UiT The Arctic University of Norway

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Can aquaculture impact the surrounding biodiversity?

A metabarcoding assessment

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Acknowledgement

I dedicate this research to my parents, my heroes, who have pushed the limits within me.

I would like to express my gratitude to both of my supervisors Kim Præbel and Owen Wangensteen that opened their door towards me and the doors towards my future academic career.

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Paraphrase

Due to COVID-19 situation, the present study has been reduced from what initially intended. This limitation mainly concerns the bioinformatic workflow for not being able to send the samples to an external sequencing provider for higher sequencing depths. Additionally, the closure of UiT terminated the laboratory work steps that I started. Therefore, the following steps after the DNA extraction such as PCR amplification, library preparation for high-throughput sequencing and bioinformatics pipeline workflow were conducted by the Research Genetics Group (RGG) lab technician Julie Bitz-Thorsen and researcher Owen Wangensteen.

Abstract

World's population growth and rise in food consumption per capita have led to increased food demand and overexploitation of natural resources in recent decades. Such increase has threatened the global feeding schemes to maintain a balance between food supply and demand. Although "The Blue Revolution" promised to fill such gap and simultaneously alleviate the overexploitation of the oceans, deterioration of biota in the surrounding marine environment from aquaculture pollution has been reported. To investigate the effects of this pollution in the biodiversity of benthic communities, I applied a metabarcoding surveillance method before and after the establishment of a salmon aquaculture facility at Dyrøya Island, Norway. Twelve monitoring stations were established and divided into three transects, each containing four stations at increasing distance from the cages. To distinguish the patterns of impact, I estimated alpha and beta diversity for each station using two metabarcoding markers (COI and 18S). Analysis showed a significant increase of alpha biodiversity after the establishment of the aquaculture where such increase occurred only in the North transect (aligned with the main current) at all distances from the cages. Alpha diversity analysis suggested that the spread of impact was heterogeneous throughout the transects and homogeneous throughout the sampled distances. Significant differences in community composition and beta diversity (only for COI marker) after the establishment of the aquaculture were observed. The spread of such change occurred homogeneously among all the monitoring stations, transects and distances from the cages. These findings support the hypothesis that the establishment of the aquaculture activities, alone, did not lead to these changes in beta diversity, which could rather be a result of seasonal variability. This study stresses the need for high sequencing depth, broad study area, and a combination of traditional surveys with metabarcoding approaches when conducting molecular biodiversity assessments.

Key words: metabarcoding, aquaculture impacts, benthic communities, biodiversity

1 Introduction

1.1 Food supply and demand state

The right to food is a human right recognised in the Universal Declaration of Human Rights proclaimed by the United Nations in 1948. Such a right is prone to misinterpretation. It does not mean that governments are obliged to provide food free of costs but rather to implement the infrastructure for food accessibility and secure humans from hunger, food insecurity and malnutrition (UN General Assembly, 1948).

World meat production in 2013 was estimated to be 315 million tons per year while the fish and shellfish production from catches and aquaculture production was estimated to be 188 million tons per year (FAO, 2014). Worldwide animal source food production has increased throughout time as a result of food demand boost. As more people would require more food, such demand has expanded due to the world's population growth (York & Gossard, 2004).

Despite the population growth, food consumption per capita has also increased, which has led to higher food demand (European Commission, 2019). Studies suggest that such outrun has occurred firstly due to a general wealth increase in countries with emerging economies (Delgado, Wada, Rosegrant, Meijer, & Ahmed, 2003). Secondly, due to changes in customer preferences leading more families to afford high-value products such as meat and dairy products (Bélanger & Pilling, 2019). From 1961 to 2015, meat consumption has nearly doubled from 23 kg per capita per year to 42 kg per capita per year (Sans & Combris, 2015). Fish consumption, including both fisheries and aquaculture, has followed a similar path, starting with an average 9 kg per capita per year in 1961 to 20.3 kg per capita per year in 2015 (FAO, 2018). Both trends display a significant increase and outrun the population growth (1.6 per cent; FAO, 2014), where fish supply leads with an average annual increase of 3.2 per cent (figure 1), while meat supply increases 2.8 per cent (FAO, 2018). Together, population growth and increased food consumption per capita lay an increased pressure on the world's food supply (York & Gossard, 2004).

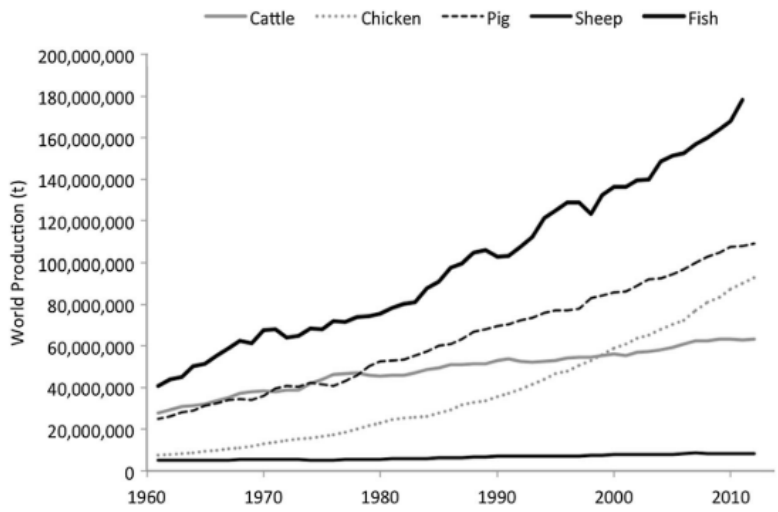

Figure 1. Global production (t) of food commodities (cattle, chicken, pig, sheep and fish) over the period 1960-2010 (Béné et al., 2015)

1.2 Importance of fish as a food commodity

Fish as a commodity covers only 1 per cent of human intake (Bélanger & Pilling, 2019), but on the contrary, it is a significant contributor in animal protein intake. Bennett et al. (2018) stated that fish and aquaculture alone provide, on average, 17 per cent of the world's consumption of animal protein per capita and such percentage being higher (sometimes exceeding to roughly 50 per cent) in developing countries (HLPE, 2014). Being one of the most expanding food commodities, fisheries and aquaculture promise steady growth and have premises for filling up the global gap between supply and demand on food consumption (Ahmed & Thompson, 2019).

Animal-derived proteins, including fish, contain crucial amino acids such as lysine and methionine. These proteins are vital for counterbalancing the plant-based diet (HLPE, 2014). Aside from amino acids, fish is a particular food source that regulates phosphorous and B-vitamin deficiency (Roos, Wahab, Hossain, & Thilsted, 2007). Compared to other food sources, fish is rich in essential micronutrients such as A-vitamin, D-vitamin, omega-3 fatty acid and minerals (Roos et al., 2007). Roughly three billion people are chronically malnourished (Cole & McCoskey, 2013). The importance of fish in the human diet is highlighted, as it can be a significant protein source to end the malnutrition (Bennett et al., 2018) and minimise non-communicable diseases (HLPE, 2014). The role and the contribution of fisheries together with aquaculture are stressed as a tool to end hunger and maintain food security (Bennett et al., 2018; Garcia & Rosenberg, 2010).

Besides the protein and micronutrients contribution, for a substantial amount of time, fishing and aquaculture have played a critical role in people's source of income, with an up to date number of 60 million people employed in the industry (FAO, 2018) and more than 520 million people who rely their income on fishing and aquaculture (Martinez-Porchas & Martinez-Cordova, 2012). Such importance, together with the added pressure from increasing trends in fish and shellfish consumption, brings the attention for political engagement and rises the need for regulations on socio-economical impacts (Cole & McCoskey, 2013).

1.3 History and the future of aquaculture

Aquaculture activities date back to ancient civilisation, at roughly 4000 years back (Costa-Pierce, 2002). The practice of aquaculture continued and got spread on other ancient civilisations such as the Egyptians and the Romans, but such practice was rather demanding and not viable (Costa-Pierce, 2002). Similar activities have been observed earlier than just 4000 years ago in the form of "proto-aquaculture" practice, involving settlements on game management and stock protection (Costa-Pierce, 2002).

The intensity of aquaculture activity was inferior compared to other types of food cultivation and therefore, was always underestimated (Holmer, Black, Duarte, Marba, & Karakassis, 2008). This underestimation was due to lack of knowledge and minor studies in the field (Ahmed & Thompson, 2019). The picture of aquaculture changed drastically after WWII as industrialisation pushed the boundaries of technological achievements (Costa-Pierce, 2002). These technical improvements allowed aquaculture to cultivate an increasingly enormous amount of fish. The intensification of aquaculture, differently called "The Blue Revolution" (Ahmed & Thompson, 2019), became increasingly attractive during the 1970s, where the fish catch started declining as a result of overfishing (Costa-Pierce, 2002). As a counteract, scholars projected that rapid advancement in aquaculture would, therefore, diminish overfishing, promote fish stocks rehabilitation (Waite et al., 2014) and supply the increasing demand for animal-based protein (Costa-Pierce, 2002; Costello et al., 2016). Governance agreements and regulations rapidly facilitated and revolutionised aquaculture production (Aarset & Jakobsen, 2009; Costa-Pierce, 2002; Garcia & Rosenberg, 2010). As an example, Norwegian government established the Aquaculture Act (1973) which gave birth to two research stations, Institute of Marine Research and Norwegian University of Life Sciences, aimed to research nutrition, selective breeding and production techniques on aquaculture (Torrissen et al., 2011).

As the unemployment rates raised due to fish stock decline (Bennett et al., 2018), “The Blue Revolution” seized the opportunity to relocate the workforce into aquaculture (Boissevain & Selwyn, 2019). Such relocation did not occur, and the entire workforce of aquaculture consisted of nearly no fishermen (Boissevain & Selwyn, 2019).

1.4 Importance of Aquaculture

Although the measures to moderate overfishing through boosting aquaculture development were rapid, the status of overfishing and fish quotas faced minor changes (Bennett et al., 2018). In their report, FAO (2018) confirms that the catch of wild fish has remained arguably stable, being roughly 95 million metric tons per year (figure 2). Costello et al. (2016) discussed the possibility that fisheries have nowadays reached the exploitation limits of ocean food provision service, and no more could be fished from our oceans.

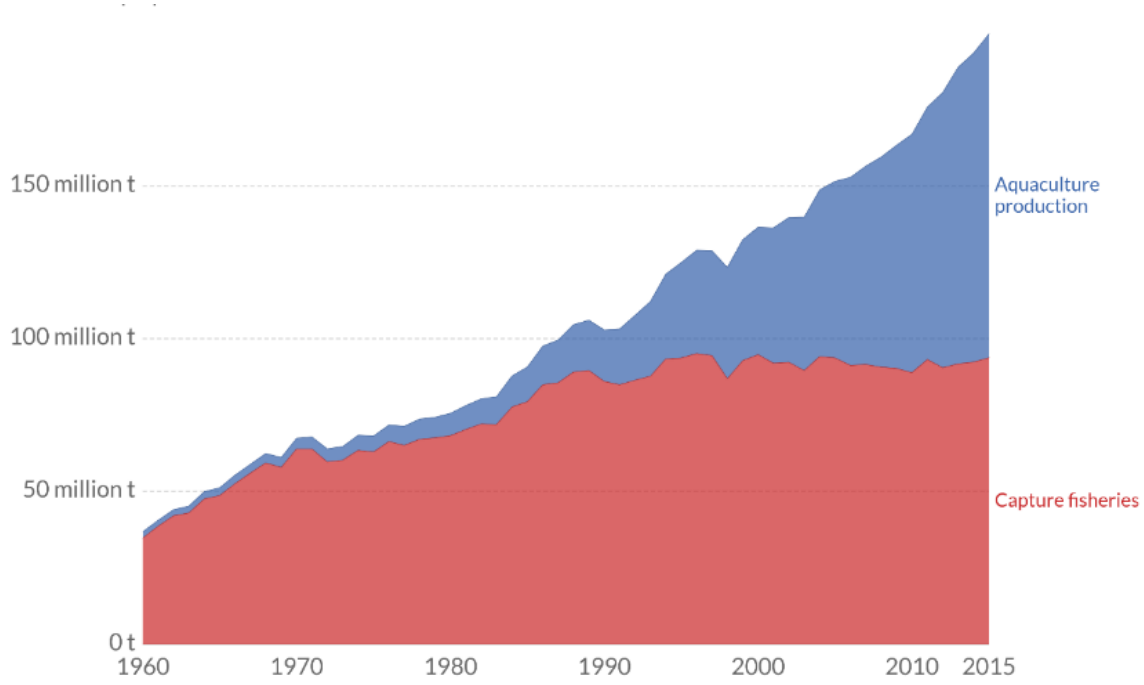

Figure 2. Global seafood production. Wild fish catch (red) and aquaculture (blue; FAO, 2014)

Due to the urgent nature of ocean food crisis, increasing pressure from population growth, climate change issues and overfishing, global feeding schemes are threatened. This led to an emerging world gap between fish protein supply and demand in the last two decades (Bennett et al., 2018; Costa-Pierce, 2002). European Commission (2013) affirmed an existing gap

between the amount of seafood consumed in the European Union (EU) and the amount caught from wild fisheries. For filling such gap, studies underline the importance of aquaculture production as it can also cope with the future food demand from population growth (Boland et al., 2013; Costello et al., 2016). With an increase on the production rate of 31.5% from 2004 to 2009 (figure 3), aquaculture alone is not only responding to world's population growth, (6.3% for the same period) but also to the increase of food consumption per capita per year (Martinez-Porchas & Martinez-Cordova, 2012).

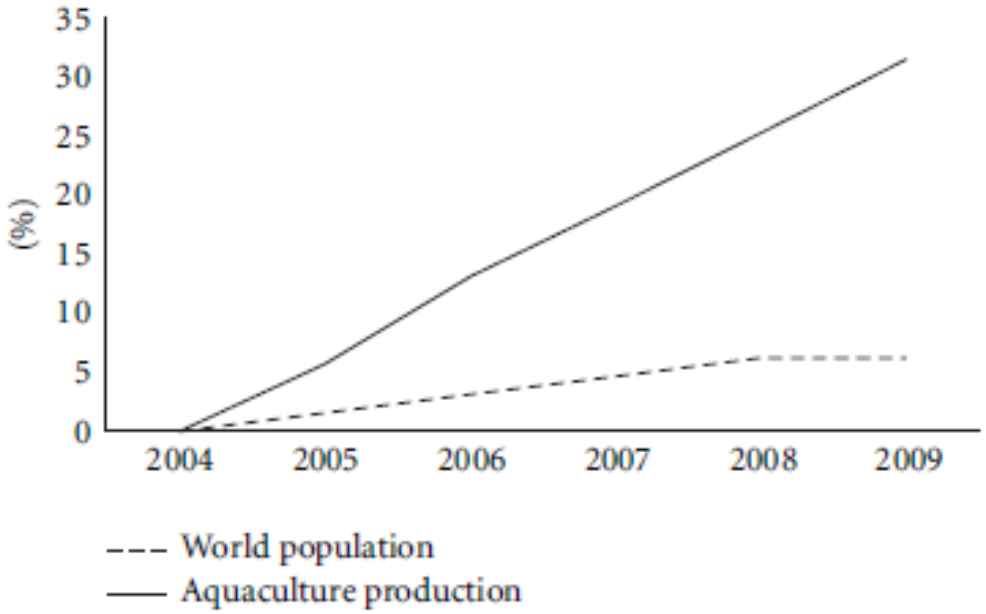

Figure 3. World's population growth compared to global aquaculture production (Martinez-Porchas & Martinez-Cordova, 2012)

Technological and scientific advances have led to the development of different types and forms of aquaculture (Ahmed & Thompson, 2019). Pond aquaculture is one of the dominating kind of production in South-East Asia (Alongi, Chong, Dixon, Sasekumar, & Tirendi, 2003). Open sea-cage aquaculture, mariculture or cage culture, is mainly being used in marine coastal waters with high exchange rate and inland aquaculture in sporadic areas (Ahmed & Thompson, 2019). In Norway, the most used type is the open sea-cage aquaculture due to immensely favourable water conditions and access to a long coastline (Ahmed & Thompson, 2019).

1.5 Impacts of open sea-cage aquaculture

The rapid development of the aquaculture industry has brought new challenges to natural resources and environmental status of coastal waters (Taranger et al., 2015). Many studies raise the concern for a range of ecological impacts from aquaculture production such as eutrophication from waste discharge (effluents), heavy metal discharge, habitat destruction, use of antibiotics, fish escape and plastic pollution (Alongi et al., 2003; Midtlyng, Grave, & Horsberg, 2011; Qiu, Lin, Liu, & Zeng, 2011; Taranger et al., 2015). Although all the problems will be briefly described, this study will focus only on issues emerged from effluents.

Water quality problems deriving from open sea-cage aquaculture arise due to uneaten feed and excess of animal faeces exposed in the marine environment as waste (Lazzari & Baldisserotto, 2008). This organic waste, being high in macronutrients concentration such as nitrogen and phosphorous, generates the thriving conditions for algae growth and algae bloom (Sarà et al., 2011). In their research, Lazzari and Baldisserotto, (2008) stated that phosphorous loss to the environment as a result of food waste from aquaculture ranged between 70-86% of the dietary phosphorous. Furthermore, they stated that the loss of nitrogen, from gill excretion, sloppy feeding, urine, and faecal output is ca. 78% of the dietary nitrogen. As consequences, enrichment of nutrients facilitates a rapid algae growth and may lead to algae blooms, depending on the hydrography of the site. Algae blooms have a cascading effect in eutrophication as the dissolved oxygen is depleted by microbial breakdown of algae after they have sedimented at the bottom (Alongi et al., 2003). Much of the same fate follows the biofouling, which after being formed around the cages, sediments at the bottom. In cases of low water retention, such as inner fjords, the sedimentation can lead to anoxic water condition where the microbes and bacteria, in the complete absence of oxygen, start producing hydrogen sulphide (Alongi et al., 2003).

Many studies indicated elevated concentration of heavy metals such as Fe, Zn, Cu, and Cd, being observed on the surface of sediments in the proximity of the aquaculture and it gradually decreased with the distance from the cages (Kalantzi et al., 2013). The significant source of zinc and potentially copper, are anti-fouling chemicals that contain such active ingredients (Dean, Shimmield, & Black, 2007). Also, fish feed contributes to spreading iron, zinc, copper and cadmium (Kalantzi et al., 2013). Few heavy metals are essential for biological processes but overreaching the threshold may become toxic (Dean et al., 2007). Depending on species tolerance, heavy metals might be a considerable danger for species health. Since heavy metals

can be bio-accumulated and bio-magnified (Akwiri, Raburu, Okeyo, Ramesh, & Onyangore, 2016), they might end up in higher trophic levels in increased concentrations. Therefore, heavy metals may alter the ecosystem function in the surroundings, having a deadly effect on species health as aftermath (Hornberger et al., 2000).

A combination of excessive amounts of heavy metals and oxygen depletion due to eutrophication leads to altering of habitats and changes in ecosystem services. In the cases of prolonged conditions, it might lead to habitat destruction (Naylor et al., 2000). For example, aquaculture accounts for ten per cent of mangrove forest loss (Stickney & McVey, 2002), which is a critically important coastal habitat in subtropical waters (Islam & Wahab, 2005). Such destruction has been caused by the rapid and sloppy development of aquaculture to fill the gap of seafood demand (Islam & Wahab, 2005).

The application of antibiotics is another threat coming from aquaculture production. Immobilisation of the fish (not freely moving around in the ocean) and overstocking of fish in high densities inside the cages threatens their wellbeing and impacts the stress levels of animals (Furones & Rodgers, 2009). Such conditions have consequences of parasitic development in aquatic animals, and while not being able to escape, the spread of it can quickly advance towards the entire stock (Furones & Rodgers, 2009). Therefore, the use of drugs and antibiotics is indicated to control the health of cultured animals. However, differently from terrestrial cultivated animals, the spread of drugs in water and its ability to affect non-targeted organisms, are higher and inevitable (Park, Hwang, Hong, & Kwon, 2012). Antimicrobial drugs used in aquaculture acquire toxic elements which can issue devastating consequences for non-targeted species and can, therefore, result in the development of antimicrobial resistance of the local cultured animals (Park et al., 2012). The most used type of medical treatment is through metaphylaxis (group medication) and a mixture of drugs with feed (Carvalho, David, & Silva, 2012). In Norway, although the production has intensified significantly (Aarset & Jakobsen, 2009), the current use of antibacterial drugs has declined drastically since the 1980s (Midtlyng et al., 2011).

