point for blue mussels**	**Water**	**Sampling points for water**
12.02.2020	12	Control	8	H, I, J
11.03.2020	6	Treatment	12	G, H, I, J
17.04.2020	4	Treatment	12	G, H, I, J
22.05.2020	13	Treatment	12	G, H, I, J
10.06.2020	12	Treatment	11	G, H, I, J
06.07.2020	2	Treatment	12	G, H, I, J
30.07.2020	11	Treatment	12	G, H, I, J
04.09.2020	10	Control	12	G, H, I, J
28.09.2020	12	Control	12	G, H, I, J
27.10.2020	12	Treatment	12	G, H, I, J
23.12.2020	12	Control	6	G, J
26.01.2021	12	Treatment	12	G, H, I, J
24.02.2021	12	Treatment	12	G, H, I, J

25.03.2021	12	Treatment	12	G, H, I, J
21.04.2021	12	Treatment	12	G, H, I, J
21.04.2021	12	Control	12	G, H, I, J

At collection, the shell of the blue mussels was carefully cracked, to allow for preservative penetration, and then drained of water, and conserved in bottles containing 96% ethanol. The bottles were transported to the university where they were kept in a freezer at -20 ºC. The ethanol was replaced after 12h, 24h, and 48-72h of the collection with ice-cold 96% ethanol, to ensure proper preservation of the samples.

2.2.3 Collection of seawater for eDNA analysis

Seawater samples were collected twice a month for all the sampling points between February 2020 and April 2021, but only water samples from the dates blue mussels were collected was used for this study (Table 1). The water samples were collected and processed using a clean protocol (Appendix A). The sampling equipment was sterilized with bleach before use and the personnel handling the samples used protective and sterile gloves to prevent contamination. The filtering station and associated equipment were cleaned thoroughly between each sample using 10% bleach solution and MilliQ water. The water samples were collected at 2 m depth using a 2.5L Model 1010 Niskin Water Sampler. The water was then transferred to a sterilized 2.04L Whirl-Pak™ Stand Up Bag. A filtering station was set up after all the samples were collected. Each sample was filtered using three 0.22 μm Sterivex tm filter units. At 0.5 L output volume the filter was removed to ensure a standard volume between the biological pseudo-replicates of each sample. The filters were then dried by pumping air through them before they were placed in sterile 50 ml Falcon tubes in closed zip-lock plastic bags and transported to UiT. The filters were long term stored at -80 ºC in a freezer only for eDNA samples.

2.3 Laboratory practices

All laboratory work was done with strict cleanness protocols. When working with eDNA all the equipment was thoroughly cleaned with bleach, ethanol, flame sterilization, and UV treatment. The same applied to the working space, except for flame sterilization. eDNA extraction from water samples had a special protocol, that included rules about showering and

eating for lab personnel before entering the specific clean lab that only was used for water eDNA extraction.

2.3.1 Dissection of blue mussels

The blue mussels were dissected before DNA extraction. The dissection was done using a clean protocol, which included changing gloves and cleaning tools with bleach and ethanol between each blue mussel. Dissecting the blue mussels was done by carefully opening the blue mussel in the anterior end with a pair of scissors and then snipping over the posterior adductor muscle (Figure 6). The interior part of the blue mussels was then separated from the shell, and as little as possible tissue including the stomach was preserved (Figure 7) in a glass bottle with 96 % ethanol in a freezer at -20ºC until DNA extraction.

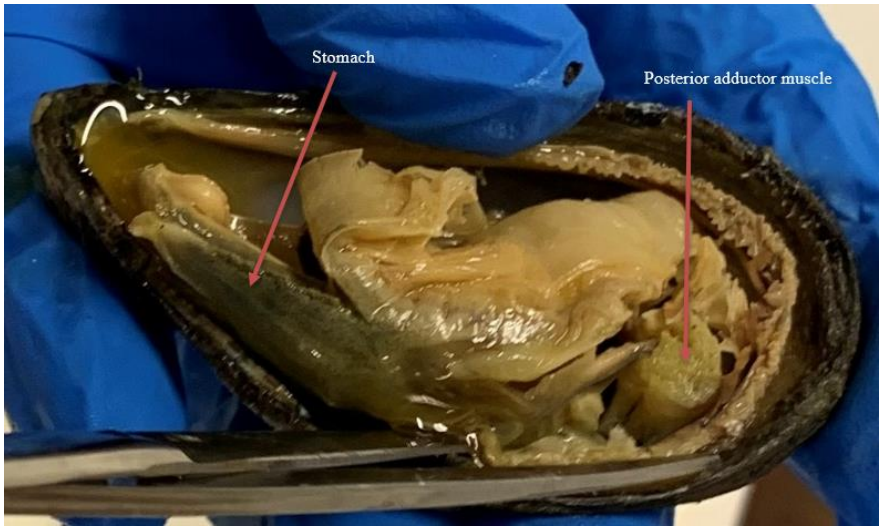

Figure 6. Internal parts of one blue mussel that has been stored in 96% ethanol in a freezer at -20ºC. Arrows showing the posterior adductor muscle that got snipped to open the blue mussel and the stomach that is used further in the study.

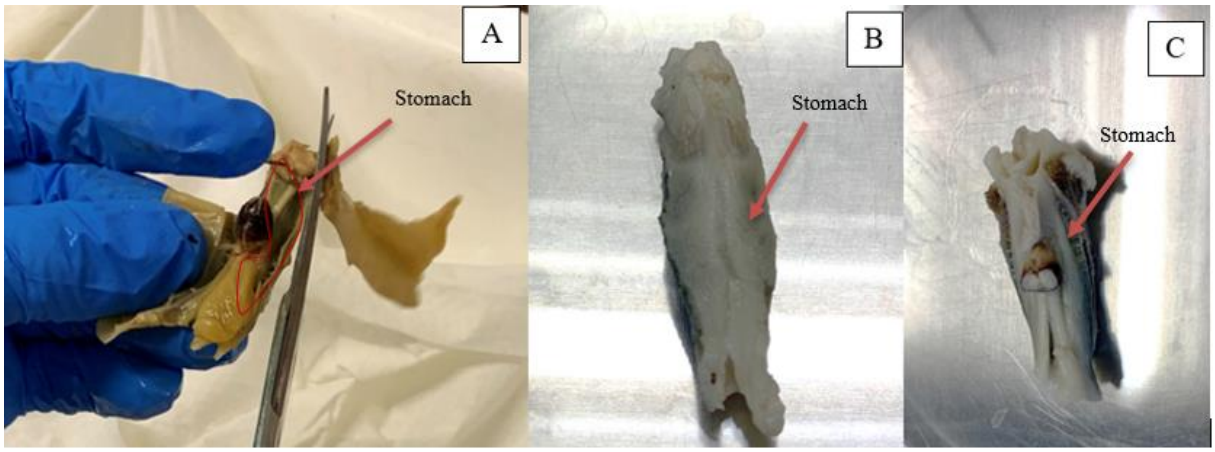

Figure 7. The interior part of the blue mussel got cut out from the shell. A: The red area shows the location of the stomach, and the picture also shows some parts that are removed from the samples. B and C: The part of the blue mussels that were used for DNA extraction from two angles. The stomach is located underneath the white tissue.

2.3.2 DNA extraction

2.3.2.1 Blue mussel samples

eDNA from blue mussels were extracted using DNeasy PowerSoil® Kit (Qiagen, Germany) with a modified protocol (Appendix B). The modifications of the manufacturers' protocol consisted of; a) vortexing for 60 min. (Step 4), b) centrifuging for 1 min. (Steps 5, 16, and 20), c) no incubation (Step 7 and 10) and d) small changes in volume, 700 μl of supernatant (Step 12) and 630 μl supernatant (Step 14). Before the extraction, the PCR workstation and dissection tools were sterilized with bleach solution, MilliQ water, ethanol, and a 30 min UV light sterilization. All dissection tools were sterilized between the dissection of each specimen using bleach, ethanol and flame sterilization. The extraction samples were collected by carefully cutting up the stomach, using forceps to open up the cut, and then a spatula to scrape out stomach content (Figure 8). The extracted DNA was stored in an 2ml Eppendorf tube in a cryobox at -40 ºC. An extraction blank was included for every round of extraction to check for contamination that may have been introduced to the DNA samples during the extraction process. A total of 159 blue mussel samples and seven blanks (166 samples) were extracted and sequenced.

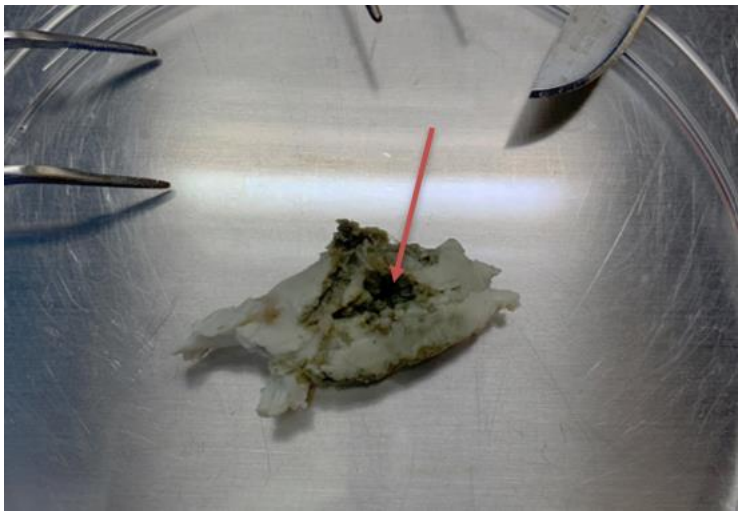

Figure 8. A blue mussel stomach after retrieving stomach content for extraction. The arrow is pointing to the opening of the stomach created and where the sample was collected from.

2.3.2.2 Water samples

The DNA extraction from the Sterivex filters containing the water samples took place in an over-pressured eDNA clean-lab where trace eDNA extraction protocols and clean-lab working routines were applied (Appendix C). This protocol was designed to prevent contamination from all airborne DNA, including DNA from the lab personnel's skin, hair, or breath. This protocol was important to ensure that the results were reliable and of good quality with the high risk of contamination that is present when working with eDNA. The extraction of DNA was performed in a pressure positive eDNA extraction room, including an airlocked changing and sampling room. Before entering the sluice, strict entrance rules were followed and the cleaning protocol for samples and equipment that were brought into the extraction room was performed here. Extractions of DNA were performed using a modified eDNA extraction protocol of the DNEasy Blood and Tissue ® kit (Qiagen). Due to the enclosed state of the Sterivex filers, an extended incubation time (24hr) was used for full lysis of the particulates captured within the filter membrane. The lysed solution was then centrifuged out of the filter casing and into 2ml Eppendorf tubes. The protocol hereafter followed the DNEasy Blood and Tissue ® kit standard steps except the volume AL added was equal to the approximate volume measured of 2-3 samples and that the eDNA was eluted in 75 μl AE (Appendix D).

2.4 PCR amplification, library preparations, and sequencing

Aliquots from each sample were pipetted into PCR well plates for 1 step amplification of the Leray-XT fragment in the COI gene, following the RGG standard protocol (Appendix F). The PCR-mix that was used for amplification contained 10 μl of AmpliTaq Gold Master Mix, 0.16

μl of Bovine Serum Albumin (20µg/µl), 5.84 μl H_2O, 1 μl each of forward and reverse primer (5μM) and 2 µl of DNA template, a total of 20 μl per sample. The amplification and PCR temperature profile are described in Appendix E. Once all the samples were amplified, they were pooled together in a single Eppendorf tube and vortexed thoroughly. The pool was then purified using MinElute columns for removing DNA fragments below 70 bp, and at the same time concentrating the amplified DNA around 10 times. Library preparations were performed using NEXT flex PCR-free library preparation kit (BIOO Scientific) and the DNA concentrations were measured by qPCR using the NEBNext Library Quant Kit.

The library pool was sent for sequencing at a commercial sequencing platform (NOVOGENE), using a partial S4 lane (20 Gb for the blue mussel libraries) with 250 bp paired-end chemistry on an Illumina Novaseq6000. The libraries for water samples had been previously sequenced, and multiplexed with other water samples, in several Illumina Novaseq6000 sequencing runs by the same company.

2.5 Bioinformatics

The Leray-XT primer set was used for COI (Wangensteen et al., 2018). The MJOLNIR Pipeline (https://github.com/uit-metabarcoding/MJOLNIR) with the recommended setup parameters for the Leray-XT fragment (Lmin=299, Lmax=320, d=13, remove singletons before clustering, and remove prokaryote sequences from the final dataset) was used to convert the raw fastq files from sequencing into the final metabarcoding table. All samples from blue mussels, water, and control were processed together in a single MJOLNIR pipeline. The DUFA-Leray-XT reference sequence database version 2021-07-20 (https://github.com/uit-metabarcoding/DUFA) was used for taxonomic assignment, using Ecotag, as implemented in MJOLNIR. The molecular taxonomic units (MOTU) table, retrieved after the bioinformatic workflow was corrected for potential TAG-jumps. MOTUs assigned to the order Primates and Mytiloida were removed, in addition to MOTUs identified only as Eukaryota (without assignment to at least the Kingdom level).

2.6 Statistical Analysis

Statistical analysis was performed in R version 4.2.0 (R Core Team, 2021). Rarefaction curves were drawn using the *rarecurve* function and species accumulation curves were drawn using the *specaccum* function both in the *vegan* package (Oksanen et al., 2022). Water samples with less than 1000 reads and blue mussel samples with less than 100 reads were filtered out.

