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Biodiversity baseline of the Norwegian coastline using eDNA metabarcoding

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Abstract

Existing research about biodiversity baseline in Norway focuses on localized studies or specific species. This thesis aims to study the entire Norwegian coastline to fill this gap by conducting the first large-scale biodiversity assessment of the entire Norwegian coastline. Environmental DNA (eDNA) metabarcoding, that targeted the cytochrome oxidase I (COI) gene, was used to analyse marine water samples from 69 different locations across the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak management plan regions.

The research questions addressed the variations in species composition and the influence of environmental gradients along the coastline. Using eDNA extraction, COI gene sequencing, and bioinformatic analyses, the study revealed significant biodiversity variations among the management regions, with higher species richness in the warmer, nutrient-rich southern regions compared to the colder northern Barents Sea-Lofoten region.

The findings showed the presence of biodiversity hotspots within each region, influenced by local environmental conditions and oceanographic processes. The study also highlighted the inadequacies of current management plans and the potential oversight of localized biodiversity patterns, suggesting the need for more refined management strategies.

This study establishes a comprehensive biodiversity baseline for the Norwegian coastline, demonstrating the use of eDNA metabarcoding in marine biodiversity assessment. The study showcases the importance of integrating molecular techniques with traditional surveys for a sufficient conservation and management plan and recommends future research to increase sampling intensity and rethink regional management plans to better preserve marine biodiversity in a changing environment.

Acknowledgement

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Going on a 5-day cruise joining the crew of the boat Prinsesse Ingrid Alexandra and learning how to collect eDNA surface samples with a handheld CTD, and samples at varying depth using a deepwater functioning CTD. I am also grateful for learning how to do DNA extraction from the eDNA samples in a clean lab environment and afterwards being hired as a student helper to help in the DNA extraction of samples collected in Greenland as part of the TUNU project. I would also like to thank Melissa Brandner for taking me with her as a helper in the processes of the PCR amplification, library preparation and sequencing process for samples from the TUNU project.

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Table of Contents

List of figures

Introduction

Biodiversity

What is biodiversity and what is known about the Norwegian marine biodiversity

Biological diversity or for short, biodiversity, is a term used for describing the genetic variability in biological species found in an area or habitat and their biological interactions (Meinard et al., 2014). Biodiversity is thus directly related to how ecosystems functions and how resilient they are towards stressors. High biodiversity often shows greater ecosystem production, stability and resilience against environmental changes and anthropogenic impacts, whereas the loss of biodiversity can potentially diminish ecosystem services and impact the vulnerability against disturbances (Cardinale et al., 2012).

The Norwegian coastline is the second longest in the world, with a length of 100,915 km. in length (Regjeringen, 2015). This vast marine area covers different climatic zones and many different habitat types (Havforskningsinstittutet, 2019) and display a changing biodiversity along the coastline, due to different biotic and abiotic factors (Mashwani, 2020). However, not much is known about the biodiversity and how it changes along the complete coastline. This is primarily because there have not been made any large-scale biodiversity studies that researches the entire coastline to ascertain a baseline of the marine environment, and the changes in biodiversity from the Barents Sea to Skagerrak.

Individual studies focusing on specific species, groups, or areas have been conducted with various interests and goals. For instance, Husa et al. (2008) investigates biodiversity changes in sublittoral zones in southwest Norway due to temperature changes and introduced species. Mecklenburg et al. (2011) examines the taxonomy and biodiversity of marine arctic fishes. Hamre (1994) focuses on two important species for the Norwegian fishing industry, Atlantic herring (*Clupea harengus*) and capelin (*Mallotus villosus*), highlighting their significance for Norway due to their economic worth.

Another examples of commercially important species that have been thoroughly studied is the Atlantic salmon (*Salmo salar*) and Norwegian lobster (*Nephrops norvegicus*). Research on salmon includes its life cycle, migration patterns, genetic diversity, and impacts of aquaculture on wild salmon,

while research on Norwegian lobster includes its population biology, habitat and management (Olsen et al., 2010; Bell et al., 2013). These species demonstrate a focus on species that have economically importance and highlights the negligence of smaller organisms.

This imbalance suggests that much of the biodiversity along the Norwegian coastline is still unknown or poorly understood. Notably, research often targets prominent or larger-bodied species such as cod, while the distribution and presence of smaller organisms are largely unknown, likely due to the difficulties in identifying and studying them. This bias in research focus leaves gaps in our understanding of overall biodiversity (Ottersen et al., 2006).

The fragmented nature of existing studies further indicates that biodiversity studies in many areas of the Norwegian coastline remains insufficient for a fuller understanding. To address the gap a broader approach should be used, which should include studies of species and ecosystems that are overlooked, as shown by Skern-Mauritzen et al. (2015) where they reveal that many fisheries plans in industrial fisheries leave out approaches to protect the existing ecosystem processes.

The definition on what a species is are still discussed. There are definitions based on several species concepts, the biological, the phylogenetic (Mayr, 2000; Cracraft 1983) and the ecological (Van Valen, 1976) are examples of this, and highlights the ongoing debate of what the definite definition of a species is. Generally, there is a lack of recognizing genetic biodiversity as a measure to differentiate species, which could potentially lead to misinformation, unintentionally harming specific species, or giving a wrong understanding of the current biodiversity (Laikre et al., 2016). An example could be the killer whale (*Orcinus orca*), which is currently only acknowledged as one species, even though there are several ecotypes differentiated by habitat, size, and predation patterns. The different types of killer whales do not interbreed, and genetic studies have shown that three of the types are so significantly genetically different that they could be perceived as different species (Morin et al., 2010). However, all these ecotypes do not occur in Norwegian waters. In Norwegian waters, the Atlantic cod (*Gadus morhua*) presents a similar story. While traditionally considered a single species, there have been extensive research on its population dynamics which suggests the presence of distinct genetic populations, each adapted to specific environmental conditions. Despite this, there is a lack of

recognition of genetic diversity within cod populations. This possible oversight may lead to mismanagement and a failure to protect genetically distinct populations, which could have significant implications particularly in marine environments where distinct populations may have unique adaptations and ecological roles. (Hutchings, 2015; Barth et al., 2017).

Dividing of the Norwegian coastline and habitats

The Norwegian coastline have been divided into different areas, regions and zones depending on the aims of the study. In a coastline wide study of nudibranch species, the coastline were divided into 26 zones, to the south zones 1-10 from the border with Sweden, to the northern part of Sogn and Fjordane County, the Western region containing zones 11-20, from Romsdal County to central part of Troms, and the Finnmark region to the north containing zones 21-26 from northern Troms to the border towards Russia (Brattegard and Holthe 1997). However, the government divide the coastline into three different management regions, termed: Barents Sea-Lofoten, the Norwegian Sea, and the North Sea-Skagerrak (Figure 1).

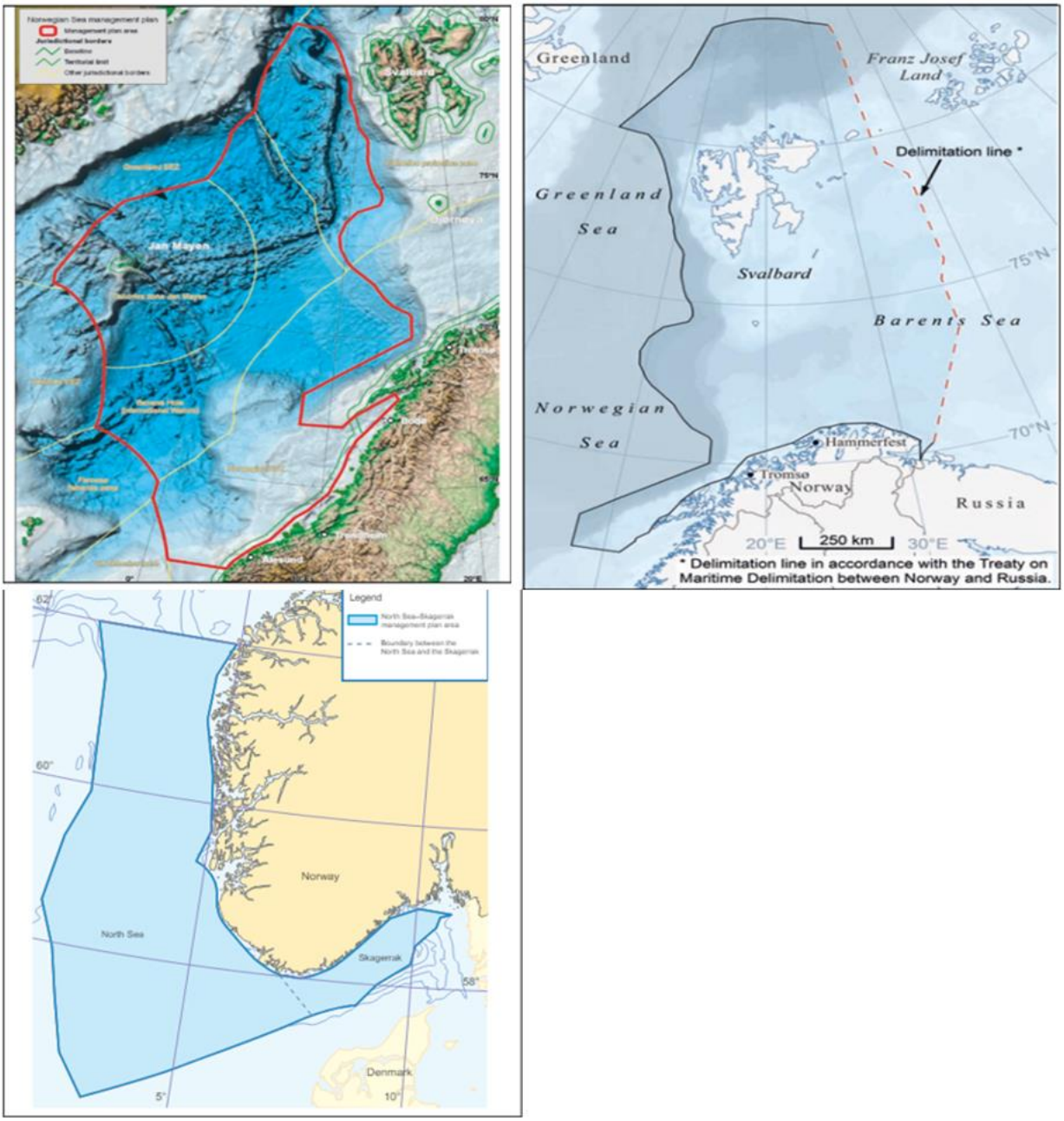

Figure 1: Norwegian marine borders, divided into three separate governmental management regions. Barents Sea-Lofoten region (Top right). (Norwegian Polar Institute 2011. Depth data: IBCAO). Norwegian Sea region (Top left). (Norwegian Hydrographic Service). North Sea-Skagerrak region (Bottom left). (Norwegian Mapping Authority)

The biodiversity along the Norwegian coastline could be much more diverse than currently understood. Studies in the last decades have shown an increase in biodiversity in the waters of northern Norway. From 1990 to 2010 there was an increase in discovered species by 15% (Narayanaswamy et al., 2010). This highlights the potential for discovering new species when research efforts are increased. There could be several reasons to why the biodiversity in the north seems to be increasing, such as rising sea temperatures and shifting ocean currents, which can lead to the migration of species to new areas, altering the already established community composition, and potentially lead to an increase in species richness in certain regions while decreasing in others (Bellard et al., 2012).

The temperature gradient along the coastline, ranges from the colder waters in the northern part of Norway to the relatively warmer southern regions. This creates habitats with much diversity and supports many different species (Wienerroither et al., 2011). Anthropogenic activities such as fishing, and aquaculture can have a negative impact on the biodiversity. Some of the species in the affected habitats may thrive in the changed environments, which could lead to changes in the ecosystem structure (Ottersen et al., 2010). Pollution from industrial and agricultural sources can also damage habitats and harm marine life this way (Norderhaug et al., 2005). Monitoring and comprehensive studies are crucial to understand the habitat dynamics to ensure sustainability (Halpern et al., 2008; Thrush & Dayton, 2010).

Conservation efforts

Only about 3.5% of the Norwegian waters are protected areas even though almost 80% of the Norwegian population lives within 10 km of the sea and many Norwegians are dependent on the ocean for their livelihood. (OECD iLibrary, 2022). This limited protection is a concern given the diverse and unique marine ecosystems present along the Norwegian coastline. Atlantic cod (*Gadus Morhua*), haddock (*Melanogrammus aeglefinus*) and several seabirds have protected areas such as spawning grounds and nesting areas during important periods to protect the populations (Norwegian Directory of Fisheries, 2021; Anker-Nilssen et al., 2013). Efforts to establish marine protected areas (MPAs) are

ongoing, with initiatives aimed at expanding protected regions to safeguard biodiversity and promote sustainable use of marine resources (Kelleher, 1999).