Another environmental and biological risk coming from aquaculture is the escape of fish in captivity. As a result of the escape, new specimens might be introduced, and its interaction with the surroundings might lead to adverse wildlife consequences (Ford et al., 2012). Examples of such escape were reported in Chile, where it resulted in non-native species thriving in low fish density and degraded habitats (Soto et al., 2006). Typically, cultivated fish, through selective

breeding, have genetical characteristics favouring production purposes such as fast growth and low aggression (Naylor et al., 2005; Science for Environment Policy, 2015). Spreading of such genes through interbreeding with the wild population can potentially lead to fatal consequences for the ecosystem (Danancher & Garcia-Vazquez, 2011), as it can lead to genetical interferences of species evolution (Jensen, Dempster, Thorstad, Uglem, & Fredheim, 2010). Cultured fish can outcompete local specimens for food sources and can lead to starvation of the latter. This competition causes habitat destruction and subsequently, a cascade of changes in the food chain of the ecosystem (Naylor et al., 2005).

Plastic pollution from aquaculture is a concern that has recently become of interest among scholars. Much lack of knowledge is currently on this particular topic. Studies show that a considerable amount of microplastic is found in the vicinity of aquaculture compared to areas further away from the spot (Krüger et al., 2020). Studies conclude that all the materials used for sustaining the facilities such as ropes, nets and cables ties are therefore the source of pollution. Exceptions are made for the rubber particles, which are denser than plastic and therefore, sediment at the bottom (Chen et al., 2018). Some studies show that up to 50 per cent of the microplastic detected in the water has originated from the aquaculture facility (Chen et al., 2018).

1.6 Regulation on Norwegian aquaculture

After “The Blue Revolution”, when production got intensified and increased in importance, aquaculture activities got regulated as sidelines of common fisheries policy in EU and fish directorate in Norway (Aarset & Jakobsen, 2009). Today, each country establishes its laws and regulation on seafood production (Holmer et al., 2008), which has led to the heterogeneity of policies and legislations. In developed countries, such regulations are made in compliance with the state’s environmental agency. However, in a few developing countries, these instances are still neglected while promoting unsustainable growth of aquaculture (Martinez-Porchas & Martinez-Cordova, 2012). This is also supported by (Abate, Nielsen, & Tveterås, 2016), which stated that countries with higher stringency on environmental legislation have slower aquaculture growth or, in few cases, even a decline in production.

Nowadays, Marine Strategy Framework Directives (MSFD), Water Framework Directives (WFD), Birds and Habitats directives, Environmental Impact Assessment and Strategic

Environmental Assessment (SEA) and when located offshore (>12 NM), Marine Spatial Planning (MSP), serve as guidance of aquaculture management in all EU member states. These agencies ensure European standard maintenance of environmental pollution and health issues (Science for Environment Policy, 2015).

Norway, on the other hand, although not being an EU member state, follows the vast majority of the European regulations and frameworks (Alexander et al., 2015). In Norway, at the national level, aquaculture production is regulated and monitored mostly through three different acts and three branches of ministries (Aarset & Jakobsen, 2009).

Ministry of Fisheries and Coastal affairs, being the principal authority, regulates and controls the development of aquaculture in land and water, focusing on assigning environmental standards, establishing locations for sea ranching and defining the licensing through Aquaculture Act 2005 (Wilson, Magill, & Black, 2009). It can, therefore, propose and change regulations through amendments to the Aquaculture Act, with the input of other ministries also partially responsible for aquaculture (Wilson et al., 2009). The Directorate of Fisheries is, thereafter, the institution within the Ministry which implements such regulations and supervises the Act, meaning that it coordinates and makes the respective surveillance within the Act (Alexander et al., 2015). Second in importance, a list of ministries conducts complementary duties to standardise aquaculture production. The Ministry of Environment sets up environmental quality standards through regulations in Pollution Act. Norwegian Food Safety Authority regulates the conditions of the hatchery for achieving food safety and quality through Food and Safety Act. Finally, the Ministry of Agriculture and Food ensures the treatments of cultured animals in an appropriate manner and controls for diseases through Animal Welfare Act 2009.

1.7 Environmental monitoring of aquaculture in Norway

The Ministry of Environment as a regulatory body and its control agency, Directorate of Environment, is the responsible entities for setting the environmental standard for the aquaculture production through the Pollution Act. In the Licensing Act 2004, within the Aquaculture Act 2005, it is required that the applicant for aquaculture production must conduct environmental surveillance of the sea bottom and that the results should not exceed those prescribed in seabed impact standard NS 9410, “Environmental survey of marine fish farms”.

Although in Norway, Environmental Impact Assessment is not required for aquaculture facilities, assessments are made through "Modelling – Ongrowing fish farm – Monitoring" (MOM) which combines benthic surveys with modelling for assessment of environmental quality standards (Wilson et al., 2009). MOM defines three zones of environmental impact: the local zone (0 – 15 m), the intermediate zone (15 m – 200 m), and the regional zone (beyond the intermediate zone), with decreasing in impact tolerance (Hansen et al., 2001). The monitoring program (MOM) consists of three different types of surveys: A-, B-, and C-type investigations, with an increase in complexity of surveillance.

A-investigations consists of surveys conducted for monitoring the sedimentation rate of organic material below the fish farm. A-surveys are handled by the fish farmers themselves by arranging two sediment traps two meters above the seafloor vertically from the cage. This survey does not define the status of the environmental conditions but rather serves as a complementary survey to B-surveys (Hansen et al., 2001).

B-investigations are surveys executed at the highest expected impact area. This area is defined as the local impact zone and does not extend from 5 to 15 meters (Hansen et al., 2001). Depending on the degree of exploitation, a number of 10 or more sediment samples are randomly taken within the local impact area using the van Veen grab. Type B-investigations are mainly focused on benthic macrofauna preservation (regardless of biodiversity) and eutrophication indication.

C-inspection is an extensive study of the seabed condition based on macrofauna community and abiotic parameters such as hydrography (temperature and salinity), sediment condition (pH and redox potential) and environmental pollutants (P, Cu, Zn; Holmer et al., 2008). This survey is conducted in three to five sampling points, during the peak in aquaculture production, at a gradual distance from the aquaculture site in the local impact and intermediate zone (Hansen et al., 2001). The quality of the environment on each sampling point is assessed through the number of macrofauna species and dominance community. However, diversity and sensitivity indexes (e.g. Norwegian sensitivity index, Shannon-Wiener's index, Density index, ES100. and International sensitivity index) are used in the report (Hansen et al., 2001).

1.8 Molecular environmental monitoring (eDNA)

Environmental DNA (eDNA) is the DNA present in soil, water and air that is continuously expelled from all living organisms, both plants and animals, through respiration, liquid excretion, and movement in the surrounding environment. In their paper, Taberlet, Coissac, Hajibabaei, and Rieseberg (2012) state that "Environmental DNA refers to DNA that can be extracted from environmental samples (such as soil, water or air), without first isolating any target organisms" (p.1789). Free DNA molecules in environment that come from mucus, sweat, skin, urine, sperm, pollen and rotting of cells can thus be captured (Bohmann et al., 2014), amplified by the polymerase chain reaction (PCR), sequenced and traced its origin to the species levels (Ji et al., 2013). The way PCR works is through using a set of primers, which are short single-stranded DNA molecules that can specifically bind with the targeted DNA string and amplify it. The amplification is often called a "molecular photocopying" process, where the enzyme DNA-polymerase recognises the end of the primers that are bound to the DNA string and reproduces the same string multiple times (Anglès D'Auriac, 2016). Thus, specific sequences present in the DNA sample can be amplified millions of times. Without the amplification, studies in microbiology would be impossible due to the low amount of available DNA in samples (Anglès D'Auriac, 2016).

The eDNA sampling method is extensively used nowadays for inventorying and locating species with high accuracy since it is very cost- and labour efficient (Lodge et al., 2012; Taberlet et al., 2012). Its applications are predominantly found in monitoring and inventorying aquatic biota in water bodies, identification of invasive species, and community composition surveillance (Takahara, Minamoto, & Doi, 2013). The use of eDNA analysis is vastly found among conservation studies due to its low impact on the environment and high accuracy compared to traditional methods (Civade et al., 2016). In their research, Lacoursière-Roussel et al. (2018) stressed the efficiency of eDNA metabarcoding as an important tool for water bodies biomonitoring as they studied the community composition and biodiversity pattern in Canadian Arctic using eDNA analysis.

1.9 Limitations and advantages of eDNA analysis

Previous studies on the robustness of available DNA in environment indicates limitations on eDNA analysis as many biotic and abiotic factors may affect its degradability (Moushomi, Wilgar, Carvalho, Creer, & Seymour, 2019; Tsuji, Ushio, Sakurai, Minamoto, & Yamanaka,

2017). Abiotic factors such as temperature, currents, stratification and concomitant microbial activity can diffuse (isolate or transport) and degrade the available DNA (Strickler, Fremier, & Goldberg, 2015). Jeunen et al. (2019) conclude that water column stratification and biological processes can affect eDNA metabarcoding surveys. Although the pace and ratio of such activities varies, Tsuji et al. (2017) show in their experiment that these factors should be considered when interpreting the results of eDNA analysis.

In their research experiment, Moushomi et al. (2019), established an exponential decay of eDNA through time with a maximal detection time of up to one month. Due to high degradability rates of eDNA through time (Strickler et al., 2015), the captured DNA would be very accurate for revealing the taxonomic presence in the water column and/or sediments (Bakker et al., 2017). This information is crucial to infer robust local-scale species distribution patterns from eDNA inventories.

Due to differences in DNA shedding rates between individuals of the same species and between different communities (Ushio et al., 2018), eDNA reads cannot be fully quantified (Bakker et al., 2017). Furthermore, due to stochasticity of PCR amplification, it is not fully proportional to available DNA in the water or sediment sample and therefore does not mirror the complete quantitative biota (Bakker et al., 2017). Although few steps are suggested by Wangensteen, Cebrian, Palacín, and Turon (2018) to transform the numbers of eDNA reads into a semi-quantitative abundance index (rank abundance), it is currently still arguable if this method is accurate to infer species abundances. Other scholars agree that presence-absence (P/A) type of data would be a more conservative way to treat eDNA reads (Bohmann et al., 2014, 2018).

Regardless of its limitations, the applicability of eDNA analysis has established advantages when used for environmental biomonitoring (Bohmann et al., 2014). Due to the nature of eDNA analysis, long term biomonitoring projects that require a high number of personnel can be cost-efficiently run and become low dependent on taxonomists (Aylagas, Borja, Muxika, & Rodríguez-Ezpeleta, 2018; Bohmann et al., 2014; Frontalini et al., 2018). When there are more than a few target species in a study – common in biomonitoring studies – eDNA metabarcoding can quickly become advantageous (Goldberg et al., 2016). Another advantage of this method is the reduced likelihood for human error when assigning species to their respective taxonomy (Bohmann et al., 2014; Frontalini et al., 2018).

Recently, an increase on the application of eDNA analysis to monitor aquatic pollution from anthropogenic activity has been documented, as methods and approaches got more diverse to address such issues (Goldberg et al., 2016; Laroche et al., 2018; Pochon et al., 2015; Stoeck et al., 2018; Yang & Zhang, 2020). Specifically, for aquaculture impact assessment, Stoeck et al. (2018) developed a new approach for assessing the environmental impact from salmon aquaculture using metabarcoding of benthic bacterial communities around aquaculture as bioindicators for environmental assessment. Pochon et al. (2015) tested the use of foraminiferal-specific metabarcoding for monitoring environmental impact of salmon farms on macrobenthic infaunal communities.

1.10 Diversity indices

Alpha diversity is a measure of the richness in species, and it can be appropriately measured as the number of species found in a sample of standard size (Whittaker, 1972). In other words, alpha diversity is a one dimensional summary of high dimensional composition data (Willis, 2019). Some describe alpha diversity as the simplification index for the ecological composition of a sampled ecosystem (Willis, 2019).

Beta diversity is the variation in community composition within a given site and as Whittaker (1972) suggested, it can be measured as the proportion by which a given area is richer than the average of samples within it. Elseways, Anderson, Ellingsen, and McArdle (2006) have developed another approach, where beta diversity can be measured as the variability in species composition among sampling units for a given area. Beta diversity can be used to address mainly two ecological questions such as species composition variation among sites of different treatment and dissimilarities between sites along an environmental gradient (Antão, McGill, Magurran, Soares, & Dornelas, 2019).

1.11 Purpose of this study.

Due to high concentration of biomass and nutrients (as fish feed and fish waste) during aquaculture production, fish farming will inevitably have some impact on the surrounding environment (Carroll, Cochrane, Fieler, Velvin, & White, 2003). In regard to effluents, previous studies on aquaculture have indicated disturbances on the benthic macrofaunal community (Karakassis, Tsapakis, Hatziyanni, Papadopoulou, & Plaiti, 2000; Mente, Pierce, Santos, &

Neofitou, 2006), concluding that such effect is negligible beyond 50 m from the cages. Conversely, studies have also indicated that implementation of aquaculture resulted in increased overall biomass and abundance, while traditional diversity indices increased in dominance (Machias et al., 2005, 2004). Similar findings were observed by Soto and Norambuena (2004). These studies have been traditionally designed with spatial comparison as control and impact, using benthic communities as environmental quality indicators (Brown, Gowen, & McLusky, 1987; Carroll et al., 2003; Kalantzi & Karakassis, 2006; Karakassis et al., 2000). Nevertheless, many other studies on fish farming environmental impact do not only lack the comparison of before-after (as supplementary to control and impact) in the search for sources of impact, but also have circumvented the relation of these impacts with abiotic factors (e.g. currents, temperature, season). In their research project, Carroll et al. (2003) acknowledged the importance of consistent temporal monitoring in order to detect environmental impact from aquaculture production.

This study was particularly designed considering the above-mentioned questions, approaches and standpoints. The main purpose of this study was to examine if the establishment of salmon aquaculture affects alpha and beta diversity of eukaryotic benthic communities in the vicinity of the farm site using a molecular biodiversity assessment (metabarcoding) approach. Supplementary to the primary purpose, I hypothesised that such changes would occur heterogeneously in space, expecting the change to be greater at closer distance to the cages and lower at further distance from the cages. In contrast, such change would occur homogeneously among different directions, studied as transects, from the aquaculture facility.

To investigate these hypotheses, two metabarcoding primer sets were used, targeting a fragment of the mitochondrial gene region cytochrome c oxidase subunit I (COI) and a fragment of small eukaryotic ribosomal subunit (18S rRNA), as metabarcoding markers. I conducted a “before and after” study design as an effective method for evaluating the anthropogenic impact on the marine environment.

2 Methods

2.1 Description of the aquaculture site

This study took place in northern Norway (figure 4). The studied coastal area located at the 69º latitude is characterised by a sub-Arctic climate and water regime. Although high latitude Norwegian coastal areas are defined as ice-free, they have strong seasonality due to high variability of light intensity throughout the year (Wiedmann, Reigstad, Marquardt, Vader, & Gabrielsen, 2016).

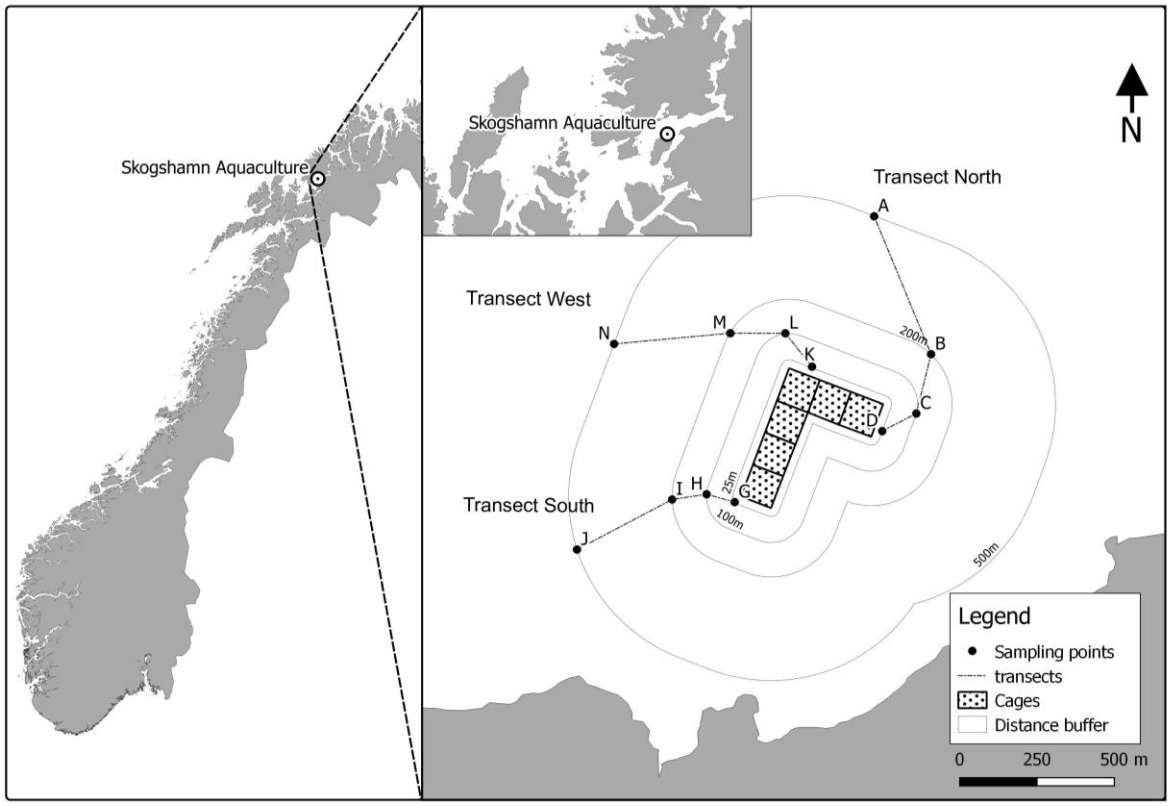

Figure 4. Location of Skogshamn salmon farms (left) in northern Norway and the study area, showing the distribution of aquaculture cages together with sampling stations (transects and distances from the cages also displayed).

An inshore salmon farm in Skogshamn (figure 4), in the vicinity of Dyrøya island, was selected for this study. The aquaculture site has a license for installing eight cages and a production of 6000 tons per production cycle, but only six cages have been installed. The permit for the salmon farm is owned by University of Tromsø, the Arctic University of Norway (UiT), for research purposes and operated by the SalMar company. The reason for choosing this site is the convenient accessibility for UiT master students to the aquaculture site. This study is part of a

bigger research project where UiT is monitoring fish health and disease outbreaks in the salmon farm.

The seabed around the aquaculture site is characterised by clay bottoms dominance with parts of silt and sandy bottom. The main active underwater current flows north-east and the second most frequent current flows south-west (figure 5). The study area has an average depth of 280m.

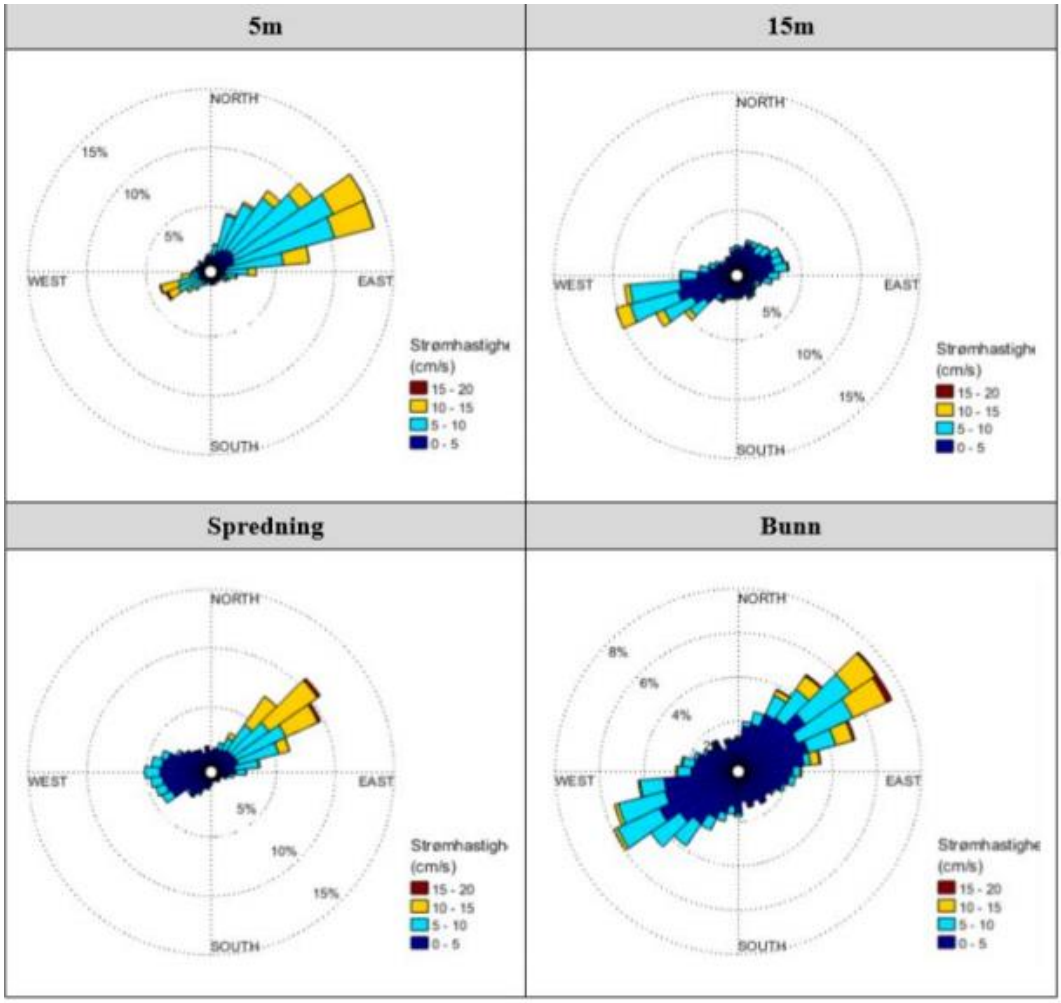

Figure 5. Current direction, speed, and intensity at 5m (top left), 15m (top right), at the bottom (bottom right) and the average current direction speed, direction, and intensity (bottom left) for the entire aquaculture site. The diagram belongs to the previous aquaculture production cycle (2016-2017). Figure obtained from SalMar ASA.