Reads were then transformed into relative abundance, with water replicates pooled together, to build a Bray-Curtis dissimilarity matrix. Non-metrical multidimensional scaling (nMDS) was performed using the Bray-Curtis dissimilarities and *metaMDS* function with 20 random start iterations to visually check for differences in the two mussel groups and between water samples and blue mussel samples. Mussel group "Control" was only used for comparison between blue mussel groups, not for further analysis between water and blue mussel. The variance between sampling method, and between sampling method and season were then assessed using Permutational Multivariate Analyses of Variance (PERMANOVA) using *adonis* function in *vegan* package (Oksanen et al., 2022) with 999 permutations. Permutation multivariate dispersion test (PERMDISP) was performed to determine the significance between sampling method and season using function *betadisper* and *pairwise permutest* in *vegan* package (Oksanen et al., 2022). Dufrene-Legendre indicator species analysis on relative abundance (IndVal) (Dufrêne & Legendre, 1997) from the *labdsv* package (Roberts, 2019) was performed to identify MOTUs that contributed the most to dissimilarity in sampling method and season. Total reads of four selected pathogens (*Pseudo-nitzschia sp, Margalefidinium polykrikoides, Paramoeba sp,* and *Pseudochattonella farcimen)* in the two different sampling methods were presented with violin plot using the *ggplot2* package (Wickham, 2016).

3 Results

This study investigated blue mussels as a natural sampler of environmental DNA by comparing COI metabarcoding data from blue mussel and water samples. The dataset retrieved from MJOLNIR consisted of a total of 88,374,564 sequence reads recovered from the COI metabarcoding, 60,074,293 from the blue mussel dataset, and 28,300,271 from the water dataset. After filtering out bacterial reads the number of sequence reads was reduced to 78,415,128 reads. Of these, 58,665,959 reads were recovered from blue mussel samples, of which 56,741,205 reads (96,7%) were host DNA (Mytiloida). 4605 MOTUs were recovered from 377 PCR samples (159 blue mussel samples and 218 water samples). The final refinement (removal of contaminants, blank correction, and minimal abundance threshold setting) resulted in a total of 17,127,435 reads and 2890 MOTUs remaining in the final dataset (Table 2).

Table 2. Overview of bioinformatic treatment of COI metabarcoding dataset for blue mussel and water samples and how the different steps in the bioinformatic pipeline affected the number of reads and subsequently number of MOTUs.

Data treatment	**COI**	
Total read nr.	78,415,128 reads	
Taxonomic assignment	4605 MOTUs	
	Blue mussel	**Water**
Dataset	58,665,959 reads	19,746,175 reads
Mytiloida	56,741,205 reads (96,7%)	
Final refinement	1,147,962 reads	15,979,473 reads
MOTUs	718	2250

From the original 190 water samples, 165 samples were included after final refinement that consisted of 15,979,473 reads and 2250 MOTUs, and 99 blue mussel samples from the original 159 samples that consisted of 1,147,962 reads and 718 MOTUs.

To examine the relationship between sequencing depth and the number of MOTUs detected I used rarefaction analysis (Figure 9). Both blue mussel samples and water samples reached an asymptotic course in the rarefaction curves, which indicated that the sequencing depth was sufficient for representing the taxonomic complexity. Even though, water samples had a higher number of MOTUs than blue mussel samples, the shape of the curves was relatively similar given that most reads (96.7%) in blue mussel samples were host DNA.

The species accumulation curves did not reach a plateau for either sampling methods (Figure 10), which indicated that increased sampling effort would increase the number of MOTUs and better represent the biodiversity at the aquaculture site. There was a clear difference in MOTU richness between sampling method, water samples detected approximately three times higher number of MOTUs than blue mussel samples.

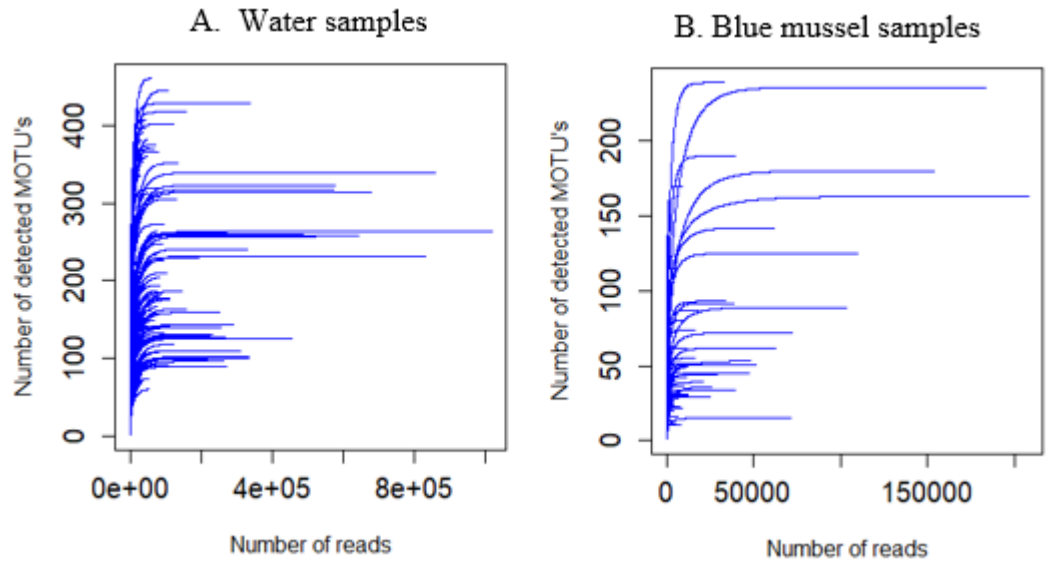

Figure 9. Rarefaction curves derived from water samples (A) and blue mussel samples (B). Each curve represents one sample.

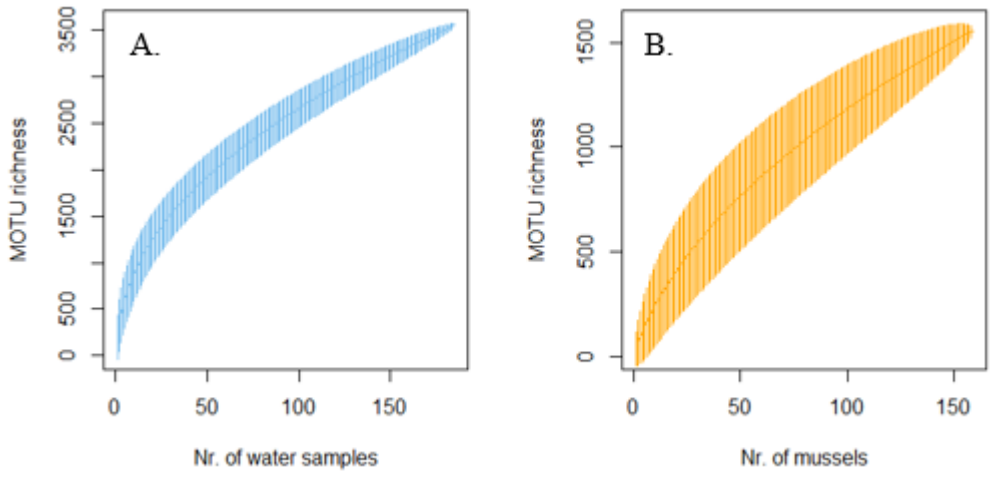

Figure 10. Species accumulation curve with 95% confidence intervals for the water samples (A) and blue mussel samples (B). The water samples are in replicates of three.

3.1 Comparison of "control" and "treatment" blue mussels

Taxonomic composition in "control mussels" and "treatment mussels" differed slightly (Figure 11). In treatment mussel samples Bacillariophyta was the dominating kingdom (Figure 11B), while most of the control mussel samples had a larger amount of Dinoflagellata and Arthropoda (Figure 11A).

Displaying dissimilarities between "control" and "treatment" blue mussels using nMDS displayed a difference in centroids between groups but with an overlap of the ellipses. The PERMANOVA indicated a significant difference (Table 3), but with only 1,7% of the variance

explained by the different groups. PERMDISP indicated no significant difference in dispersion between “control” and “treatment” mussels.

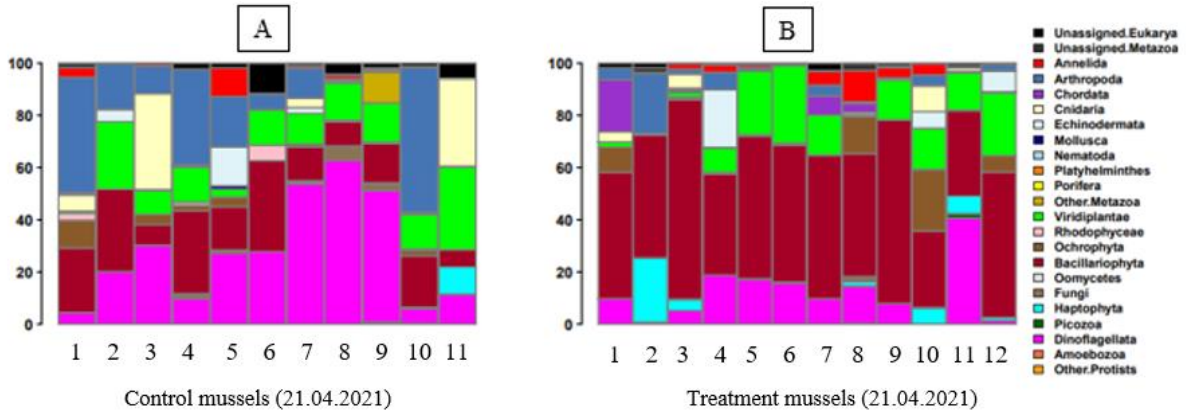

Figure 11. Diversity in kingdoms retrieved from eDNA of “control mussels” (A) and “treatment mussels” (B) sampled 21.04.2021. Numbers on x-axis refers to separate individuals of blue mussels.

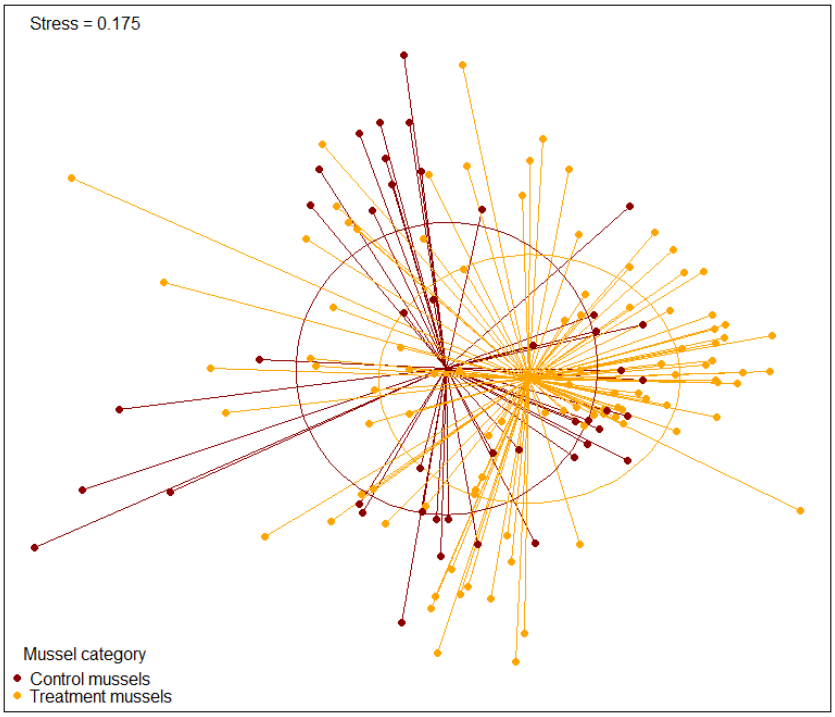

Figure 12. non-metric multidimentional scaling (nMDS) of difference between “treatment mussels” and “control mussels” with relative abundance, Bray-Curtis distance, and K=3. “Control mussels” are blue mussels collected at the harbor, “Treatment mussels” are collected from the blue mussel station next to the aquaculture site.

Table 3. PERMANOVA analysis comparing eDNA community in "control mussels" and "treatment mussels". PERMDISP probability for homogeneity of dispersion are also shown. Significant values are indicated with bold. Number of permutations was 999.

Factor	*Df*	*SS*	*R2*	*F*	*P-value*	*PERMDISP*
Mussel Category	1	1.009	0.017	1.493	**0.002**	0.239
Residual	142	57.479	0.983			
Total	143	58.488	1.000			

3.2 Comparison of relative abundance estimates between water and blue mussels

The nMDS plot based on relative abundance displayed a significant difference between MOTU diversity captured in the water and blue mussel samples (Figure 13). The two sampling methods were distinctly separated with no overlap of ellipses. PERMANOVA analysis with sampling methods as a factor confirmed a significant difference between eDNA retrieved with mussel samples and water samples (Table 4). PERMDISP indicated a higher variation in the community sampled by blue mussels compared to water samples, which is also supported by the nMDS. The ellipse in the blue mussel samples was larger than the ellipse in the water samples and displayed a larger variation within blue mussel samples than in water samples.

Visual representation of the taxonomic composition in summer and wintertime showed somewhat distinct centroids in both sampling types simultaneously with overlap between seasons (Figure 14). PERMANOVA revealed an interaction between the sampling method and season (Table 4). PERMDISP indicated a significant difference in dispersion within sampling type and season, with the seasonal difference in opposite directions for the two sampling types. Pairwise comparison tests indicated a significant difference between "Mussel summer" and "Water summer" (p=0.001), between "Mussel winter" and "Water winter" (p=0.001), and between seasons within sampling methods (p=0.001 and p=0.001) (Suppl. Table 2).