A new governmental management plan for the three regions Barents Sea-Lofoten, Norwegian Sea and North Sea-Skagerrak was started in 2001, the goal was to utilize and conserve these diverse marine regions. Several interest such as the economic aspect from the fishing and oil industry and conservative efforts played a part in the plans. The management plans were approved by the Ministry of Climate and Environment, and the first plan for the Norwegian Sea was completed in 2009 (Olsen et al., 2016; Regjeringen, 2021), followed by the plan for the Barents Sea-Lofoten region in 2011 (Hoel, 2014), and lastly, the plan for the North Sea-Skagerrak in 2013 (Skjoldal et al., 2013).

The management plans aimed to protect both economic and environmental interests. The management plans identify specific areas within each region that is crucial for upholding the sustainability of the regional habitats and are vulnerable or important (Douvere and Ehler, 2011).

Biodiversity hotspots

Some habitats or areas may have a higher abundance of species than other and are termed biodiversity hotspots. A biodiversity hotspot is defined by either having a very high biodiversity, many endangered or rare species, or a mix of them (Reid, 1998). Over time, marine biodiversity hotspots have changed due to global climate change and geological changes. Studies from marine biogeography, has discovered that in the last 50 million years, global marine biodiversity hotspots have shifted from southwestern Europe to the Middle East and are now concentrated in the Indo-Australian archipelago (Renema et al.,2008).

Even though there is a global marine hotspot, regional hotspots exist all around the world, including in colder places like Norway. Along the Norwegian coastline, several areas are recognized as having a high biodiversity, such as Lofoten, Trøndelag coast and Skagerrak coast. The high biodiversity in these areas is due to unique and diverse habitats, well suited for many different species making them important areas for conservative efforts (Gulliksen & Kåven, 2000; Skjoldal et al., 2013).

The location of biodiversity hotspots changes through time due to several factors such as environmental changes and anthropogenic activity. Shifts in climate, such as temperature changes and precipitation, have an impact on species and habitat distribution (Parmesan, 2006). For example, glacial and interglacial cycles have historically caused significant shifts in biodiversity patterns, leading to the emergence and disappearance of hotspots (Hewitt, 2000). Anthropogenic activities are an increasing factor in the disappearance of biodiversity hotspots, due to habitat destruction, pollution, and exploitation of resources. (Sala et al., 2000). Conservation efforts and habitat restoration can help rebuild destroyed areas and help biodiversity (Chape et al., 2005).

Traditional observation methods of biodiversity

Traditional observation methods for marine animals include various monitoring techniques, such as trawling, visual observations, underwater visual censuses, sonar, and more (Murphy and Jenkins, 2010). These methods are essential for understanding and conserving species communities, each with its own advantages and limitations. Trawling has been extensively used to monitor fish and benthic communities (Olsgard et al., 2008; Sant'Ana and Alvarez Perez, 2016). However, bottom trawling can damage the seabed and disrupt benthic habitats, while pelagic trawling can harm or kill fish, even if they are released again (Petović and Marković, 2013; Metcalfe, 2009). Additionally, there is an ongoing ethical debate about whether fish feel pain (Rose et al., 2014). Visual observations can be conducted from shore, boats, or planes. This method requires a good knowledge of the targeted species to ensure accurate identification. The observation timing is also important, but. Some species, particularly marine mammals, have been shown to actively avoid boats (Schaffar et al., 2009). Underwater visual census involves various techniques, including divers in the water or stationary cameras (Samoilys and Carlos, 2000; Richards et al., 2011). Stationary cameras can be deployed on boats or fixed positions like buoys, capturing continuous footage of marine life cost-effective. Mobile techniques offer broader coverage but may scare away animals (Menza et al., 2011; Colton and Swearer, 2010). Sonar uses hydroacoustic technology to collect data, providing detailed images and maps of underwater environments. It is particularly useful for detecting species at greater depths and in areas with poor visibility due to murky water (Fornshell and Tesei, 2013; Moursund et al., 2003).

While sonar is effective in locating fish, it often struggles with species identification, necessitating supplementary methods (Horne, 2000).

Biodiversity baseline

A marine biodiversity baseline is a snapshot of data derived from a specific area and can provide a comprehensive understanding of the biodiversity and can include information about habitat, species, populations and ecological interactions in the area. It can serve as reference for conservative efforts or future studies, helping to understand the biodiversity or changes within (Gullison et al., 2015). Establishing a marine biodiversity baseline involves gathering various types of data, including information about the abundance and density of each species, which can provide insight into population sizes and distribution patterns (Calbet and Landry, 2004). By examining the genetic diversity, it is possible to ascertain if populations within the same species have genetic variability (Tedersoo et al., 2014) and make a species inventory list of the species that are found in the moment of sample collection (Sherr and Sheer, 2007). The collected data can be used for conservation efforts, helping to monitor changes, assess impacts, set conservation priorities, and help inform the ones in charge of the management to make more informed decisions (Gullison et al., 2015).

In the Norwegian seas, only a few biodiversity baselines exist, typically covering specific smaller areas or particular groups of species, such as certain fish species in the Arctic waters or Northern Norway (Bull et al., 2014). A biodiversity baseline for the entire coast of Norway is not currently existing, but developing such a baseline could provide essential information for conservation efforts and help identify the presence of unwanted or invasive species (Kopf et al., 2015), or to identify species at risk of extinction (Worm et al., 2006).

Establishing a biodiversity baseline could be crucial for effective marine conservation and sustainable management of marine resources. A baseline not only helps in understanding and preserving marine biodiversity but also ensures that new projects or activities are carried out sustainably, with minimal negative impact on the marine environment.

Abiotic and biotic factors and their influence on marine habitats and biodiversity

Abiotic factors

Abiotic factors are non-living factors influencing marine habitats and the biodiversity. Key abiotic factors in marine ecosystems can include sunlight and temperature, salinity, pH, nutrient availability, and ocean currents. They can form distinct marine habitats, supporting different communities of organisms (Smith et al., 2008). Sunlight is essential for photosynthesis, and are primarily performed by phytoplankton, which forms the base of the marine food web. (Gattuso et al., 1999). Sea surface temperature varies with latitude and depth, significantly influencing marine life distribution. Most marine species are adapted to specific temperature ranges and salinity levels (Elliott and Whitfield, 2011). A species in Norway that could be affected in the future due to climate change is the Norway lobster (*Nephrops norvegicus*). They are normally found in habitats with a salinity of 33 psu and prefer a water temperature of 13-14 °C and have been shown to not thrive if either of them changes to drastically (Harris and Ulmestrand, 2004). Ocean pH influences the solubility of minerals and ocean acidification, caused by increased atmospheric CO2 absorption, leads to lower pH levels, affecting calcifying organisms, which can alter marine habitats and reduce biodiversity (Doney et al., 2009). Nutrients such as nitrogen and phosphorus are essential for the growth of marine phytoplankton and can be influenced by upwelling currents, which bring nutrient-rich waters from the deep ocean to the surface, supporting high primary productivity and a diverse marine life. Nutrients washed off from anthropogenic sources can lead to eutrophication, causing harmful algal blooms and hypoxic zones that can be catastrophic to marine habitats (Diaz and Rosenberg, 2008). The Ocean currents play an important role in distributing heat, nutrients, and organisms, influencing ecosystem patterns and the distribution of marine species. For instance, the Gulf Stream transports warm water from the Gulf of Mexico all the way to northern Norway (Wunsch, 2001). Changes in abiotic factors can lead to the loss of biodiversity, shifts in species distributions, and the emergence of new, and potentially less diverse marine habitats (IPCC, 2014) and abiotic factors interactions and full impact on habitat biodiversity are generally poorly understood (Morley and Lewis, 2004).

Biotic factors

Biotic factors in marine habitats are the living elements in the ecosystem, they interact with each other and with the abiotic factors. These factors are essential for a well-functioning ecosystem and can be divided into several categories such as producers, consumers, and decomposers (Danovaro et al., 2008). Microscopic plants and algae are the primary producers in marine ecosystems, they form the base of the marine food web, supporting a vast spectrum of marine life (Falkowski et al., 1998). Larger autotrophic organisms, such as kelp and seagrasses, also contribute significantly to primary production in coastal areas and provide habitat and food for various marine species (Orth et al., 2006). The consumers consist of herbivores like zooplankton, small fish and sea urchins, and are organisms that feed on primary producers. They play an important role in transferring energy from producers to higher trophic levels (Cebrian, 1999; Lawrence, 2001). Consumers also consist of carnivores. Which are organisms that consume other animals. Marine carnivores include a wide range of species from small predatory fish and fish larvae to large apex predators like sharks and marine mammals. They help regulate the population sizes of other organisms, maintaining ecosystem balance (Baum and Worm, 2009). Decomposers break down dead organic material, recycling nutrients back into the ecosystem. In marine habitats, bacteria and fungi are the primary decomposers, along with detritivores. These organisms are essential for nutrient cycling and maintaining the health of marine ecosystems (Tedersoo et al., 2014; Kruse et al., 2011).

Abiotic and biotic factors impact each other and create marine ecosystems composed by different biodiversity (Kumar, 2021). Biotic and abiotic factors and their interrelationship are changing many places due to global warming (Walther et al., 2002). Every species in a functioning ecosystem has a specific role, and if any factors alters more than what the ecosystem can cope with, there will be changes in both the composition and biodiversity among the ecosystem community, this can be both beneficial, harming or with no significant effects (Matyssek and Luttge, 2013; Souza and Luttge, 2015). The complexity of ecosystems is everchanging but have an equilibrium state they cannot surpass, before a normalization is impossible to achieve, either because of arrival of new better suited species, human interaction, or evolutionary variabilities (Bang et al., 2018; Classen et al., 2015). An example of biotic change is how parasitism in many ecosystems is expected to change (Harvell et al., 2002).

Temperature increases will lead to higher stress in hosts since many parasites have a positive correlation with shedding rates and warmer temperatures (Meer, 2006).

Molecular biomonitoring

Environmental DNA (eDNA) refers to the total DNA isolated from an environmental sample, consisting of two main components. Trace DNA, which includes extra-organismal and extracellular DNA, and community DNA, representing the genetic material of actual animals such as microeukaryotes (Deiner et al., 2017).

eDNA analysis is increasingly utilized for DNA-based species identification, mitochondrial DNA (mtDNA) is targeted due to its stability compared to nuclear DNA or RNA, and its abundance (Turner et al., 2014). MtDNA is chosen for its higher genomic abundance per cell, compared to a single genome per nuclear DNA cell. This makes it more detectable in environmental samples. Moreover, mtDNA databases are often more diverse and comprehensive than nuclear DNA databases, because of a history of use in genetic studies (Thomsen & Willerslev, 2015). In marine environments, eDNA analysis typically targets metazoan cell fractions (Taberlet et al., 2018). This method has been successfully used to assess the biodiversity of fish communities, invertebrates, and even microbial communities in various aquatic ecosystems (Bohmann et al., 2014).

The first example of a method similar to the modern eDNA technique used today, was first used by microbiologist Andrew Ogram, Gary Sayler and Tamar Barkay in 1987 trying to find microbial DNA in different sediment samples (Ogram et al., 1987). The use of eDNA from eukaryotes as a genetic analysis to identify species, started in the 2000's, two separate example studies used permafrost cores up to 10.000 years old to confirm the hypothesis of plants and animal DNA being resilient and able to be preserved in the permafrost for thousands of years, and Gentile Ficetola in the second study, where it was used from water samples in conservation biology to detect the presence of a specific type of frog (Willerslev et al., 2003, Ficetola et al., 2008).

To analyse eDNA, environmental samples are collected. They could be from both marine, terrestrial or limnic environments, and can then be analyzed using several different techniques, with pros and cons associated.