2.2 Sampling

In order to testify that the development of the aquaculture has an impact on the surrounding biodiversity, before-after sampling design was introduced, with the time periods accounting for the time that the fish started to be cultured (table 1). The time period "before" is defined as the time before the aquaculture was operating and/or any fish was introduced in the cages and/or

no anthropogenic source of pollution had derived in the aquatic environment. The time period "after" is while the aquaculture was operating and/or the fish got introduced and/or anthropogenic pollution can occur in the marine environment and therefore potentially shift the species composition. As part of a bigger project between UiT and SalMar, a continuous site monitoring was carried on over the course of ten months (June – March), when seawater was sampled with an average sampling frequency of two times per month (September - March). However, in this study, I will present just the results regarding the impact on benthic communities.

Twelve monitoring stations were established and divided into three transects. Transects were named "North", "South" and "West" for simplicity and do not fully represent geographical directions. Transect North, being aligned with the main current direction, transect South, being against the main current's direction and transect West, being perpendicular to the main current. Each transect was monitored at four different distances, namely 25 m, 100 m, 200 m, and 500 m, from the cages (table 1). Benthic communities from all twelve monitoring stations were sampled before and after the operation of the aquaculture.

*Table 1. Aquaculture monitoring program and sampling design. *Station ID respectively represents the distances from the cage. From each station, three biological replicate sediment samples were retained*

Time period	*Sampling date*	*Transect*	*Distance from the cages (m)*	*Station ID**
Before	20th September	North	25, 100, 200, 500	D, C, B, A
		South	25, 100, 200, 500	G, H, I, J
		West	25, 100, 200, 500	K, L, M, N
After	12th March	North	25, 100, 200, 500	D, C, B, A
		South	25, 100, 200, 500	G, H, I, J
		West	25, 100, 200, 500	K, L, M, N

At each benthic sampling station, a van Veen grab of 250 cm^3 (model 12.110, KC-Denmark) was deployed to the bottom of the sea. From each sample site, roughly 100 cm^2 of sediment was retrieved where I sub-sampled three biological replicates from the top 2 cm of the surface sediment using Falcon tubes. A total of 72 benthic sediment samples were collected and used for eDNA metabarcoding analysis. Benthic sediment samples were stored at -80^oC until DNA extraction process. To reduce the risk of cross-contamination during sampling, all the sampling

equipment were sterilised with 10% NaClO (bleach) and then rinsed with distilled water before and after every fieldwork. Sterile nitrile gloves were used when in contact with sediment samples.

Four feed samples (feed pellets), used for feeding the cultured salmon, were collected to account for potential bias that might occur due to sinking of food waste (uneaten feed; Stigebrandt, Aure, Ervik, & Hansen, 2004).

2.3 DNA workflow

2.3.1 DNA extraction

For extracting DNA from sediment samples and feed samples, I used DNeasy PowerSoil Kit (Qiagen, Germany). For each replicate sample, I took 0.3 g of sampled soil and together with 60 µL of solution C1 I added them into the PowerBead tube where afterwards it got vortexed for 2h at room temperature. To recover the purified DNA, a sequence of solutions (250 µL of solution C2, 200 µL of solution C3 and 1,200 µL of C4) was added, the precipitates were discarded, and then the final supernatant was transferred into MB Spin Column, where the DNA get selectively bonded to the silica membrane of the column. Solution C5 (500 µL) was then added to wash away the residual salt and inorganic waste. Lastly, the DNA got eluted from the filter using 100 µL of solution C6.

To minimise the risk of sample cross-contamination, before and after each extraction round, the extraction kit and extraction hood got decontaminated with 10% bleach and was exposed to UV (Ultraviolet light) for 30 min as Goldberg et al. (2016) suggests. For each extraction round of 12 samples, I added a negative sample (no sediment) and extracted one extraction blank by using only the solutions mentioned above, to control for eventual contaminations in the extraction room. Lastly, to reduce the risk of laboratory cross-contamination, procedures for sediment DNA extractions and PCR steps were performed in different laboratories.

2.3.2 Metabarcoding and bioinformatic pipeline

The following steps after the DNA extraction such as PCR amplification, library preparation for high-throughput sequencing and bioinformatics pipeline workflow were conducted by the Research Genetics Group (RGG) lab technician Julie Bitz-Thorsen and researcher Owen

Wangensteen. For COI, the Leray-XT primer set was used (Wangensteen, Palacín, Guardiola, & Turon, 2018), which is capable to amplify a 313-bp COI fragment of most marine eukaryotic groups. For 18S, the All_Shorts primer set was used (Guardiola et al., 2015) which amplifies a fragment of 18S of around 130-bp, although the length is variable among eukaryotic taxa. The PCR amplifications, library preparations and bioinformatics pipelines followed published protocols from the RGG (Atienza et al., 2020; Garcés-Pastor et al., 2019).

2.4 Data analysis

The molecular operational taxonomic units (MOTU) table, retrieved after the bioinformatic workflow, was filtered from spurious MOTU. Sample biases are generated due to PCR and next-generation sequencing errors (Beltman et al., 2016) that can lead to the occurrence of spurious MOTU, which might alter the conclusions. Beltman et al. (2016) state that specific sequencing errors occur at an approximately constant rate across different samples and this process is stochastic, leading to samples with a few reads having a higher error rate than samples with many reads. To filter spurious sequences from the dataset, a general threshold (cut-off) of 0.1% in relative read abundance was established, and MOTUs with a relative abundance of lower than or equal to the threshold were removed from each sample. Although this approach is claimed to be insufficient (Beltman et al., 2016), it minimises the risk for the inclusion of spurious sequences.

Furthermore, MOTU table was corrected for blanks. MOTUs present in blank samples (eleven samples in total) with more than 10% of the total MOTU reads were removed as an indicator of potential cross-contamination. To account for potential bias resulting from uneaten feed, the same procedure (as for blank correction) followed where MOTUs with more than 10% of the reads present in feed samples (four samples in total) were removed. Moreover, since this study targets only eukaryotic diversity, all MOTUs assigned to bacteria or to the root of the tree of life were removed from the analysis.

2.4.1 Sequencing and eDNA sampling effort

Although using the same sampling protocol, differences in eDNA sampling effort (total number of reads) can occur among samples. To investigate such variation, two types of curves for each time period (sampling time) were plotted: rarefaction curves and species accumulation curve.

The former curves, which represents the total number of different MOTUs recovered as a function of the number of reads per sample, were generated to examine the quality of sequencing effort for detecting genetic diversity (Brose, Martinez, & Williams, 2003) using *rarecurve* function in vegan package in R (Oksanen et al., 2019; R Core Team, 2020). On the other hand, species accumulation curves, which represent MOTU richness as a function of sample size (the dependability on the volume of sediment collected; Brose et al., 2003) were generated using *specaccum* function in *vegan* package in R (Oksanen et al., 2019; R Core Team, 2020).

2.4.2 Alpha diversity

To account for unequal sample sequencing depth, samples with total number of reads lower than 2,000 reads for COI and 500 reads for 18S were discarded as an indication of too low sequencing depth and potentially bias-generating samples for alpha diversity comparison. The remaining samples were then rarefied to the lowest sequencing depth using *rrarefy* function in *vegan* package in R (Oksanen et al., 2019; R Core Team, 2020). This process was iterated 50 times, and the mean values of rarefied MOTU richness per sample were assigned as alpha diversity indices. This approach is more robust and lowers the possibility of biases generated by a single rarefaction. The rarefied samples from both primers were used for alpha diversity comparison. The following analyses were conducted in *R v.3.6.3* (R Core Team, 2020).

Due to unbalanced number of samples and the inability to conduct paired t test, mixed effects model was conducted using *lmer* function in *lme4* package (Bates, Mächler, Bolker, & Walker, 2015) to estimate the change of alpha diversity among time periods. The Kenward-Roger approximations of degrees of freedom (df), F value and p values were obtained via *anova* function from *lmerTest* package (Kuznetsova, Brockhoff, & Christensen, 2017). Time period was treated as fixed effect and stations nested within time period, were treated as random effects. Two separate analysis of variance (ANOVA) tests, using *Anova* function in *car* package with type III sums of squares (Fox & Weisberg, 2019), were carried to investigate spatial patterns of alpha diversity temporal changes among distances and transects. The first test compared MOTU richness among time period and distances and the second test compared MOTU richness among time period and transects. Pairwise t-tests with Bonferroni correction (to correct p-values for multiple comparisons) were conducted when the ANOVA found significant interactions. When the tests (mixed effects model, ANOVA and pairwise t-test) were

found significant, means were estimated using *lsmeans* function in *emmeans* package with Tukey's adjustment method (Lenth, 2020).

Assumptions of the analysis were checked using Shapiro test for normality of residuals, and Levene's test for homogeneity of variances (homoscedasticity in case of mixed effects model) in combination with visual inspections by plotting standardised residual towards theoretical quantiles and the residuals towards the predicted values, respectively. Sphericity is always met since the factor "time period" has only two levels (before and after).

2.4.3 Beta diversity

For beta diversity analysis, biological replicates (three per each station) were pooled (added) together with all the samples included (including samples with low sequencing depth). Since the correlation between true biomass abundance of species and eDNA reads through metabarcoding is uncertain (Ushio et al., 2017), both MOTU tables (COI and 18S) were converted into qualitative data table with only presence-absence. All the following analysis were conducted on both datasets, COI and 18S simultaneously.

To investigate temporal changes and spatial patterns of changes in community composition, dissimilarity matrices for presence-absence data (replicates pooled) were obtained using Jaccard index, through *vegdist* function in *vegan* package (Oksanen et al., 2019). Both dissimilarity matrices (COI and 18S) were visually represented through non-metric multidimensional scaling (nMDS) ordination using *metaMDS* in *vegan* package (Oksanen et al., 2019), with three dimensions (k=3) and using 20 random starts in search of a stable solution.

Three separate permutational analysis of variances (PERMANOVA) were used to compare the temporal and spatial changes of community composition using *adonis* function in *vegan* package (Oksanen et al., 2019) with 999 permutations. First, changes among time period were compared, where the factor was considered as fixed. Second, changes among time period and distances were compared, where both factors were considered as fixed factors. Lastly, changes among time period and transects were tested, where both factors were considered as fixed factors.

Where PERMANOVA was found significant, permutational multivariate dispersion test (PERMDISP) and permutational pair-wise comparisons with Benjamini–Yekutieli FDR (False

Discovery Rate) correction were conducted, via *betadisper* and *pairwise.adonis* functions, respectively, in *vegan* package (Oksanen et al., 2019). PERMDISP and visual inspection from nMDS were used to indicate if such significance was due to a different multivariate mean or due to the different heterogeneity of the groups, while pair-wise comparison tested the differences within the groups, equivalent to post hoc test. Beta diversity, measured as multivariate dispersion (Anderson et al., 2006), was compared among time periods, transects and distances using pairwise PERMDISP. Note that time periods have only two levels, therefore PERMDISP and pairwise PERMDISP on time periods will have exactly the same results.

Lastly, Mantel test was used to investigate the correlation between dissimilarity matrices deriving from both primers (COI and 18S) using *mantel* function in *vegan* package (Oksanen et al., 2019).

2.4.4 Supplementary analysis

Supplementary, analysis of similarity percentage (SIMPER) and estimation of ecological quality (as Marine Biotic Index) were conducted. To identify MOTU that contributed the most to the dissimilarity of samples between time periods (before and after), SIMPER analysis was conducted via simper function in vegan package in R (Oksanen et al., 2019; R Core Team, 2020) using Bray-Curtis dissimilarity index on relative read abundance data (replicates pooled). Even if read abundance may not truly reflect the real abundance of species, this analysis has proven useful to identify the most important MOTUs contributing to changes in beta-diversity (Bakker et al., 2019; Wangensteen, Cebrian, et al., 2018).

To indicate the level of pollution derived from aquaculture AMBI (AZTI's Marine Biotic Index) values were assessed for each replicate using presence-absence only from COI data (Aylagas, Borja, & Rodríguez-Ezpeleta, 2014). MOTUs assigned to the species and genus level were matched in AMBI species reference list (accession: May 2019; the list can be obtained here http://ambi.azti.es) to respective ecological group (I: very sensitive, II: indifferent, III: tolerant, IV: second-order opportunist, and V: first-order opportunist) according to their sensitivity towards disturbance (Borja, Franco, & Pérez, 2000). Thereafter, the matched MOTUs were incorporated in AMBI *v.5.0* software (http://ambi.azti.es) to determine the level of disturbance.

3 Results

The MOTU table retrieved after bioinformatics workflow and removal of bacterial MOTUs consisted of 1,473,868 reads and 195,485 reads for COI and 18S metabarcoding primers, respectively. A number of 230,261 reads for COI and 17,617 reads for 18S were discarded after spurious MOTU filtration, blank sample and feed sample correction resulting on 1,243,607 reads for COI dataset and 177,868 reads for 18S dataset.

3.1 Sequencing and eDNA collection effort

Rarefaction curves were performed to examine the relation between sequencing depth (DNA sequencing effort) and genetic diversity (MOTU richness) on stations among different time periods (figure 6). Sequencing effort differed among primers (COI and 18S) and between time periods (before and after) only for COI marker. For the COI dataset, the saturation of sequencing effort for time period "before" was achieved, where after ca. 12,000 reads per sample, the majority of samples reached a plateau (figure 6a). The majority of samples belonging to the time period "after" did not reach a plateau, meaning that higher sequencing depth would have yielded more MOTUs (figure 6a). With an average sequencing depth of 17,277 reads per sample, COI represents most of the MOTUs detected for the time period "before".

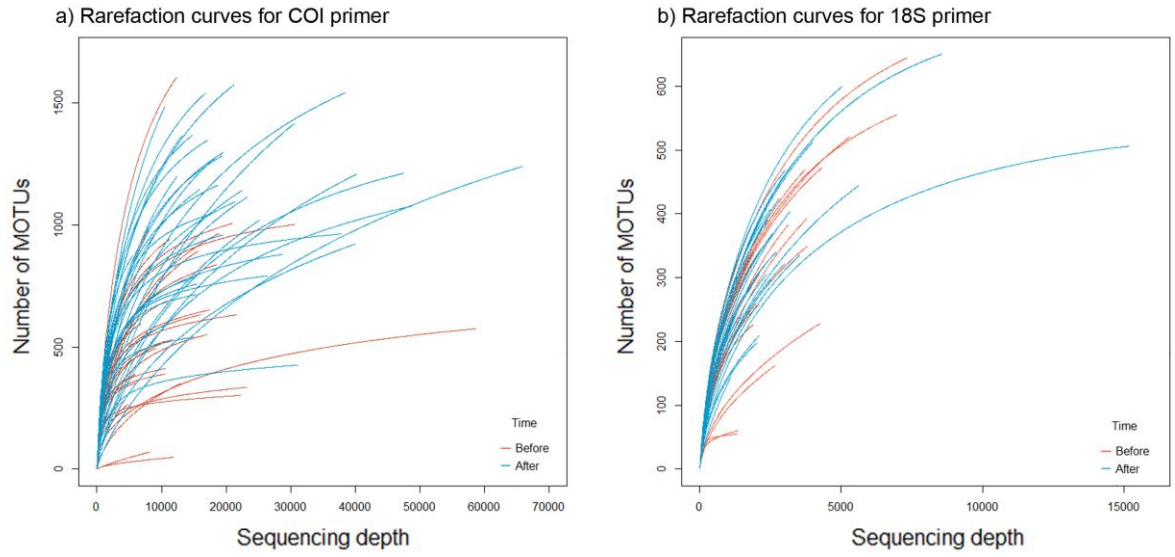

Figure 6. Rarefaction curves on the number of MOTUs obtained at the increasing number of reads per sample for time period "before" (red) and time period "after" (blue) on both metabarcoding markers (COI on the left and 18S on the right).

The rarefaction curves did not reach a plateau for 18S in either of time periods, and consequently, the majority of samples continued detecting new MOTUs with increasing sequencing effort (figure 6b). Having an average sequencing depth of just 2,473 reads per sample, the 18S dataset results in poor resolution for representing all MOTUs.

Species accumulation curves among sampling times (before and after) and between primers (COI and 18S) were performed to investigate eDNA sampling effort (figure 7). The curves varied between sampling times, suggesting a considerable variability of eDNA collection effort. In both datasets and for both time periods, the accumulation curves did not plateau at the total sample number of 36, and consequently, more samples would have detected more MOTUs. Comparatively, both primers detected more MOTUs for the time period "after".

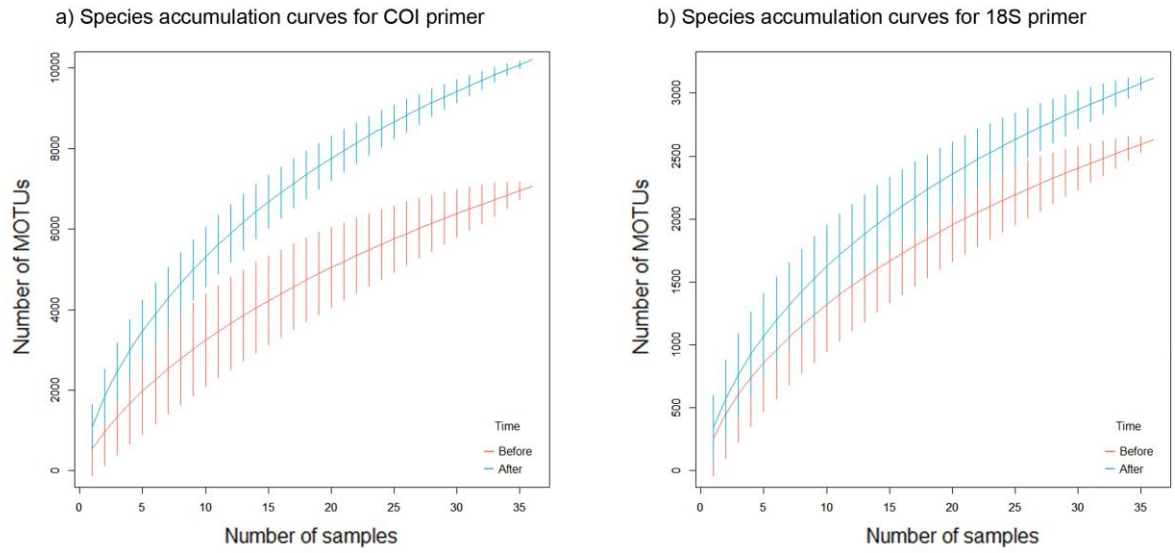

Figure 7. Species accumulation curve of the number of new MOTUs obtained after adding an increasing number of samples to the analysis, for time period "before" (red) and time period "after" (blue) on both metabarcoding markers (COI on the left and 18S on the right), with the confidence interval (95%) represented (vertical lines).

3.2 Taxonomic assignments of MOTUs

Primers differed on taxonomic assignments. After the filtration process, COI primers yielded 11,924 MOTUs, where 5,888 (49.4%) remained unassigned at the kingdom level (figure 8a). The most genetically diverse were Kingdom Metazoa, with the highest number of unique MOTUs (3,660; 30.7%), where 1,937 of those (52.9% of all Metazoans MOTU) remained unassigned at the phylum level. Conversely, 18S primers yielded 3,955 MOTUs, where 1,732 (43.8%) remained unassigned to the kingdom level (figure 8b). The highest MOTU diversity

was found within the Alveolata Kingdom, with 678 unique MOTUs detected (17.1%). Out of these, only 61 MOTUs (9% of Alveolata MOTUs) remained unassigned to the phylum level.