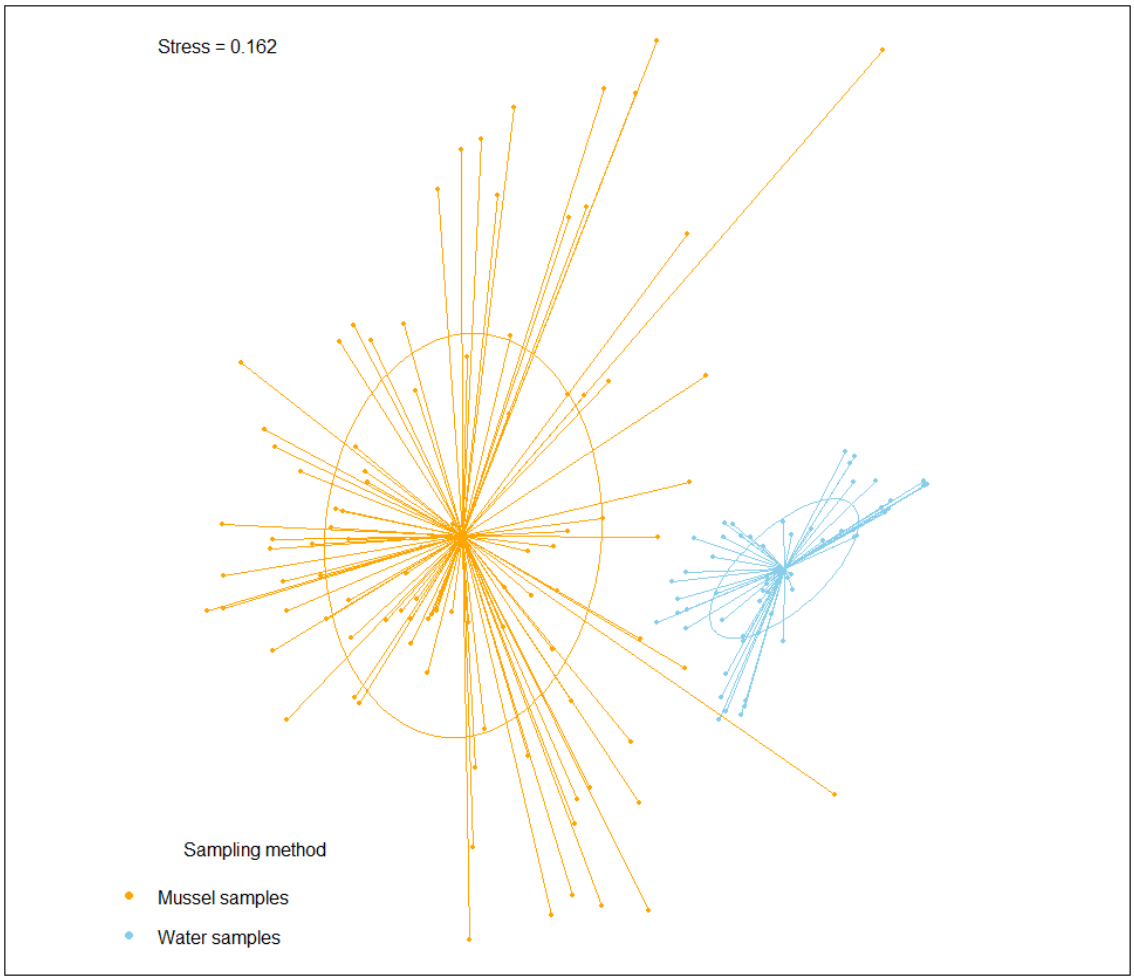

Figure 13. nMDS plot of eDNA community obtained by water samples and blue mussel samples based on relative abundance. K=3

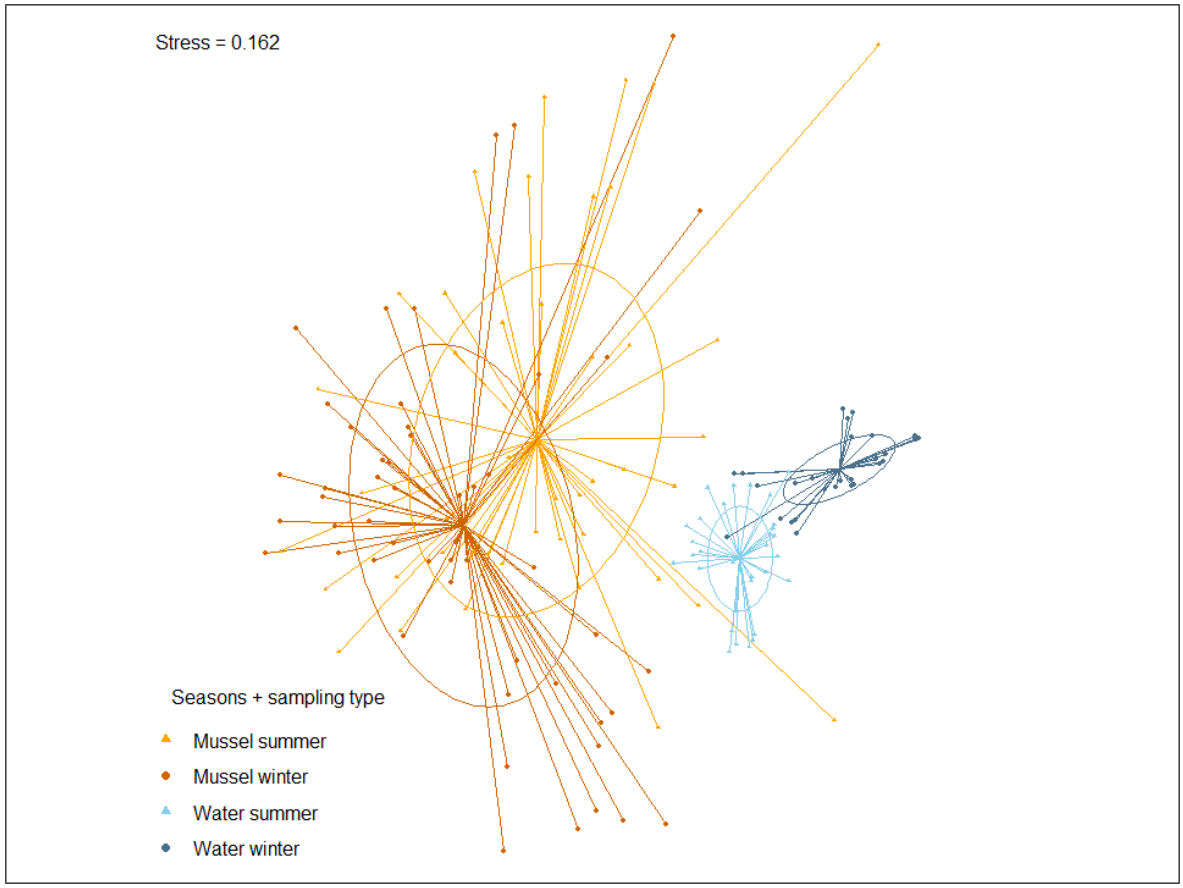

Figure 14. nMDS plot of biodiversity obtained from eDNA sampling with two different sampling methods, blue mussels and water and seasonal trends on relative abundance and K=3. Summer samples are set to the months May-October, and winter samples includes months November-April.

Table 4. PERMANOVA analysis comparing eDNA community in blue mussel samples and water samples, and in between seasons summer (May-October) and winter (November-April). PERMDISP probability for homogeneity of dispersion are also shown. Significant values are indicated with bold. Number of permutations was 999.

Factor	*df*	*SS*	*R2*	*F*	*P-value*	*PERMDISP*
Sampling method	1	10.276	0.159	32.033	**0.001**	**0.001**
Season	1	1.760	0.027	5.485	**0.001**	0.848
Sampling method*Season	1	2.653	0.041	8.270	**0.001**	**0.001**
Residuals	156	50.044	0.77309			
Total	159	64.732	1.00000			

3.3 Comparison of molecular diversity from water and blue mussels

Differences in species contribution to seasonal change in blue mussel samples and water samples displayed different MOTUs as main contributors in the two sampling methods. The Indicator Value analysis (IndVal) showed that a total of 163 MOTUs had significant p-values (Suppl. Figure 3), divided into 27 MOTUs in the blue mussel samples ($p<0.05$) and ~140 MOTUs in the water samples ($p<0.001$). The 30 most contributing MOTUs were selected for further analysis which showed that the water samples had more MOTUs contributing to seasonal changes than blue mussel samples (Figure 15). Both sampling methods have fewer MOTUs in the winter samples than in the summer samples. The ten most contributing species were detected with both sampling methods (Suppl. Table 3.). Seven of these MOTUs were significant in seasonal changes in blue mussel samples and six MOTUs were significant in seasonal changes in water samples. *Micromonas pusilla* (identity 0.97) was only significant for seasonal changes within blue mussel samples and had a higher relative read abundance in blue mussel summer samples while the relative read abundance in water samples was similar in both seasons. *Bathycoccus prasinos* (identity 1.00) influences seasonal differences in water samples the most and had a higher relative abundance in water samples collected in winter months (November-April) than summer months (May-October) and was present in low relative abundance in blue mussel samples.

Platyhelminthes (identity 0.77) was the MOTU that contributed most to the difference between seasons in blue mussel samples (Figure 15) that was not present in the water samples. In the water samples *Kareniaceae* (identity 0.98), *Oomycota* (identity 0.86), and *Picobiliphyte sp. MS584-11* (identity 0.81) were the species promoting most differences between seasons that were unique MOTUs for water samples.

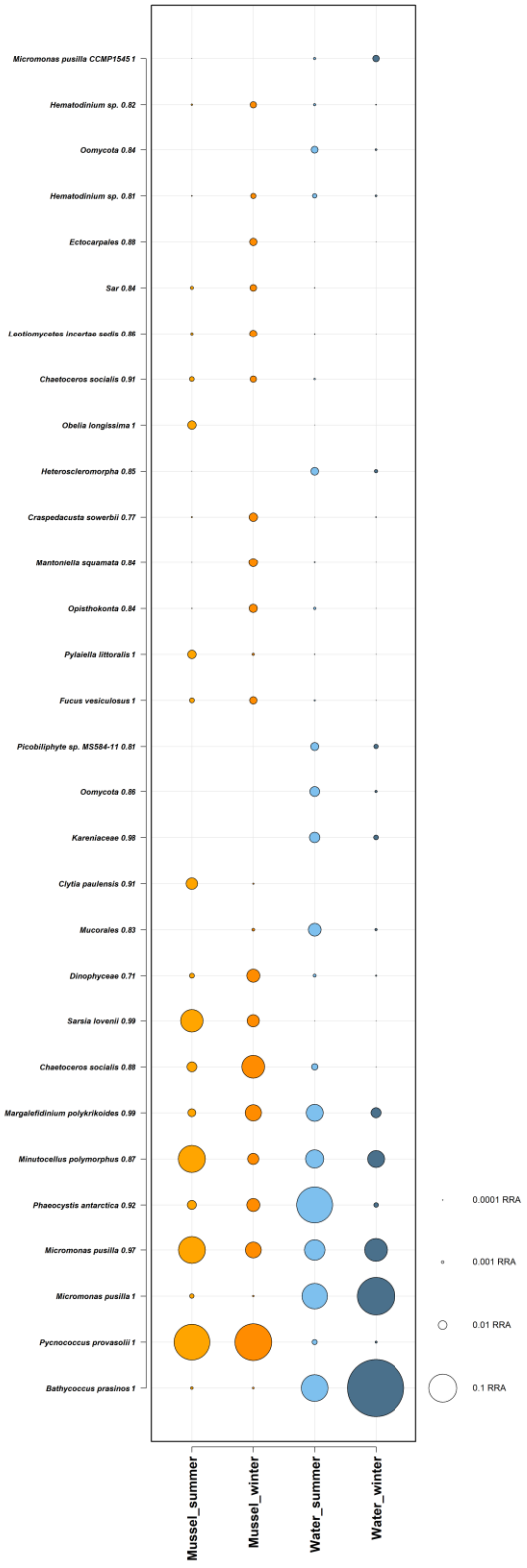

Figure 15. IndVal analysis of MOTUs contribution to seasonal changes in blue mussel samples and water samples. MOTUs are selected by significant difference in the IndVal analysis ($p<0.001$ for water samples, and $p<0.05$ for blue mussel samples) and plotted with the relative read abundance from 1-100. MOTUs are shown with best identity in parentheses. Summer months = May-October, winter months=November-April.

3.4 Pathogen detection in water versus blue mussels

To assess the possibility that blue mussels can detect eDNA from pathogens better than water samples, all known aquaculture pathogens and absolute read abundance detected in blue mussel (21 MOTUs) and water (58 MOTUs) samples were identified (Suppl. Table 4). Four species were selected and compared for the two sampling methods (Figure 16). *Pseudo-nitzschia* sp, *Margalefidinium polykrikoides*, and *Pseudochattonella farcimen* were detected with more reads in water samples, while *Paramoeba* sp. was the only one with more reads in blue mussel samples.