Metabarcoding

Metabarcoding is a method that uses high-throughput sequencing to identify species or groups within an environmental sample. This method involves the amplification of specific genetic markers, such as cytochrome c oxidase subunit I (COI), that is a mitochondrial gene, used together with universal primers, followed by sequencing and taxonomic identification (Taberlet et al., 2012; Valentini et al., 2016). Collected samples and the subsequently DNA extraction using environmental samples, such as water or sediment, are collected and filtered to concentrate eDNA. DNA is then extracted using specialized protocols to ensure minimal contamination (Mauvisseau et al., 2022). The extracted DNA is amplified using Polymerase Chain Reaction (PCR) with primers targeting conserved regions of mitochondrial DNA or ribosomal RNA (rRNA) genes. These markers are chosen for their high copy number and taxonomic resolution. Mitochondrial DNA is particularly useful because it is more abundant than nuclear DNA, making it easier to detect and sequence (Deiner et al., 2017). Additionally, mtDNA databases are extensive, aiding in accurate species identification (Taberlet et al., 2012). To sequence and then analyse, high-throughput sequencing platforms, as Illumina MiSeq, are used to sequence the amplified DNA. Bioinformatic tools then match the sequences to reference databases to identify the species present in the sample. This process allows for the simultaneous identification of multiple species from various taxonomic groups in a single analysis (Taberlet et al., 2012). Because of this, metabarcoding is particularly effective for biodiversity assessments and monitoring community composition (Deiner et al., 2017).

Application and advantages of using eDNA for a biodiversity baseline in marine habitats

eDNA techniques have diverse applications in biodiversity studies. They can detect rare or elusive species, confirm the presence of endangered or invasive species, and monitor spawning events and migrations (Thomsen et al., 2012). It is possible to identify and monitor targeted species spawning events due to spikes in eDNA concentration from the species (Tsuji and Shibata, 2021) and monitor migrations of migratory species (Duda et al., 2021). eDNA can also identify important breeding, feeding, or nursery grounds by detecting gatherings of species (Orzechowski et al., 2019; Wang et al., 2021). Additionally, eDNA analysis is a valuable tool for conservation measures, providing data on

species presence and aiding in biodiversity monitoring and ecosystem management (Beng & Corlett, 2020).

Some of the advantages of using eDNA is that it is non-invasive and allows to collect samples without needing to disturb the organisms themselves (Thomsen and Willerslev, 2015). eDNA can detect a wide range of species from a single sample, this can provide at great species overview with minimal sampling effort (Taberlet et al., 2012). Compared to traditional methods which are in many cases reliable of taxonomic experts in different fields to get the same overview, eDNA can potentially provide information at a faster rate (Rees et al., 2014). It is also possible to use sediment core samples to get a historic overview of the habitat, to compare with the current composition of species (Turner et al., 2014).

Objectives

The main objective is to create the first biodiversity baseline of the entire Norwegian coastline using eDNA metabarcoding analysis of seawater samples. Trying to establish the species present in the three management plan regions, Barents Sea-Lofoten, Norwegian Sea and North Sea-Skagerrak and compare the biodiversity. Also to ascertain if only three management regions on such a vast coastline is sufficient for conservation efforts.

Does any of the three management regions show as a biodiversity hotspot, and if so, is it for all phyla or specific ones. Could eDNA bring light to species not previously, and potentially new species not known in Norwegian waters.

Hypotheses

i) eDNA will give a varied biodiversity baseline stretching across the Norwegian coastline with differences between the three management regions.

ii) Biodiversity hotspots or key regions will be observed for specific groups or species from the water samples.

iii) Three management regions are not sufficient for conservation efforts for such a vast coastline.

iv) eDNA will show new species not known in Norwegian waters.

Material and methods

Sampling of environmental DNA

The biodiversity baseline will be constructed using eDNA techniques from water samples taken from surface water from 69 locations along the Norwegian coastline.

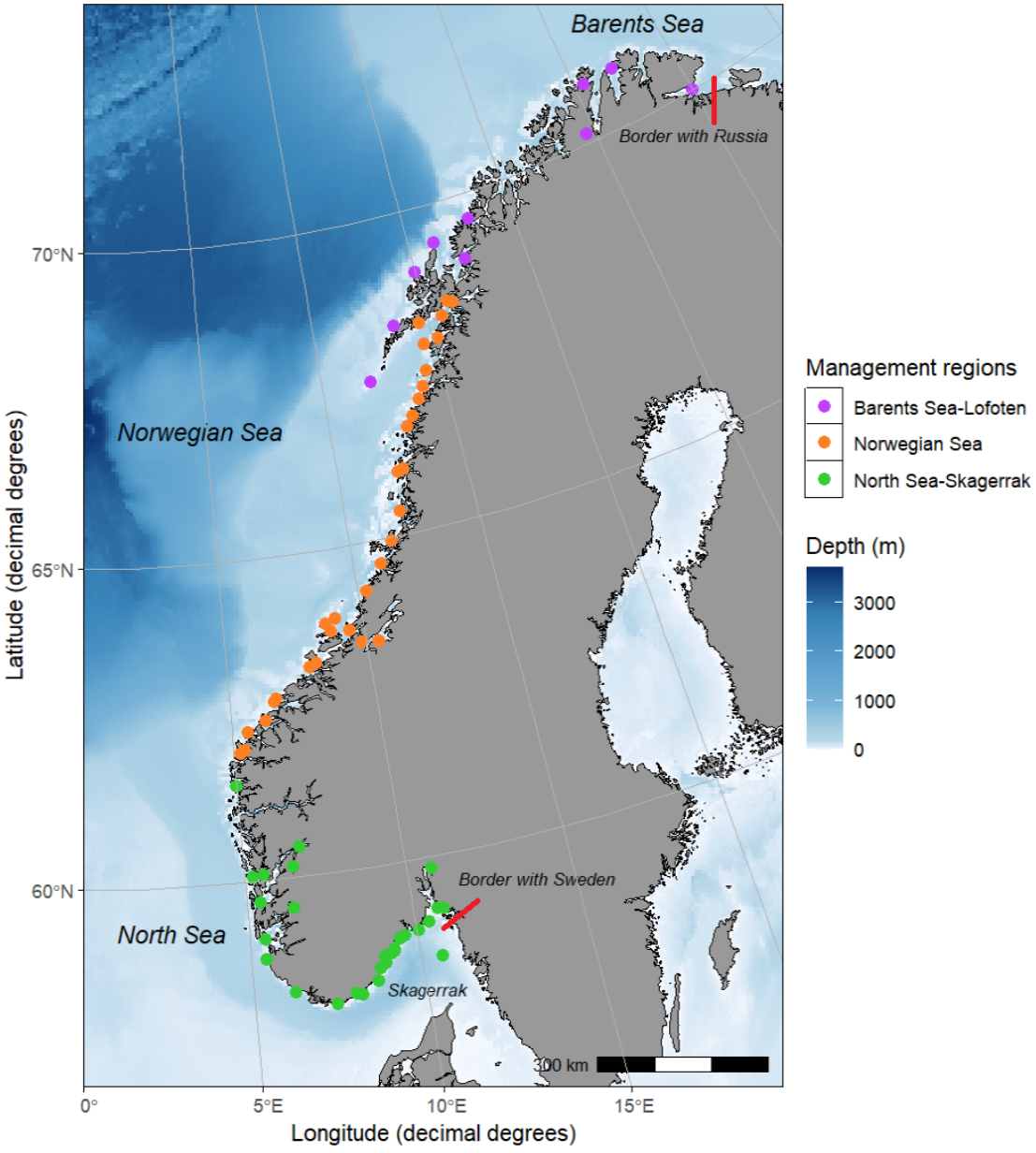

Figure 2: Bathymetric map of sample locations from 2019 along the Norwegian coastline, collected by The Norwegian Directory of Fishery (FDIR) and divided into the three management regions, with red lines for marking land borders with Russia to the north and Sweden to the south.

The samples were collected by the Norwegian Directory of Fishery (FDIR) during 2019 in a Collaboratory project with the Norwegian College of Fishery Science, The Artic University of Norway (UiT) in Tromsø. The samples were collected onboard two different boats owned and operated by FDIR. The two boats, Eir and Rind (Figure 3), normal purpose is to monitor and protect fishery by making sure fishing vessels and the public operate within the law.

Figure 3: Eir and Rind outside Bergen. Foto: Sjøfartsdirektoratet.

The sea water samples were collected following the protocol (Environmental DNA sampling protocol FDIR) appendix a. A metal bucket that had been disinfected thoroughly with bleach, MilliQ and ethanol in a three-step process. First, the bucket was sprayed inside and outside with 10% diluted bleach and wiped down with paper towels. Next, it was rinsed with distilled water and wiped down again. Finally, it was covered with ethanol and wiped down once more. With a rope tied to the handle, the bucket was lowered from the side of the boat to a depth of 1-2 meters below the surface collection 3-5 liters of sea water.

The collected water was then pumped through sterile Sterivex filter using handheld syringes, capturing all DNA within the filter. This process was repeated three times at each location to obtain three replicate samples. The filters were subsequently double-bagged and placed in a freezer until they were handed over to UiT, where they were stored at -80°C.

DNA extraction

The day before the samples is to be processed, they are removed from the -80° Celsius freezer and moved to the fridge in the lock as described in (Extraction Protocol for Sterivex Filters) appendix b to thaw. When the extraction is to be done, clean suits and gloves are put on to do the lock protocol from (Clean Lab Routines – C358) appendix c, before entering the clean lab extraction room with the samples and putting them in the fridge in the extraction room, and then cleaning and preparing the room and flowhood for extraction work, all this is done according to protocol from (Clean Lab Routines – C358) appendix c to prevent contamination in all processes of the extraction, so no unwanted Human or airborne DNA is detected in the samples.

To do the extraction of the DNA from the filters, the (Extraction protocol for Sterivex Filters) appendix b from Dneasy Blood and Tissue Kit (Qiagen) was used to maximize DNA output from the samples. The result was 12 µl aliquot of extracted DNA that were transferred to a PCR plate and kept in the freezer until further processing.

Amplification, library pooling and concentration

When amplifying the DNA, mitochondrial cytochrome c oxidase I (COI) was used. The Leray-XT primer set (Wangensteen et al. 2018) was used to amplify a 313 basepair (bp) fragment of COI that is in most eukaryotic groups. A one step PCR protocol is used to amplify the Leray fragment. The primers used in the metabarcoding have an eight-base sample tag, and numbers 2-4 of leading N's, this is to increase sequence variability which improves the illumina sequencing. The forward and reverse primer have the same sample tag attached to both of its ends. Then protocol was carried out according to (Protocol COI Leray-XT Metabarcoding) appendix d.

When all the samples have been amplified, they need to be pooled and concentrated. 2 µl of the sample can be used to check if the amplification has been successful, by using gel electrophoresis in 1% agarose, the remaining 18 µl from the sample is pooled together in one Eppendorf tuba and homogenized through vortexing. To remove unwanted DNA fragments of below 70 bp, the pooled samples are purified by using MinElute columns, this concentrates the amplified DNA about 10 times. The concentrated columns are eluted in 12-15 µl of elution buffer, and then all the columns are polled

together and vortexed again to be homogenized. The DNA concentration in the pool is then measured using Qubit fluorimeter with the Broad-Range DNA qualification kit. For the best performance in the next litigation step, a concentration of 75 ng/µl or higher in the pool is needed. All details are described in the sections on DNA Amplifications, and Library pooling and concentrations in the protocol (Protocol COI Leray-XT Metabarcoding) in appendix d.

Library preparation and sequencing

In the library prep a PCR-free litigation protocol was used called NEXTflex PCR-Free DNA Sequencing Kit and is from BIOO Scientific, 3 µl of DNA are used as starting material, and the COI library are prepared according to the kit manual for the NEXTflex PCR-Free DNA Sequencing Kit. This exact manual covers fragments between 300-400 bp, which match with the Leray-XT primer set previously used. The kit will ligate the amplicons to the illumina adapters and a 6 base library tag. The sequencing is done by using the library prepped DNA with the described illumina adapters. The steps above are described in detail in the section Library preparation in the protocol (Protocol COI Leray-XT Metabarcoding) appendix d.

Bioinformatics

The sequenced raw data was processed by Owen Simon Wangensteen Fuentes, former postdoc at The Norwegian College of Fishery Science. To convert the raw data to the final working data file the bioinformatic MJOLNIR pipeline was used (https://github.com/uit-metabarcoding/MJOLNIR). The MJOLNIR pipeline trimmed, filtered and clustered the data. This removed all prokaryotes, low-quality reads and sequence errors if any happened during PCR amplification. The data file then contained all eukaryotes found and assigned to species level if possible, using the DUFA sequencing database for taxonomic assignment, with reads from each sample and total reads. The data file was then manually processed to remove terrestrial species.

