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Assessing the effect of salmon aquaculture on fungal diversity in seawater and sediments through eDNA metabarcoding

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Abstract

Fungi have been found to play important roles in marine ecosystems, e.g., symbionts of plants and animals, and as decomposers of organic materials. The fungal communities, however, are often considered less studied and their functional and taxonomic diversity in aquatic environments remains poorly understood. Since the 1970s the Norwegian salmon industry developed from a small-scale industry to becoming the world's leading producer of Atlantic salmon (*Salmo salar L.*). To investigate the impact of aquaculture on fungal diversity, fungal DNA was extracted and amplified from environmental samples collected at an active commercial scale aquaculture facility in Arctic Norway. In this study, environmental samples from water and sediment surrounding the aquaculture were collected and investigated for the determination of fungal community diversity and composition variance. Through 18S metabarcoding, a high-level fungal diversity was revealed by 4,554 Molecular Operational Taxonomic Units (MOTUs) that spanned nine phyla. Over a grid of 12 sampling points at increasing distance from the farm, all effected the fungal diversity, and combined with known physical factors (season), provided evidence that fungal community diversity and composition are highly influenced by seasonal variations. To distinguish the patterns of impact, alpha and beta diversity for each sampling point were analyzed. Analysis showed that the aquaculture only affected the alpha diversity within 250m from the farm in water samples. Although, alpha diversity suggested impact in water, no such observations were observed for sediments. The study also finds that the beta diversity indicated a higher spatial variation in fungal diversity in water samples compared to sediments. These findings support the hypothesis that the fungal diversity decreases with increased distance from the aquaculture cages for water samples but is rejected for sediment samples. The discoveries highlight the capability of 18S metabarcoding to assess the spatiotemporal trends of fungal community diversity and composition, encouraging the need for further biodiversity assessments.

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1 Introduction

1.1 Origins of aquaculture

Aquaculture is defined as "the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants" (Food and Agriculture Organization of the United Nations (FAO, 2017)). Another key point in the FAO definition is that farming implies some form of intervention in the rearing process to enhance production, such as regular stock, feeding and protection from predators (FAO, 2017). Aquaculture has existed for thousands of years and has been adopted among many people for food production (Costa-Pierce, 2002). The development of aquaculture is correlated with the nutritional demand of fishes and other marine organisms. The organization of fisheries dates back to the tenth century where Europeans started catching fish for sale, which was the start of a small-scale commercial fishery industry (Hoffmann, 2004). Following the start of aquaculture, the attempts to increase fish yields by engineering cages and netted-off areas in the ocean and lakes started (Costa-Pierce, 2002). In Norway, aquaculture was a relative unknown area until around 1850 when the first hatchings of brown trout (*Salmo trutta*) took place. The first attempts at pond culture were initiated in 1900 with the import of rainbow trout (*Oncorhynchus mykiss*) from Denmark (FAO, 2005). The major breakthrough came in the 1970s when the onshore tanks were replaced with the first cages following the sprout of the commercial aquaculture. Since that time, aquaculture has developed into a major industry with significant fish farming operations in the Arctic area (Figure 1) (Aarset et al., 2009; FAO, 2005; Stien et al., 2020). As a result, salmon aquaculture has increased its production from thousands of tonnes in 1980 to around 1.3 million tonnes in 2019 (Larsen & Asche, 2011; Directorate of fisheries, 2020).