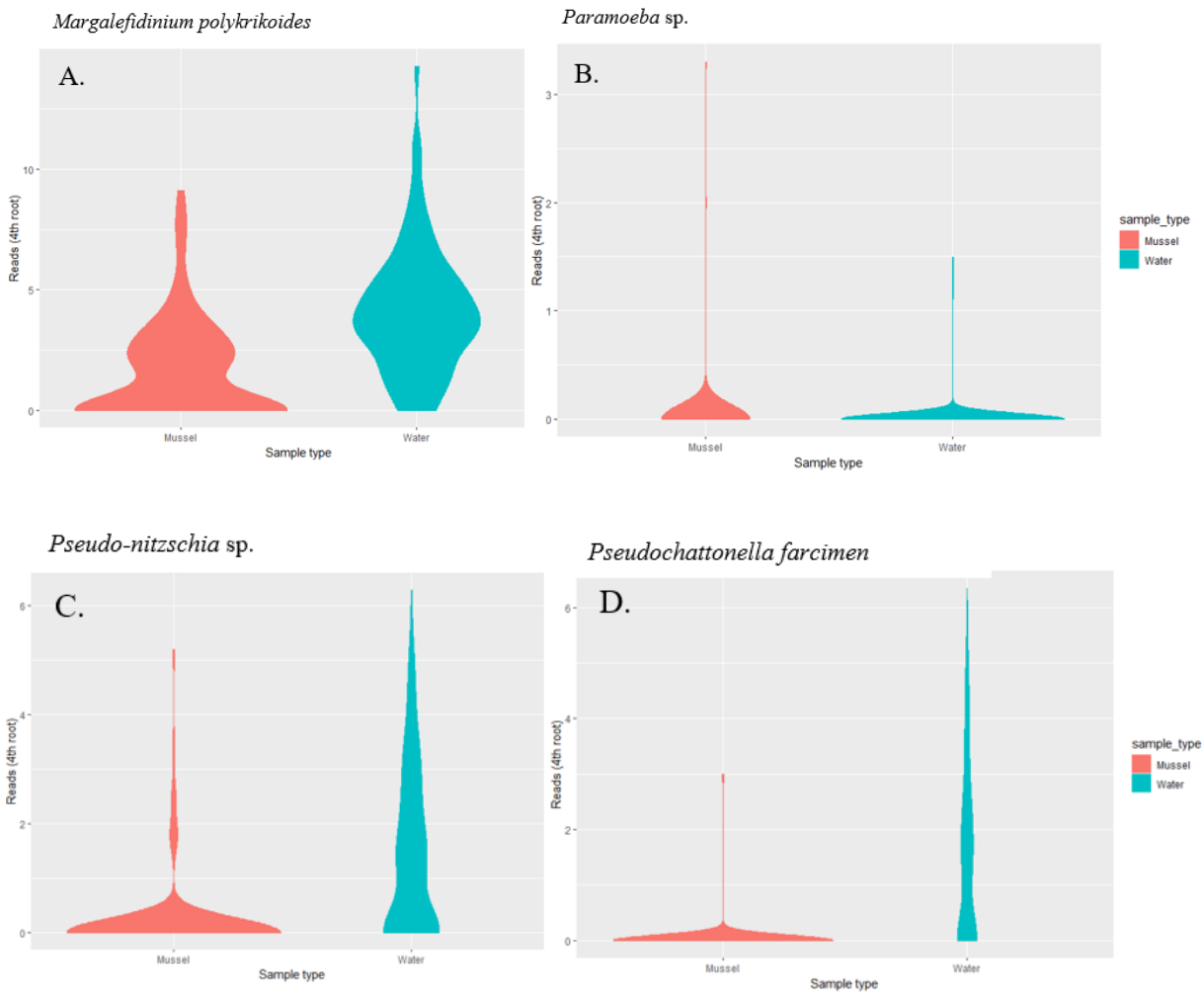

Figure 16. Forth root of absolute read abundance of Margalefidinium polykrikoides (A*),* Paramoeba *sp. (B),* Pseudo-nitzschia *sp (C) and* Pseudochattonella farcimen *(D) in blue mussel samples and water samples.*

4 Discussion

Natural samplers of eDNA have been forwarded as a promising new avenue for surveying biodiversity in nature due to their presumed ability to integrate eDNA signals through time (Siegenthaler et al., 2018; Mariani et al., 2019; Turon et al., 2020; Jeunen et al., 2021). This suggests that eDNA extracted from natural samplers should enable more consistent determinations of e.g., harmful species in aquaculture, compared to eDNA extracted from seawater. The overall objective of this thesis was to investigate blue mussels as a natural sampler of eDNA in seawater. This study was the first to attempt to use blue mussel stomachs as a natural sampler of eDNA diversity over a longer period. Similar comparisons of eDNA retrieved from blue mussels versus eDNA from water samples have been done only in "snapshots", but with blue mussel gill tissue and pooling of the individuals (Jeunen et al., 2021; Weber et al., 2021) (both studies are in preprint, not peer-reviewed). In contrast to these two studies, my study was done with COI primer and is the first study to do so on eDNA collected from blue mussels, and will contribute to a better picture of eDNA signals detected by blue mussels. The results from the two previous studies are conflicting, with one observing no reliable detection of eDNA in mussel tissue (Jeunen et al., 2021), while the other study observed eDNA taxa overlap between mussels and water, but with lesser species richness in the mussels than in water samples (Weber, et al., 2021). My study adds to the result of more richness in water samples than blue mussel samples. The blue mussels detected the most abundant species in water samples but with lower relative abundance. Seasonal signals are picked up by the blue mussel, but with other species contributing to the seasonal difference than in water samples. Three out of four selected pathogen MOTUs were better detected in water samples than in blue mussel samples. The only exception was *Paramoeba* sp. which had a higher number of reads in the blue mussel samples. In this discussion I will achieve the main objective by discussing the secondary objectives; i) comparison of differences in diversity obtained from metabarcoding of eDNA isolated from blue mussels and water samples throughout 15 months of sampling, and ii) investigate how the blue mussels perform as a sampler of pathogens and harmful species in aquaculture compared to water samples.

4.1 Comparison of eDNA diversity obtained from blue mussels and water through time

eDNA retrieved from the filter feeder *M. edulis* had lower species richness and did not reflect the eDNA community retrieved from water samples. This finding leads to a rejection of the hypothesis that eDNA from blue mussels provides higher species richness compared to eDNA

from water samples. Sponges are the most studied filter feeder as a natural sampler and have proven to detect differences between habitats (Mariani et al., 2019) since they detect fish species that are known to a specific habitat (Turon et al., 2020), and they detect eDNA signals that are also detected in eDNA water samples (Jeunen et al., 2021). Metabarcoding of stomach content from brown shrimp *(Crangon crangon)* and northern shrimp (*P. borealis*) has also proven to recover fish diversity efficiently compared to traditional survey techniques (Siegenthaler et al., 2018; Urban, 2019). An issue with the detection of eDNA when using stomach content is the overabundance of host DNA in the extraction (Krehenwinkel et al., 2017). In the blue mussel samples, Mytiloida accounted for 96,7% of the total reads, and thus was removed from the final OTU table and the total amount of reads used in the analysis was therefore considerably lower than in the water samples. The host DNA might be a reason for a lower number of reads of other MOTUs due to high competition during PCR (Weber et al., 2021), thus dominant sequences will prevail (Vestheim & Jarman, 2008). A possible solution to this issue would be to use blocking primers, to prevent the amplification of the dominant DNA from the host itself and it has been shown that the use of blocking primers can enable the detection of less abundant DNA fragments when using universal primers (Vestheim & Jarman, 2008). Primers that suppress mussel DNA fragments have successfully been used in a natural sampler diet study previously (Weber et al., 2021). However, it has also been shown that blocking primers may unspecifically inhibit the detection of some other taxon groups, which is very difficult to predict and should be assessed using extensive empirical testing (Tan & Liu, 2018). The sampling of both blue mussels and water was done close to the aquaculture site where more than a million farmed salmon were reared. There were a greater number of *Salmo salar* reads in the water samples than in the blue mussel samples, but the amount was still low considering the number of farmed salmon present. The location of the blue mussel net and the water sampling points were both on the south side of the aquaculture site, while the current normally has a northbound direction, suggesting that eDNA and pelagic communities could be drifting away from the sampling site. This might be one reason for the low detectability of *S. salar*. Another possible reason for the lack of salmon reads is the challenge in metabarcoding for quantification of abundance in community samples (Peters et al., 2018), and the biases that could be introduced by extraction method, PCR conditions, or marker/primer selection (Bista et al., 2018; Collins et al., 2019). Most studies on natural samplers have been done using vertebrate-specific markers (12S,16S) (Mariani et al., 2019; Urban, 2019; Turon et al., 2020; Jeunen et al., 2021), where this study used the COI Leray-XT marker that is amplifying the eukaryotic community. These studies focused on the detection of fish MOTUs, where universal

COI primers are known to detect small amounts of fish reads (Grey et al., 2018). Comparison between primers should therefore be done with caution (Siegenthaler et al., 2018). To be able to compare this study with other natural sampler studies comparison between different primers is done to a certain extent, which has to be taken into account when reading and interpreting this discussion. An example of the difference in fish diversity obtained from shrimp stomach using 12S and COI found that the 12S detected about 200 MOTUs but only 62 was assigned to species or genus level while the COI had fewer fish MOTUs detected but 25 out of 27 was identified to species or genus level (Siegenthaler et al., 2018). Using more specific taxon markers such as 12S and 16S also opens up the possibility to pool the samples (done in several natural sampler studies) (Sato et al., 2017), and if blocking primers are added the dissection does not have to be done with such caution because host DNA will not be amplified. As done with shrimp stomachs it would be interesting to see the results provided from the blue mussel samples with vertebrate-specific markers, to investigate their potential for monitoring fish diversity.

The water samples had more than three times higher number of MOTUs detected (2250 MOTUs) than the number of MOTUs derived from blue mussels (718 MOTUs). Both sampling methods could provide more MOTUs with increased sampling effort according to the species accumulation curve. Only two studies have been done on eDNA obtained from a possible natural sampler versus eDNA obtained from water samples (Jeunen, et al., 2021; Weber, et al., 2021). Similar to the results from this study, the largest number of taxa was observed in water samples compared to filter feeder samples. The size of eDNA in water ranges from smaller than 0.2 μm to several 100 μm but is most abundant below 0.2 μm (Turner et al., 2014). The particle retention of the mussel is most efficient around 3-35 μm (Vahl, 1972) (Strohmeier et al., 2012), which indicates that the mussel has a narrow window of eDNA size that they retain. Retrieved eDNA from stomach content will also be affected by gut passage time, if there are separate "meals" a day then the eDNA content may be different depending on what was ingested in the "meal" before the sampling, as seen in other species (Deagle et al., 2005). In such case, blue mussels might capture the eDNA community better than water samples if the sampling interval matches the gut passage time, of approximately 10-15 hours depending on blue mussel size (Hawkins et al., 1990). Because of the selective size range of particle retention in the blue mussels their eDNA detection performance might differ between species. Further investigation into which species and what kind of eDNA they capture can clarify if blue mussels capture some species better than water samples. Weber et al. (2021) reported that the mussels retained

metazoans equally as well as the water samples in their study. Cnidaria was one of the phyla that were abundant in the blue mussel samples, a closer look at this group revealed that some species were only found in the water samples, and some were only found in the blue mussel samples. In this study, it is not possible to conclude that the blue mussels sample Cnidarians better than water, but IndVal analysis indicated that there were species that were present at a high frequency in the blue mussel samples that were not detected in the water samples.

Seasonal change was detected with metabarcoding of eDNA from blue mussel samples, but the seasonal change detected in the water samples and blue mussel samples were significantly different. The species that were most dominant in the seasonal changes within water samples were *Bathycoccus prasinos* (identity 1.00), *Pycnococcus provasolii* (identity 1.00), and *Phaeocystis antarctica* (identity 0.92). One of the MOTU with the largest relative read abundance in the water samples was *Micromonas pusilla* (identity 1.00) but it did not have a significant contribution to seasonal difference. *B. prasionos* (identity 1.00) and *M. pusilla* (identity 1.00) had a large relative abundance in both seasons of water samples. *M. pusilla* has a worldwide distribution and has been recorded as most abundant in Skagerak/Kattegat region in the spring (Sahlsten, 1998), which indicates a bloom in North Norway later and probably within what is defined as "summer months" in this study. The relative read abundance of *P. antartica* (identity 0.92) in summer samples of water was significantly larger than in the winter samples. *P. antartica* has previously been reported as present in the Barents Sea but has mostly been studied in the Ross Sea where it is associated with extensive phytoplankton blooms (Mathot et al., 2008). Of the two species with a significant effect on season in water samples (*B. prasionos* and *P. provasolii)* and *M. pusilla* (identity 1.00) with a high relative read abundance, only *M. pusilla* (identity 1.00) was detected as a species that contributed to seasonal changes in blue mussel samples but with a low relative read abundance. In addition, the blue mussel samples had another OTU assigned as *M. pusilla* (identity 0.97), *Minutocellus polymorphus* (identity 0.87), and *M. polykrikoides* (identity 0.99) that were the four species contributing mostly to seasonal change in the blue mussel samples. Even though seasonal signals were picked up in the blue mussel samples, they were driven by different MOTUs than the MOTUs that contributed to the seasonal changes in the water samples. Nevertheless, the seven species that were the main contributors to seasonal changes in the water samples were all detected in the blue mussel samples. The largest difference between the sampling methods was the relative abundance of these species, that most likely was highly affected by a large amount

of host reads in the blue mussel samples, and/or by the difference in capture selectivity due to the different size range of these microalgae.

A noticeable difference between seasons in the blue mussel samples was that the eDNA retrieved in the winter samples was more evenly distributed between MOTUs, while the summer samples were dominated by the four species: *M. pusilla* (identity 0.97), *M. polymorphus* (identity 0.87*), P. provasolii* (identity 1.00) and *Sarsia Lovenii* (identity 0.99). The same pattern was not seen in the water samples, but in the summer months, the water samples are highly dominated by pelagic green algae species, while in the winter months the samples are more evenly distributed between species from several kingdoms. Large amounts of green algae in both sampling methods in the summer such as *P. provasolii* and *M. pusilla* is coherent with the bloom of the primary production in the Arctic and sub-Artic Ocean (Pabi et al., 2008; Coguiec, et al., 2021). Green algae have a wide range of sizes from 0.2 μm for picoplankton up to 2 mm for mesoplankton (Not et al., 2012). The blue mussels should not efficiently retain species or particles below a size of 3 μm, which is consistent with my findings that the two green algae species *P. provasolii* and *M. pusilla* with the highest relative read abundances in the blue mussel samples are picoplankton that usually is less than 2 μm in length. A possible explanation for why these MOTUs were present in such a high abundance in the blue mussel samples could be that the blue mussel does retain the largest individuals of these picoplankton and that blue mussels do efficiently retain particles with a smaller size than 3 μm. It could also be a result of secondary predation; the two picoplankton species are eaten by other species that are in the size range that the blue mussel efficiently retains. The green algae species *Ostreococcus tauri* was very abundant in the water samples but was only detected in one blue mussel sample with a total of 22 reads. As *O. tauri* has a cell diameter of less than 1 μm (Not et al., 2012). This could indicate that the actual distinction between the size that blue mussels retain efficiently and the size they do not retain efficiently is somewhere between 1 μm and 2 μm. The difference in MOTUs that was detected within and between the blue mussel samples and the water samples do show that the blue mussels have a certain selectivity in what they digest, both in terms of the size of the particles they retain and what MOTUs they prefer. However, when performing a natural sampler study with stomach content it is difficult to determine if the detected MOTUs are a part of the diet or secondary predation, and therefore knowledge about predator-prey relations and secondary predation is important (Calvignac-Spencer et al., 2013; Siegenthaler, et al., 2018) to be able to interpret the results from this kind of natural sampler study.