Plots and statistical analysis

Plots and statistical analysis were done in R version 4.3.3 (February 2024). The Venn diagram and ANOVA analysis were made using the packages tidyverse (Wickham, 2017), phyloseq (McMurdie & Holmes, 2013), stringi (Gagolewski, 2022), VennDiagram (Chen & Boutros, 2011), MiEco (Raymond,

2023), and grid (R Core Team, 2023), and plotted using the venn_plot and geom_jitter functions. Rarefaction curves and bubble plots were made using tidyverse (Wickham, 2017), phyloseq (McMurdie & Holmes, 2013), stringi (Gagolewski, 2022), ggplot2 (Wickham, 2016), and vegan (Oksanen et al., 2019) packages, creating them using the plot_rarefaction and ggplot functions. Non-metric multidimensional scaling (nMDS) and stacked barplots were created using the vegan (Oksanen et al., 2019) and MASS (Venables & Ripley, 2002) packages, and plotted using the draw_mds and barplot functions. Bray-Curtis dissimilarities index was chosen for the nMDS plots.

Results

This study investigated the possibility to establish a large-scale biodiversity baseline of the entire Norwegian coastline using marine water samples collected along the entire coastline and divided into the three management plan regions Barents Sea-Lofoten, Norwegian Sea and North Sea-Skagerrak as set by the Norwegian government. It was done by using COI metabarcoding data from 69 different sample locations each with three sets of replicas, collected in 2019. The raw data was transformed into a working dataset with all eukaryote data and removed blanks, this was done by Owen Simon Wangensteen fuentes in 2020 and handed to me in 2023. I then manually removed terrestrial species. The final working dataset consisted of 8584 MOTUs with 9.490.558 reads (Table 1).

The metadata file consisted of the 69 sample locations from 2019, but two samples were removed because of possible misassignment. The two samples that were removed was FDIR048 and FDIR062. The final metadata contained 67 sample locations in total from the three management regions, 10 samples from Barents Sea-Lofoten (Supplementary table 1), 30 Samples from Norwegian Sea (Supplementary table 2) and 27 samples from North Sea-Skagerrak (Supplementary table 3).

Data Treatment	COI
Total number of reads in final working dataset	9.490.558 reads
Total number of MOTUs in final working dataset	8584

Table 1: Overview of number of reads and MOTUs in final working dataset after COI metabarcoding treatment.

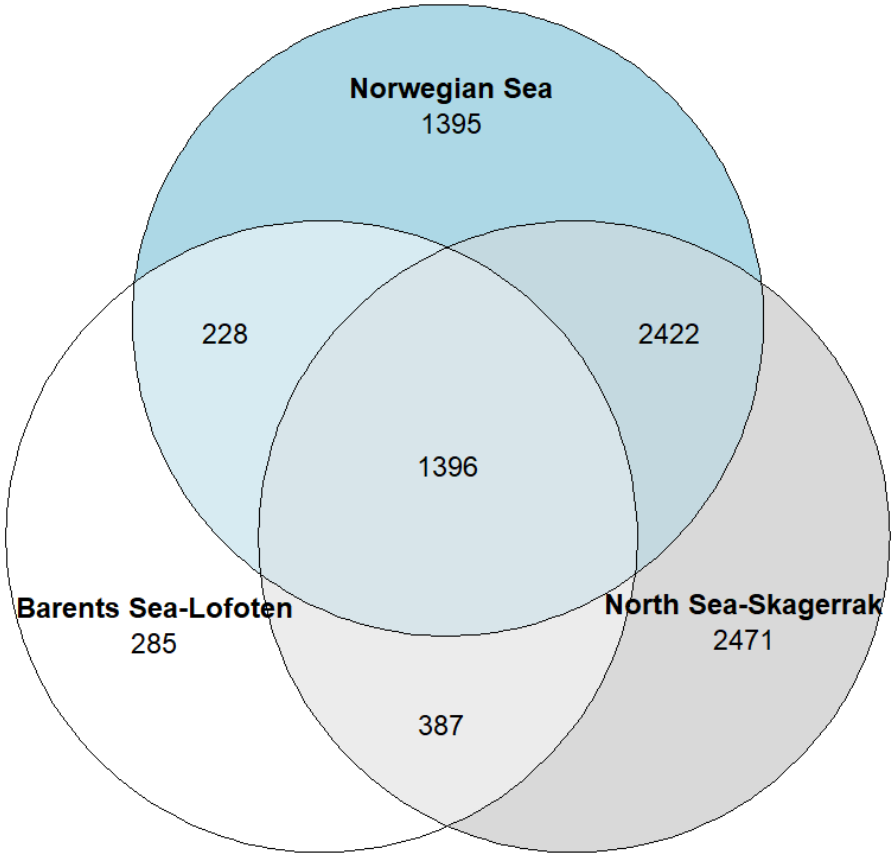

Figure 4: Venn diagram of the three management regions of Barents Sea-Lofoten (placement), Norwegian Sea (Placement) and North Sea-Skagerrak (Bottom right). Each section showcases the number of MOTUs detected in each or several regions.

A Venn diagram (Figure 4) displays the number of MOTUs detected in the three different management regions: Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak. The overlapping sections of the circles represent where MOTUs are detected in more than one region, illustrating shared detections. Non-overlapping sections show where MOTUs are unique to each specific region. A total of 8584 MOTUs were identified across these regions. Specifically:

- The overlap between the Barents Sea-Lofoten and Norwegian Sea shows 228 MOTUs.
- The overlap between the Norwegian Sea and North Sea-Skagerrak shows 2422 MOTUs.
- The overlap between the Barents Sea-Lofoten and North Sea-Skagerrak shows 387 MOTUs.
- The central overlap, representing MOTUs detected in all three regions, shows 1396 MOTUs.

- Unique detections were 285 MOTUs in Barents Sea-Lofoten, 1395 MOTUs in Norwegian Sea, and 2471 MOTUs in North Sea-Skagerrak.

Rarefaction

To investigate the correlation between sequencing depth and the number of MOTUs, rarefaction curves were generated for each of the three management regions (Figure 5). None of the rarefaction curves reached an asymptote, indicating that the sequencing depth was not sufficient to capture all present MOTUs in the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak regions.

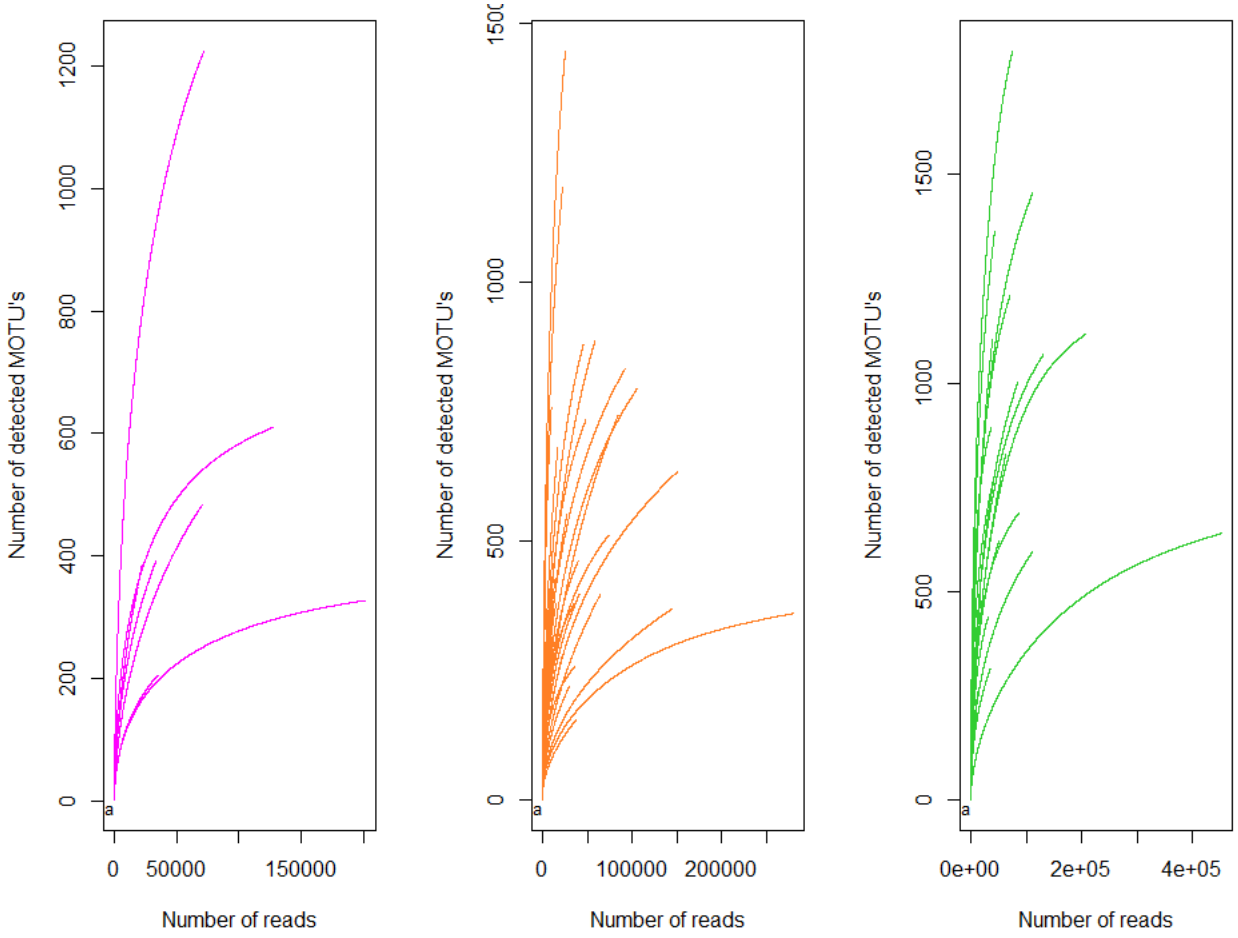

Figure 5: Rarefaction curves from each of the three management regions showing number of detected MOTUs on the y-axis and number of reads on the x-axis. Barents Sea-Lofoten (Left), Norwegian Sea (Middle) and North Sea-Skagerrak (Right). Every curve is representing a single sample.

nMDS analysis

Two nMDS plots were created to visualize the similarities and differences in community composition of all eukaryota across the three regions.

- The first nMDS plot (Figure 6) includes all the samples from each region along with their three replicates. This plot has a stress value of 16.82%, indicating the goodness of fit of the reduced dimensions to the high-dimensional data. The spread of points shows the variability and clustering patterns among the samples from Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak, reflecting ecological distinctions among these regions.
- The second nMDS plot (Figure 7) shows the same data but with the sample replicates collapsed into one for each sample. This plot has a stress value of 15.6%, slightly lower than the first plot, indicating a marginally better fit. This visualization provides a clearer comparison of the overall community composition among the regions without the additional variability introduced by the replicates.

The sample (FDIR057) is isolated in the nMDS plots, showing irregularity to any of the other samples. This sample was taken in Fredrikstad close to an elv.

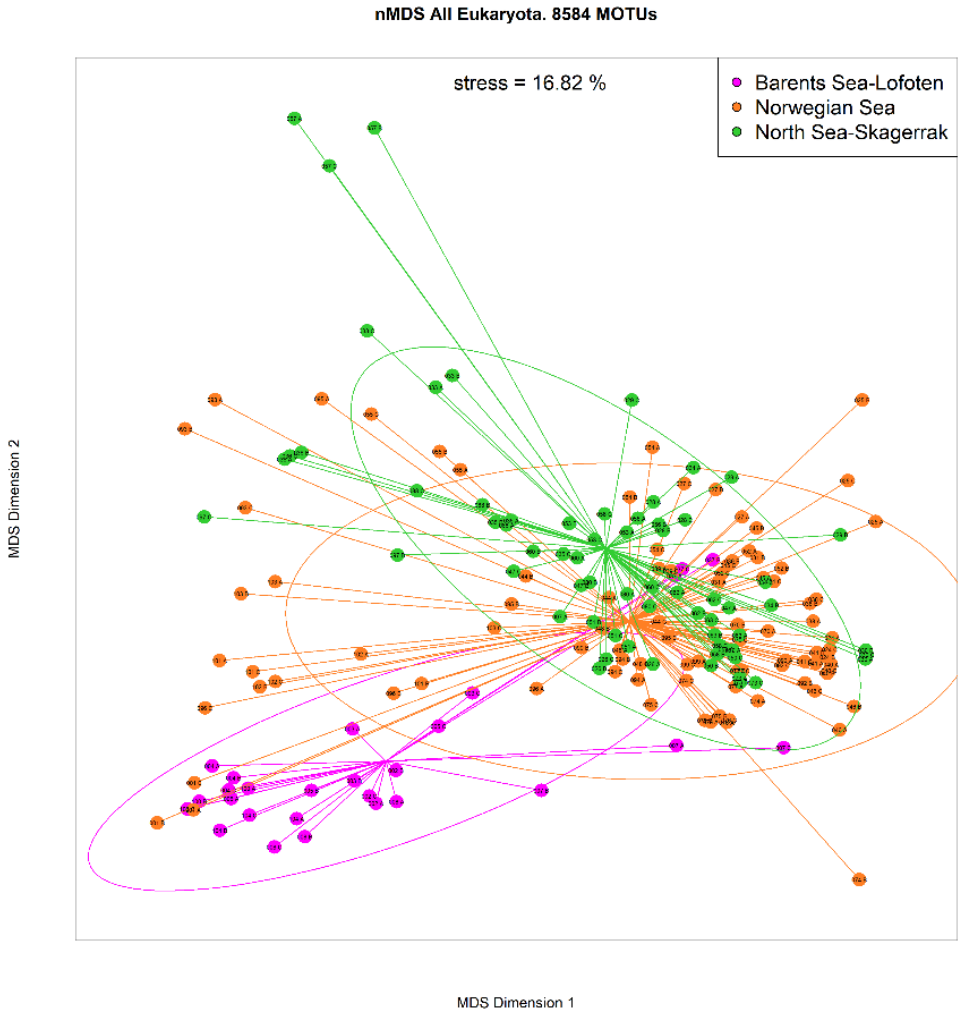

Figure 6: nMDS plot of all eukaryota, 8584 MOTUs. Differences between the three management regions using Bray-Curtis. All three replicates of each sample showing.