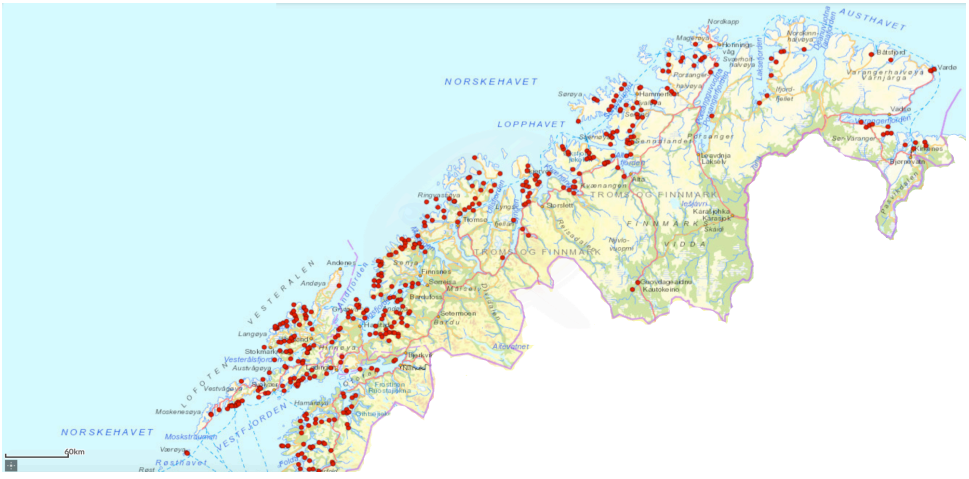

Figure 1. Detailed map of aquaculture sites in Norwegian Arctic area. Red circles indicate sites with current production (Directorate of fisheries; retrieved from portal.fiskedir.no).

1.2 Impacts of open-sea cage aquaculture on seabed and benthic community

Since the 1970s the Norwegian salmon industry has developed from a small-scale industry to become the world's leading producer of Atlantic salmon (*Salmo salar L.*). Although the impact of aquaculture on fungal diversity is not known, many studies have investigated benthic impacts of fish farming on soft sediment benthic systems (Taranger et al., 2014). In general, due to high levels of water circulation and relatively deep fjords, discharges of nutrients, salts and organic material from fish farming are a minor environmental problem in Norway (Anon, 2009). However, as a result of essentially being an open system, the discharge of nutrient salts and organic materials are usually characterized as being a significant factor in many environmental and ecological impacts on seabed (Anon, 2009; Islam, 2005; Martinez-Porchas & Martinez-Cordova, 2012). A major bulk of waste foods and faeces deriving from salmon farming seabeds result in water quality problems as both faeces and food waste are denser than sea water and sink to the sediments (Dowle et al., 2015; Lazzari & Baldisserotto, 2008). Fish farming is carried out in different geographic areas, at sites varying in depth over seabed and various sediment types. The size of farms in terms of hydrodynamic regime, production and management play important roles in determining the effects of fish farming on seabed (Kalantzi & Karakassis, 2006). Also, there is a large variation in current velocities along the Norwegian coast and in the fjords, which means greater variation in dispersion of organic material from fish farms (Buhl-Mortensen et al., 2013).

Direct effects of changes in chemistry and ocean temperature, caused for example by climate change or by aquaculture activities, are having profound and diverse consequences (Doney et al., 2012). Several studies have reported that eutrophication of the ecosystem and mortality of benthos and fish is the result of the excessive nitrogen caused by fish farming productions (Islam, 2005). Conversely, Lazzari & Baldisserotto (2008), states that approximately 78-80% of the dietary nitrogen is lost to the environment as a result of food waste. Significant quantities of phosphorus (P) and nitrogen (N) are incorporated into fish feeds to balance essential nutrients (Lazzari & Baldisserotto, 2008). Similarly, like protein and other nutrient requirements, fish are dependent on a definable dietary P requirement for hard tissue development and structure of the digestive tract (Herath & Satoh, 2015). Hence, the main end-product of protein metabolism in teleost fish is ammonia, while in some species nitrogenous waste is excreted as urea (Herath & Satoh, 2015; Lazzari & Baldisserotto, 2008). However, N and P can not only affect the environment as a whole, but also rearing water (Lazzari & Baldisserotto, 2008). For instance, external supply of N and P can lead to excessive plant growth (eutrophication) and

change the indigenous flora and fauna in aquatic ecosystems. In addition, dissolved N and P can accelerate the growth of primary producers such as phytoplankton. Consequently, increased phytoplankton production will limit the light penetration and interrupt aquatic vegetation due to enhanced turbidity of the upper water levels (Herath & Satoh, 2015). However, according to a study done by Guo et al., (2015), the concentration of dissolved silicate in surface water together with N:P in porewater, were the most significant abiotic variables shaping the planktonic and benthic communities. The data suggested that the N:P ratio indicated benthic fungi having an active role in anaerobic processes such as nitrogen mineralization and organic matter decomposition (Guo et al., 2015). Yet, due to the ultimate fate of suspended particular matter sinking and settling on the sea floor, sediment systems are object to influences in the sediment stratigraphy. Hence, increase in organic matter input can produce important changes in the sediment chemical parameters, as well as deleterious consequences for the seabed communities (Sanz-Lázaro & Marin, 2008). However, benthic communities are supported only by a relatively minor rate of organic matter and nutrient flux to the seabed. As a result, great quantities of organic matter input can greatly influence and exceed the carrying capacity of the ecosystem, producing important changes in the sediment chemical parameters. Consequently, organic matter overload can result in anoxic conditions and introduce bacteria to the seafloor. For instance, *Beggiatoa*, a type of bacteria that produces sulphides, are toxic to the inhabiting fauna at high concentrations and may lead to depletion of sensitive species (Sanz-Lázaro & Marin, 2008).