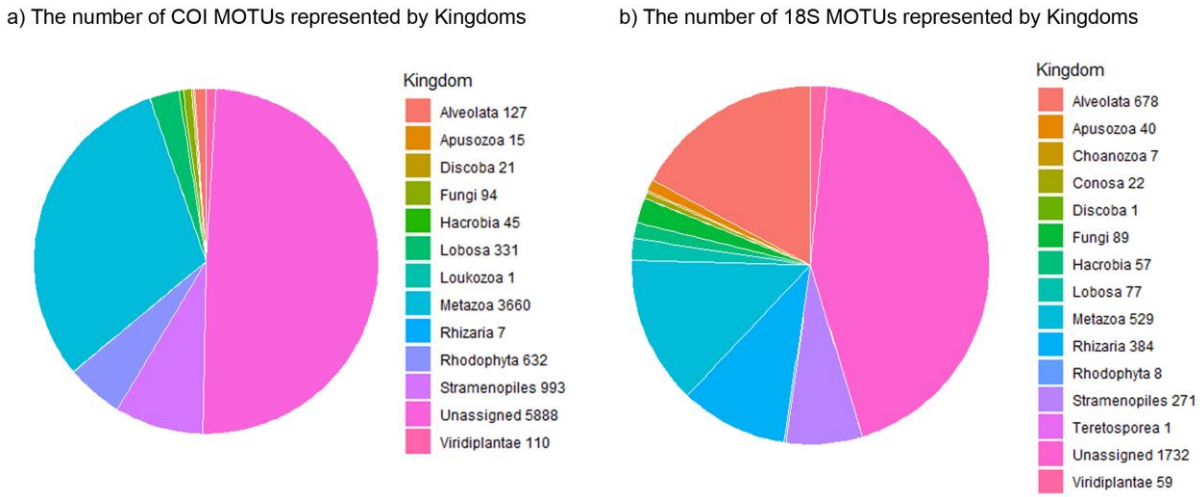

Figure 8. Number of MOTUs belonging to each of the Kingdoms, for both metabarcoding markers (COI on the left and 18S on the right). The total number of unique MOTUs per group are presented to the right of the pie-chart.

3.3 Alpha diversity

3.3.1 COI

Five samples were rejected for alpha diversity estimation due to insufficient sequencing depth. The remaining 67 samples (31 in time period "before" and 36 "after") ranged between 56 and 1,578 of unique MOTUs per sample. The highest genetic diversity was found during the time period "after" with 9,693 unique MOTUs while diversity during time period "before", consisting of 6,728 MOTUs, was the lowest (figure 9). Relatively, both time periods shared 38% of MOTUs (n=4,519) detected. A more detailed exploration of MOTUs in each time period can be found in supplementary material S1 (appendix 7.1).

For alpha diversity comparison, samples were repetitively rarefied (n=50) to the lowest sequencing depth of 4,030, and the mean value was assigned as MOTU richness per sample. Mixed effects model indicated a significant difference on the overall alpha diversity among time periods (p=0.029), where the estimated mean ± confidence interval (CI) of MOTU richness was 553 ± 75 and 422 ± 80 for time period "after" and "before", respectively (supplementary material S2; appendix 7.2).

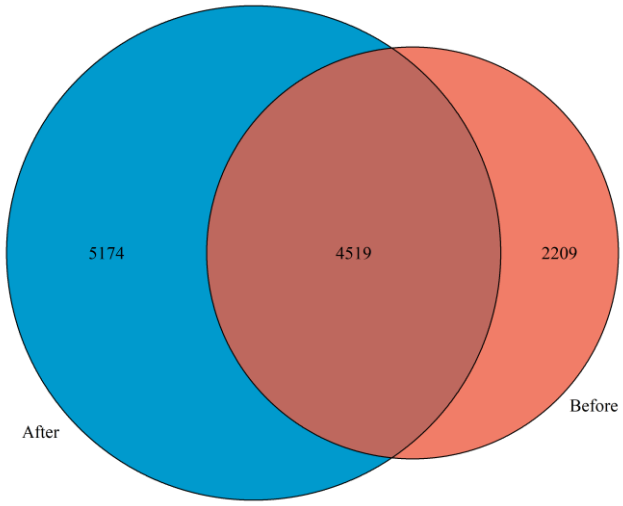

Figure 9. Venn diagram representing the total number of MOTUs found only in time period "before" (red), only in time period "after" (blue), and the number of MOTUs found in both time periods (the overlap surface) for COI marker.

The first ANOVA test showed significant changes among time periods (p=0.007) but no changes among distances (p=0.720) and no interaction between the two factors (p= 0.960; table 2 and figure 10a). The second test indicated significant changes among time periods (p=0.004), no changes among transects (p=0.557) and revealed a significant interaction among time periods and transects (p= 0.027; table 3 and figure 10b). Pairwise t-test indicated a significant increase within transect North (p=0.003) between time periods. The estimated mean ± standard error (SE) of MOTU richness for transect North was 637 ± 53.1 for time period "after" and 339 ± 55.7 for time period "before".

Table 2. Two-way ANOVA analysis table with type III SS (Sums of Squares) on the effects of time periods (as factor) and distances (as factor) on MOTU richness derived from COI dataset. Significant values are indicated in bold. Shapiro and Levene's test passed (p=0.485 and p=0.986, respectively).

Factors	*df*	*SS*	*F*	*P-value*
Time period	1	282270	7.7620	**0.007**
Distance	3	48729	0.4467	0.720
Time period * Distance	3	10849	0.0994	0.960
Residuals	59	2145564		

Table 3. Two-way ANOVA analysis table with type III SS (Sums of Squares) on the effects of time periods (as factor) and transects (as factor) on MOTU richness derived from COI dataset. Significant values are indicated in bold. Shapiro and Levene's test passed (p=0.486 and p=0.053, respectively).

Factors	*df*	*SS*	*F*	*P-value*
Time	1	276294	8.7947	**0.004**
Transect	2	37101	0.5905	0.557
Time period * Transect	2	240104	3.8214	**0.027**
Residuals	61	1916369		

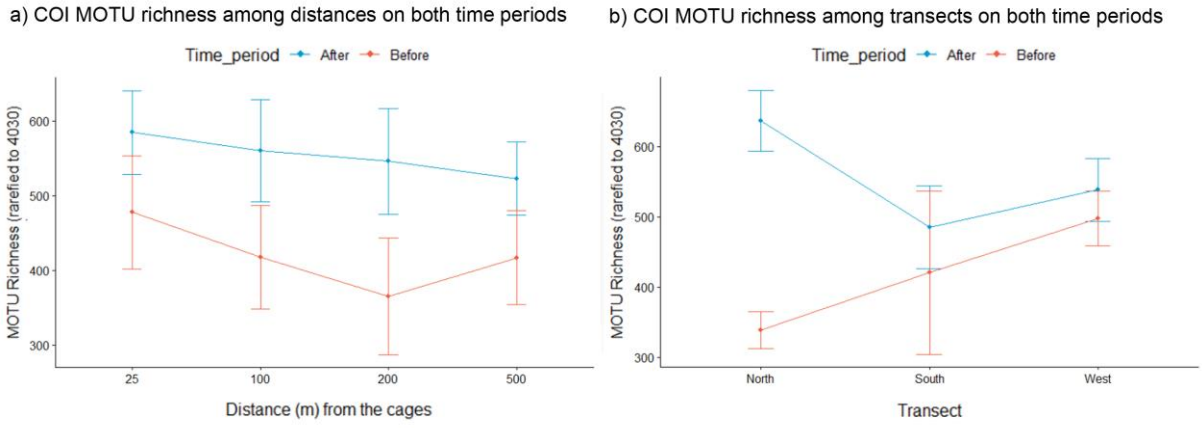

Figure 10. Mean MOTU richness (rarefied to 4030) for time period "before" (red) and "after" (blue) among distances (a) and transects (b) derived from COI dataset. Bars indicate standard error of the precision of the sample means.

3.3.2 18S

Six samples were discarded for alpha diversity analysis due to insufficient sequencing depth. The remaining 66 samples (32 for time period "before" and 34 "after") ranged between 55 and 647 of unique MOTUs per sample. The highest diversity was found during the time period "after" with 3,072 unique MOTUs while diversity during time period "before", consisting of 2,582 MOTUs, was the lowest (figure 11). Relatively, both time periods shared 44% of all MOTUs (n=1,726) detected. A more detailed exploration of MOTUs in each time period can be found in supplementary material S1 (appendix 7.1).

For alpha diversity comparison, samples were repetitively rarefied (n=50) to the lowest sequencing depth (n=511), and the mean value was assigned as MOTU richness per sample. To fit all the model assumptions (normality of residuals and homogeneity of variances) the response variable (MOTU richness) was box-cox transformed (raised in the power of 3). Mixed

effects model indicated a non-significant difference on overall alpha diversity among time periods (p=0.084) where the estimated mean ± confidence interval (CI) of MOTU richness was 158 ± 10.28 and 146.8 ± 12.46 for time period "after" and "before", respectively (supplementary material S2; appendix 7.2).

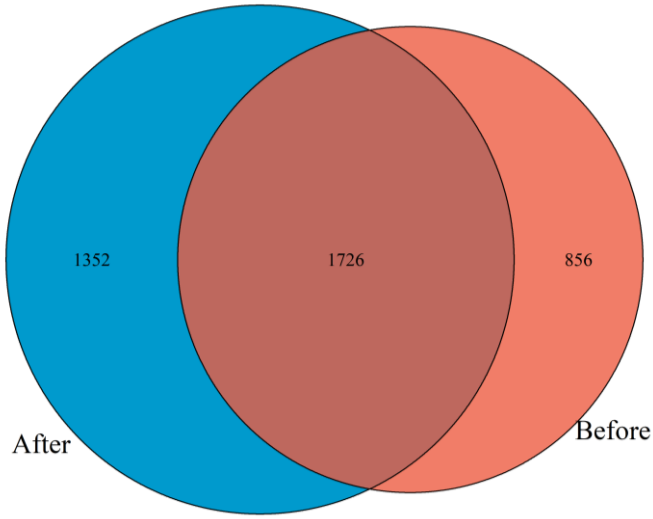

Figure 11. Venn diagram representing the total number of MOTUs found only in time period "before" (red), only in time period "after" (blue), and the number of MOTUs found in both time periods (the overlap surface) for COI marker.

The first ANOVA test found no significant changes among time periods (p=0.078), distance (p=0.383), and no interaction between the two factors (p=0.957; table 4 and figure 12a). The second test found no significance among time periods (p=0.058) and transects (p=0.055) but revealed a significant interaction among the factors (p<0.001; table 5 and figure 12b). Pairwise t-test revealed a significant increase in transect North (p=0.012) between time periods. The estimated mean ± standard error (SE) of MOTU richness for transect North was 172 ± 14 for time period "after" and 132 ± 18 for time period "before".

Table 4. Two-way ANOVA analysis table with type III SS (Sums of Squares) on the effects of time periods (as factor) and distances (as factor) on MOTU richness derived from 18S dataset. Shapiro and Levene's test passed (p=0.174 and p=0.472, respectively).

Factors	*df*	*SS*	*F*	*P-value*
Time	1	16356	1.427	0.078
Distance	3	13322	0.771	0.383
Time period * Distance	3	2062	0.003	0.957
Residuals	58	57490		

Table 5. Two-way ANOVA analysis table with type III SS (Sums of Squares) on the effects of time periods (as factor) and transects (as factor) on MOTU richness derived from 18S dataset. Significant values are indicated in bold. Shapiro and Levene's test passed (p=0.051 and p=0.566, respectively).

Factors	*df*	*SS*	*F*	*P-value*
Time period	1	3195	3.7461	0.058
Transect	2	4180	2.4508	0.055
Time period * Transect	2	9988	5.8556	**0.001**
Residuals	60	51169		

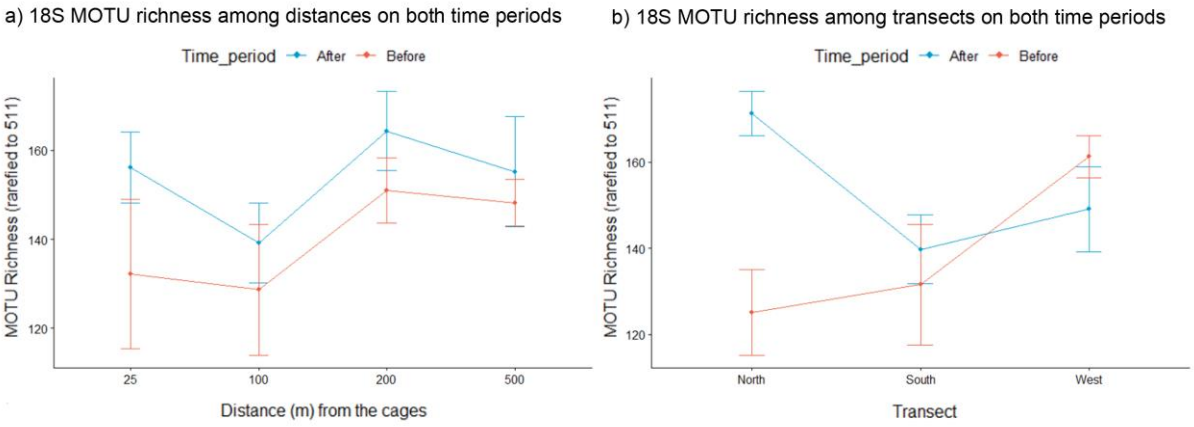

Figure 12. Mean MOTU richness (rarefied to 511) for time period "before" (red) and "after" (blue) among distances (a) and transects (b) derived from 18S dataset. Bars indicate standard error of the precision of the sample means.

3.4 Beta diversity

3.4.1 COI

Non-metric multidimensional scaling ordination plot was generated, displaying dissimilarities of species composition among stations (replicate pooled), based on Jaccard (presence-absence) dissimilarity index (figure 13). The stations were distinctly separated by time periods (colours red for time period "before" and blue for "after"). Ordination plot indicated that in the time period "before", transects differed between each other with generally no overlap of ellipses while in time period "after" only transect South differed from the transect North and West.

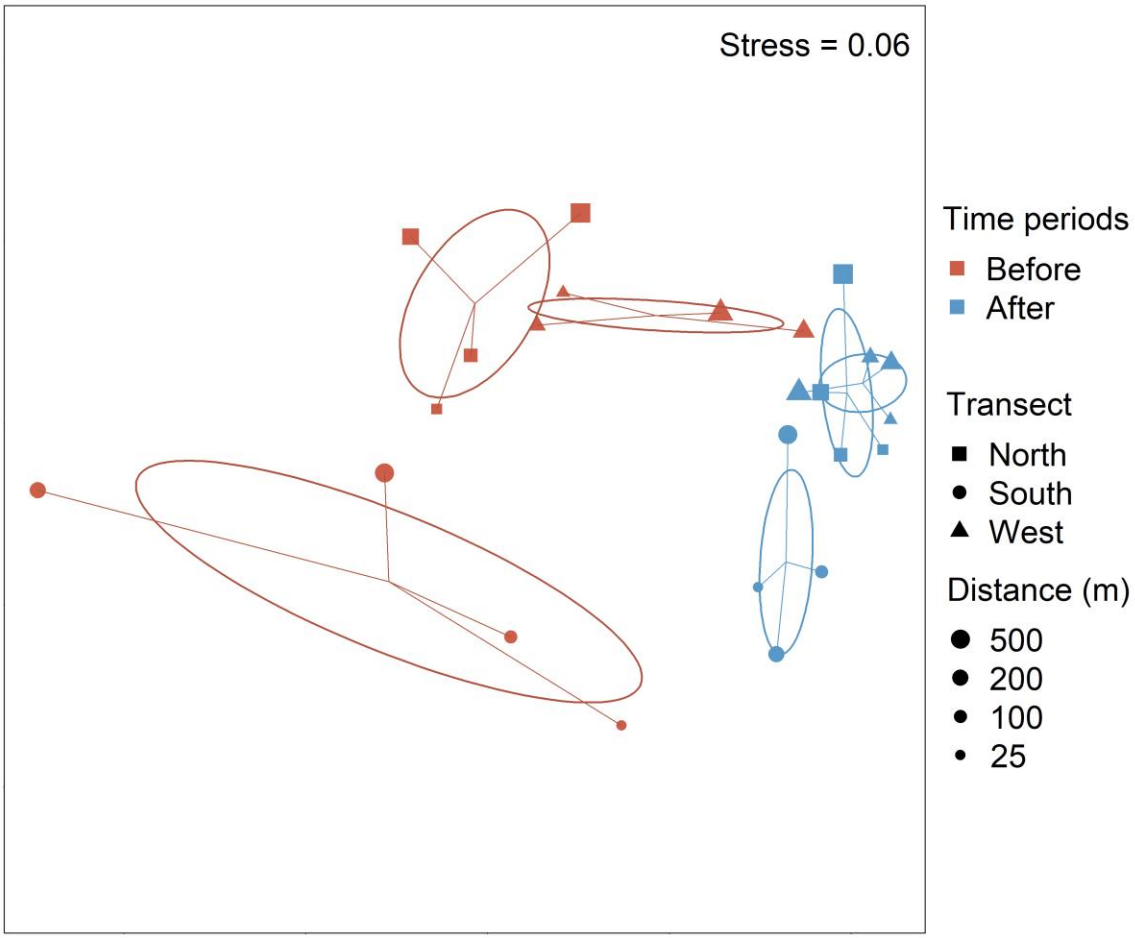

Figure 13. Non-metric multidimensional scaling (nMDS) ordination of samples (three replicates pooled) obtained using Jaccard dissimilarity index on COI dataset. The centroids for the transects and their corresponding time period are indicated. Ellipses indicate 95% confidence interval of the group centroid dispersion.

The first PERMANOVA analysis indicated a highly significant difference in the composition between time periods ($p<0.001$; table 6). Differences in the composition among distances were found non-significant ($p=0.594$) and no significant level of interaction ($p=0.633$) was indicated (table 7). The third test showed a significant difference between transects ($p=0.002$) and no interaction between transects and time periods was found ($p=0.148$; table 8).

Table 6. PERMANOVA analysis comparing community composition among time periods (fixed factor) on COI presence-absence dataset using Jaccard index. PERMDISP probabilities for homogeneity of dispersion are also shown. Significant values are indicated in bold. Number of permutations for each test was set to 999.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*R2*	*P-value*	*Permdisp*
Time	1	0.848	0.848	2.601	0.106	**0.001**	**0.003**
Residuals	22	7.172	0.326		0.894		
Total	23	8.021			1.000		

Table 7. PERMANOVA analysis comparing community composition among time periods (fixed factor) and distances (fixed factor) on COI presence-absence dataset using Jaccard index. PERMDISP probabilities for homogeneity of dispersion are also shown. Significant values are indicated in bold. Number of permutations for each test was set to 999.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*R2*	*P-value*	*Permdisp*
Time	1	0.848	0.848	2.572	0.106	**0.001**	**0.003**
Distance	3	0.951	0.318	0.961	0.119	0.594	
Time period * Distance	3	0.945	0.315	0.952	0.118	0.633	
Residuals	16	5.278	0.329		0.658		
Total	23	8.020			1.000		

PERMDISP indicated significant heterogeneity of dispersions within time periods (p=0.003), where within time period "before" the heterogeneity was higher. No significant difference of dispersions within transects was found (p=0.251; table 8).

Table 8. PERMANOVA analysis comparing community composition among time periods (fixed factor) and transects (fixed factor) on COI presence-absence dataset using Jaccard index. PERMDISP probabilities for homogeneity are also shown. Significant values are indicated in bold. Number of permutations for each test was set to 999.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*R2*	*P-value*	*Permdisp*
Time	1	0.841	0.841	2.7198	0.105	**0.001**	**0.003**
Transect	2	0.947	0.473	1.5308	0.118	**0.002**	0.251
Time period * Transect	2	0.682	0.341	1.1036	0.085	0.148	
Residuals	18	5.569	0.309		0.693		
Total	23	8.041			1.000		

Pairwise comparison tests indicated that transect North and transect West were significantly different from transect South (p=0.046 and p=0.005, respectively; table 9). The difference between transect North and transect West was found non-significant (p=0.283). Pairwise PERMDISP indicated no significant changes within distances (p=0.280) and transects (p=0.165) between time periods (supplementary material S3; appendix 7.3).

Table 9. Pairwise PERMANOVA analysis comparing community composition within transects (fixed factor) group in both time periods (before and after) on COI presence-absence dataset using Jaccard index. Significant values after FDR correction (False Discovery Rate) are indicated in bold. Number of permutations was set to 999.

Transects	*df*	*SS*	*Pseudo-F*	*P-adjusted*
N-S	1	0.4312	1.6374	**0.046**
N-W	1	0.2515	1.0637	0.283
S-W	1	0.5694	2.3305	**0.005**

3.4.2 18S

Non-metric multidimensional scaling ordination plot was generated, displaying dissimilarities of species composition among stations (replicate pooled), based on Jaccard (presence-absence) dissimilarity index (figure 14).