For a filter feeder to reflect the whole eDNA community it needs to have the morphological and physiological ability to ingest and accumulate both extracellular eDNA from water and the total pelagic community. The particles in the water are a part of the blue mussels' diet and do either get digested or filtrated out as pseudofaeces. The eDNA extracted from the blue mussels' stomachs has gone through the digestive system and has been impacted by enzymes and other processes related to digestion. How that affects the degradation and detectability of DNA in the stomach is not known and would require an experimental study in a controlled environment to investigate. The particles that get filtrated out as pseudofaeces will not be a part of the DNA extracted from the stomach. These particles might be detected if the DNA extraction is done on gill tissue or the entire blue mussel tissue. In further investigation into blue mussels as a natural sampler, it would be interesting to compare eDNA detected in blue mussel stomach and gill tissue to determine where the MOTU detection is highest.

4.2 Pathogens in aquaculture

Traditionally, important pathogens for aquaculture are surveyed through monthly routine visits by veterinarians and visual observations by the farmers working at the aquaculture sites. Detection of pathogens in environmental samples or from natural samplers has the potential to become a method to continuously monitor the pathogens present in the surrounding water of an aquaculture site. The water samples had both a higher richness and absolute read abundance of pathogens than the blue mussel samples except for *Paramoeba sp.* Even though the blue mussels had one species that was better detected than in water samples, the overall results lead to rejection of the hypothesis that blue mussels detect a higher richness of pathogens at a higher absolute abundance than water samples. Herein I discuss the difference in pathogen detection between blue mussel samples and water samples and the applicability of detecting pathogens with metabarcoding of eDNA retrieved from natural samplers in the future.

Continuous pathogen detection with non-invasive molecular methods in aquaculture has shown potential in both this and other studies. Although the marker used in this study is not specifically selected for pathogen detection there were several MOTUs of pathogens, parasites and harmful algae detected in the samples that can negatively affect aquaculture production. Multiple pathogens related to aquaculture have previously sensitively and specifically been retrieved from water with eDNA metabarcoding of the 18S SSU V9 region (Peters et al., 2018). 22 out of 39 MOTUs of salmon pathogens (viral, bacteria, and eukaryotic) were detected with qPCR of eDNA in water sampled nearby several aquaculture sites (Shea et al., 2020), and salmon lice

(*Lepeophtherirus salmonis)* have been detected in eDNA from water samples using qPCR and metabarcoding (Peters et al., 2018; Krolicka et al., 2021). All these studies prove that it is possible to detect pathogens and other harmful species in environmental samples using qPCR or metabarcoding. My study is the first to use metabarcoding of eDNA retrieved from a natural sampler to detect aquaculture-related pathogens. All MOTUs detected in association with organisms and potential disease or distress for farmed salmon were found with both sampling methods, except for *Chrysochromulina simplex* and *Anisakis simplex* which were only identified in water samples. The most abundant pathogen detected with both sampling methods was *M. polykrikoides* (previously named *Cochlodinium polykrikoides* (Aquino-Cruz et al., 2020)), which is a dinoflagellate related to harmful algal blooms and extensive fish death in Latin America (López-Cortés et al., 2019) and Asia (Kim et al., 1999). *M. polykrikoides* can bloom in a wide temperature range (17-32°C) (López-Cortés et al., 2019). How it affects farmed salmon is not known, but it has affected several marine organisms such as fish, crabs, and shrimps (López-Cortés et al., 2019). The high abundance of *M. polykrikoides* in the samples despite the non-optimal temperature for a possible bloom indicates that this species is present at a high abundance without causing any detectable distress for the farmed salmon in the area. This can be explained by the relatively low water temperature (2-12ºC) in the area where the samples are taken. In the southern part of Norway, however, where the temperature in the water can reach 20°C in the summertime, a bloom of *M. polykrikoides* could pose a threat to the aquaculture production. Additionally, with global warming causing higher temperatures in the water along the Norwegian coast, the areas with suitable environmental conditions for this species to cause a harmful algal bloom will increase. The relative read abundance of *Paramoeba sp.* was higher in the blue mussel samples than in the water samples, but the *Paramoeba sp.* MOTUs were not detected very frequently in the blue mussel samples. One possible explanation for a higher number of reads in the blue mussel samples could be that the time of sampling was done within a "pulse" of digestion and that the signal of *Paramoeba sp.*, therefore, was well integrated in the blue mussel stomach. Even though the water samples only represent snapshots of the eDNA present in the water, the sampling was done with replicates at several points and over a long period which should make the margin of error small. The read abundance for most pathogens in the blue mussel samples was lower than in water samples. Still, the fact that the blue mussels do detect pathogens can in some cases be sufficient as using natural samplers can ease the sampling process. Instead of collecting and filtering a large volume of water, the alternative is to pick up a blue mussel at the area of interest, extract DNA and most likely find several hundred MOTUs. This is one of the key reasons to use blue mussels

as natural sampler, they are easy to access and can be found in most places along the Norwegian coast. Blue mussels are already being used as biological indicators for monitoring coastal water pollution in mussel watch programs (Beyer et al., 2017). In such mussel watch programs, it would be easy to integrate eDNA extraction from the blue mussels that are collected and increase the information obtained from the mussels about the surrounding environment beyond contaminants.

Despite the high number of host reads in the blue mussel samples in my study the taxonomic diversity is high relative to the limited effort that it required. When comparing results from natural sampler studies to traditional survey methods or eDNA retried from water the results should be considered with cost-efficiency in mind (Turon et al., 2020). Further investigations into the species that the blue mussels can sample will proved insights to decide where the applicability of blue mussels as a natural sampler is a suitable option. In addition, for environmental samples and metabarcoding to become a commercial method for pathogen detection in aquaculture the processing and sequencing need to be efficient, cheap, and easy to access (Peters et al., 2018). Regardless of the lower relative abundance of most species in the blue mussel samples, the results from my study suggest that blue mussels could be a promising natural sampler because in some cases the most important result is not the abundance of a species but merely the detection of the species' presence.

5 Conclusion

This study adds to the promising results from other natural sampler studies and confirms that filter feeders can be used for the retrieval of eDNA from seawater. The blue mussel samples succeeded in detecting seasonal signals. The seven species contributing mostly to the distinction between seasons in water samples were detected in the blue mussel samples, although the two species with the highest contribution to seasonal difference in water samples *(B. prasinos* and *P. provasolii*) not were the same as the two species contributing most to seasonal difference in blue mussel samples (*M. pusilla* (identity 1.00) and *M. pusilla* (identity 0.97)). Detection of pathogens was generally better in water samples, but six out of eight pathogen genus' were detected in blue mussel samples with better detection of *Paramoeba sp.* than in water samples. A possible next step could be to investigate eDNA diversity obtained from blue mussel tissue versus blue mussel stomach and a monitoring study of pathogens in aquaculture with specific primers or a focus on *Paramoeba sp.* detection in blue mussels to further verify their ability to detect this species better than water samples. All in all, the results

state that blue mussels can be used as natural samplers of eDNA, but they do not reflect the same species richness and relative abundance of species as the eDNA from water samples.

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7 Supplementary plots and materials

Suppl. Table 1. Concentration of libraries used on the blue mussel samples and the proportion of how they were pooled.

	Qubit cons	µl DNA pool	µl water
MUS 2	378 ng/µl	7,94	32,06
MUS 3	436 ng/µl	6,88	33,12

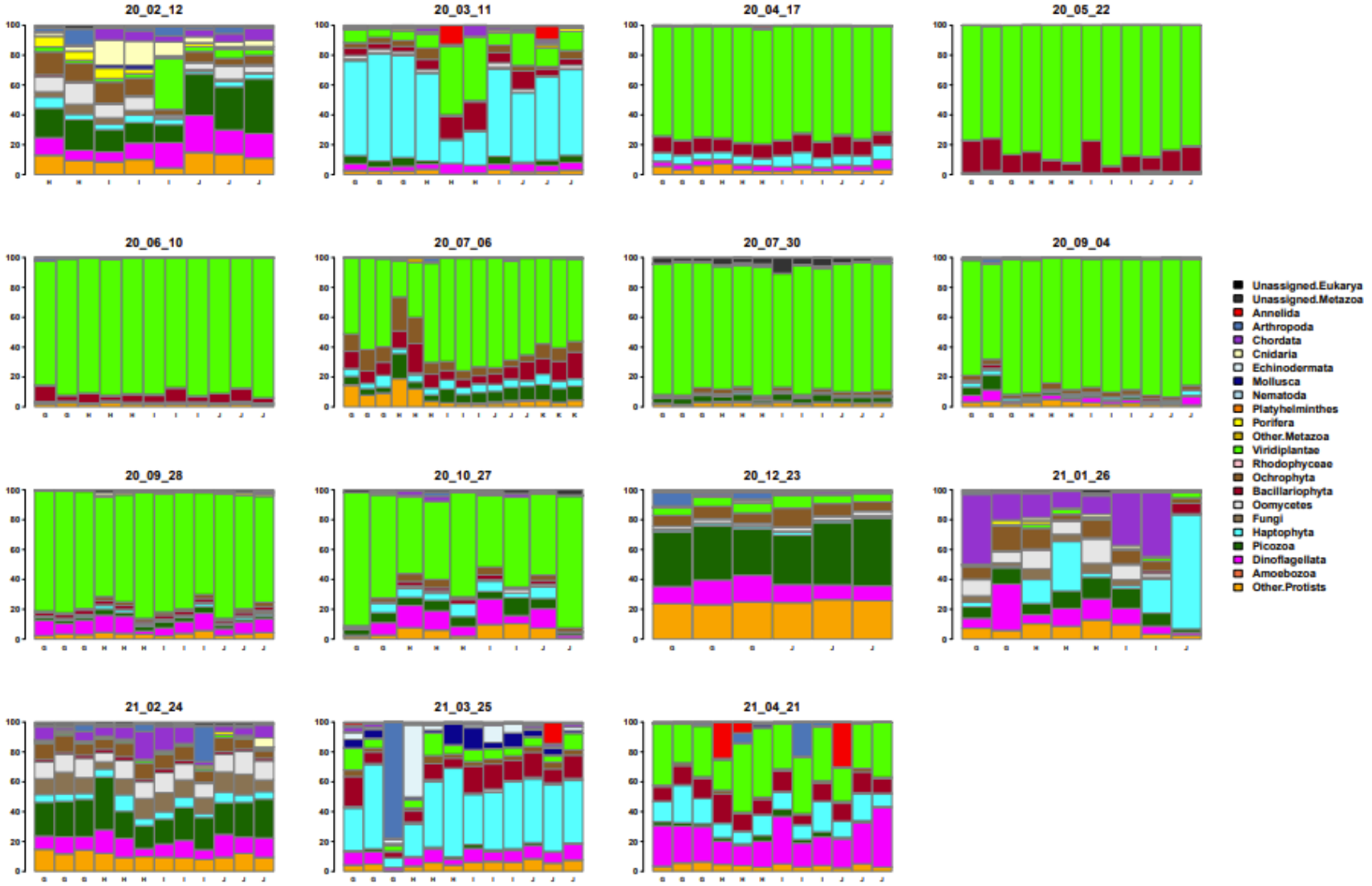
Suppl. Table 2. Pairwise PERMANOVA (Adonis) on water sampler and blue mussel samples for seasons "summer" (May-October) and "winter" (November-April).

Sample type+season	*df*	*SS*	*R2*	*F*	*P*
Mussel-Winter vs. Mussel-Summer	1	1.111	0.028	2.844	**0.001**
Mussel-Winter vs. Water-Winter	1	7.907	0.244	25.818	**0.001**
Mussel-Summer vs. Water-Summer	1	5.041	0.165	14.999	**0.001**
Water-Winter vs. Water-Summer	1	3.302	0.213	12.011	**0.001**

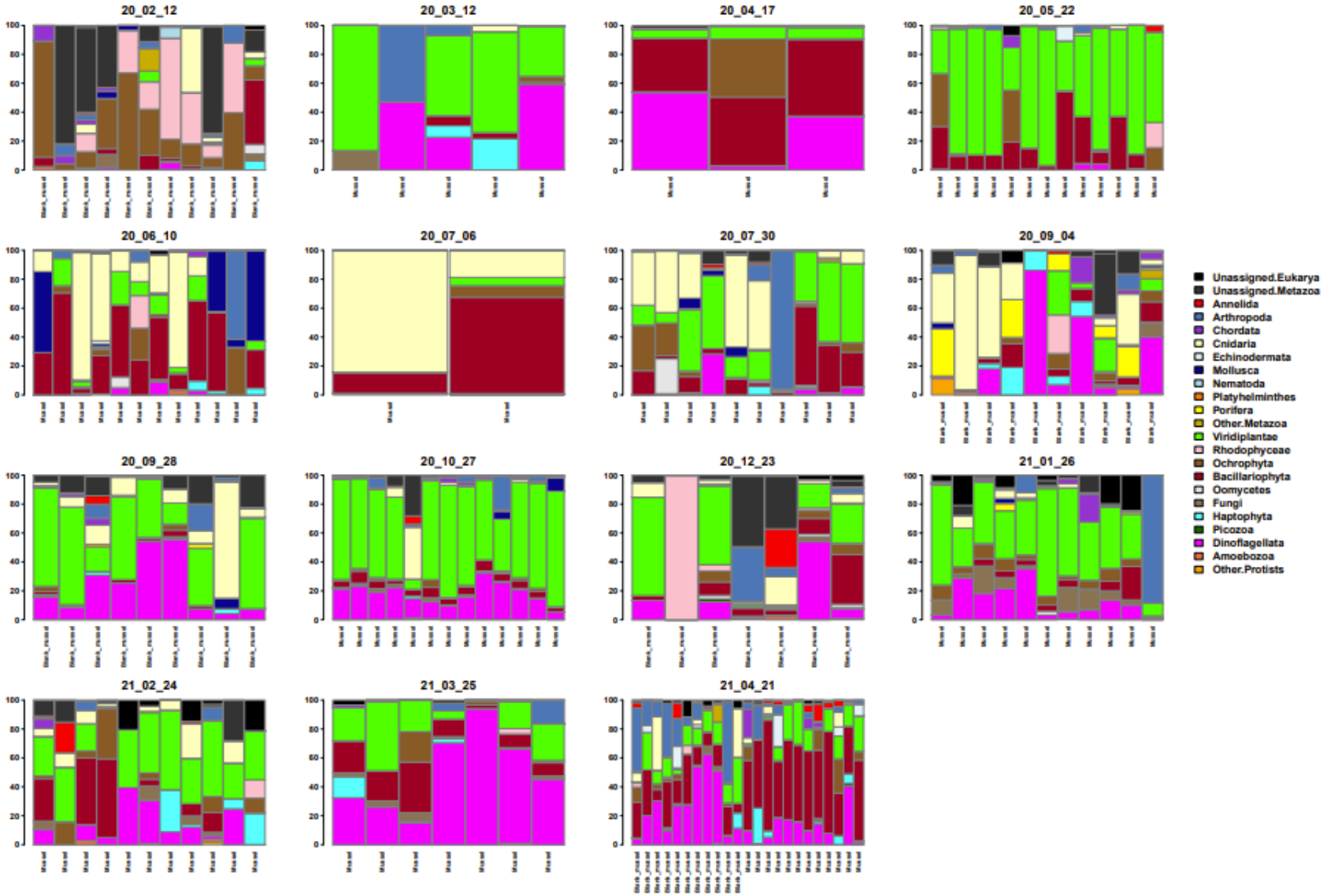
Suppl. Table 3. Species contribution to seasonal difference and relative abundance in blue mussel samples and water samples. P-value retrieved from IndVal analysis. Summer= May-October. Winter=November-April.