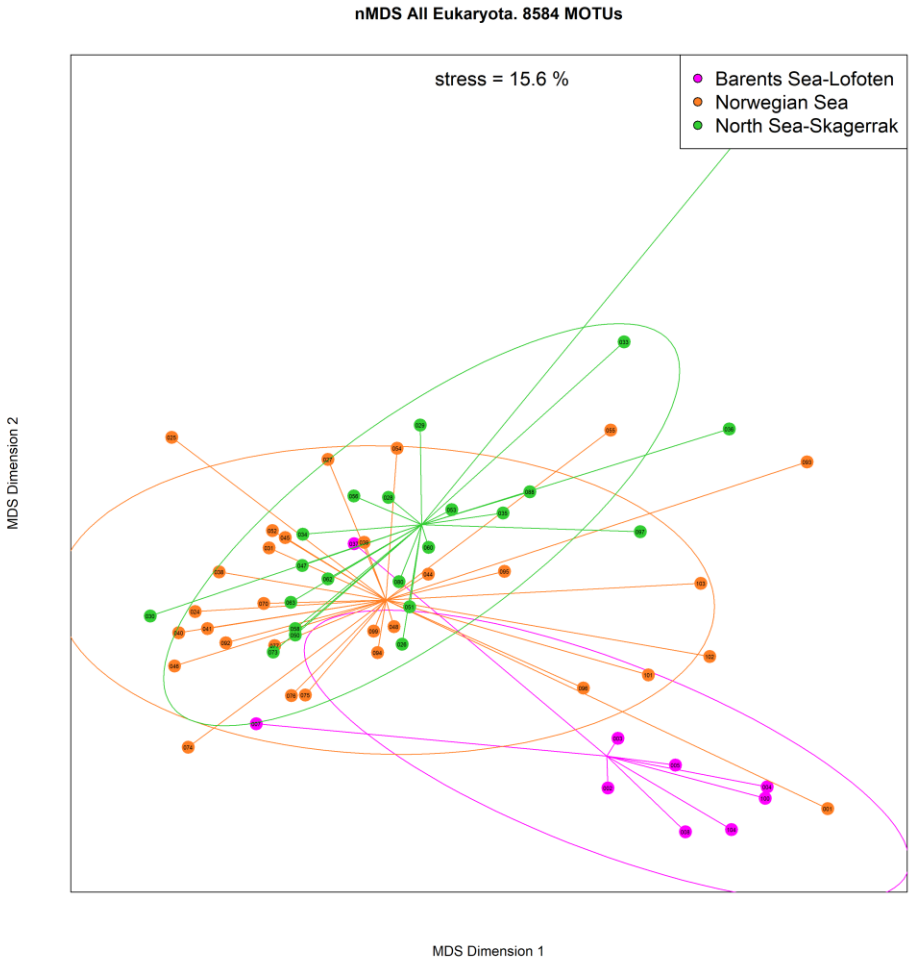

Figure 7: nMDS plot of all eukaryota, 8584 MOTUs. Differences between the three management regions using Bray-Curtis. Showing the three replicates of each sample collapsed into one.

Analysis of variance (ANOVA)

ANOVA was performed to assess the differences in observed values across the three regions and ANOVA using the Shannon diversity index. The analysis yielded the following results (Table 2).

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Management region	2	1838073	919036	7.4714	0.001932
Residuals	36	4428252	123007		

Table 2: Results table for comparing the three Management regions Barents Sea-Lofoten, Norwegian Sea and North Sea-Skagerrak when using ANOVA to assess observed values.

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Management regions	2	11.656	5.8278	9.218	0.0005855
Residuals	36	22.760	0.6322		

Table 3: Results table for comparing the three Management regions Barents Sea-Lofoten, Norwegian Sea and North Sea-Skagerrak when using ANOVA to assess variation in biodiversity.

The ANOVA for observed values (Table 2) compares the mean observed values among the three regions: Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak. It shows that there is a statistically significant difference in observed values among these regions (p = 0.001932), indicating that the mean observed values vary significantly across the regions. The ANOVA for the Shannon diversity index (Table 3) assesses the variation in biodiversity among the three regions. It demonstrates a significant difference in the Shannon diversity index across the regions (p = 0.0005855), suggesting that there are significant differences in biodiversity levels among Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak.

Barplot and bubble plot analysis

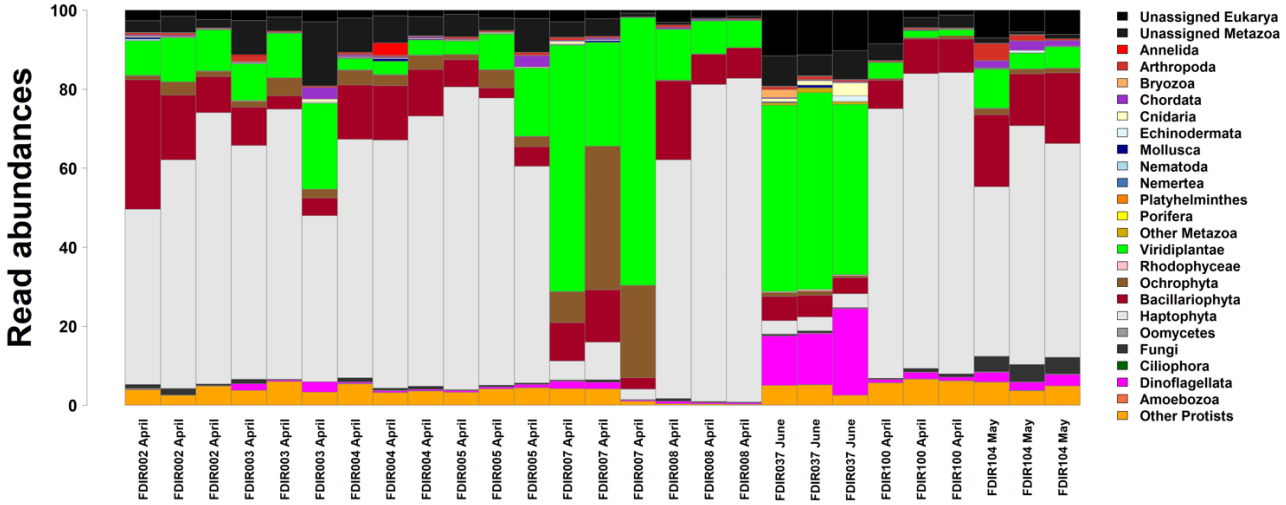

Figure 8: Stacked bar plot displaying phyla from samples collected in the Barents Sea-Lofoten management region, with read abundance on the y-axis and samples on the x-axis.

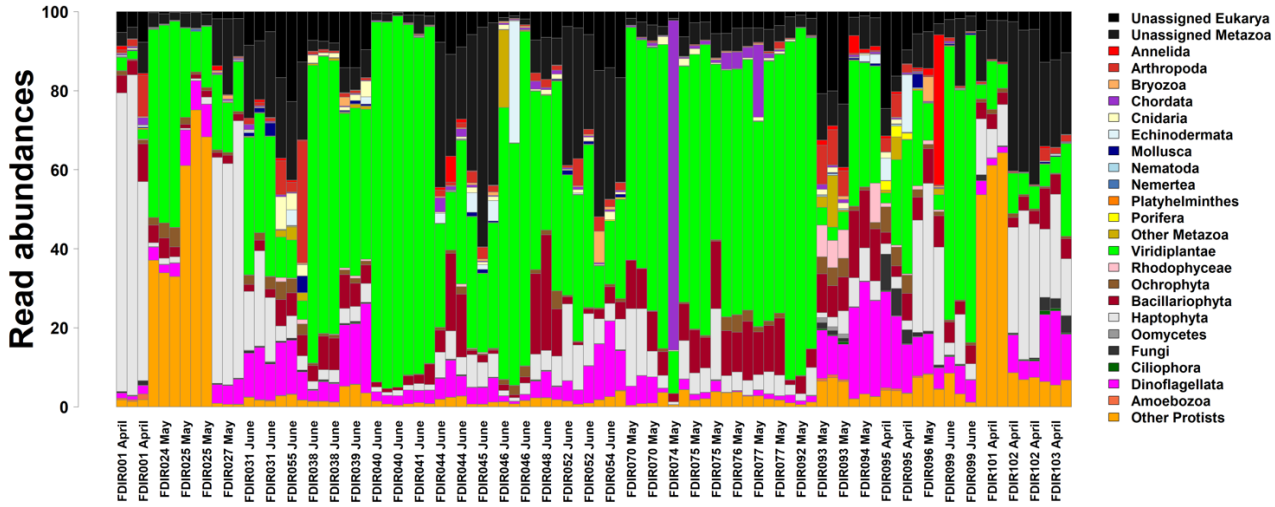

Figure 9: Stacked bar plot displaying phyla from samples collected in the Norwegian Sea management region, with read abundance on the y-axis and samples on the x-axis.

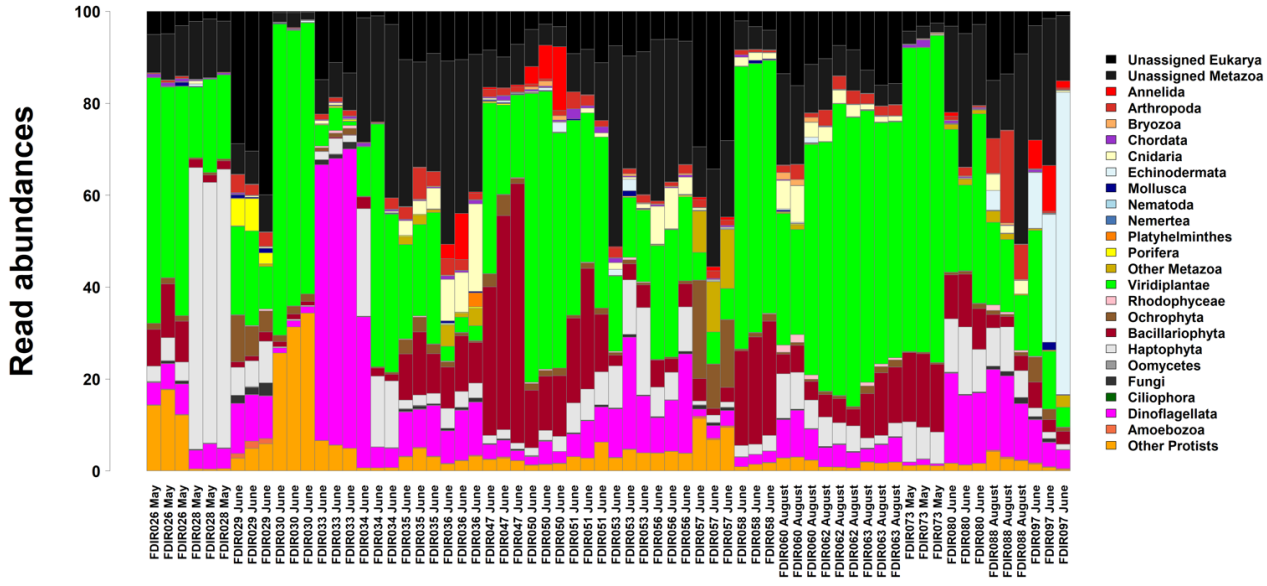

Figure 10: Stacked bar plot displaying phyla from samples collected in the North Sea-Skagerrak management region, with read abundance on the y-axis and samples on the x-axis.