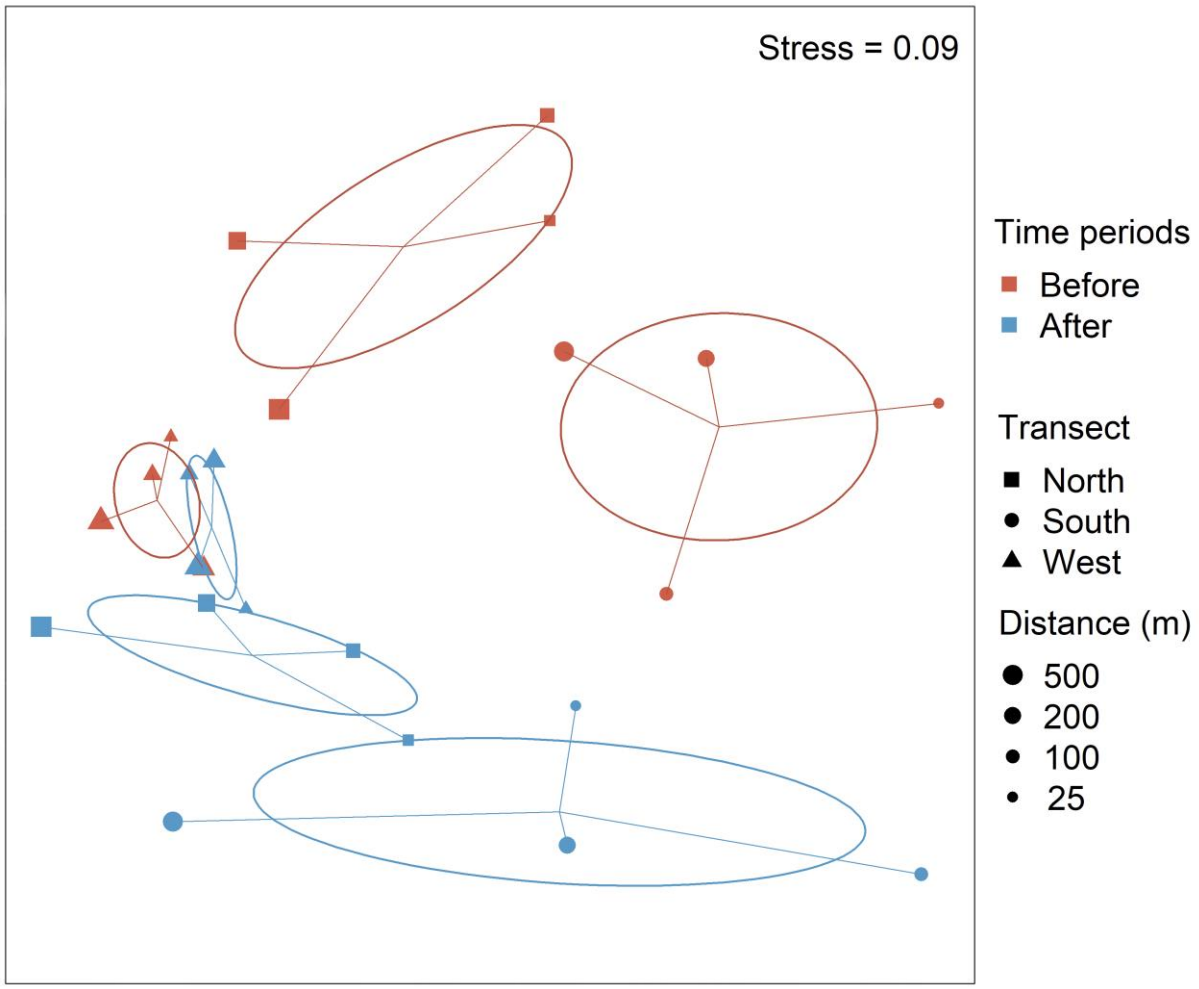

Figure 14. Non-metric multidimensional scaling (nMDS) ordination of samples (three replicates pooled) obtained using Jaccard dissimilarity index on 18S dataset. The centroids for the transects and their corresponding time period are indicated. Ellipses indicate 95% confidence interval of the group centroid dispersion.

The stations were distinctly separated by transects with no overlap of ellipses and the transects were separated clearly by time period. Ordination plot indicated that stations belonging to transect West were less variable in changes of MOTU composition among time compared to the other two transects (transects South and North).

The first PERMANOVA indicated a highly significant difference in the composition between time periods (p=0.014; table 10). Differences in the composition among distances were found non-significant (p=0.762) and no significant level of interaction (p=0.994) was indicated (table 11). The third test showed a significant difference between transects (p=0.006), but no interaction between the transects and time periods was found (p=0.166; table 12). PERMDISP indicated no significant difference of dispersions within time periods (p=0.459). However, significant heterogeneity of dispersion within transects was revealed (p=0.006). Pairwise comparison tests indicated that transect North and transect West were significantly different from transect South (p=0.006 and p=0.002, respectively; table 13). The difference between transect North and West was found non-significant (p=0.102). Pairwise PERMDISP indicated no significant changes within distances (p=0.953) and transects (p=0.089) between time periods (supplementary material S3; appendix 7.3).

Table 10. PERMANOVA analysis comparing community composition among time periods (fixed factor) on 18S presence-absence dataset using Jaccard index. PERMDISP probabilities for homogeneity of dispersion are also shown. Significant values are indicated in bold. Number of permutations for each test was set to 999.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*R2*	*P-value*	*Permdisp*
Time period	1	0.389	0.389	1.357	0.058	**0.014**	0.459
Residuals	22	6.309	0.286		0.941		
Total	23	6.699			1.000		

Table 11. PERMANOVA analysis comparing community composition among time periods (fixed factor) and distances (fixed factor) on 18S presence-absence dataset using Jaccard index. PERMDISP probabilities for homogeneity of dispersion are also shown. Significant values are indicated in bold. Number of permutations for each test was set to 999.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*R2*	*P-value*	*Permdisp*
Time period	1	0.389	0.389	1.325	0.058	**0.021**	0.893
Distance	3	0.838	0.279	0.951	0.125	0.762	
Time period * Distance	3	0.771	0.257	0.875	0.115	0.994	
Residuals	16	4.699	0.293		0.701		
Total	23	6.699			1.000		

Table 12. PERMANOVA analysis comparing community composition among time periods (fixed factor) and transects (fixed factor) on 18S presence-absence dataset using Jaccard index. PERMDISP probabilities for homogeneity are also shown. Significant values are indicated in bold. Number of permutations for each test was set to 999.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*R2*	*P-value*	*Permdisp*
Time period	1	0.389	0.389	1.442	0.058	**0.006**	0.459
Transect	2	0.866	0.433	1.605	0.129	**0.001**	**0.006**
Time period * Transect	2	0.583	0.291	1.080	0.087	0.166	
Residuals	18	4.859	0.269		0.725		
Total	23	6.699			1.000		

Table 13. Pairwise PERMANOVA analysis comparing community composition within transects (fixed factor) group in both time periods (before and after) on COI presence-absence dataset using Jaccard index. Significant values after FDR correction (False Discovery Rate) are indicated in bold. Number of permutations was set to 999.

Transects	*df*	*SS*	*Pseudo-F*	*P-adjusted*
N-S	1	0.329	1.698	**0.008**
N-W	1	0.195	1.182	0.102
S-W	1	0.498	2.845	**0.001**

3.4.3 Mantel test

When comparing the dissimilarity matrices for COI and 18S, Mantel test showed a significant correlation between the ordinations resulting from both primers ($r=0.558$, $p<0.001$) stating that the matrix entries are positively associated.

3.4.4 Supplementary analysis

The results of SIMPER analysis and estimation of AMBI values are found in the supplementary material S4 and S5 (appendix 7.4 and 7.5, respectively). The reasons for exclusion of such analysis from the results are explained in the discussion sections 4.4.

4 Discussion

The aim of this study was to investigate the impact of the aquaculture on benthic biodiversity with respect to effluents using eDNA metabarcoding as a monitoring tool. Marine sediments are particularly species-rich, yet remain poorly studied due to complicated taxonomic work using traditional means of sampling (Guardiola et al., 2015). Changes in benthic macrofauna communities due to effluents are well documented (Dowle, Pochon, Keeley, & Wood, 2015; Stoeck et al., 2018), but very few studies have investigated changes in eukaryotic richness and composition as a result of aquaculture impact on the environment. Thus, this study is the first of its kind conducted in Norwegian waters with before-after comparison, providing an informative picture of molecular biodiversity patterns as a result of aquaculture establishment.

4.1 Alpha diversity

Specific patterns were observed despite the fact that 18S marker did not detect a significant change of the overall alpha diversity after the establishment of the aquaculture. Rarefaction curves derived from COI marker (figure 6a) concluded that sequencing depth was adequate and covered nearly all of the MOTUs for stations sampled before the aquaculture establishment, but it did not cover all the MOTUs in stations sampled during the period "after", thus providing unreliable MOTU richness values. This suggested that more MOTUs could be detected in the time period "after" if the sequencing depth were to be increased. Conversely, the rarefaction curves derived from 18S marker (figure 6b) showed that the genetic complexity of the sediment community for both time periods was not fully represented due to insufficient sequencing depth.

Nonetheless, for both markers, a similar pattern was observed in species accumulation curves (figure 7). Despite the failure of MOTU numbers reaching a plateau, more MOTUs in time period "after" were detected compared to time period "before" for the same number of samples, therefore resulting in a higher rate of accumulation for the former time period. The curve suggests that with increasing the number of samples, more MOTUs would be detected in both time periods, hence resulting in even higher number of MOTUs in stations sampled after aquaculture establishment. This is also supported by results from mixed effects model. The results indicated a significant increase on the overall MOTU richness after the establishment of aquaculture only for COI marker, thus mirroring the species accumulation curve. Such increase in alpha diversity was not found significant for 18S marker.

While most of the studies on benthic macrofauna have documented eutrophication and decrease of diversity (Diana, 2009; Sarà et al., 2011), few studies have reported enrichment of fish biodiversity not only in species richness but also in accordance with increased biomass and abundance (Machias et al., 2005, 2004; Soto & Norambuena, 2004). Machias et al. (2005) conclude that the reason for such increase is likely the increase in nutrients supply and the consequences related to the latter factor. Furthermore, they support the hypothesis that oligotrophic marine systems are likely to increase the local biodiversity around the fish farm due to enrichment of nutrients. In most cases it is reported that the concentration of the elements derived from uneaten feed (food waste) tends to accumulate at the bottom, directly beneath the cages (Kalantzi et al., 2013), thus leading to a localised homogenous distribution of impacts where the impacted area being few meters (0-30 m) from the edges of the cages.

In both markers, MOTU richness increased significantly within transect North (transect aligned with the main current direction) after the development of the aquaculture, compared to respective reference transects (transect South and West) where such change was found insignificant, pointing to the conclusion that the impact of aquaculture was heterogeneous and might be influenced by the dominant direction of ocean currents. Furthermore, MOTU richness changed homogeneously among distances as ANOVA found no significant interaction. Thus, this result contradicts the well-established hypothesis of strictly localised impact (e.g. 25-30 m) and its decrease with the increasing distances from the edge of the cages (Mente et al., 2006; Stoeck et al., 2018). Although this might have alternative interpretations, the present results strongly indicate that such effect is mainly driven by currents. Nevertheless, for a definitive conclusion of the statement, accurate measurement of current speed and direction is required.

Supporting that aquaculture can impact heterogeneously, previous studies show that currents will shift the deposition of nutrient waste and therefore they can alter the ecological impact of the aquaculture due to such shift (Hamoutene, Salvo, Donnet, & Dufour, 2016; Stoeck et al., 2018). Chamberlain, Fernandes, Read, Nickell, and Davies (2001) concluded that slight variations in current velocity and direction around the farms could have a significant effect on the dispersion of organic load, thus potentially leading to heterogeneous species richness favoured by the currents.

Despite the fact that increased level of disturbance from anthropogenic sources often results in increased levels of stress on natural communities (Machias et al., 2004), the present data outlines that excess level of nutrients can enrich the biodiversity. However, greater species

richness does not necessarily mean improved ecological status as the abundances of species could decline due to species replacement process. Generally, characteristics of pollution are set to drive the population size smaller (Machias et al., 2005) on average by replacing some large-sized species with smaller ones, meaning that irrespective to richness increase, the biomass might decrease. Due to the nature of the tools used for this study, the true abundances of species are impossible to investigate, thus leaving a gap in interpreting the nature of the impact (positive or negative).

4.2 Beta diversity

PERMANOVA showed for both primers that the community composition changed significantly after the implementation of the aquaculture. Temporal comparison of the homogeneity of multivariate dispersion (PERMDISP) for COI marker showed that the distances to group centroids were higher in the time period "before", meaning that the beta diversity decreased after the establishment of the aquaculture. Moreover, this was sustained by the non-metric multidimensional scaling plot (nMDS) indicating that the differences in centroids are not due to the heterogeneity of dispersion but rather to the true multivariate mean difference (figure 13). In contrary, PERMDISP and nMDS on 18S marker failed to show heterogeneity in dispersion of community composition between time periods (figure 14 and table 10), indicating that beta diversity remained uniform despite the change in community composition.

Temporal changes in community composition, from both markers, did not differ among distances as PERMANOVA indicated no significant interaction between time periods and distances. The same findings could be concluded from ordination plots where both nMDSs (figure 13 and figure 14) showed no systematic structure among distances. No significant difference in multivariate dispersion was found among the distances. Thus, the temporal shift in community composition occurred homogeneously throughout all the distances.

Both markers indicated significant differences in community composition among transects, where transect north and west were distinct from the transect South. PERMDISP for COI marker found no significant difference suggesting that the dispersion of the temporal difference occurred homogeneously among all transects. Significant changes in dispersion occurred for

18S marker, where transect west resulted in lower dispersion, meaning that the latter transect had smaller changes in community composition compared to the reference transects (table 12).

Although a definite conclusion on beta diversity impact patterns from aquaculture requires more consistent sampling and a larger study area, it could be outlined that either the aquaculture impact stretches beyond the study area or the seasonal variability has been the driving force of such change. A combination of both of these factors could also be an alternative cause (less likely) for the observed shifts in beta diversity. The present results suggest that the decay of beta diversity (detected by COI marker) is due to seasonal variability as it occurs among all stations homogeneously and has no spatial differences.

4.3 Primers performance on revealing biodiversity patterns

Although Mantel test showed significant similarity between the results from both primers, revealing that the overall patterns were similarly recovered by both markers, substantial differences occurred. MOTUs yielded by both primers differed in taxonomic assignment. 18S primers had more uniform detection among kingdoms while in COI, the spread of detection was irregular. COI detected more MOTUs, but the majority had weak taxonomic assignments. Conversely, despite the low number of MOTUs detected by 18S, the taxonomic assignment remained relatively elevated. These differences can be strongly influenced by current gaps in reference sequence databases, where the available reference database for COI has significant gaps for other eukaryotic groups different from Metazoa, while completeness of 18S database is more homogeneous among different eukaryotic kingdoms (Wangensteen, Palacín, et al., 2018)

Nonetheless, a multigene approach is desirable for covering a broader range of taxonomy, but primer characteristics need to be underlined when doing biodiversity assessment (Wangensteen, Palacín, et al., 2018). COI fragments targeted here are longer and more variable, thus leading to the detection of more diverse MOTUs when compared to MOTUs detected by 18S where the 18S rRNA fragment is shorter and therefore several species can share the same sequence (Guardiola et al., 2015; Ruppert, Kline, & Rahman, 2019; Wangensteen, Palacín, et al., 2018). As described by Wangensteen, Palacín, et al. (2018), the former marker tends to overestimate the species richness while the latter marker tends to underestimate the species richness. This reason may explain why only COI found significant increase in MOTU richness.

Moreover, the difference of fragment's length results in the power of detecting live or dead DNA fragments (Atienza et al., 2020; Guardiola et al., 2015; Ruppert et al., 2019; Wangensteen, Palacín, et al., 2018). Atienza et al. (2020) in their research concludes that large DNA fragments (higher than 300 bp), COI fragments amplified here, can indicate DNA from living organisms or recently dead while shorter fragments (ca. 130 bp), 18S rRNA fragment amplified here, can also include DNA from dead organisms since large fragments degrade faster.

Furthermore, the attribute of detecting live organisms makes the COI fragment more desirable (Wangensteen, Palacín, et al., 2018). Drummond et al. (2015), when comparing primers performance, concluded that COI region had the strongest correlation with traditional sampling when used for biodiversity assessment. Supplementary, Atienza et al. (2020) enforced the use of COI metabarcoding marker as a high-resolution method to detect ecological shifts. The present study, aligned with previous studies (mentioned above), showed that COI had better performance in terms of showing higher contrast between time periods. However, both primers were able to indicate patterns of biodiversity changes (Grey et al., 2018). Alternatively, their performance could be related to the difference in sequencing depth between markers (Lanzén, Lekang, Jonassen, Thompson, & Troedsson, 2017).

4.4 Supplementary analysis

The reason for the exclusion of supplementary analysis (SIMPER analysis and estimation of ecological quality) from the results and conclusion is twofold. The first reason is that both markers have a high number of unassigned MOTUs, which can lead to insufficient results and uncertain interpretation (Aylagas et al., 2014). The second reason is the inability to infer real species abundances from presence-absence type of data, which can lead to biases on AMBI implementation. Although studies have proven the use of p/a AMBI (Aylagas et al., 2014), noise and biases from random occurrence of species might occur (Warwick, Robert Clarke, & Somerfield, 2010), as there are extensively more sensitive species compared to opportunistic species in AMBI reference database (n=6,972 and n=563, respectively).

Despite such facts, an attempt on estimating Marine Biotic Index (supplementary material S5; appendix 7.5) revealed no changes on ecological quality after the establishment of aquaculture. Although such results cannot be concluded with certainty (due to the reasons explained above), they can supply supplementary descriptive information on the nature of the impact.

Additionally, SIMPER analysis revealed that organisms responsible for the biggest part of the temporal change are still taxonomically unassigned, for both markers 18S and COI. Therefore, drawing conclusion might lead to misinterpretations of accurate drivers of temporal changes.

4.5 Limitations

The number of samples analysed in this study does not fully represent all MOTUs (figure 7), thus more samples and/or larger quantities of sediments (e.g. 10 g of sediment) are required for acquiring complete view of the biodiversity patterns and conducting more accurate comparisons between periods. Although Grey et al. (2018) indicate that non-parametric tests are more robust to sampling effort variation, sequencing depth could still be a limiting factor, especially for the results derived from 18S primer metabarcoding, as the curves did not saturate with increasing sequencing depth (figure 6).

As described by Machias et al. (2004), an ideal before-after study that incorporates a nested design with multiple years, areas and seasons would be nearly impossible. Additionally, both economy and willingness of the aquaculture industry to allow access to their production facilities, are limiting factors for using such an approach. However, the present study design, although at only one aquaculture site, has minimized the issues arising from the seasonal and spatial variability by collecting samples at different time periods, different distances from the cages and at different orientations (transects). Conversely, the impact of aquaculture can be concealed under the seasonal variability, thus leading to a failure of detection (type II error) for such impact. Although very unlikely, the seasonal variability could occur irregularly in space. Besides the irregularity of seasonal variability, having a narrow time span (five months) between periods before-after may potentially result in misleading conclusions if the detected impact were to reverse in the near or unknown future, knowing that the impact is often expected to be highest during the peak of production (Hansen et al., 2001).

Estimating true species abundance using the number of reads is not yet possible as biological factors (e.g. variations in mitochondria copy numbers and/or individual shedding rates) will lead to biases (Guardiola et al., 2015). Although being the most used method (Ruppert et al., 2019), working with presence-absence (P/A) data could arguably lead to limitations on the interpretation of results. Furthermore, when using P/A sets of data, questions of particular species dominance remain unanswered. Such estimate can define and interpret, in-depth, the

nature of the observed impact (positive or negative). In that regard, adding measurements of abiotic factors such as depth, sediment type, sediment chemical properties, and accurate measurement of current speed and direction will lead to a thorough investigation of the response behaviour. Additionally, management variables (e.g. feeding rates, fish biomass and operating time) are considered necessary for clarifying responses (Carroll et al., 2003). Lastly, current gaps in both markers (COI and 18S) reference database limits the capacity of analysis for conducting through taxonomic investigation in the search for ecological explanations.