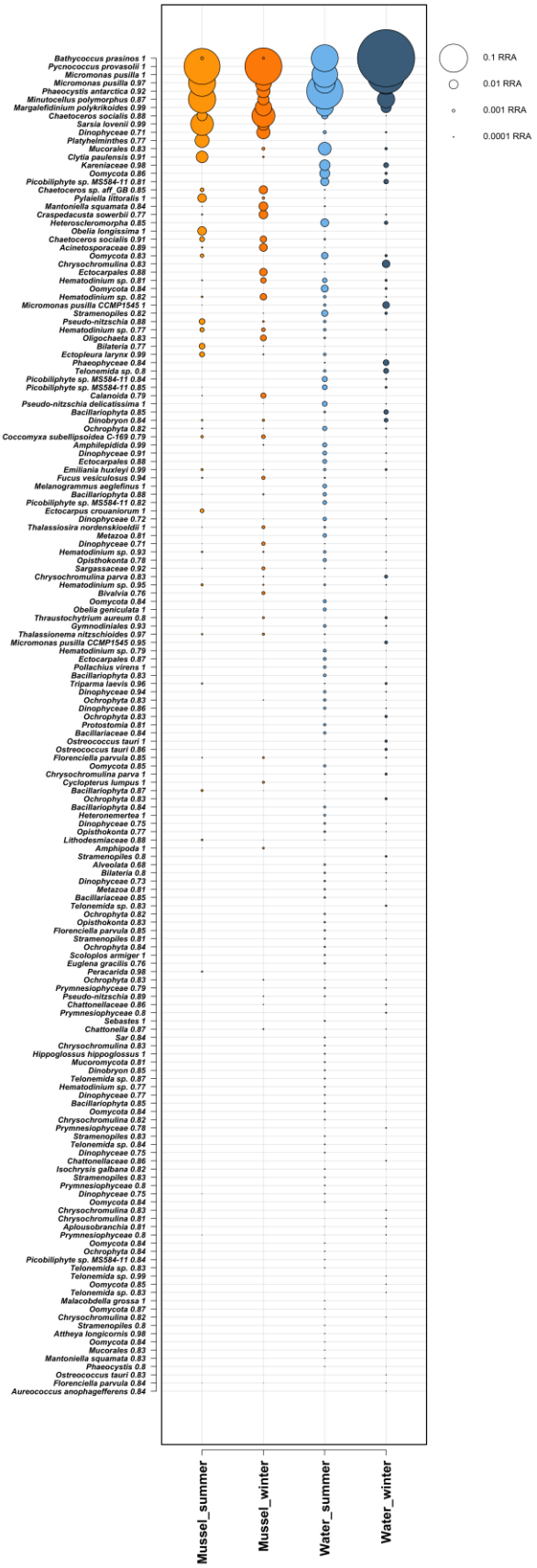
MOTU	*Mussel summer*	*Mussel winter*	*P-value*	*Water summer*	*Water winter*	*P-value*
Bathycoccus prasinos (1)	1.038747e-01	5.708705e-02	0.447	9.166157e+00	4.143819e+01	**0.001**
Pycnococcus provasolii (1)	1.634630e+01	1.729886e+01	0.637	3.479583e-01	7.079239e-02	**0.001**
Micromonas Pusilla (1)	2.756103e-01	4.259987e-02	**0.038**	8.310635e+00	1.789643e+01	0.002
Micromonas Pusilla (0.97)	9.208460e+00	3.302464e+00	**0.003**	5.434614e+00	6.649250e+00	0.655
Phaeocystis antarctica (0.92)	1.055591e+00	2.241640e+00	0.553	1.645844e+01	2.934355e-01	**0.001**
Minutocellus polymorphus (0.87)	9.377248e+00	1.614876e+00	**0.001**	4.265810e+00	3.746925e+00	0.765
Margalefidinium polykrikoides (0.99)	8.332969e-01	3.433172e+00	**0.002**	3.686723e+00	1.316276e+00	**0.001**
Chaetoceros socialis (0.88)	1.268973e+00	6.666583e+00	**0.008**	5.358644e-01	3.051827e-03	**0.001**
Sarsia lovenii (0.99)	6.474355e+00	1.949262e+00	**0.038**	7.620844e-04	7.741975e-05	0.147
Dinophyceae (0.71)	3.287017e-01	2.224564e+00	**0.004**	1.296064e-01	2.947363e-02	**0.001**
Platyhelminthes (0.77)	2.493112e+00	0.000000e+00	**0.047**	0.000000e+00	0.000000e+00	1
Mucorales (0.83)	0.000000e+00	1.107226e-01	1	2.092706e+00	8.172196e-02	**0.001**
Kareniaceae (0.98)	0.000000e+00	0.000000e+00	1	1.414776e+00	2.992671e-01	**0.001**
Oomycota (0.86)	0.000000e+00	0.000000e+00	1	1.283157e+00	7.689899e-02	**0.001**
Picobiliphyte sp. MS584-11 (0.81)	0.000000e+00	0.000000e+00	1	8.639386e-01	2.720698e-01	**0.001**

Suppl. Figure 1.Contribution of different kingdoms to water samples from different sampling months based on COI metabarcoding. The sequence reads were normalized by relative read abundance.



Suppl. Figure 2. Contribution of different kingdoms to stomach content in blue mussels sampled through 15 months based on COI metabarcoding. Sequencing reads were normalized using relative read abundance





Suppl. Figure 3. Molecular diversity in blue mussel samples and water samples between seasons summer (May-October) and winter (November-April) based on p-value retreived from IndVal analysis and normalization of data with realtive read abundance (0-100).

Suppl. Table 4. Pathogen MOTUs detected in blue mussel samples and water samples collected near an aquaculture site in Skogshamn, Dyrøya. Number in parenthesis is the identity nr of the MOTU retreived from dataset produced by MJOLNIR.

	Absolute read nr	
Scientific name	**Mussel**	**Water**
Margalefidinium polykrikoides (0.987)	24 889	272 578
Pseudo-Nitzscia delicatissima (1)	7	9555
Pseudo-Nitzscia sp (0.883)	880	2877
Pseudo-Nitzscia sp (0.895)	43	740
Pseudo-Nitzscia sp (0.877)	44	-
Pseudo-Nitzscia sp (0.898)	-	11
Pseudo-Nitzscia sp (0.904)	-	4
Parvamoeba rugata (0.848)	121	-
Parvamoeba rugata (0.851)	120	1
Parvamoeba rugata (0.870)	86	-
Parvamoeba rugata (0.846)	-	44
Parvamoeba rugata (0.882)	-	40
Parvamoeba rugata (0.847)	25	11
Parvamoeba rugata (0.853)	20	-
Parvamoeba rugata (0.876)	9	3
Parvamoeba rugata (0.874)	11	-
Parvamoeba rugata (0.849)	-	39
Parvamoeba rugata (0.830)	37	-
Parvamoeba rugata (0.850)	10	-
Parvamoeba rugata (0.854)	-	7
Parvamoeba rugata (0.873)	-	6
Parvamoeba rugata (0.847)	-	6
Parvamoeba rugata (0.848)	-	5
Parvamoeba rugata (0.856)	-	5
Parvamoeba rugata (0.895)	-	4
Parvamoeba rugata (0.876)	-	4
Parvamoeba rugata (0.831)	-	3

Parvamoeba rugata (0.846)	-	3
Parvamoeba rugata (0.846)	-	3
Parvamoeba rugata (0.852)	-	3
Parvamoeba rugata (0.8495)	6	2
Parvamoeba rugata (0.883)	-	3
Parvamoeba rugata (0.855)	-	3
Parvamoeba rugata (0.875)	-	3
Parvamoeba rugata (0.889)	-	3
Parvamoeba rugata (0.843)	-	3
Paramoeba pemaquidensis (0.914)	98	-
Paramoeba pemaquidensis (0.898)	29	2
Paramoeba sp (0.867)	118	-
Paramoeba sp (0.911)	-	33
Paramoeba sp (0.838)	-	9
Paramoeba sp (0.849)	-	5
Paramoeba sp (0.824)	-	3
Chrysochromulina parva (0.794)	-	24
Chrysochromulina parva (0.840)	-	12
Chrysochromulina parva (0.820)	-	2
Chrysochromulina parva (0.826)	81	18 286
Chrysochromulina parva (0.831)	8	548
Chrysochromulina parva (0.799)	-	2224
Chrysochromulina parva (0.819)	-	743
Chrysochromulina parva (0.809)	-	99
Chrysochromulina parva (0.811)	-	48
Chrysochromulina parva (1)	-	6036
Chrysochromulina parva (0.811)	-	709
Chrysochromulina parva (0.801)	-	282
Chrysochromulina parva (0.819)	-	278
Chrysochromulina parva (0.808)	-	62
Chrysochromulina parva (0.795)	-	8

Chrysochromulina simplex (0.838)	-	21
Chrysochromulina simplex (0.837)	-	5
Chrysochromulina simplex (0.844)	-	2
Chrysochromulina simplex (0.838)	-	233
Chrysochromulina simplex (0.987)	-	126
Chrysochromulina simplex (0.857)	-	755
Chrysochromulina simplex (0.860)	-	478
Anisakis simplex (1) *(parasite)*	-	46
Pseudochattonella farcimen (0.974)	80	13 479

7.1 Appendix A- Water sampling protocol

Skogshamn eDNA project sampling guidelines

Water sample collection:

Before navigating to a sampling point, clean (10% klor mixture) and rinse (saltwater) the Niskin bottle and attached line in the black storage bucket and make them ready for sampling. Then clean and rinse the surfaces of the boat where sampling will occur.

Once at the intended sampling point:

1. Spring load the bottle lids and rinse the Niskin bottle with saltwater before use.
2. Lower the Niskin bottle to the surface depth (~2m), send the weight to close the lids, then retrieve the bottle.
3. If the lids are not fully closed, reload and try again.
4. Label a sterile plastic bag with the sample location(A-Z) and collection depth (2, 15, or 200), remove the plastic seal, then open it and fill it by carefully pouring collected seawater directly from the top of the Niskin bottle.
5. Close the bag by squeezing out the remaining air, rolling the top 3-5 times, then twist the wire ties.
6. Bring the sealed bag to the plastic bin and place it upright to prevent leakage.
7. Reload the lid spring and slide weight back up the line in preparation for the next collection.
8. Navigate back to the same GPS point for the second depth collection if any drift has occurred or on to the next sampling site.
9. Repeat water sample collection protocol from step 1 for each remaining sample, then immediately return to the float to begin filtration process.

Static pump setup, water sample filtration, and storage protocols

Filtration station setup and pump assembly:

Bring container of water samples from boat to pumping area.

Set up folding table.

Spray with bottles and wipe with lint free paper towels to clean (klor mixture) and rinse(dH2O):

Table surface

Outside of tubing

Inside and outside of all 500mL and 1L plastic containers

Any other materials or surfaces used during the pumping process

Unpack pump and place on raised surface above table. Carefully mount pump head with Allen wrench and 4 screws provided. Plug in. Test for any noises or visible issues.

Unpack 3 cassettes and attach to pump with tubing running through them. Cassette tube size markers should be set to 17 on both sides and pressure applicator loose enough for cassettes to click into place with gentle downward pressure.

Fill a 1L container with klor mixture and another with dH2O and place both on input side of pump.

Place 3rd 1L container for waste on output side. This will be used for Klor, dH2O, and priming saltwater

Pumping procedures:

1. Clean surgical tubing of residual DNA by pumping klor mixture through all 3 tubes into the waste container. Run pump until tubes are completely full of klor mix, then shut off and let sit for 2min.
2. Remove input tubes from solution and pump remaining liquid from all 3 tubes.
3. Rinse chlorinated tubing by pumping dH2O through all 3 tubes and into the waste container. Use ~200mL dH2O, then repeat step 2
4. Select a sterile water bag and note location and depth labels. Place it in an empty filters box (for better stability) on the input side, carefully open it, and place all 3 pieces of tubing in it.
5. Prime all 3 tubes with saltwater by pumping ~200mL through them into the waste container, then shut off pump.
6. Change nitrile gloves.
7. One at a time, remove filters from sterile packaging and attach filters to output end of each tube. The exposed end of each filter should rest on the rim of an empty 500mL container, pointing downwards so all filtered water is collected in the container without immersing and contaminating the filter.
8. Begin pumping. Keep at 60rpm to prevent excess pressure from popping the filters off of the tubes.
9. When filtered water levels near the 500mL mark, remove the input end of each tube from the water sample bag. Continue pumping air until the filter becomes visibly dry.
10. Once all filters appear dry, turn off pump, remove filters, and place each into a separate pre-labeled falcon tube. Each should be labeled with location letter and depth number from the water sample bag. Also include a replicate number(r1, r2, or r3) for each of the 3 filters.
11. Place all 3 falcon tubes into a small ziplock bag labeled with the sampling letter, depth number, and date of collection.
12. Place that bag into a large ziplock bag with all water sample filters from that same sampling date. Close the larger bag between samples to reduce contamination from other DNA sources.
13. Repeat from step 1 for each water sample.