Resuspension and consumption of feed pellets by wild fish is considered to be important modifiers of dispersion of waste products, that further enlarge the waste area (Holmer et al., 2007; Sarà et al., 2004). A study done by Holmer et al., (2007), revealed the sedimentation of waste particulate products was higher under the net cages. However, Buhl-Mortensen et al., (2013) and Holmer et al., (2007), both emphasized that the particle size associated with waste products and pellets from fish farms are much larger than the ambient seston. As a result, even at relatively high current velocities, fast sinking rates (>5 cm/s^{-1}) of the materials derived from fish farms will settle underneath or close to the cages (Holmer et al., 2007). Conversely, Sarà et al., (2004) emphasizes the role of wild and escaped fish functioning as “attracting devices”, consuming the waste products dispersed in the environment around the cage and depositing them elsewhere by defecation. Thus, food pellets being eaten around the cages will indirectly slow down the settling speed of waste particles. As a result, the permanency time of waste particles in the water column is increased, hence allowing water currents

to move the particles far from the cages (Sarà et al., 2004). Additionally, Broch et al., (2017) emphasizes the effects of organic effluents on temporal and spatial patterns in infauna community composition. In their study, all farm sites investigated showed that the distribution of organic matter in the bottom layer was non-homogenous, with significant temporal variations and settling of matter up to 0.5 km away from the studied farms (Broch et al., 2017). Similarly, Kutti et al., (2007) investigated the impact of organic effluents around a fish farm at a deep site over one production cycle. They found that the benthic community had a higher diversity of species close to the farm than the reference station 3 km away where the benthic community was unaffected. Several of the species dominating near the studied farm indicated that the sea bottom within 250 m from the fish farm was affected by a moderate loading of organic matter (Kutti et al., 2007). In addition, Kutti et al., (2007) revealed that the highest abundance of species was twice as high at peak production at 550-900 m from the farm. The peak in fungal diversity observed at an intermediate distance from the salmon farm, corresponds with the fact that fungal diversity and species richness can often be found in areas where organic enrichment is sufficient to provide a rich food source (Kutti et al., 2007).

1.3 Impacts of aquaculture on seabed and benthic community: management approaches

The need to regulate the environmental impacts affecting the benthic communities, dates back to the 1970s when there was a major industry setback after a disagreement over the future access to resources and growth. In the aftermath of the setback in 1970s, the Norwegian government emphasized the need for the government to monitor and regulate the Norwegian aquaculture (Aarset et al., 2009). Thus, Norway has in recent years had the primary objective of promoting sustainable aquaculture development to ensure that the procedures are regulated and managed (FAO, 2009). The administration of the Norwegian aquaculture industry is fragmented into different bodies, examining the fish welfare and environmental management. In addition, the different management areas are allocated to six different ministries and regulatory authorities, each responsible for the regulations regarding fish welfare, environment and fish farm technical standards (Holmen et al., 2018).

In order to implement global standards of environmental quality, most monitoring programs include examination of the benthic environment and water quality (Holmer et al. 2008: Kalantzi & Karakassis, 2006). In particular, Norway is strongly regulated by a monitoring scheme referred to as the Modelling-Ongrowing fish farms-Monitoring (MOM). The MOM-B investigations are performed regularly in close vicinity of the fish cages and are based on

indicators such as pH, redox potential and presence and/or absence of macro-infauna. In addition, the monitoring scheme follow predefined thresholds, categorizing the farming operations into different environmental conditions (1; low, 2; medium, 3; high-organic loading, 4; organic overloading). By doing so, the sediment chemistry around the fish farm is evaluated, either indicating an acceptable or unacceptable state of the sediment and whether the production can proceed. Although the MOM-B estimates the sediment chemistry in the production zone, the MOM-C system has been implemented as an extended investigation to perform quantitative measurements of the organic enrichment and the impact on biodiversity in infauna communities. As with the predefined thresholds in MOM-B, MOM-C categorizes the farming sites into different environmental states (i.e., very good, good, moderate, poor and very poor) (Taranger et al., 2014; Wilson et al., 2009).