In the Barents Sea-Lofoten region (Figure 8), Haptophyta predominates in most samples, accompanied by varying levels of Arthropoda. Notably, two samples exhibit distinct compositions, one sample is primarily composed of Viridiplantae and Ochrophyta and another sample is dominated by Viridiplantae and Dinoflagellata. The Norwegian Sea (Figure 9) reveal a predominant presence of Viridiplantae across most samples. However, several samples also exhibit dominance by Haptophyta,

Chordata, or other protists. Similarly, in the North Sea-Skagerrak region (Figure 10), Viridiplantae are prevalent across the majority of samples. Some samples show dominance by Haptophyta, Dinoflagellata, Bacillariophyta, or Echinodermata. Both the Norwegian Sea and North Sea-Skagerrak indicate significant reads from unassigned Eukarya and unassigned Metazoa. These visualizations provide insights into the taxonomic composition and relative abundance of phyla within each region, highlighting both typical patterns and unique sample compositions across the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak areas.

The bubble plot (Figure 11) illustrates the relative abundance of different phyla across the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak management regions. The size of the bubbles corresponds to the relative abundance of each phylum within each region, with values ranging from 0.00 to 0.60. The colors of the bubbles indicate the importance of each phylum within the region compared to the others (Blue: High importance, Yellow: Medium importance, Red: Lowest importance).

Figure 11: Relative abundance of different phyla across the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak regions. The size of the bubbles represents the relative abundance of each phylum within each region (values ranging from 0.00 to 0.60). Bubble colors indicate the importance of each phylum within the region (Blue: High importance, Yellow: Medium importance, Red: Lowest importance). This visualization highlights the dominant phyla and the distribution of biodiversity across the three regions.

Caveats of eDNA analysis

The potential for contamination and the presence of non-target species is present when using such sensitive methods as eDNA samples. DNA from freshwater fish was detected in the marine samples (Table 4). Showing possible contamination from nearby freshwater sources or during sample handling. Additionally, DNA from various terrestrial mammalian species was also identified (Table 4).

	id	rank	scientific_name	best_identity	total_reads	total_reads_1	superkingdom_name	kingdom_name	phylum_name	class_name
2	FDCO_000000432	genus	Anguilla	0.993610224	3	3	Opisthokonta	Metazoa	Chordata	Actinopterygii
3	FDCO_000026514	species	Abramis brama	1	26	26	Opisthokonta	Metazoa	Chordata	Actinopterygii
4	FDCO_001092707	species	Hypophthalmichthys molitrix	0.996805112	2	2	Opisthokonta	Metazoa	Chordata	Actinopterygii
5	FDCO_000087699	species	Leuciscus idus	0.993630573	2	2	Opisthokonta	Metazoa	Chordata	Actinopterygii
6	FDCO_001079862	species	Phoxinus phoxinus	1	8	8	Opisthokonta	Metazoa	Chordata	Actinopterygii
7	FDCO_001080780	species	Lota lota	0.996805112	2	2	Opisthokonta	Metazoa	Chordata	Actinopterygii
8	FDCO_000023767	species	Cottus poecilopus	1	3	3	Opisthokonta	Metazoa	Chordata	Actinopterygii
9	FDCO_000035148	species	Gymnocephalus cernua	0.9968	6	6	Opisthokonta	Metazoa	Chordata	Actinopterygii
10	FDCO_000026775	species	Bos taurus	1	219	223	Opisthokonta	Metazoa	Chordata	Mammalia
11	FDCO_000639188	species	Ovis aries	1	3	3	Opisthokonta	Metazoa	Chordata	Mammalia
12	FDCO_000543480	species	Alces alces	1	51	51	Opisthokonta	Metazoa	Chordata	Mammalia
13	FDCO_000349857	species	Cervus elaphus	1	40	41	Opisthokonta	Metazoa	Chordata	Mammalia
14	FDCO_000002057	species	Sus scrofa	1	583	590	Opisthokonta	Metazoa	Chordata	Mammalia
15	FDCO_000102029	species	Canis lupus	1	80	81	Opisthokonta	Metazoa	Chordata	Mammalia
16	FDCO_000205452	species	Vulpes vulpes	1	5	5	Opisthokonta	Metazoa	Chordata	Mammalia
17	FDCO_001279108	species	Felis catus	1	98	98	Opisthokonta	Metazoa	Chordata	Mammalia

Table 4: Freshwater fish and terrestrial mammalian species found in the collected water samples.

Discussion

Establishing a biodiversity baseline

The study supports Hypothesis i, demonstrating that eDNA metabarcoding provides a nuanced biodiversity baseline across the Norwegian coastline. Through the analysis of Molecular Operational Taxonomic Units (MOTUs), significant variations in species composition were identified among the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak management regions. Statistical analyses including ANOVA for observed values and Shannon diversity index confirmed these differences, highlighting the complex ecological dynamics and species distributions influenced by environmental gradients and oceanographic processes (Valentini et al., 2016; Deiner et al., 2017).

The presence of localized biodiversity hotspots within each region was evident from the study findings. Unique MOTUs were identified, with the Barents Sea-Lofoten region exhibiting 285 unique MOTUs, compared to 1395 in the Norwegian Sea and 2471 in the North Sea-Skagerrak. These distinct biodiversity patterns highlighted the influence of local environmental conditions, species distributions, and ecological interactions on marine ecosystems along the Norwegian coastline. The nMDS plots visually depicted differences in biodiversity between the Barents Sea-Lofoten and North Sea-Skagerrak regions, further supporting the hypothesis of varied biodiversity baselines across management regions.

The southward increase in detected species richness could be attributed to several factors, including nutrient enrichment from ocean currents and agricultural runoff, influencing the ecological productivity of southern regions. However, rarefaction curves indicated that sampling efforts were insufficient to fully capture the biodiversity potential in each region, with the Barents Sea-Lofoten region particularly underrepresented due to fewer samples collected compared to the Norwegian Sea and North Sea-Skagerrak regions. This highlights the critical role of sampling intensity in eDNA metabarcoding studies for accurately assessing biodiversity (Valiere and Taberlet, 2000; Collins et al., 2018).

Climate gradients along the Norwegian coastline significantly influenced species distributions and biodiversity patterns observed in the study. Warmer temperatures in southern regions of the Norwegian Sea and North Sea-Skagerrak supported higher species richness compared to the colder northern Barents Sea-Lofoten region. These temperature gradients impact species physiology, reproductive cycles, and habitat preferences, shaping biodiversity hotspots along the coastline (Long and Perrie, 2017; Zhao et al., 2023).

Oceanographic processes, including currents, upwelling events, and nutrient availability, played pivotal roles in shaping biodiversity hotspots and favorable conditions along coastal regions (Boitsov et al., 2012). They influenced nutrient availability and primary productivity, supporting diverse biological communities. Ocean currents facilitated species dispersal and colonization, enhancing biodiversity stability under stable oceanographic conditions. Conversely, fluctuations in ocean pH, nutrient levels, and sea ice dynamics in the Barents Sea-Lofoten region could disrupt ecological balance and species distributions (Rock et al., 2022). Integration of oceanographic data with eDNA metabarcoding provided insights into marine biodiversity patterns, aiding in effective conservation strategies tailored to regional dynamics (Wang et al., 2024).

Identifying biodiversity hotspots

Hypothesis ii was supported by the study's findings, which identified key biodiversity hotspots within the Norwegian coastal regions. Each region exhibited distinct taxonomic compositions dominated by different phyla, as illustrated by stacked bar plots and bubble plots. The Barents Sea-Lofoten region showed dominance of Haptophyta, while Viridiplantae were prominent in the Norwegian Sea and North Sea-Skagerrak. These phyla-specific dominance patterns highlighted unique ecological niches and species assemblages across the Norwegian coastline, necessitating targeted conservation efforts and adaptive management strategies (Thomsen et al., 2012; Stat et al., 2020).

The presence of these biodiversity hotspots provides crucial insights for conservation planning and management, emphasizing the need to preserve ecological integrity in key habitats and species assemblages. Effective conservation strategies should consider region-specific ecological dynamics revealed by eDNA metabarcoding, ensuring adaptive responses to environmental changes and

anthropogenic pressures (Thomsen et al., 2012; Stat et al., 2020). By focusing on preserving these biodiversity hotspots, conservation efforts can maximize their impact in safeguarding biodiversity and promoting ecosystem resilience along the Norwegian coastline.

Assessing the sufficiency of three management regions

The study findings provide support for Hypothesis iii by revealing significant biodiversity differences among the three management regions, indicating their capacity for a wide range of biodiversity. However, the presence of many unique MOTUs in each region suggests that the current three-region management plan may not fully capture localized biodiversity patterns. The Venn diagram analysis highlighted substantial species exclusivity in each region, emphasizing the need for more nuanced conservation strategies or additional management regions.

For example, the Barents Sea-Lofoten region encompasses both the northern area of the Norwegian Sea and the Barents Sea, each influenced by distinct environmental factors. This geographical complexity challenges the efficacy of a single management plan encompassing such different areas. Current conservation frameworks often outline management regions based on political, economic, or administrative boundaries rather than ecological considerations (Fernandes et al., 2018). This approach can lead to oversights regarding local biodiversity hotspots or ecological connectivity across broader scales.

Integrating eDNA metabarcoding with traditional ecological surveys can provide a fuller understanding of the complex biodiversity dynamics along the Norwegian coastline (Taberlet et al., 2012). This integration enhances our understanding of species distributions, environmental gradients, and habitat connectivity, providing a comprehensive basis for effective management strategies. Studies by Elbrecht and Leese (2017) and Jane et al. (2021) highlights the importance of genetic data from eDNA metabarcoding in guiding marine spatial planning and conservation initiatives.

Therefore, while the current study supports the notion that the three management regions exhibit significant biodiversity, the presence of distinct MOTU compositions and ecological factors across these regions challenges the adequacy of the existing management framework. Thus, Hypothesis iii is

accepted, suggesting that more refined and ecologically informed management regions are necessary to effectively conserve the diverse marine biodiversity along the Norwegian coastline.

Discovering new or previously undetected species

The study identified DNA from non-target species, including the silver carp (*Hypophthalmichthys molitrix*) and various terrestrial mammals, suggesting either new species introductions or contamination. This highlights the potential of eDNA metabarcoding to detect previously undetected biodiversity but highlights the need for quality control measures to distinguish true positives from contaminants. Because the DNA from the silver carp cannot be confirmed as not being a new species in Norwegian waters, the hypothesis iv is confirmed.

Advances in eDNA technology have facilitated the discovery of cryptic species and novel biodiversity in aquatic ecosystems (Deiner and Altermatt, 2014; Thomsen and Willerslev, 2015). The detection of unexpected DNA signatures and many unassigned species in my study prompts further investigation into species distributions, biological invasions, and ecological interactions along the Norwegian coastline, and could prove as more new species in the Norwegian waters.

eDNA for biodiversity assessment and associated challenges

Environmental DNA (eDNA) is a powerful tool for assessing biodiversity, but its application comes with several challenges and caveats. When using eDNA to establish a biodiversity baseline, several issues can arise that may impact the accuracy and reliability of the results.

One significant concern is the sensitivity of Sterivex filters used in eDNA sampling. These filters can capture DNA from a wide range of sources, including unwanted contaminants. For instance, in some of the samples, DNA from terrestrial animals was recorded and only 5 of the 13 Mammalia MOTUs found were marine species. The DNA (*Canis lupus*) could originate from both dogs and wolves, and current eDNA analysis techniques often lack the resolution to distinguish between these closely related species. The presence of canine DNA could be due to various factors, such as a crew member's clothing carrying dog hair or environmental contamination from precipitation washing terrestrial DNA into marine environments (Deiner et al., 2017; Goldberg et al., 2016). This highlights a broader issue with eDNA and its susceptibility to contamination from external sources. The inability to differentiate

between these sources limits the technique's potential for certain terrestrial applications, such as tracking invasive species. The Swedish boar and domestic pigs could both be (*Sus scrofa*) like the Swedish boar. Boars can have significant ecological impacts, and if eDNA methods could reliably distinguish between domestic pigs and wild boars, they could be highly effective for monitoring and managing these populations (Boivin-Delisle et al., 2021).