After completing filtration of all samples, double bag the samples and place in the eDNA only freezer onsite as soon as possible.

Freezer guidelines:

The freezer should only be used for eDNA water filters. Avoid opening it unless adding samples for storage or removing bags for immediate transportation to UiT.

Before leaving the float:

Clean(klor mixture) and rinse(freshwater) all sampling equipment in black buckets and pack away neatly in the designated area.

Spray/wipe clean(klor mixture) and rinse(dH2O) all pumping and filtration equipment before placing it in eDNA storage boxes(aluminum) and packing them away neatly in designated area.

Ensure doors between workshop and living areas are closed, interior lights are off, and rolling door is completely shut and bolted.

7.2 Appendix B- DNA extraction blue mussels

Modified DNeasy PowerSoil Kit Protocol

1. Add 0.3 g of sample to the PowerBead tube provided.
2. Take the Solution C1 from the heating cabinet and add 60 µl to the sample and invert several times or vortex briefly.
3. Secure PowerBead tubes horizontally on the vortex using a Vortex Adapter for 24 tubes.
4. Vortex at speed 3 for 1 hour at room temperature inside the cabinet.
5. Centrifuge tubes at 10 000 x g for 1 min
6. Transfer the supernatant to a new collection tube.
7. Add 250 µl of Solution C2 and vortex for 5 seconds.
8. Centrifuge the tubes at 10 000 x g for 1 min
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a new collection tube.
10. Add 200 µl of Solution C3 and vortex briefly.
11. Centrifuge the tubes at 10 000 x g for 1 min
12. Avoiding the pellet, transfer up to 700 µl of supernatant to a larger 2 ml tube.
13. Shake to mix Solution C4 and add 1200 µl (2x600 µl) to the supernatant. Vortex for 5 seconds.
14. Load 630 µl into an MB Spin column and centrifuge at 10 000 x g for 1 min. Discard flow through.
15. Repeat step 14 until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge at 10 000 x g for 1 min.
17. Discard flow-throgh but place the spin column back into the collection tube. Change gloves after discarding. Dry spin, centrifuge at 10 000 x g for 1 min.

18. Carefully place the MB Spin Column into your final and well labelled 1.5 ml tube. Avoid getting any of the Solution C5 on the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane.
20. Centrifuge at room temperature at 10 000 x g for 1 min. Keep the flow-through. Discard the MB Spin Column.
21. Transfer 30 µl of your DNA as an aliquot into a PCR-plate. This eases the downstream lab work of PCR, library preparations etc. Freeze the remaining 70 µl as stock in the freezer.

7.3 Appendix C- Clean lab routines

Norwegian College for Fishery Science
Genetics Group
Last updated: April 2021

CLEAN LAB - RULES

The clean labs are used only for extracting eDNA from water samples and setting up those samples for metabarcoding. The labs are split into an extraction room and a Primer/PCR-setup room. Only people who have completed the specific local training for clean lab work in the eDNA-Labs, are allowed to enter the lab. People who have local training for Lab B310 still needs local training for the clean labs. Please book your lab training with Julie Bitz-Thorsen.

GENERAL RULES IN THE CLEAN LABS

There are a few major rules for these labs, which must always be followed:

1) **You must be freshly showered** (hair and beard too) and wear a clean set of clothes that has not been in any other labs.

2) **You must <u>not</u> enter the clean labs if you have been to any other labs beforehand!**
When entering the lab area, you must only enter in the lab corridors from the door between stairs and elevator. You must have been in any of the high-risk contamination areas before entering the lab (see next point).

3) **You must have entered the NFH-building so that you avoid certain high-risk contamination areas.**
Enter the NFH building from the main entrance or the east side entrance (by the new building). The high-risk contamination areas are our genetic lab B310, PCR-room B307 and adjacent rooms and corridors, 'Fiskemottak' area on the first floor and adjacent rooms and corridors, labs C106, C112, C114 and adjacent rooms and corridors on 1st floor just before reaching the store.
The only way to access the store is to use the staircase in the middle of the building and only walk on the corridors by the workshop and computer workshop. Preferably, you buy from the shop the day before extractions and then shower before entering the next day.

4) **You cannot have eaten any fish on the day of entry to the clean labs.** Or bring food into the lab with you.

5) **There is a one way-rule,** going from the extraction room to the primer/PCR-setup room or any other lab. You cannot return to the extraction room if you have been to any other lab.

CLEAN LAB ROUTINES – C358

Firstly, before you enter the lab, do you have everything you need to perform the lab task at hand? We do not want you to leave in the middle of the process. Remember to visit the restroom as well.

GENERAL CLEAN LAB ROUTINES

1. Take your shoes off in the corridor and bring with you the plastic box (if there is any stuff in it) to bring into the labs. Enter the lock.
2. Leave personal belonging in the small box (i.e., phone, access card, rings etc.) Wash your hands, put on clean suit and blue shoe covers, and finally a pair of gloves.
3. Make fresh 10% bleach in the bucket, and more 50% EtOH solution in the spray bottles if needed. Take a new dish cloth.
4. Clean what was in the plastic box from the corridor and place them where it belongs.
5. If you have samples to bring into a clean lab:
    a. Remove the outer bag and clean the next bag with bleach before entering room. If your samples are not double-bagged (which means only one bag around you triplicates samples), then you clean the bag very thoroughly with bleach, but not ethanol since it will wipe off the label.
    b. Enter the room with only the bags of samples. Leave the sample box in the lock. Put samples in another box inside the extraction room box and put in the fridge.
    c. Exit back to the lock. Clean box used for samples and also the box from the corridor.
    d. Change gloves. Re-enter the extraction room with bleach, ethanol and lab equipment you may wish to bring in.
6. When inside the extraction room put on lab shoes. Put your lab equipment in assigned box with project name. Keep all your things in this box always and keep it clean.
7. Clean you gloves and put a second pair on.
8. Clean the flow hood and all equipment that are to be used for the protocol, i.e., vortex, centrifuges, pipettes, racks on so forth. Clean the orange 50ml tube adapters from the centrifuge and place inside the flowhood. Always clean everything.
9. Place everything in the flow hood and UV-treat it for 10 mins. At this step you can even place the tubes, tips, bags, markers etc. needed for the lab protocol in the hood to be UV'ed too. NEVER UV the chemicals from the kits.
10. While waiting for the UV to finish, first clean the heating cabinet and switch it on, the proceed to clean the large falcon centrifuge, sample wheel (rotator), chair and any other surfaces. Put bags in the two trash cans – one for regular waste and one for lab waste.
11. Change the second pair of gloves and clean them.
12. Proceed to the appropriate lab protocol.

When extractions are done, your samples should be stored in the stock and aliquot freezers respectively. Remember, do not freeze your aliquots if they are to be used within 4 weeks for downstream protocol (i.e., lib.prep., PCR etc.). Store in fridge if that is the case. Store your aliquots in PCR plates or strips. Put two bags around and make sure to label it properly. The rest of your extracted DNA (stock) is stored in 1.5 ml tubes in a cryobox that you have purchased at the store and brought with you. Make sure to label the box properly. Put the cryobox in two bags before storing it in the stock freezer.

AFTER LAB TIME AND EXITING CLEAN LAB

1. Clean your own stuff and put back in assigned box. Clean the sample box that you used for your samples in in the fridge.
2. Clean the flow hood and all equipment that has been used, i.e., racks, vortex, centrifuges, pipettes. Clean everything!
    a. Use the same rack always and keep it in your box of lab stuff. When the project is done, then you bring it out to the slues and give it a proper wash/cleaning.
3. Place everything back in the flow hood and UV treat it for 30 mins.
4. Clean the large falcon centrifuge, chair and all other table surfaces.
5. Empty both trash bins and put in new bags. You will take both bags with you out of the room when you are completely done.
6. Go through the checklist before you leave.
7. Take off lab shoes and exit to the lock. Bring with you the bleach bucket, ethanol bottle, MilliQ bottle and the trash.
8. Bag with lab trash goes in the yellow bin, the other you take with you out of the lab.
9. Empty bucket with bleach. Throw out the dish cloth in regular trash. Put bucket, MilliQ and ethanol spray bottles back where they belong.
10. Take off the blue shoe covers and throw them out in regular trash. Take off your clean suit and put in bag with you name. Place in bookcase.
11. If needed, empty the regular trash bin in the lock and put in new bag. If a yellow bin is full, put lid on. Bring trash, yellow bin and box from the corridor out with you.
12. Leave the trash in the hallway for the short time while you go to the store to get the things needed for your lab protocol or the lab. Remember to not pass any high-risk areas. In case the store is closed when you are finished you can buy the necessary things the next morning.
13. Label the things that are yours and put all things in the box in the corridor to bring in one of the next days.
14. If you are not to re-enter the clean lab the rest of the day, bring the trash to lab B310. Remember to fill out blue label for the yellow bin. Ask Julie if you have any questions.

Remember to shower and fresh clean clothes on the day you wish to work in the clean lab. It is possible to shower at the NFH-building right next to the SIMFISH-meeting room on floor 0.

7.4 Appendix D- DNA extraction water

Modified DNeasy Blood and Tissue kit protocol

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Norwegian College for Fishery Science
Research Group for Genetics, K. Præbel
Last updated: November 2019, edit. J. Bitz

EXTRACTION PROTOCOL FOR STERIVEX FILTERS

DAY 1:

1. Follow the descriptions in the 'Clean Lab Routines' of how to enter the labs.
2. Clean the outside of the 50ml falcon tubes containing the filters with bleach. Alternatively, if your filters are in ziplock bags, clean the outside of the bag. Do not use ethanol if there is any labelling on the tube/bag.
3. To remove excess water inside the filters, place the inlet of the filter (narrow end) in a 1.5 ml Eppendorf tube and gently slide filter and tube into the 50 ml falcon tube that contained the filter (or in a new 50 ml tube if samples were stored in ziplock bags). If more than one filter is in the tube, label a new tube for the second filter. When done with filters from one species/station, clean everything again (forceps, gloves, working surface) with bleach, MilliQ water and ethanol, before proceeding to the next species/station.
4. Centrifuge the tubes at 1500 x g for 3 minutes to remove the remaining seawater from the filters.
5. Make extraction buffer solution for adding 2.5X the recommended volume = 500µl per filter.
   - Recommended volume is 20µl Proteinase K + 180µl Buffer ATL per sample:
     - 2.5 * 20µl ProK = 50µl
     - 2.5 * 180µl ATL = 450 ul
     - Total amount of extraction buffer per sample = 500µl
     - E.g. for 20 samples: 1000µl ProK, 9000µl ATL. First, pipette the 9ml with a sterile glass pipette into a clean 50ml falcon tube. Then pipette 1 ml of ProK into the same tube. Close with lid and invert solution, avoiding foaming.
6. Add 500µl of the extraction solution to each filter, starting with blanks, by pushing the 1000µl tip tight **into the outlet** end of the filter and gently aspirating the solution into the filter. Take care that all the solution goes into the filter. If the filter is clogged, then aspirate from the inlet end of the filter.
7. Cap the filters with sterile caps. Make sure that its **completely sealed**.
8. MAKE SURE YOU LABEL ALL THE FILTERS CORRESPONDING TO THE TUBES, by writing the label and the replicate letter (A, B, C etc.) on the filter and **cover with tape**.
9. Place the filters in rotator and fasten them with the elastic band.
10. When done with all filters, move the rotator to the incubator oven (56°C). Make sure that the rotator is moving at 6 rpm and not hitting the oven. Check the filters after a couple of hours and leave them overnight for the 2nd day of extractions. Minimum 8-12 hours incubation.
11. Note: Always use similar incubation time for all filters within a project. Note the time for when incubation in the incubator oven started.

DAY 2

12. Enter lab and clean according to the Clean Lab Routines.
13. Label all tubes needed for the process: 2ml Eppendorf tubes, spin columns and the final 1.5ml Eppendorf tubes that will hold the eluted DNA (sample ID on top, and more details on the side including replication (A,B,C), depth, date of collection, date of extraction and your initials).
14. Note the time when the filters are removed from the incubator oven.
15. Reopen the sealed filters and transfer them to a marked 2ml tube inside a new 50ml falcon tube with the inlet facing down into the 2ml Eppendorf tube.
16. Centrifuge the 50ml tubes containing the 2ml tubes and the filters at 1700 x g for 3 minutes.
17. Remove the filter from the 50ml tube and discard it. Then carefully remove the 2ml tube from the bottom of 50ml tubes with a tweezer holding the root of the cap, without touching the cap itself or the edge of

the tube opening. Close the 2ml tube and place it in a rack. Again, start with the lowest concentration (e.g. air-> blank -> real samples).