4.6 MOM, metabarcoding and the future of aquaculture

Many studies suggest that traditional sampling for biodiversity assessments are biased (Fonseca et al., 2010; Wangensteen, Palacín, et al., 2018) and focused on large organisms (Fonseca et al., 2010). This bias occurs due to circumvention of meiofaunal assemblages which executes substantial tasks in the marine ecosystem. This is typically present in traditional benthic surveys (type C-investigations) used for aquaculture's environmental monitoring in Norway. Type C-investigations are primarily focused on taxonomic distribution of the benthic macrofauna in the intermediate impact zone, the zone where the tolerance for expected impact is much lower than those in the local zone (Wilson et al., 2009). Fonseca et al. (2010), in their study, confirmed that metabarcoding enabled them to detect greater diversity when compared to traditional morphological sampling despite the similarity of the patterns between the methods. This conclusion leads to believe that incorporation of metabarcoding during type C-investigation could, therefore, be beneficial and resolve biased answered questions as it adds strength to the results originated from the latter surveys. This study, in accordance with other studies (Atienza et al., 2020; Fonseca et al., 2010; Grey et al., 2018; Guardiola et al., 2015; Ji et al., 2013; Ruppert et al., 2019), provides strong evidence that metabarcoding can be used to detect ecological shifts due to anthropogenic impacts when primer characteristics are well defined from the start of the project.

Furthermore, adding an external application, such as genomic biotic index, as suggested by Aylagas et al. (2018), could lead to remarkable resolution of environmental assessment. Additionally, as this study documents, significant spatial variability around the aquaculture can occur. Sampling only three to five samples during type C-investigation is rather low (Fernandes, Miller, & Read, 2000) and can exhibit stochastic results due to large biases from random sampling. Conducting metabarcoding surveys prior to type C-monitoring would screen the

spatial heterogeneity within the sampling area and therefore, can easily circumvent such biases. Finally, as traditional monitoring techniques are highly relied on taxonomist expertise, making such surveys extremely sensitive to human errors, metabarcoding can minimize the human error and yield high-resolution taxonomic coverage (Bohmann et al., 2014).

Environmental DNA has quickly become one of the fastest developing and utilized tools for biodiversity surveillance. Many authors confirm that steady progress is made on updating the reference database, and the lack of specificity will be a problem of the past (Ruppert et al., 2019). Its applicability is also expanding due to the non-invasive nature of metabarcoding sampling, as many conservational studies highly rely on the low environmental impact (Civade et al., 2016). This sampling characteristic could be beneficiary also for biomonitoring surveys on anthropogenic impact. As biodiversity surveys are becoming crucial for understanding the nature and sources of impacts (Grey et al., 2018), metabarcoding surveys reveal the power to quickly and accurately assess the ecological communities response to environmental changes, making it a reliable toolbox for biodiversity assessment (Ruppert et al., 2019). Aylagas et al. (2018) concluded that metabarcoding surveys are twice as cheap and three times faster compared to the traditional ones. Massive sample multiplexing which can be achieved using improved sequencing platforms, such as the new Illumina NovaSeq, will undoubtedly contribute to decreasing the cost of metabarcoding even further. Furthermore, shifting to metabarcoding surveys instead would accelerate the decision-making process in marine environmental management (Aylagas et al., 2018).

Due to technological advances, increasing global population, and increasing food consumption per capita, there is a rise of dependability on aquaculture in resolving such emerging concerns. Being the fastest growing food production system, aquaculture faces many challenges regarding ecosystem management and sustainability. This increased pressure urges for adequate monitoring techniques, leading to a better understanding of the nature and characteristics of the environmental impact. Furthermore, a continuous update on makers reference database is required to make metabarcoding assessments more efficient for impact monitoring.

5 Conclusion

This study confirmed that the establishment of the aquaculture farm affected the alpha diversity, as a significant increase in MOTU richness was observed during production. The increased MOTU richness values were observed at all sample distances along the transect that followed the prevailing current direction. However, changes were mostly non-significant along the other two sampled directions. Combined, this concludes that the spread of impact was heterogeneous throughout the transects and homogeneous throughout the distances. Additionally, the present study suggested that the establishment of the aquaculture alone did not affect the beta diversity. Thus, the decrease in beta diversity might be as a result of seasonal variability. This conclusion is based on the fact that beta diversity changes occur homogeneously among all the monitored stations, transects and distances from the cages.

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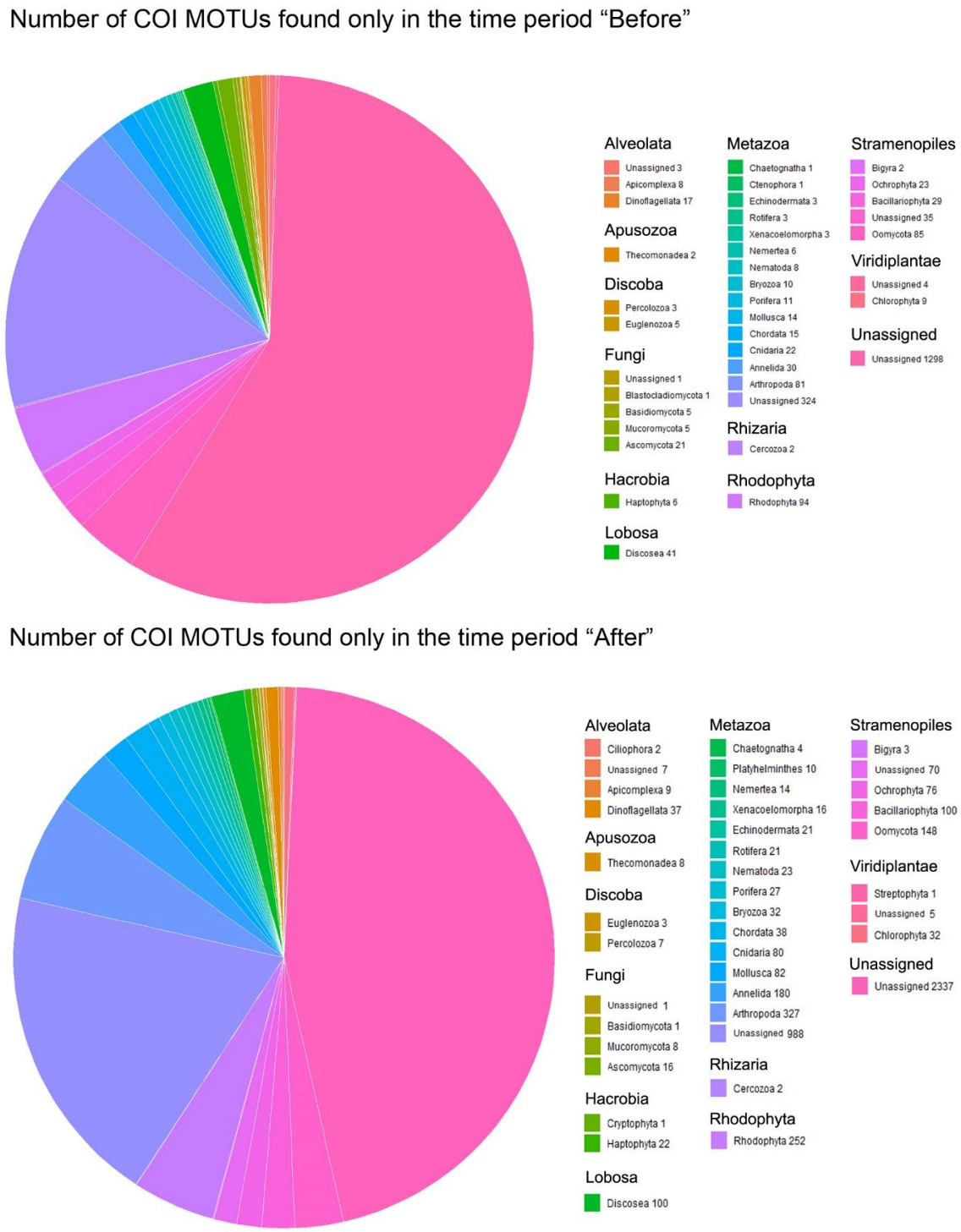
7 Appendix

7.1 Supplementary material S1. Figures and tables of MOTU distribution among time periods

7.1.1 COI marker



Appendix 7.1.1.1. Pie chart indicating the number of MOTUs found only in time period “before” (top) and “after” (bottom) represented by Phylum and grouped by Kingdom. Chart derived from COI dataset.

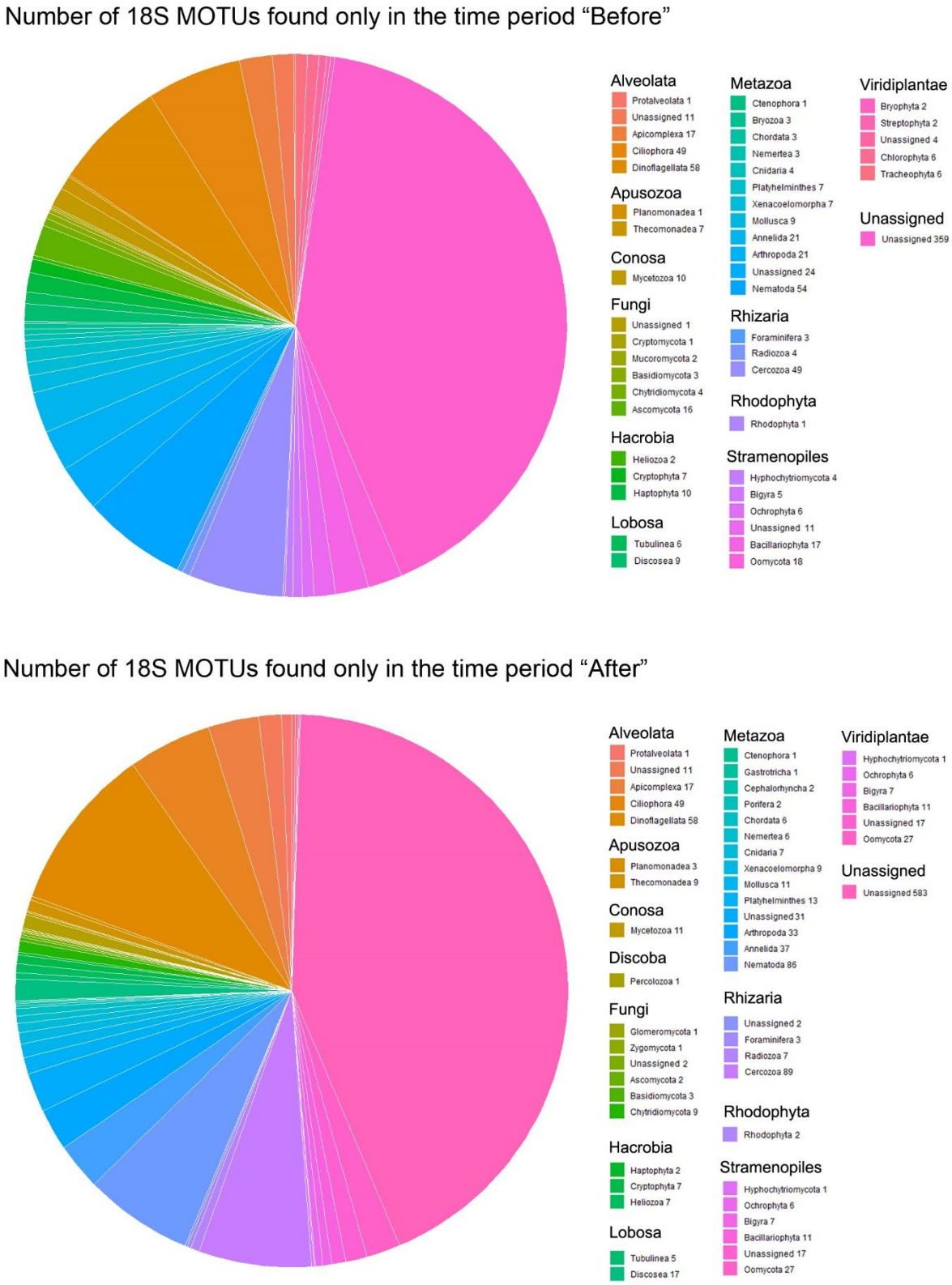
Appendix 7.1.1.2. Table indicating the number of MOTUs found only in time period "before" represented by Phylum and grouped by Kingdom. Chart derived from COI dataset.

Kingdom	*Phylum*	*Number of MOTU*
Alveolata		
	Unassigned	3
	Apicomplexa	8
	Dinoflagellata	17
Apusozoa		
	Thecomonadea	2
Discoba		
	Percolozoa	3
	Euglenozoa	5
Fungi		
	Blastocladiomycota	1
	Unassigned	1
	Basidiomycota	5
	Mucoromycota	5
	Ascomycota	21
Hacrobia		
	Haptophyta	6
Lobosa		
	Discosea	41
Metazoa		
	Chaetognatha	1
	Ctenophora	1
	Echinodermata	3
	Rotifera	3
	Xenacoelomorpha	3
	Nemertea	6
	Nematoda	8
	Bryozoa	10
	Porifera	11
	Mollusca	14
	Chordata	15
	Cnidaria	22
	Annelida	30
	Arthropoda	81
	Unassigned	324
Rhizaria		
	Cercozoa	2
Rhodophyta		
	Rhodophyta	94
Stramenopiles		
	Bigyra	2
	Ochrophyta	23
	Bacillariophyta	29
	Unassigned	35
	Oomycota	85
Viridiplantae		
	Unassigned	4
	Chlorophyta	9
Unassigned		
	Unassigned	1298

Appendix 7.1.1.3. Table indicating the number of MOTUs found only in time period "after" represented by Phylum and grouped by Kingdom. Chart derived from COI dataset.

Kingdom	*Phylum*	*Number of MOTU*
Alveolata		
	Ciliophora	2
	Unassigned	7
	Apicomplexa	9
	Dinoflagellata	37
Apusozoa		
	Thecomonadea	8
Discoba		
	Euglenozoa	3
	Percolozoa	7
Fungi		
	Basidiomycota	1
	Unassigned	1
	Mucoromycota	8
	Ascomycota	16
Hacrobia		
	Cryptophyta	1
	Haptophyta	22
Lobosa		
	Discosea	100
Metazoa		
	Chaetognatha	4
	Platyhelminthes	10
	Nemertea	14
	Xenacoelomorpha	16
	Echinodermata	21
	Rotifera	21
	Nematoda	23
	Porifera	27
	Bryozoa	32
	Chordata	38
	Cnidaria	80
	Mollusca	82
	Annelida	180
	Arthropoda	327
	Unassigned	988
Rhizaria		
	Cercozoa	2
Rhodophyta		
	Rhodophyta	252
Stramenopiles		
	Bigyra	3
	Unassigned	70
	Ochrophyta	76
	Bacillariophyta	100
	Oomycota	148
Viridiplantae		
	Streptophyta	1
	Unassigned	5
	Chlorophyta	32
Unassigned		
	Unassigned	2337

7.1.2 18S marker



Appendix 7.1.2.1. Pie chart indicating the number of MOTUs found only in time period “before” (top) and “after” (bottom) represented by Phylum and grouped by Kingdom. Chart derived from 18S dataset.

Appendix 7.1.2.2. Table indicating the number of MOTUs found only in time period "before" represented by Phylum and grouped by Kingdom. Chart derived from 18S dataset.

Kingdom	*Phylum*	*Number of MOTU*
Alveolata		
	Protalveolata	1
	Unassigned	11
	Apicomplexa	17
	Ciliophora	49
	Dinoflagellata	58
Apusozoa		
	Planomonadea	1
Apusozoa		
	Thecomonadea	7
Conosa		
	Mycetozoa	10
Fungi		
	Cryptomycota	1
	Unassigned	1
	Mucoromycota	2
	Basidiomycota	3
	Chytridiomycota	4
	Ascomycota	16
Hacrobia		
	Heliozoa	2
	Cryptophyta	7
	Haptophyta	10
Lobosa		
	Tubulinea	6
	Discosea	9
Metazoa		
	Ctenophora	1
	Bryozoa	3
	Chordata	3
	Nemertea	3
	Cnidaria	4
	Platyhelminthes	7
	Xenacoelomorpha	7
	Mollusca	9
	Annelida	21
	Arthropoda	21
	Unassigned	24
	Nematoda	54
Rhizaria		
	Foraminifera	3
	Radiozoa	4
	Cercozoa	49
Rhodophyta		
	Rhodophyta	1
Stramenopiles		
	Hyphochytriomycota	4
	Bigyra	5
	Ochrophyta	6
	Unassigned	11
	Bacillariophyta	17
	Oomycota	18
Viridiplantae		
	Bryophyta	2
	Streptophyta	2
	Unassigned	4
	Chlorophyta	6
	Tracheophyta	6
Unassigned		
	Unassigned	359

Appendix 7.1.2.3. Table indicating the number of MOTUs found only in time period "after" represented by Phylum and grouped by Kingdom. Chart derived from 18S dataset.

Kingdom	*Phylum*	*Number of MOTU*
Alveolata	Protalveolata	8
	Unassigned	18
	Apicomplexa	40
	Dinoflagellata	66
	Ciliophora	131
Apusozoa		
	Planomonadea	3
	Thecomonadea	9
Choanozoa		
	Cristidiscoidia	1
	Choanofila	2
Conosa		
	Mycetozoa	11
Discoba		1
	Percolozoa	
Fungi		
	Glomeromycota	1
	Zygomycota	1
	Ascomycota	2
	Unassigned	2
	Basidiomycota	3
	Chytridiomycota	9
Hacrobia		
	Haptophyta	2
	Cryptophyta	7
	Heliozoa	7
Lobosa		
	Tubulinea	5
	Discosea	17
Metazoa		
	Ctenophora	1
	Gastrotricha	1
	Cephalorhyncha	2
	Porifera	2
	Chordata	6
	Nemertea	6
	Cnidaria	7
	Xenacoelomorpha	9
	Mollusca	11
	Platyhelminthes	13
	Unassigned	31
	Arthropoda	33
	Annelida	37
	Nematoda	86
Rhizaria		
	Unassigned	2
	Foraminifera	3
	Radiozoa	7
	Cercozoa	89
Rhodophyta		
	Rhodophyta	2
Stramenopiles		
	Hyphochytriomycota	1
	Ochrophyta	6
	Bigyra	7
	Bacillariophyta	11
	Unassigned	17
	Oomycota	27
Viridiplantae		
	Bryophyta	1
	Tracheophyta	1
	Unassigned	2
	Chlorophyta	3
Unassigned		
	Unassigned	583

7.2 Supplementary material S2. Tables for alpha diversity analysis

7.2.1 COI marker

Appendix 7.2.1.1. ANOVA table with Kenward-Roger approximations of the mixed effects model comparing MOTU richness between time periods (fixed effect). Stations (random effect) are nested within time period. Data derived from COI dataset. Significant values are indicated in bold. Shapiro and Levene's test passed (p=0.103 and p=998, respectively).

Factors	*SS*	*MS*	*df*	*Den DF*	*F*	*P-value*
Time	184843	184843	1	10.922	6.243	**0.029**

Appendix 7.2.1.2. Estimated means for time period "before" and "after" using COI dataset. Standard error (SE) and 95% confidence interval indicated (lower.CL and upper.CL).

Time	*Estimated means*	*SE*	*df*	*lower.CL*	*upper.CL*
After	553	37.4	20.1	478	628
Before	422	39.7	23.3	342	502

Appendix 7.2.1.3. Pairwise t test comparing MOTU richness for levels of time period (before and after) and levels of transect (North, South, and West) using COI dataset. P values corrected with Bonferroni method. Significant p values are indicated in bold.

Pairwise comparison	*p-adjusted*
After North – After South	0.664
After North – After West	1.000
After North – Before North	**0.003**
After North – Before South	0.167
After North – Before West	0.980
After South – After West	1.000
After South – Before North	0.860
After South – Before South	1.000
After South – Before West	1.000
After West – Before North	0.154
After West – Before South	1.000
After West – Before West	1.000
Before North – Before South	1.000
Before North – Before West	0.583
Before South – Before West	1.000

Appendix 7.2.1.4. Estimated means for transects (North, South, and West) on each time period (before and after) using COI dataset. Standard error (SE) and 95% confidence interval indicated (lower.CL and upper.CL).

Time	*Transect*	*Estimated means*	*SE*	*df*	*lower.CL*	*upper.CL*
After	North	637	53.1	34.5	529	745
Before	North	339	55.7	37.1	226	452
After	South	485	53.1	34.5	377	592
Before	South	409	66.4	45.9	275	542
After	West	539	53.1	34.5	431	647
Before	West	498	53.1	34.5	390	606

7.2.2 18S marker

Appendix 7.2.2.1. ANOVA table with Kenward-Roger approximations of the mixed effects model on MOTU richness between time periods (fixed effect). Stations (random effect) are nested within time period. Data derived from 18S dataset. Shapiro and Levene's test passed (p=0.105 and p=0.853, respectively).

Factors	*SS*	*MS*	*df*	*Den DF*	*F*	*P-value*
Time	18294	18294	1	10.881	2.973	0.084

Appendix 7.2.2.2. Estimated means for time period "before" and "after" using 18S dataset. Standard error (SE) and 95% confidence interval indicated (lower.CL and upper.CL).