Another challenge is the unexpected detection of non-native species. For example, the find of the silver carp collected from the Nidelve area. This is a freshwater species originating from eastern Asia and not previously recorded in Norway, was detected. This finding is surprising in a marine context and could have several explanations. It most likely results from food waste from nearby restaurants, contamination from someone handling the fish, but could be an unrecorded introduction of the species into the area (Ficetola et al., 2008; Deiner et al., 2015). The sample collected close to an elv in Fredrikstad showed many of the freshwater species that were found, and the fact that the sample was significantly different in community composition, combined with the silver carp find, confirms that the eDNA metabarcoding method works. Such instances highlight the need for careful interpretation of eDNA data and consideration of potential contamination sources and the importance of implementing rigorous protocols.

A fundamental limitation of eDNA is its inability to distinguish between DNA from living organisms and DNA from dead or decayed sources. This limitation means that eDNA can only confirm the presence of a species' DNA in the environment, not the current population size or health of the species (Pilliod et al., 2014). For instance, detecting DNA from a species does not indicate whether it is currently living in the area or if the DNA is from a decayed organism that drifted from elsewhere. The spatial and temporal variability of eDNA concentrations also poses challenges. DNA can disperse through water and may not accurately represent the local biodiversity at the sampling site. This variability can lead to inconsistent results, particularly in dynamic environments such as estuaries or coastal zones where water movement can transport DNA over considerable distances (Collins et al., 2018).

This study represents the first large-scale investigation of the entire Norwegian coastline using eDNA metabarcoding from seawater samples. Making such an extensive survey showcases unique

challenges, including logistical difficulties in sample collection across such vast and diverse marine environments, and to ensure consistent sample handling and processing, and managing potential contamination. Despite these challenges, this effort provides insights into the biodiversity of Norwegian coastline and establishes a baseline for future monitoring and conservation efforts.

Future perspectives and research

Future research should implement increased sampling intensity and collect samples throughout the calendar year to achieve a more comprehensive biodiversity baseline, particularly in the underrepresented Barents Sea-Lofoten region. Also improving sequencing depth will be crucial to ensure more accurate and comprehensive data collection Integrating eDNA metabarcoding with oceanographic data will enhance our understanding of species distributions. Continuous sampling and further studies will provide a fuller understanding of biodiversity along the Norwegian coastline and better track potential changes, as well as capture rare or elusive species. Additionally, training in eDNA sample collection is essential to reduce potential contamination and improve data accuracy.

Conclusion

This study successfully established a biodiversity baseline along the Norwegian coastline using eDNA metabarcoding, revealing significant variations in species composition across different management regions. The findings showed the presence of regional biodiversity hotspots influenced by local environmental conditions and oceanographic processes. However, the study was limited by insufficient sequencing depth and underrepresented sampling in the Barents Sea-Lofoten region, which may have impacted the detection of some species. The research highlighted the complex ecological dynamics and species distributions shaped by environmental gradients, oceanographic processes, and climate gradients along the coastline. The significant biodiversity differences among the three management regions suggest that region-specific strategies are necessary to efficiently conserve marine ecosystems, and dividing the Norwegian coastline into more ecologically based regions would be more efficient. Overall, this research provides a foundation for future monitoring and conservation efforts, emphasizing the importance of integrating eDNA metabarcoding with traditional ecological surveys.

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Supplementary plots and tables

1	object_id	UiT_ID	Date	Region	Location	Latitude	Longitude
2	FDIR_RIND	FDIR002	24-04-2019	Barents Sea-Lofoten	Undstad	6827310	1353430
3	FDIR_RIND	FDIR003	25-04-2019	Barents Sea-Lofoten	Hekkingen	6957610	1795390
4	FDIR_RIND	FDIR004	25-04-2019	Barents Sea-Lofoten	Andenes	6938290	1619090
5	FDIR_RIND	FDIR005	25-04-2019	Barents Sea-Lofoten	Nyksund	6901550	1498090
6	FDIR_EIR	FDIR006	25-04-2019	Barents Sea-Lofoten	Røst	6747680	1202550
7	FDIR_RIND	FDIR007	26-04-2019	Barents Sea-Lofoten	Syd av Arnøy	7000670	3000120
8	FDIR_RIND	FDIR008	28-04-2019	Barents Sea-Lofoten	Vest av Hjelmsøy	7014270	2459030
9	FDIR_RIND	FDIR014	04-05-2019	Barents Sea-Lofoten	Laksefjorden	7092830	2705000
10	FDIR_RIND	FDIR037	08-06-2019	Barents Sea-Lofoten	Vågsfjorden	6901000	1727420
11	FDIR_RIND	FDIR069	19-10-2019	Barents Sea-Lofoten	Lafjorden	7087750	2547420

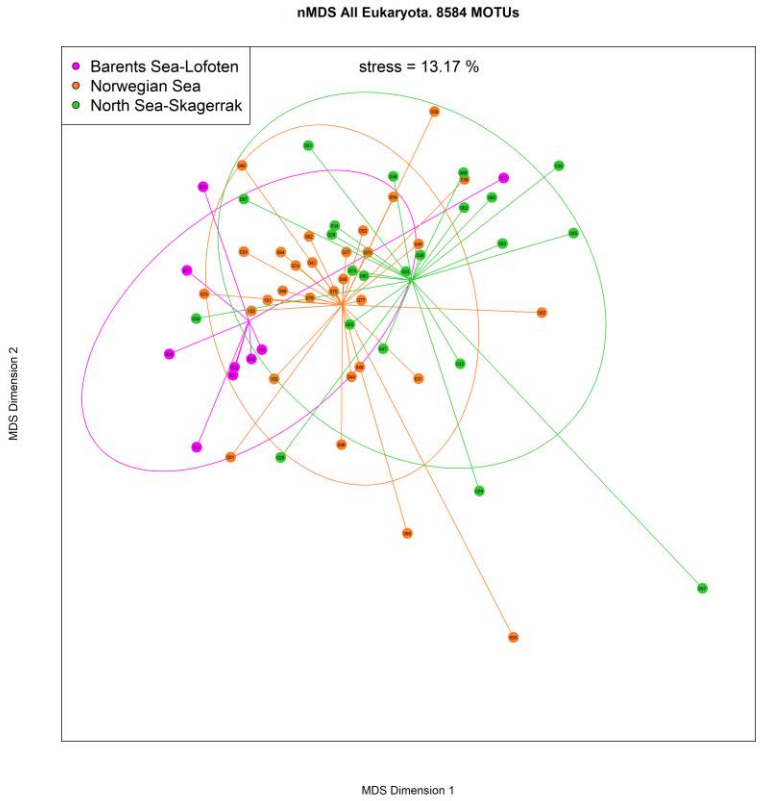
Supplementary table 1. Sample locations data from samples collected in the Barents Sea-Lofoten region.

1	object_id	UiT_ID	Date	Region	Location	Latitude	Longitude
2	FDIR_RIND	FDIR001	23-04-2019	Norwegian Sea	Svolvær	6823570	1457470
3	FDIR_EIR	FDIR009	28-04-2019	Norwegian Sea	Saltfjorden	6721830	1412170
4	FDIR_EIR	FDIR010	29-04-2019	Norwegian Sea	Meløyfjorden	6680770	1345170
5	FDIR_EIR	FDIR011	29-04-2019	Norwegian Sea	Alstenfjorden	6598920	1237450
6	FDIR_EIR	FDIR012	30-04-2019	Norwegian Sea	Folda	6463300	1100480
7	FDIR_EIR	FDIR013	01-05-2019	Norwegian Sea	Sistranda	6373080	883450
8	FDIR_EIR	FDIR015	12-05-2019	Norwegian Sea	Nidelven	6343820	1040870
9	FDIR_EIR	FDIR016	15-05-2019	Norwegian Sea	Frohavet	6392130	906220
10	FDIR_EIR	FDIR017	15-05-2019	Norwegian Sea	Mausundfjorden	6385600	867820
11	FDIR_EIR	FDIR018	15-05-2019	Norwegian Sea	Edøyfjorden	6322030	794280
12	FDIR_EIR	FDIR019	15-05-2019	Norwegian Sea	Harøyfjorden	6275480	653630
13	FDIR_EIR	FDIR020	16-05-2019	Norwegian Sea	Herøyfjorden	6232250	553570
14	FDIR_EIR	FDIR021	17-05-2019	Norwegian Sea	Selje	6204580	534370
15	FDIR_EIR	FDIR022	18-05-2019	Norwegian Sea	Sildefjorden	6200600	521670
16	FDIR_RIND	FDIR024	28-05-2019	Norwegian Sea	Stabben	6839670	1624830
17	FDIR_RIND	FDIR025	28-05-2019	Norwegian Sea	Hammarøygrunn	6823500	1565330
18	FDIR_RIND	FDIR026	30-05-2019	Norwegian Sea	Bogøya	6790000	1519050
19	FDIR_RIND	FDIR030	02-06-2019	Norwegian Sea	Lødingen	6843000	1601330
20	FDIR_RIND	FDIR038	08-06-2019	Norwegian Sea	Vestfjorden	6787880	1457850
21	FDIR_RIND	FDIR039	08-06-2019	Norwegian Sea	Landegode	6746180	1440180
22	FDIR_RIND	FDIR040	09-06-2019	Norwegian Sea	Fugløyfjorden (Nordl.)	6702830	1379830
23	FDIR_RIND	FDIR041	09-06-2019	Norwegian Sea	Rødøyfjorden	6667500	1313830
24	FDIR_RIND	FDIR042	09-06-2019	Norwegian Sea	Sandnessjøen	6602450	1255830
25	FDIR_RIND	FDIR043	09-06-2019	Norwegian Sea	Brønnøysund	6539500	1211830
26	FDIR_RIND	FDIR044	09-06-2019	Norwegian Sea	Morvika	6496830	1155500
27	FDIR_RIND	FDIR046	10-06-2019	Norwegian Sea	Bessaker	6425500	1033500
28	FDIR_RIND	FDIR047	10-06-2019	Norwegian Sea	Brekstad	6370180	950970
29	FDIR_RIND	FDIR049	10-06-2019	Norwegian Sea	Sør av Edøya	6327670	814170
30	FDIR_RIND	FDIR050	10-06-2019	Norwegian Sea	Sandøy	6280670	662250
31	FDIR_RIND	FDIR051	10-06-2019	Norwegian Sea	Ålesund	6247280	615470

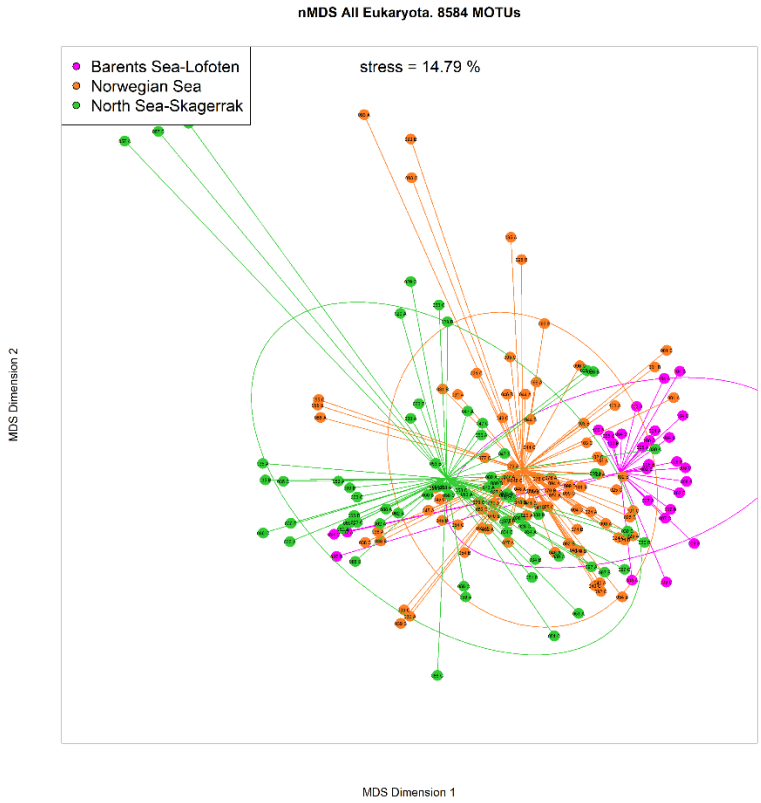
Supplementary table 2. Sample locations data from samples collected in the Norwegian Sea region.