18. "Measure" the approximate volume of 2-3 samples using a pipette with NEW tips for each sample. Round the mean volume to nearest 50µl.
19. Add an equal volume of the Buffer AL as the one determined above (7.) and ensure to mix it with the pipette immediately using new tips for each sample.
20. Add an equal volume of 100% EtOH as the one determined above (7.) and ensure to mix it with the pipette immediately using new tips for each sample.
21. Vortex and spin down the samples to make sure it is mixed and liquid from the cap is removed.
22. Place the spin columns in front of the samples in the rack.
23. Transfer 630µl of the sample into corresponding spin column. Be careful not to make any bubbles but at the same time try not to leave liquid in the tip because it is precious DNA.
24. Centrifuge the columns at 15.000 x g for 2 mins.
25. Discard the collection tube with the flow-through and transfer the spin column to a new collection tube. Make sure the flow-through has not spilled back to the column when you removed it from the centrifuge.
26. Transfer the rest of the sample to the corresponding spin column. If more than 630µl, three rounds of spinning are required.
27. Centrifuge the columns at 15.000 x g for 2 mins.
28. Discard the collection tube with the flow-through and transfer the spin column to a new collection tube. Make sure the flow-through has not spilled back to the column when you removed it from the centrifuge.
29. Add 500µl Buffer AW1 (check EtOH has been added to buffer) using new tips for each tube.
30. Centrifuge at 15000 x g for 2 mins.
31. Discard the collection tube with the flow-through and transfer the spin column to a new collection tube. Make sure the flow-through has not spilled back to the column when you removed it from the centrifuge.
32. Add 500µl Buffer AW2 and centrifuge for 4 mins at 20.000 x g.
33. While centrifuging, clean flowhood, pipettes, and pens with bleach, MilliQ and ethanol.
34. TAKE GREAT CARE that no flow-through is present on the sides of the spin columns. If so, spin the columns again in a new collection tube at 20.000 x g for 2 mins. Note what samples that have been centrifuged twice.
35. Transfer the spin-columns to the corresponding Eppendorf tubes. Make sure that the lid/tap of the spin column does not touch the cap of the Eppendorf tube to avoid contamination.
36. Add 75µl of Buffer AE to each spin columns. Make sure to add the buffer at the center of the membrane without touching the membrane. Incubate for 1 min, then spin the samples at 20.000 x g for 2 mins.
37. Discard the spin columns and transfer a 20µl aliquot of the extracted DNA from each sample to a PCR plate or PCR strips. It is very important the plate/strip is labeled properly with all necessary information and with unique names (not just 'Plate 1'!). If you are using strips, use an empty pipette tip box as rack. Wrap aliquots in two bags before temporary storage. Place the aliquot in the fridge at 4°C if you are certain it will be processed within the next 2-3 weeks or in the aliquot freezer if longer.
38. Store the rest of the DNA as stock in the freezer located in the extraction lab. Store the 1.5ml tubes in a cryobox that you have purchased at the store and brought with you. Make sure to label the box properly. Put the cryobox in two bags before storing it and **ONLY** thaw the stock if absolutely necessary.
39. Clean flowhood and all equipment according to the guidelines.

7.5 Appendix E- PCR and library prep

Protocol for COI metabarcoding using Leray-XT primers and Metafast library preparation (PCR-free ligation procedure)

Owen S. Wangensteen. January 2018.

METABARCODING PRIMERS

We use the Leray-XT primer set (Wangensteen et al., 2018). This is a highly-degenerated primer pair able to amplify a 313 bp fragment of cytochrome *c* oxidase subunit I (COI) from a wide array of eukaryotic groups, including virtually all metazoans. The sequences (where "I" stands for deoxy-inosine) are:

Forward, **miCOIint-XT**: 5'-GGWACWRGWTGRACWITITAYCCYCC-3'
Reverse, **jgHCO2198**: 5'-TAIACYTCIGGRTGICCRAARAAYCA-3'

DNA AMPLIFICATION

We use a simple 1-step PCR protocol to amplify the Leray fragment. The metabarcoding primers have an 8-base sample-tag attached (each tag with at least 3 differences out of 8 bases). Also, we add a variable number (2-4) of leading Ns, in order to increase sequence variability to improve Illumina sequencing. Each forward and reverse primer has the same sample-tag attached in both ends. E.g.:

Primer F1: NNaacaagccGGWACWRGWTGRACWITITAYCCYCC
Primer R1: NNNNaacaagccTAIACYTCIGGRTGICCRAARAAYCA

Primer F2: NNNggaatgagGGWACWRGWTGRACWITITAYCCYCC
Primer R2: NNNggaatgagTAIACYTCIGGRTGICCRAARAAYCA

Primer F3: NNNNaattgccgGGWACWRGWTGRACWITITAYCCYCC
Primer R3: NNaattgccgTAIACYTCIGGRTGICCRAARAAYCA

We have 96 such different pairs, so we can multiplex up to 96 samples in one library.

The PCR protocol uses Amplitaq Gold 360 master mix (ThermoFisher)
https://www.thermofisher.com/order/catalog/product/4398886
and bovine serum albumin (BSA)
https://www.thermofisher.com/order/catalog/product/B14?ICID=search-B14

The PCR mix is as follows:

AmpliTaq Gold Master Mix	10.00	µl
BSA 20 µg/µl	0.16	µl
H2O	5.84	µl
Forward primer 5 µM	1	µl
Reverse primer 5 µM	1	µl
DNA Template	2	µl

Note that the primers cannot be added to the PCR master mix for aliquoting (as is common practice for preparing normal PCRs). They have to be added to every individual sample, since every sample will be amplified with a different version of the primer set.

The PCR programme is:

95°C	10 min	(needed for denaturing the blocking antibody of Taq polymerase)
94°C	1 min	
45ºC	1 min	x 35 cycles
72°C	1 min	
72°C	5 min	(extension time)

LIBRARY POOLING AND CONCENTRATION

Once all samples are amplified, the success of amplifications may be checked by gel electrophoresis in 1% agarose. Note that the samples must be prepared in a clean room to avoid contaminations. They should never be opened in a common electrophoresis laboratory. We routinely use 2 µl of the PCR products for the electrophoresis. The rest (18 µl per sample, including the blank samples) will be pooled together in a single Eppendorf tube and this pool is then thoroughly homogenized by vortexing.

The pool is then purified using MinElute columns for removing DNA fragments below 70 bp. This step will also concentrate the amplified DNA around 10 times. https://www.qiagen.com/qdm/aw/cup/pcr-purification/

These MinElute columns have a maximum sample volume capacity of 130 µl per sample. So you will probably need to use 10 or 12 of such columns, depending on the total volume of your pool. Follow the protocol in the kit. In the final step, you can elute every column in 12-15 µl of elution buffer. Then pool all the eluates together and homogenize thoroughly by vortexing.

You can measure the DNA concentration in the final pool using a Qubit fluorimeter with the Broad-Range DNA quantification kit. You need a minimum concentration of 75 ng/µl in the final pool for a best performance of the next ligation step.

LIBRARY PREPARATION

For library preparation, we use a PCR-free ligation protocol, the NEXTflex PCR-Free DNA Sequencing Kit from BIOO Scientific: http://www.biooscientific.com/Next-Gen-Sequencing/Illumina-Library-Prep-Kits/NEXTflex-PCR-Free-DNA-Sequencing-Kit

We use 3 µg of DNA (up to 40 µl of the previous pool) as starting material. The instructions for preparing a COI library are exactly the ones described in the kit manual: http://www.biooscientific.com/Portals/0/Manuals/NGS/5142-01-NEXTflex-PCR-Free-DNA-Seq-Kit.pdf

Note this protocol is valid for selecting fragment sizes of 300-400 bp, exactly the right size for the Leray fragment. If you want to use a different metabarcoding marker with a shorter fragment, then you need to change Step B of the protocol (size selection).

With this kit, you will get to ligate your amplicons to the Illumina adapters and a 6-base library tag. The basic kit includes just one such library-tag, which is enough for multiplexing 96 samples with our set of 96 sample-tags. If you wish to multiplex over 96 samples, you could use two or more library tags. For this, you would need to buy an extra box of BIOO barcodes, which come in 6, 12, 24, 48 or 96 versions: http://www.biooscientific.com/Next-Gen-Sequencing/Illumina-Adapters/DNA-Seq/NEXTflex-DNA-Barcodes

You will need to use magnetic beads for some steps of this protocol. The original Agencourt AMPure XP beads are quite expensive, but they are most convenient. http://uk.beckman.com/nucleic-acid-sample-prep/purification-clean-up/pcr-purification?geolocation=gb

LIBRARY CHECKING

We usually analyse the final library using either an Agilent TapeStation or Bioanalyzer, in order to check that the ligation has gone well. If you don't have any of these analyzers available, then you could use just a gel electrophoresis to check the right migration of the fragment. Note that the library fragments are the result of a special Y-shaped adapter ligation and they will not be linear DNA. So they will migrate anomalously in all this analytical methods. The library peak will not appear at the expected size of ~ 510 bp, but it will produce a broad peak of ~ 800 bp. This strange migration behaviour is normal and won't interfere with the MiSeq sequencing.

LIBRARY QUANTIFICATION

In order to load the right concentration of the library in the MiSeq, it is essential to check the exact concentration of the library using a specific qPCR method. This method will use a specific probe for the Illumina adapter sequence, so it allows to quantify exactly which molarity of adapter you will be loading into the MiSeq, whih is crucial for not overclustering the Illumina flow-cell.

For this purpose, we use the NEBNext Library Quant Kit from New England Biolabs: https://www.neb.com/products/e7630-nebnext-library-quant-kit-for-illumina

We usually analyse library dilutions of 1:5000, 1:10,000 and/or 1:50,000.

You will need to use a qPCR machine. In Salford, we use the Rotor-Gene Q from QIAGen but, of course, any qPCR machine will work: https://www.qiagen.com/us/search/rotor-gene-q/

LIBRARY DILUTION AND MiSeq LOADING

The final target concentration for the MiSeq loading will depend if you want to use a v2 or v3 MiSeq sequencing kit. With a v2 kit, you can get up to 15 M reads, and you will use a sample with up to 10 pM DNA concentration. With a v3 kit you will get up to 25 M reads, and you will use a sample with up to 20 pM DNA concentration. We usually target at 9 pM for a v2 or 18 pM for a v3, so to prevent overclustering of the flow-cell.

We will prepare our sample including a 1% of PhiX library, which will be used as an internal sequencing control for calculating error rates per cycle. https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/phix-control-v3.html

The protocol for the final sample denaturation before loading is as follows:
- Prepare a mix of up to 10 µl of your library and PhiX-library mix (in the right molar proportions) and put it in the bottom of a 2-ml Eppendorf tube.
- Denature with the same volume of 0.2N NaOH during 5 min. During this time, you may vortex once and spin in a centrifuge for recovering the sample.
- Add HT1 hybridization buffer (included with your the MiSeq reagent kit) to a total volume of 2 ml and vortex thoroughly.
- Load 600 µl of this denatured sample into the the MiSeq for sequencing.

References:

- Wangensteen OS, Palacín C, Guardiola M, Turon X (2018) DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. PeerJ 6, e4705. https://peerj.com/articles/4705/

7.5.1 Library pooling and concentration

Minelute® PCR Purification Kit (Qiagen, Germany)

Notes before starting

This protocol is for cleanup of up to 5 µg PCR product (70 bp to 4 kb).

Add ethanol (96-100%) to Buffer PE concentrate before use (see bottle label for volume)

All centrifugation steps are carried out at 17,900 x g (13 000 rpm) in a conventional tabletop microcentrifuge at room temperature (15-25).

Add 1:250 volume pH indicator I to Buffer PB. Add pH indicatior I to the entire buffer contents. Do not add pH indicator I to bugger aliquots. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤ 7.5. The adsorption of DNA to the membrane is efficient only at pH ≤ 7.5. (If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without addition of pH indicator I.)

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix. Check that the color of the mixture is yellow (similar to Buffer PB without the PCR sample). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetat, pH 5.0, and mix. The color of the mixture will turn to yellow.
2. Place a MinElute column ● in a provided 2 ml collection tube ▲ into a vacuum manifold. See the MinElute Handbook for details on how to set up a vacuum manifold.
3. Apply the sample to the MinElute column and ● centrifuge for 1 min or ▲ apply vacuum until the entire sample has passed through the column. ● Discard flow-through and place the MinElute column back into the same collection tube.
4. Add 750 µl Buffer PE to the MinElute column and ● centrifuge for 1 min or ▲ apply vacuum. ● Dicard flow-through and place the MinElute column back in the same collection tube.
5. Centrifuge the column in a 2 ml collection tube (provided) for 1 min. Residual ethanol from Buffer PE will not completely removed unless the flow-through is discarded before this additional centrifuge.
6. Place each MinElute column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 10 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the MinElute membrane. (Ensure that the elution buffer is dispensed directly onto the

center of the membrane for complete elution of bound DNA.) Let the column stand for 1 min, and then centrifuge the column for 1 min.

8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

(The MinElute colums have a maximum sample volume capacity of 130 µl per sample. So you will probably need to use 10 or 12 of such columns, depending on the total volume of your pool. In the final step, you can elute every column in 12-15 µl of elution buffer. Then pool all the eluates together and homogenize thoroughly by vortexing.)

Measure the DNA concentration in the final pool using a Quibit fluorimeter with the Broad-Range DNA quantification kit. A minimum concentration of 75 ng/µl in the final pool for a best performance of the next ligation step.

7.5.2 Library preparation

NEXTflex™ PCR-free DNA Sequencing Kit (Bioo scientific corporation, USA). Originally made for genomic DNA, but modified for mitochondrial DNA. (DNA pool not in a PCR-plate at this stage, but in an Eppendorf tube).

STEP A: End Repair

1. For each sample, combine the following reagents on ice in a 96-well PCR (if samples are not pooled) or a PCR-strip.
   _ µl Nuclease-free water
   _ µl Fragmented DNA
   7 µl NEXTflex™ PCR-Free End Repair Buffer Mix
   3 µl NEXTflex™ PCR-Free End Repair Enzyme Mix
   50 µl TOTAL
2. Mix thoroughly by pipetting
3. Apply PCR-strip seal and incubate on a thermocycler for 30 minutes at 22°C.

STEP B: Clean-up

1. Transfer 50 µl of the DNA mix to a new 1,5 ml Eppendorf tube. Add 42.5 µL of AMPure XP Beads to each sample and mix thoroughly by pipetting. (Used 42,4 µl because the pipette did not have 42,5 as an option.)
2. Incubate the tubes at room temperature for 5 minutes.
3. Place the tubes on the magnetic stand at room temperature for 5 minutes or until the supernatant appears completely clear.
4. Set pipette to 90 µl, slowly remove and discard the supernatant taking care not to disturb beads. Some liquid may remain in tubes. This selectively removes DNA below 300 bp.
5. With the tubes on stand, add 200 µl of freshly prepared 80% ethanol to each magnetic bead pellet and incubate tubes at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
6. Repeat step 5 for a total of 2 ethanol washes. Ensure all ethanol has been removed.
7. Remove the tubes form the magnetic stand and let dry at room temperature for 3 minutes.
8. Resuspend dried beads with 53 µl of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 2 minutes.
10. Place the tube on magnetic stand at room temperature for 5 minutes or until the sample appears completely clear.

Do not discard the sample in this step. Transfer 16 µl of clear sample to a new PCR-strip.