Time	*Estimated means*	*SE*	*df*	*lower.CL*	*upper.CL*
After	158.1	4.78	20.9	147.8	168.4
Before	146.8	5.74	22.1	134.4	159.3

Appendix 7.2.2.3. Pairwise t test on MOTU richness comparing levels of time period (before and after) and levels of transect (North, South, and West) using 18S dataset. P values corrected with Bonferroni method. Significant p values are indicated in bold.

Factor	*p-adjusted*
After North – After South	0.473
After North – After West	1.000
After North – Before North	**0.012**
After North – Before South	0.085
After North – Before West	1.000

After South – After West	1.000
After South – Before North	1.000
After South – Before South	1.000
After South – Before West	1.000
After West – Before North	0.972
After West – Before South	1.000
After West – Before West	1.000
Before North – Before South	1.000
Before North – Before West	0.083
Before South – Before West	0.397

Appendix 7.2.2.4. Estimated means for transects (North, South, and West) on each time period (before and after) using 18S dataset. Standard error (SE) and 95% confidence interval indicated (lower.CL and upper.CL).

Time	*Transect*	*Estimated means*	*SE*	*df*	*lower.CL*	*upper.CL*
After	North	172	5.02	60	161	183
Before	North	132	8.94	60	114	150
After	South	143	7.80	60	127	159
Before	South	141	8.95	60	123	159
After	West	154	6.64	60	140	168
Before	West	163	5.89	60	151	175

7.3 Supplementary material S3. Tables for beta diversity analysis

7.3.1 COI marker

Appendix 7.3.1.1. Mean values of homogeneity of multivariate dispersion (average distance to the group centroid) for time periods (before and after) using COI dataset.

Time period	*Average distance to median (beta diversity)*
Before	0.5700
After	0.5202

Appendix 7.3.1.2. Pairwise PERMDISP test with 999 permutations comparing beta diversity among groups. Groups represent each distance (25, 100, 200, and 500 m) among each time period (before and after) treated as fixed factor. The table derived from COI dataset.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*P-value*
Groups	7	0.021	0.003	1.371	0.280
Residuals	16	0.035	0.002		

Appendix 7.3.1.3. Pairwise PERMDISP test with 999 permutations comparing beta diversity among groups. Groups represent each transect (North, South, and West) among each time period (before and after) treated as fixed factor. The table derived from COI dataset.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*P-value*
Groups	5	0.014	0.003	1.708	0.165
Residuals	18	0.029	0.001		

Appendix 7.3.1.4. Mean values of homogeneity of multivariate dispersion (average distance to the group centroid) for distances (25, 100, 200, and 500 m) grouped in both time periods (before and after) using COI dataset.

Distance to the cages (m)	*Average distance to median (composition dispersion)*
25	0.5367
100	0.5395
200	0.5588
500	0.5307

Appendix 7.3.1.5. Mean values of homogeneity of multivariate dispersion (average distance to the group centroid) for transects (North, South, and West) grouped in both time periods (before and after) using COI dataset.

Transect	*Average distance to median (composition dispersion)*
North	0.5488
South	0.5545
West	0.5231

Appendix 7.3.1.6. Mean values of beta diversity measured as homogeneity of multivariate dispersion (average distance to the group centroid) for distances (25, 100, 200, and 500 m) in each time period (before and after) using COI dataset.

Distance (m) on each time period	*Average distance to median (beta diversity)*
25 – After	0.4257
25 – Before	0.4842
100 – After	0.4297
100 – Before	0.4848
200 – After	0.4622
200 – Before	0.5201
500 – After	0.4553
500 – Before	0.4774

Appendix 7.3.1.7. Mean values of beta diversity measured as homogeneity of multivariate dispersion (average distance to the group centroid) for transects (North, South, and West) in each time period (before and after) using COI dataset.

Transect on each time period	*Average distance to median (beta diversity)*
North – After	0.4582
North – Before	0.4956
South – After	0.4703
South – Before	0.5219
West – After	0.4502
West – Before	0.4834

Appendix 7.3.1.8. Pair-wise permutation test of homogeneity of multivariate dispersions (beta diversity) for distances (25, 100, 200, and 500 m) in each time period (before and after) using COI dataset. P values after FDR correction are indicated.

Pairwise comparison	*p-adjusted*
After 25 – After 100	0.714
After 25 – After 200	0.851
After 25 – After 500	0.170
After 25 – Before 25	0.099
After 25 – Before 100	0.322
After 25 – Before 200	0.612
After 25 – Before 500	0.309
After 100 – After 200	0.503
After 100 – After 500	0.478
After 100 – Before 25	0.232
After 100 – Before 100	0.195
After 100 – Before 200	0.072
After 100 – Before 500	0.236
After 200 – After 500	0.854
After 200 – Before 25	0.632
After 200 – Before 100	0.614
After 200 – Before 200	0.249
After 200 – Before 500	0.722
After 500 – Before 25	0.405
After 500 – Before 100	0.323
After 500 – Before 200	0.094
After 500 – Before 500	0.442
Before 25 – Before 100	0.978
Before 25 – Before 200	0.329
Before 25 – Before 500	0.831
Before 100 – Before 200	0.279
Before 100 – Before 500	0.782
Before 200 – Before 500	0.217

Appendix 7.3.1.9. Pair-wise permutation test of homogeneity of multivariate dispersions (beta diversity) for transects (North, South, and West) on each time period (before and after) using COI dataset. P values after FDR correction are indicated.

Pairwise comparison	*p-adjusted*
After North – After South	0.714
After North – After West	0.851
After North – Before North	0.170
After North – Before South	0.099
After North – Before West	0.322
After South – After West	0.612
After South – Before North	0.309
After South – Before South	0.152
After South – Before West	0.584
After West – Before North	0.185
After West – Before South	0.103
After West – Before West	0.327
Before North – Before South	0.242
Before North – Before West	0.124
Before South – Before West	0.133

7.3.2 18S marker

Appendix 7.3.2.1. Mean values of homogeneity of multivariate dispersion (average distance to the group centroid) for time periods (before and after) using 18S dataset.

Time period	*Average distance to median (beta diversity)*
Before	0.5171
After	0.5067

Appendix 7.3.2.2. Pairwise PERMDISP test with 999 permutations comparing beta diversity among groups. Groups represent each distance (25, 100, 200, and 500 m) among each time period (before and after) treated as fixed factor. The table derived from 18S dataset.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*P-value*
Groups	7	0.004	0.0005	0.288	0.953
Residuals	16	0.032	0.0020		

Appendix 7.3.2.3. Pairwise PERMDISP test with 999 permutations comparing beta diversity among groups. Groups represent each transect (North, South, and West) as factor on each time period (before and after) as factor. The table derived from 18S dataset.

Factor	*df*	*SS*	*MS*	*Pseudo-F*	*P-value*
Groups	5	0.007	0.0014	2.334	0.089
Residuals	18	0.011	0.0006		

Appendix 7.3.2.4. Mean values of homogeneity of multivariate dispersion (average distance to the group centroid) for distances (25, 100, 200, and 500 m) grouped in both time periods (before and after) using 18S dataset.

Distance to the cages (m)	*Average distance to median (composition dispersion)*
25	0.4966
100	0.5004
200	0.4912
500	0.4842

Appendix 7.3.2.5. Mean values of homogeneity of multivariate dispersion (average distance to the group centroid) for transects (North, South, and West) grouped in both time periods (before and after) using 18S dataset.

Transect	*Average distance to median (composition dispersion)*
North	0.4979
South	0.5140
West	0.4642

Appendix 7.3.2.6. Mean values of beta diversity measured as homogeneity of multivariate dispersion (average distance to the group centroid) for distances (25, 100, 200, and 500 m) in each time period (before and after) using 18S dataset.

Distance (m) on each time period	*Average distance to median (beta diversity)*
25 – After	0.4238
25 – Before	0.4606
100 – After	0.4463
100 – Before	0.4544
200 – After	0.4270

200 – Before	0.4534
500 – After	0.4340
500 – Before	0.4331

Appendix 7.3.2.7. Mean values of beta diversity measured as homogeneity of multivariate dispersion (average distance to the group centroid) for transects (North, South, and West) in each time period (before and after) using 18S dataset.

Transect on each time period	*Average distance to median (beta diversity)*
North – After	0.4395
North – Before	0.4565
South – After	0.4706
South – Before	0.4697
West – After	0.4322
West – Before	0.4272

Appendix 7.3.2.8. Pair-wise permutation test of homogeneity of multivariate dispersions (beta diversity) for distances (25, 100, 200, and 500 m) in each time period (before and after) using 18S dataset. P values after FDR correction are indicated.

Pairwise comparison	*p-adjusted*
After 25 – After 100	0.686
After 25 – After 200	0.923
After 25 – After 500	0.632
After 25 – Before 25	0.108
After 25 – Before 100	0.060
After 25 – Before 200	0.275
After 25 – Before 500	0.806
After 100 – After 200	0.740
After 100 – After 500	0.814
After 100 – Before 25	0.769
After 100 – Before 100	0.898
After 100 – Before 200	0.899
After 100 – Before 500	0.841
After 200 – After 500	0.843
After 200 – Before 25	0.360

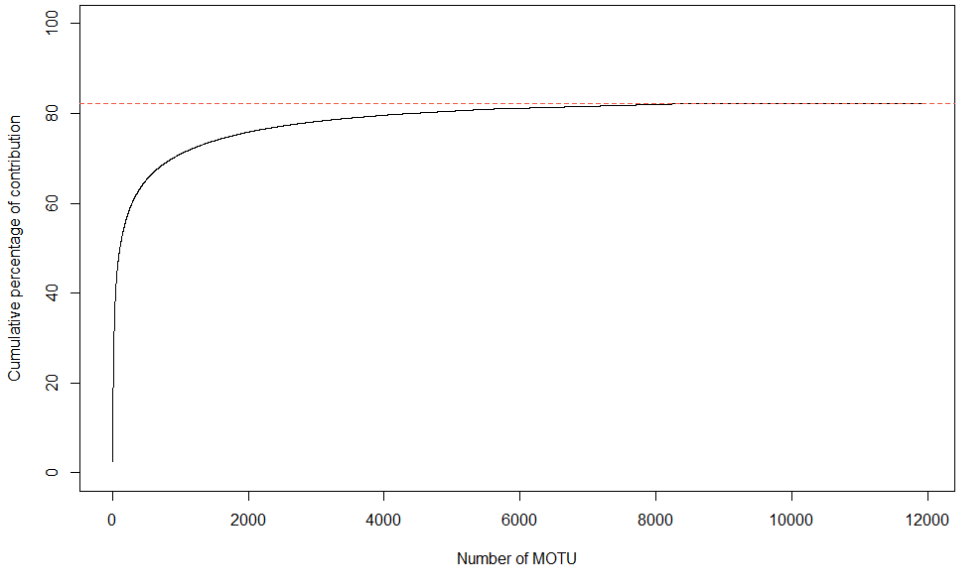
After 200 – Before 100	0.366
After 200 – Before 200	0.497
After 200 – Before 500	0.902
After 500 – Before 25	0.343
After 500 – Before 100	0.352
After 500 – Before 200	0.522
After 500 – Before 500	0.976
Before 25 – Before 100	0.723
Before 25 – Before 200	0.813
Before 25 – Before 500	0.487
Before 100 – Before 200	0.981
Before 100 – Before 500	0.566
Before 200 – Before 500	0.637

Appendix 7.3.2.9. Pair-wise permutation test of homogeneity of multivariate dispersions (beta diversity) for transects (North, South, and West) on each time period (before and after) using 18S dataset. Significant P values after FDR correction are indicated in bold.

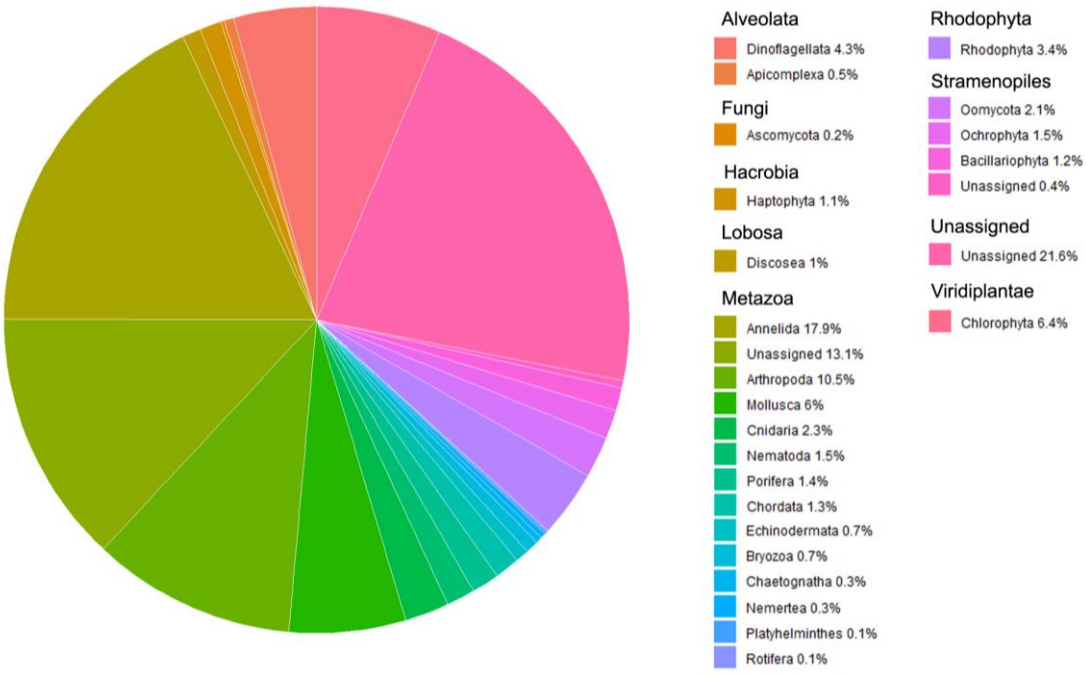
Pairwise comparison	*p-adjusted*
After North – After South	0.217
After North – After West	0.605
After North – Before North	0.339
After North – Before South	0.134
After North – Before West	0.413
After South – After West	0.110
After South – Before North	0.576
After South – Before South	0.964
After South – Before West	0.075
After West – Before North	0.147
After West – Before South	**0.037**
After West – Before West	0.572
Before North – Before South	0.480
Before North – Before West	0.100
Before South – Before West	**0.025**

7.4 Supplementary material S4. SIMPER analysis

7.4.1 COI



Appendix 7.4.1.1. Cumulative contribution to the total dissimilarity of samples (replicate pooled) between time periods (before and after) with increasing number of MOTUs. Graph derived from SIMPER analysis using Bray-Curtis dissimilarity index on relative abundances of COI dataset. The total contribution of MOTUs (peak) to the dissimilarity between time periods is indicated in red line (n=82.002%)

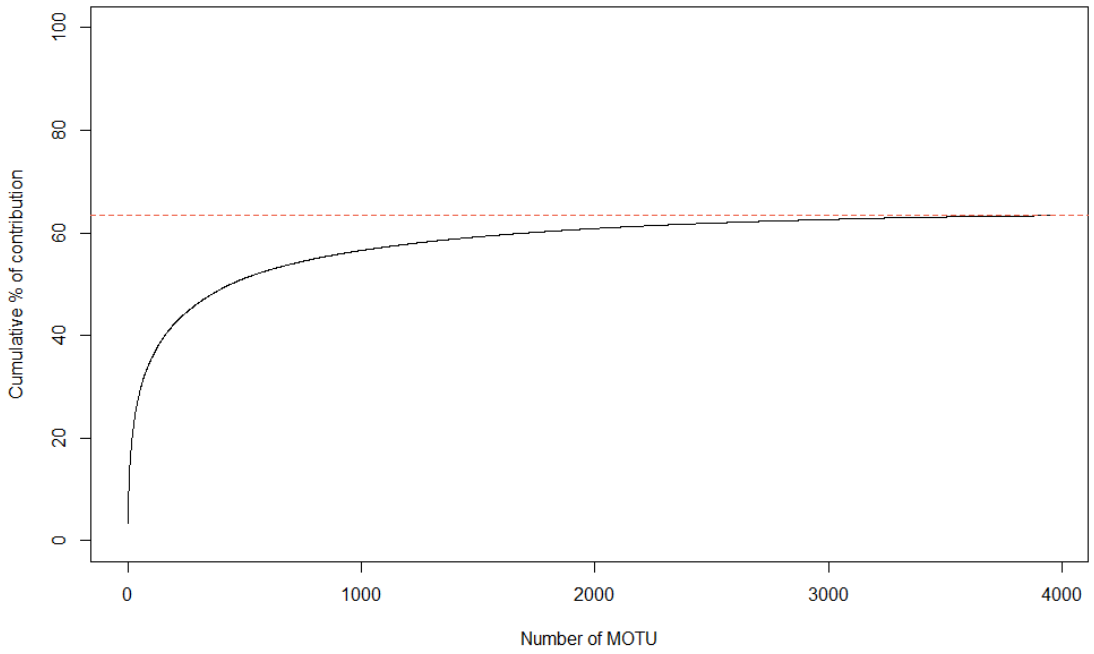


Appendix 7.4.1.2. Pie chart presentation on summary of SIMPER analysis using Bray-Curtis dissimilarity index on relative abundances from COI dataset. Cumulative contribution of Phylum grouped by Kingdom are indicated in percentage. Percentages are relative to the total contribution of MOTUs (n=82.002%). Phylum with contribution less than 0.1% were removed from the graph from simplicity.

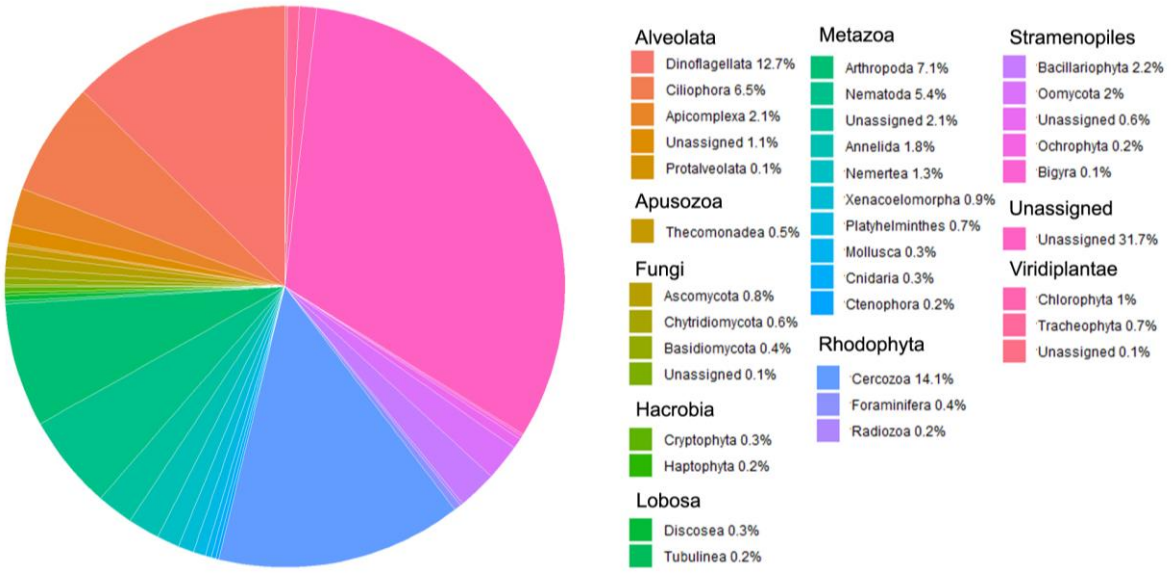
Appendix 7.4.1.3. Summary of SIMPER analysis using Bray-Curtis dissimilarity index on relative abundances of COI dataset. Cumulative contribution of Phylum grouped by Kingdom are indicated in percentage. Percentages are relative to the total contribution of MOTUs (n=82.002%).