1	object_id	UiT_ID	Date	Region	Location	Latitude	Longitude
2	FDIR_EIR	FDIR023	18-05-2019	North Sea-Skagerrak	Tranøyosen	6149180	498170
3	FDIR_EIR	FDIR027	30-05-2019	North Sea-Skagerrak	Lamøyosen	6008780	528830
4	FDIR_EIR	FDIR028	31-05-2019	North Sea-Skagerrak	Bjørangsfjorden	6009970	557000
5	FDIR_EIR	FDIR029	01-06-2019	North Sea-Skagerrak	Eidfjorden	6047170	678850
6	FDIR_EIR	FDIR031	02-06-2019	North Sea-Skagerrak	Sørfjorden	6017260	656850
7	FDIR_EIR	FDIR032	04-06-2019	North Sea-Skagerrak	Bømlofjorden	5968650	541000
8	FDIR_EIR	FDIR033	05-06-2019	North Sea-Skagerrak	Hylsfjorden	5954770	642750
9	FDIR_EIR	FDIR034	06-06-2019	North Sea-Skagerrak	Kvitsøyfjorden	5911740	545770
10	FDIR_EIR	FDIR035	07-06-2019	North Sea-Skagerrak	Skotemedgrunnen	5879830	546670
11	FDIR_EIR	FDIR036	07-06-2019	North Sea-Skagerrak	Siragrunnen	5825860	623210
12	FDIR_EIR	FDIR045	09-06-2019	North Sea-Skagerrak	Kvåsefjorden	5810330	814330
13	FDIR_EIR	FDIR052	10-06-2019	North Sea-Skagerrak	Svenner	5892990	1005550
14	FDIR_EIR	FDIR053	10-06-2019	North Sea-Skagerrak	Østregapet	5864800	917350
15	FDIR_EIR	FDIR054	10-06-2019	North Sea-Skagerrak	Jeløya	5849180	1059210
16	FDIR_EIR	FDIR055	10-06-2019	North Sea-Skagerrak	Skagerak	5827710	865260
17	FDIR_EIR	FDIR056	13-06-2019	North Sea-Skagerrak	Nesodden	5985500	1077230
18	FDIR_EIR	FDIR057	14-06-2019	North Sea-Skagerrak	Fredrikstad	5921370	1092280
19	FDIR_EIR	FDIR058	23-06-2019	North Sea-Skagerrak	Kristiansand	5813280	799500
20	FDIR_RIND	FDIR059	16-08-2019	North Sea-Skagerrak	Kilsund	5851060	894170
21	FDIR_RIND	FDIR060	18-08-2019	North Sea-Skagerrak	Lyngør	5867060	922760
22	FDIR_RIND	FDIR061	19-08-2019	North Sea-Skagerrak	Skåtøysund	5886270	944680
23	FDIR_RIND	FDIR063	25-08-2019	North Sea-Skagerrak	Hillevågen	5800570	736620
24	FDIR_EIR	FDIR064	12-09-2019	North Sea-Skagerrak	Arendal	5846170	877770
25	FDIR_EIR	FDIR065	15-09-2019	North Sea-Skagerrak	Tvedestrand	5862170	893170
26	FDIR_EIR	FDIR066	17-09-2019	North Sea-Skagerrak	Jomfruland	5890430	961400
27	FDIR_EIR	FDIR067	20-09-2019	North Sea-Skagerrak	Smaustangen	5922430	1071930
28	FDIR_EIR	FDIR068	21-09-2019	North Sea-Skagerrak	Verdensende	5901850	1042330

Supplementary table 3. Sample locations data from samples collected in the North Sea-Skagerrak region.



Supplementary figure 1. nMDS plot of the three management plan regions. 4th root transformed collapsed.



Supplementary figure 2. nMDS plot of the three management plan regions. 4th root transformed.

Supplementary figure 3. Relative abundance of different phyla across the Barents Sea-Lofoten, Norwegian Sea, and North Sea-Skagerrak regions. The size of the bubbles represents the relative abundance of each kingdom within each region (values ranging from 0.00 to 0.60). Bubble colors indicate the importance of each kingdom within the region (Blue: High importance, Yellow: Medium importance, Red: Lowest importance). This visualization highlights the dominant kingdom and the distribution of biodiversity across the three regions.

Data files, Rstudio scripts and appendixes

Data file with terrestrial species included

File too large to include as an appendix but can be sent if wanted. Contact me on email (obl1603Hotmail.com).

Data file without terrestrial species included

File too large to include as an appendix but can be sent if wanted. Contact me on email (obl1603Hotmail.com).

Metadata file with locations

File too large to include as an appendix but can be sent if wanted. Contact me on email (obl1603Hotmail.com).

Rstudios scripts

All Rstudios scripts were too large to include appendix but can be sent if wanted. Contact me on email (obl1603Hotmail.com).

Appendix – a. Environmental DNA sampling protocol FDIR

Needed equipment

If water is filtered at location with syringe

- Pack all equipment for sampling in "eDNA sampling only" Zarges boxes. Never use boxes that have been used for equipment for fish/community sampling. Avoid opening the box(es) in areas where fish/community sampling occur and strictly keep away from PCR machines/rooms.
- Water collector or bucket. If water collector with rope and weight, use a large black round bucket to carry all. Make sure it have a few holes in the bottom and that the rope is tied to one of them. If a bucket are used for surface sampling, use one in stainless steel.
- Spray flask(s) (if many stations/depths, also bring one of the 5l garden sprays we have)
- Bleach (and water)
- Gloves
- Sterivex filters (Part: SVGPL10RC. Must have luer in both ends)
- 50 ml syringes
- 50 ml falcons
- Zip lock bags (small that fit three 50 ml falcons and larger that can fit a station of bags)
- Markers, pencils, notebook, waste bag
- If far to the freezer and/or filtering in a heated place, bring a eDNA-only cooling box with blue ice elements packed in zip-loc bags that are bleached and rinsed in ddH2O before transferring into the cooling box.

Important

- Always use rubber gloves
- Avoid touching the anything but the samples with the gloves, to minimize contamination
- The buckets that were delivered to collection of water, should only be used for this purpose
- Position for every sample location needed

- Note, if possible, any sightings of marine mammals, distance to aquaculture facilities, fishing boats or other relevant information
- Note if there have been fish or other animals on the boat within 24 hours
- Note if you have eaten any fish or other animal before sample collection, and preferably use newly washed clothes

Sample collection steps

1. When arriving to the location, mark bag and three falcon tubes with location and date. A fourth falcon tube is marked with location, date and "BLANK".
2. Get 4 filters and a syringe
3. Thoroughly clean the bucket inside and outside with bleach, MilliQ and ethanol
4. Rinse the bucket a couple of times with surface water
5. Take a sea water sample with the bucket, collecting 3-5 liters of water
6. Change gloves, avoiding touching anything
7. Take a "BLANK" by suctioning 50 ml of air into the syringe and pushing it through the filter. Repeat 9 times, so the total volume of air that have passed through the filter is 500 ml
8. Put the syringe in plastic wrapping the syringe came in
9. Transfer the filter to the falcon tube marked "BLANK" only keeping the falcon tube open as briefly as possible
10. Take the same syringe and fill it with 50 ml of water from the bucket and push the water through another filter. Repeat 9 times with the same syringe to filter a total of 500 ml of sample water
11. Fill the syringe with air and push out excess water in the filter
12. Transfer the filter to one of the marked falcon tubes
13. Repeat for the other 2 samples with the same syringe
14. Place all 4 falcon tubes in a bag marked with location and date and close the bag
15. Now put the bag in another bag, so it is double bagged
16. Store bag with filters at -80 ^{o}C if possible, or -20 ^{o}C until the boat arrives to shore and can store samples at -80 ^{o}C.

Appendix – b. Extraction Protocol for Sterivex Filters

excess tubes.

- Only open the bags containing Eppendorf tubes, or other tubes inside the flowhood.
- Only use pipette tips with barriers/filters and only open the boxes inside the flowhood.
- Always follow the workflow or any precautions given for the eDNA clean lab working routines.
- Always discard tips/tubes/gloves if you have the slightest suspicion about contamination (e.g. if the tip touches the table before entering a tube or buffer bottle).

UiT
THE ARCTIC UNIVERSITY OF NORWAY

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

EXTRACTION PROTOCOL FOR STERIVEX FILTERS

EDNA EXTRACTION BASED ON QIAGEN DNEASY BLOOD & TISSUE KIT

IMPORTANT NOTES

- Make sure that the incubator is set to 56°C before starting the work. The equipment you are going to use for the extraction protocol should always be cleaned
- Always shake Eppendorf tubes out of the bag, don't put your hand inside of it. Discard any

- Always work with at least one extraction blanks per extraction round (i.e. 24 samples). However, if you are working with 22 samples to extract, then to complete the number to 24, you work with two blanks.
- Always start with the lowest concentration i.e. air blanks and water blanks (if any) except extraction blanks, which should be treated as any regular sample.
- However, the extraction blanks
- If extracting samples from several species/locations, sterilize everything between samples.
- Do not touch the ends of the Sterivex filters or the inside of the tube caps with hands or tweezers.
- Always be careful when you open the Eppendorf tubes not to touch the inside of the cap. Hold them in your hand and flick them open with the tip of your thumb.
- MAKE SURE YOU HAVE ENOUGH TIPS! You will mainly use 1000µl tips but also stock up on 20µl and 200µl ones. You also need Eppendorf tubes (both 1.5ml and 2.0ml), 50ml falcon tubes. Always have enough of these things before you start working.

DAY 0:

1. Find filters in -80°C freezer and place them in the fridge in the lock at 4°C for gentle thawing. It takes approx. 1-2 hours but since the freezer is located in a "contaminated" area the preference is to take the samples out the day before. The day after you start your extractions by showing up in clean clothes and freshly showered.

DAY 1:

2. Follow the descriptions in the 'Clean Lab Routines' of how to enter the labs.
3. 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.
4. 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.
5. Centrifuge the tubes at 1500 x g for 3 minutes to remove the remaining seawater from the filters.
6. Make extraction buffer solution for adding 2.5X the recommended volume = 500µl per filter.
   o 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 or 15ml tube (if you have 24 or less samples the smaller tube is enough). Then pipette 1 ml of ProK into the same tube. Close with lid and invert solution, avoiding foaming.
7. 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.
8. Cap the filters with sterile caps. Make sure that its **completely sealed**.
9. 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**.
10. Place the filters in rotator and fasten them with the elastic band.

11. 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.
12. 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

13. Enter lab and clean according to the Clean Lab Routines.
14. 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).
15. Note the time when the filters are removed from the incubator oven.
16. 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.
17. Centrifuge the 50ml tubes containing the 2ml tubes and the filters at 1700 x g for 3 minutes.
18. 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).

19. "Measure" the approximate volume of 2-3 samples using a pipette with NEW tips for each sample. Round the mean volume to nearest 50µl.
20. 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.
21. 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.
22. Vortex and spin down the samples to make sure it is mixed and liquid from the cap is removed.
23. Place the spin columns in front of the samples in the rack.

24. 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.
25. Centrifuge the columns at 15.000 x g for 2 mins.
26. 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.
27. Transfer the rest of the sample to the corresponding spin column. If more than 630µl, three rounds of spinning are required.
28. Centrifuge the columns at 15.000 x g for 2 mins.
29. 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.
30. Add 500µl Buffer AW1 (check EtOH has been added to buffer) using new tips for each tube.
31. Centrifuge at 15000 x g for 2 mins.
32. 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.
33. Add 500µl Buffer AW2 and centrifuge for 4 mins at 20.000 x g.
34. While centrifuging, clean flowhood, pipettes, and pens with bleach, MilliQ and ethanol.
35. 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.
36. 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.
37. 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.
38. Discard the spin columns and transfer a 12µl 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 (if using strips, use empty pipette tip boxes as racks). 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.
39. 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.
40. Clean flowhood and all equipment according to the guidelines.

Appendix – c. General Clean Lab Rules

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.

CLEAN LAB ROUTINES _ C358

BEFORE ENTERING THE CLEAN LABS

You must be freshly showered and wear a clean set of clothes, have entered the building so that you avoid certain high-risk contamination areas. You must enter the NFH building from the main entrance or the east side entrance (by the new building). When inside the lab area, you must only enter in the lab corridors from the door between stairs and elevator (opposite from lab B310). The high-risk contamination areas are our genetic lab B310 and adjacent rooms and corridors, and 'Fiskemottak' area on the first floor and adjacent rooms and corridors. Finally, you cannot have eaten any fish on the day of entry.

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.

   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. Clean the centrifuge cups. 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.

2

Appendix – d. Protocol COI Leray-XT Metabarcoding

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 8base 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-PrepKits/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-sampleprep/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/sequencingkits/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 itin 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 andspin 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 andvortex thoroughly.
- Load 600 µl of this denatured sample into the the MiSeq for sequencing.