Kingdom	*Phylum*	*Contribution (%)*
Alveolata		
	Dinoflagellata	4.254
	Apicomplexa	0.511
	Unassigned	0.012
	Ciliophora	0.003
Apusozoa		
	Thecomonadea	0.026
Discoba		
	Percolozoa	0.02
	Euglenozoa	0.005
Fungi		
	Ascomycota	0.176
	Mucoromycota	0.038
	Basidiomycota	0.037
	Unassigned	0.007
	Blastocladiomy-cota	0.002
Hacrobia		
	Haptophyta	1.117
	Cryptophyta	0.011
Lobosa		
	Discosea	0.957
Metazoa		
	Annelida	17.872
	Unassigned	13.07
	Arthropoda	10.49
	Mollusca	6.01
	Cnidaria	2.275
	Nematoda	1.478
	Porifera	1.416
	Chordata	1.284
	Echinodermata	0.687
	Bryozoa	0.667
	Chaetognatha	0.336
	Nemertea	0.278
	Platyhelminthes	0.125
	Rotifera	0.102
	Xenacoelomorpha	0.078
	Ctenophora	0.001
	Brachiopoda	0.001
Rhizaria		
	Cercozoa	0.016
Rhodophyta		
	Rhodophyta	3.432
Stramenopiles		
	Oomycota	2.119
	Ochrophyta	1.476
	Bacillariophyta	1.191
	Unassigned	0.374
	Bigyra	0.007
Unassigned		
	Unassigned	21.624
Viridiplantae		
	Chlorophyta	6.408
	Unassigned	0.007

7.4.2 18S



Appendix 7.4.2.1. Cumulative contribution to the total dissimilarity of samples (replicate pooled) between time periods (before and after) with increasing number of MOTUs. Graph derived from SIMPER analysis using Bray-Curtis dissimilarity index on relative abundances of 18S dataset. The total contribution of MOTUs (peak) to the dissimilarity between time periods is indicated in red line (n=63.35%).



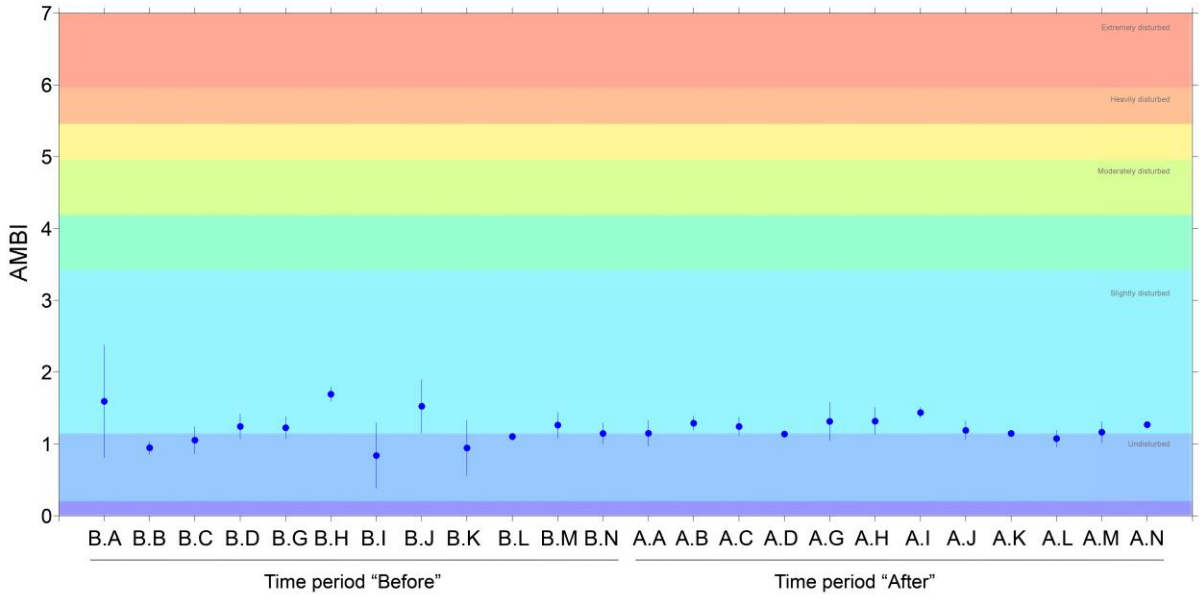
Appendix 7.4.2.2. Pie chart presentation on summary of SIMPER analysis using Bray-Curtis dissimilarity index on relative abundances of 18S dataset. Cumulative contribution of Phylum grouped by Kingdom are indicated in percentage. Percentages are relative to the total contribution of MOTUs (n=63.35%). Phylum with contribution less than 0.1% were removed from the graph from simplicity.

Appendix 7.4.2.3. Summary of SIMPER analysis using Bray-Curtis dissimilarity index on relative abundances of 18S dataset. Cumulative contribution of Phylum grouped by Kingdom are indicated in percentage. Percentages are relative to the total contribution of MOTUs (n=63.35%). The table continues in the next page.

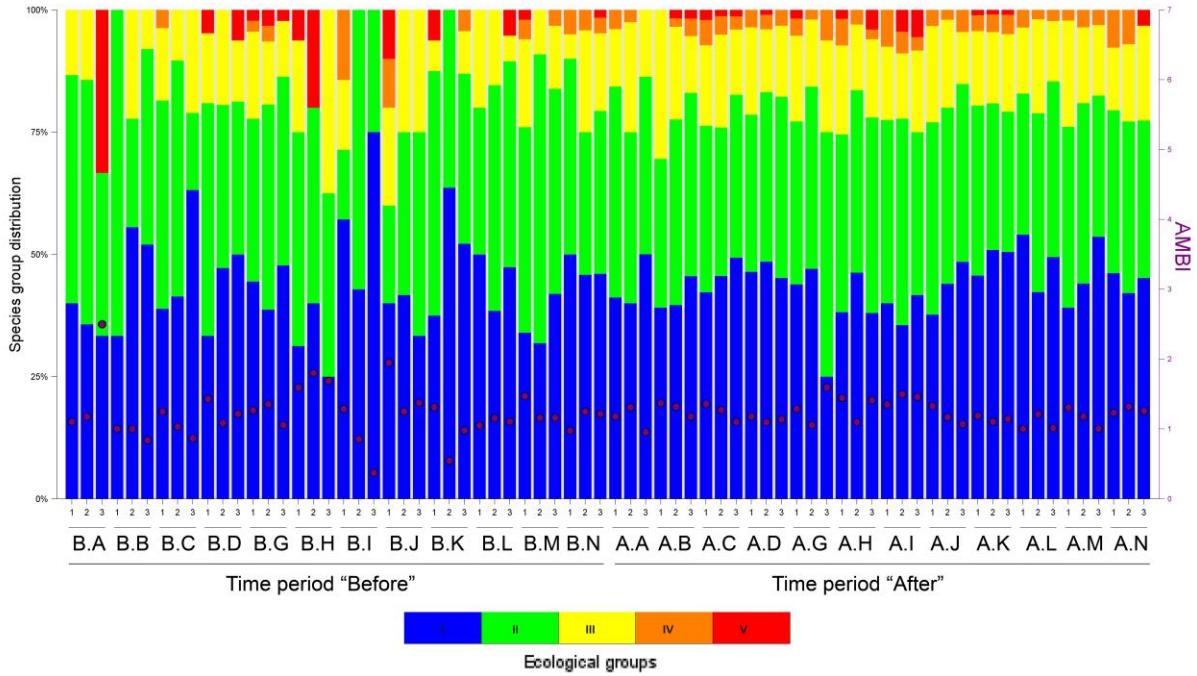
Kingdom	*Phylum*	*Contribution (%)*
Alveolata		
	Dinoflagellata	12.673
	Ciliophora	6.499
	Apicomplexa	2.095
	Unassigned	1.061
	Protalveolata	0.126
Apusozoa		
	Thecomonadea	0.484
	Planomonadea	0.019
Choanozoa		
	Choanofila	0.099
	Cristidiscoidia	0.002
Conosa		
	Mycetozoa	0.047
Discoba		
	Percolozoa	0.001
Fungi		
	Ascomycota	0.78
	Chytridiomycota	0.584
	Basidiomycota	0.389
	Unassigned	0.112
	Mucoromycota	0.099
	Glomeromycota	0.008
	Zygomycota	0.003
	Cryptomycota	0.001
Hacrobia		
	Cryptophyta	0.314
	Haptophyta	0.186
	Heliozoa	0.046
Lobosa		
	Discosea	0.304
	Tubulinea	0.17
Metazoa		
	Arthropoda	7.083
	Nematoda	5.416
	Unassigned	2.084
	Annelida	1.81
	Nemertea	1.279
	Xenacoelomorpha	0.894
	Platyhelminthes	0.713
	Mollusca	0.343
	Cnidaria	0.257
	Ctenophora	0.188
	Gnathostomulida	0.062
	Hemichordata	0.059
	Porifera	0.057
	Chordata	0.052
	Cephalorhyncha	0.009
	Echinodermata	0.007
	Bryozoa	0.007
	Blastocladiomycota	0.005
	Gastrotricha	0.003
Rhizaria		
	Cercozoa	14.052
	Foraminifera	0.398
	Radiozoa	0.158
	Unassigned	0.045
Rhodophyta		
	Rhodophyta	0.031
Stramenopiles		
	Bacillariophyta	2.228
	Oomycota	2.05
	Unassigned	0.611
	Ochrophyta	0.189

Kingdom	*Phylum*	*Contribution (%)*
Stramenopiles		
	Bigyra	0.116
	Hyphochytriomycota	0.056
Teretosporea		
	Mesomycetozoea	0.009
Unassigned		
	Unassigned	31.736
Viridiplantae		
	Chlorophyta	0.983
	Tracheophyta	0.677
	Unassigned	0.148
	Streptophyta	0.064
	Bryophyta	0.02

7.5 Supplementary material S5. AMBI values



Appendix 7.5.1. Estimated mean AMBI values (scale on the left) and disturbance classification (indicated in colours; scale on the right) among stations on both time periods (before and after). Bars indicate the total variability within each station (dispersion).



Appendix 7.5.2. Chart representing AMBI values (with dots; scale on the right) and distribution (in %; scale on the left) of ecological groups (blue, green, yellow, orange, and red) per each replicate in stations on both time periods (before and after).

Appendix 7.5.3. Table representing mean AMBI values and distribution (in %) of ecological groups (I,II,III,IV, and V) among stations on both time periods (before and after).

Time period	*Station*	*Ecological groups*					*Mean AMBI value*	*Richness*	*Diversity*
		I(%)	*II(%)*	*III(%)*	*IV(%)*	*V(%)*			
Before	B.A	38.3	46.8	12.8	0	2.1	1.593	41	5.28
Before	B.B	48.8	41.9	9.3	0	0	0.947	34	4.99
Before	B.C	44.1	39.2	14.7	2	0	1.051	66	5.89
Before	B.D	43.8	37	16.4	0	2.7	1.244	46	5.38
Before	B.G	44.2	37.5	14.2	1.7	2.5	1.226	65	5.88
Before	B.H	31	41.4	20.7	0	6.9	1.694	23	4.44
Before	B.I	55.6	33.3	5.6	5.6	0	0.839	16	3.95
Before	B.J	39.1	32.6	23.9	2.2	2.2	1.525	35	4.98
Before	B.K	50	40	6	2	2	0.945	38	5.1
Before	B.L	45.2	40.5	11.9	0	2.4	1.103	28	4.65
Before	B.M	35.9	45.6	14.6	2.9	1	1.263	71	6
Before	B.N	46.7	33.6	15	3.7	0.9	1.146	84	6.28
After	A.A	42.5	38.9	15.9	2.7	0	1.148	76	6.09
After	A.B	43	36.8	16.1	2.6	1.6	1.289	128	6.86
After	A.C	45.4	32.7	16.3	4	1.6	1.244	127	6.84
After	A.D	46.9	34.2	15.3	3.3	0.4	1.138	139	6.97
After	A.G	42.7	37.1	16.1	3.2	0.8	1.314	87	6.31
After	A.H	41.3	37.8	15.7	3.5	1.7	1.317	103	6.52
After	A.I	38.8	38	14.9	5	3.3	1.436	83	6.23
After	A.J	43.5	37.3	15.8	3.4	0	1.189	98	6.46
After	A.K	49.2	31	15.2	3.6	1	1.146	150	7.08
After	A.L	50	32.9	14.3	2.8	0	1.074	134	6.93
After	A.M	47.1	33.5	16.3	3.1	0	1.163	132	6.88
After	A.N	44.1	33.9	15.7	5.5	0.8	1.268	85	6.26

Appendix 7.5.4. List of species from COI marker matched in AMBI reference list and their ecological group value. The table continues beyond this page.

Taxa	*AMBI ecological group*	*Taxa*	*AMBI ecological group*
Acanthochitona crinita	I	*Bugula* sp.	I
Acanthonyx lunulatus	I	*Bulla striata*	II
Achelia sp.	I	*Bylgides* sp.	I
Aeolidia sp.	I	*Caecum trachea*	I
Aglaophamus malmgreni	II	*Calliostoma virescens*	I
Aglaophamus sp.	II	*Callipallene brevirostris*	I
Akera bullata	I	*Callochiton septemvalvis*	I
Alcyonium sp.	I	*Campanularia hincksii*	II
Alitta virens	III	*Capitella capitata*	V
Amblyosyllis sp.	II	*Caprella equilibra*	II
Amphilepis norvegica	I	*Caprella fretensis*	II
Amphipholis squamata	I	*Caprella* sp.	II
Amphiura filiformis	II	*Cephalothrix* sp.	III
Ampithoe ramondi	III	*Ceratocephale loveni*	II
Aphelochaeta sp.	IV	*Cestopagurus timidus*	I
Apherusa sp.	I	*Chaetopterus* sp.	I
Aplysia fasciata	I	*Chaetozone setosa*	IV
Aplysia sp.	I	*Chiton olivaceus*	II
Arbacia lixula	II	*Chlamys islandica*	I
Arca sp.	I	*Ciliatocardium ciliatum*	I
Arcopsis interplicata	I	*Cirratulus* sp.	IV
Arctica islandica	III	*Clanculus cruciatus*	I
Ascidiella aspersa	III	*Clava multicornis*	I
Aspidosiphon muelleri	I	*Cliona* sp.	III
Aspidosiphon sp.	I	*Clione limacina*	I
Athanas nitescens	I	*Clymenura borealis*	III
Balanus trigonus	II	*Clytia gracilis*	I
Barantolla americana	II	*Clytia hemisphaerica*	I
Barbatia sp.	II	*Clytia linearis*	I
Botryllus schlosseri	I	*Clytia* sp.	I

Brissopsis lyrifera	I	*Columbella rustica*	I
Bugula flabellata	I	*Corophium* sp.	III

Taxa	*AMBI ecological group*	*Taxa*	*AMBI ecological group*
Corynactis californica	I	*Euphysa* sp.	II
Coscinasterias tenuispina	I	*Eupolymnia* sp.	III
Ctenodiscus crispatus	I	*Exogone verugera*	II
Cucumaria sp.	I	*Galathea intermedia*	I
Cumella sp.	II	*Galathowenia oculata*	III
Dexamine spiniventris	III	*Gattyana cirrhosa*	III
Dexamine spinosa	III	*Gibbula turbinoides*	I
Diopisthoporus sp.	II	*Golfingia margaritacea*	I
Diplocirrus glaucus	I	*Goniada maculata*	II
Discoconchoecia elegans	III	*Grania* sp.	V
Dodecaceria concharum	IV	*Gyptis* sp.	II
Dodecaceria sp.	IV	*Haliclona* sp.	I
Donax sp.	I	*Haliotis tuberculata*	I
Doto sp.	I	*Halosydna* sp.	II
Dynamene magnitorata	II	*Haminoea* sp.	II
Echinaster sepositus	I	*Haplostylus normani*	II
Echinocardium cordatum	I	*Harmothoe* sp.	II
Ectopleura larynx	I	*Hauchiella tribullata*	I
Elasmopus sp.	III	*Henricia* sp.	I
Electra pilosa	II	*Hesiospina aurantiaca*	II
Endeis spinosa	II	*Heteroclymene robusta*	V
Epizoanthus sp.	I	*Heteromastus filiformis*	IV
Ericthonius brasiliensis	I	*Hiatella arctica*	I
Ericthonius punctatus	I	*Holothuria forskali*	I
Eriopisa elongata	I	*Holothuria* sp.	I
Eteone sp.	III	*Holothuria tubulosa*	I
Eualus cranchii	I	*Hydroides elegans*	II
Eudorella emarginata	II	*Hydroides sanctaecrucis*	III
Eumida kelaino	II	*Hydroides* sp.	III
Eumida sp.	II	*Inachus dorsettensis*	I
Eunice sp.	II	*Ischyrocerus* sp.	II
Euphrosine foliosa	I	*Isozoanthus sulcatus*	I

Taxa	*AMBI ecological group*	*Taxa*	*AMBI ecological group*
Janira maculosa	I	*Musculus subpictus*	I
Jassa pusilla	V	*Mya truncata*	II
Jassa slatteryi	II	*Myrianida longoprimicirrata*	II
Kefersteinia cirrata	II	*Myrianida* sp.	II
Kurtiella bidentata	III	*Mytilus edulis*	III
Laomedea calceolifera	II	*Mytilus* sp.	III
Laticorophium baconi	II	*Nassarius incrassatus*	II
Leodice harassii	II	*Neanthes acuminata*	III
Lepidonotus clava	II	*Nephasoma* sp.	I
Leptochelia dubia	III	*Nereis falsa*	III
Leucon nasica	II	*Nereis* sp.	III
Levinsenia gracilis	III	*Nothria conchylega*	II
Lima loscombi	I	*Nucula* sp.	I
Limaria hians	I	*Nymphon* sp.	I
Lineus bilineatus	III	*Obelia dichotoma*	II
Lumbrineris perkinsi	II	*Obelia geniculata*	II
Lumbrineris sp.	II	*Odontosyllis fulgurans*	II
Lysidice collaris	II	*Odontosyllis gibba*	II
Lysidice ninetta	II	*Oerstedia dorsalis*	III
Macoma calcarea	II	*Onoba semicostata*	I
Macrochaeta polyonyx	II	*Ophiactis* sp.	II
Malacobdella grossa	III	*Ophiocomina nigra*	I
Malacoceros sp.	III	*Ophiocten gracilis*	II
Marthasterias glacialis	I	*Ophiocten sericeum*	II
Meganyctiphanes norvegica	I	*Ophiopholis aculeata*	II
Membranipora membranacea	I	*Ophiothrix fragilis*	I
Metridium sp.	I	*Ophiura carnea*	II
Microdeutopus chelifer	I	*Ophiura robusta*	II
Micrura sp.	I	*Ophiura sarsii*	II
Modiolula phaseolina	I	*Ophryotrocha* sp.	IV
Modiolus modiolus	II	*Orbinia latreillii*	I
Monocorophium acherusicum	III	*Ostrea stentina*	I

Taxa	*AMBI ecological group*	*Taxa*	*AMBI ecological group*
Pachygrapsus marmoratus	II	*Proceraea* sp.	II
Pandalus borealis	II	*Pseudamussium peslutrae*	II
Paracentrotus lividus	I	*Pseudoprotella phasma*	III
Paramphinome sp.	III	*Pusillina inconspicua*	I
Parvicardium exiguum	I	*Quadrimaera inaequipes*	I
Patella sp.	I	*Rissoa lilacina*	I
Pectinaria auricoma	I	*Rissoa parva*	I
Pectinaria hyperborea	I	*Rocellaria dubia*	I
Pectinaria koreni	IV	*Ruditapes decussatus*	I
Phascolosoma granulatum	II	*Sabellaria spinulosa*	I
Philine aperta	II	*Sagitta elegans*	I
Pholoe baltica	I	*Scalibregma* sp.	III
Phtisica marina	I	*Scoletoma fragilis*	II
Phyllochaetopterus socialis	I	*Scoloplos armiger*	III
Phyllodoce groenlandica	IV	*Sipunculus norvegicus*	I
Pilumnus hirtellus	I	*Spadella cephaloptera*	III
Pilumnus sp.	I	*Spatangus purpureus*	I
Pinna rudis	I	*Spio* sp.	III
Pisa carinimana	I	*Spiophanes kroyeri*	III
Pisa sp.	I	*Spisula subtruncata*	I
Pisidia longicornis	I	*Stegopoma plicatile*	I
Pista cristata	I	*Stenothoe monoculoides*	II
Planocera sp.	II	*Striarca lactea*	I
Platynereis dumerilii	III	*Strongylocentrotus droebachiensis*	I
Podocerus variegatus	III	*Styela* sp.	II
Polycera sp.	I	*Syllidia armata*	II
Polycirrus carolinensis	IV	*Syllis armillaris*	II
Polycirrus medusa	IV	*Syllis ferrani*	II
Polydora cornuta	IV	*Syllis gerlachi*	II
Polyphysia sp.	III	*Syllis gracilis*	III
Porcellana platycheles	I	*Syllis hyalina*	II
Proceraea aurantiaca	II	*Syllis* sp.	II

Taxa	*AMBI ecological group*	*Taxa*	*AMBI ecological group*
Synalpheus sp.	II	*Thyasira obsoleta*	I
Tectura testudinalis	I	*Thyasira* sp.	II
Terebella lapidaria	I	*Trichobranchus* sp.	II
Terebellides gracilis	I	*Tricolia pullus*	I
Terebellides sp.	I	*Unciola planipes*	I
Tetrastemma flavidum	III	*Verruca stroemia*	I
Thelepus cincinnatus	II	*Yoldiella* sp.	I
Themisto gaudichaudii	III	*Thyasira obsoleta*	I
Thyasira equalis	III	*Thyasira* sp.	II