Additionally, Norwegian aquaculture is highly regulated by the Ministry of Fisheries and Coastal affairs, working as the main principal authority in regulating and controlling the development of the aquaculture industry (Directorate of fisheries, 2005; Wilson et al., 2009). By being the main authority, the ministry may prescribe detailed provisions to ensure an environmentally responsible aquaculture through administrative decisions or regulations (Directorate of fisheries, 2005). However, since the 1990s the so-called Ecosystem Approach to Fisheries Management (EAFM) has been adopted by the government to sustain healthy marine ecosystems. By generating knowledge of ecosystem processes and consequences following aquaculture, EAFM is able to sustain healthy marine ecosystems, minimize the risk of irreversible change to ecosystem processes and assemblage of species (Gullestad et al., 2017). Conversely, a new Marine Resources Act entered into force in Norway in 2009, ensuring sustainable and economical management of wild living marine resources and the genetic material derived from them (Directorate of Fisheries, 2008; Gullestad et al., 2017). The management principle in the Marine Resources Act confers to the Ministry and reads: “The Ministry shall evaluate which types of management measures are necessary to ensure sustainable management of wild living marine resources” (Gullestad et al., 2017). The principle emphasizes that the Ministry is of obligation to evaluate which types of management measures are necessary to ensure sustainable management of wild living marine resources (Directorate of Fisheries, 2008; Gullestad et al., 2017).

1.4 Diversity and the ecological roles of marine fungi

Marine fungi are known for their contribution to nutrient cycling in ecosystems with an important role as symbionts associated with animals and plants. Fungi are heterotrophs as they get their energy needed from other organisms, and they use organic matter produced by living (pathogens and parasites) or dead organic matter (saprotrophs) for their growth and reproduction (Khoo, 2000). Being key players in terrestrial environments and performing vital functions as decomposers, fungi represent a noteworthy proportion of the microbial diversity on Earth (Richards et al., 2012). Although Hawksworth (1991, 2001) estimated the total diversity of fungi to be 1.5-1.6 million species, a revision of global fungal diversity increased Hawksworth's estimate of 1.5 million species to 3.5-5.1 million species (Richards et al., 2012).

The first historical definition of marine fungi was based on the effect of salinity on the growth of fungi in seawater and freshwater. Johnson and Sparrow (1961) defined marine fungi as capable of exhibiting growth with a range of salinities of 20% or more. However, many fungi are known for their salinity tolerance, preserving their reproductive maturity and growth in both saline and freshwater. Hence, using these physiological parameters on highly adaptable microorganisms is problematic when defining a marine fungus (Johnson & Sparrow, 1961; Pang et al., 2016). Extensive studies on the salinity tolerance of marine fungi, led to Kohlmeyer and Kohlmeyer (1979) proposing a definition dividing marine fungi into obligate and facultative groups: "obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, and facultative marine fungi are those from freshwater and terrestrial milieus able to grow and possibly also sporulate in the marine environment". This definition has been cited and adopted for the last 35 years by most studies, providing the first classification of higher orders of marine fungi (Jones et al., 2015; Pang et al., 2016). The first classification of higher orders of marine fungi were incorporated before molecular techniques and general high throughput sequencing. As a result, Jones (2011) and Jones et al., (2015) listed an updated scheme of classification of marine fungi that included recent higher order nomenclature changes and accounts of new families and genera. Therefore, Jones et al., (2015) suggest that the definition of what constitutes a marine fungus proposed by Kohlmeyer and Kohlmeyer (1979) is too narrow and restrictive. Both Jones et al., (2015) and Pang et al., (2016) states that the definition on 'marine-derived fungi' by Overy et al., (2014) is the first attempt to list true marine fungi to distinguish them from those of terrestrial/freshwater origin. Hence, Overy et al., (2014) implements the term 'marine-derived fungi', that simply indicates that a marine fungus actively grows and sporulate in a marine substratum. By adopting the term

marine in the long-standing Kohlmeyer definition, this will redirect the confusion regarding 'facultative marine' fungi, whether or not a terrestrial strain should be considered as marine (Overy et al., 2014). Consequently, Pang et al., (2016) states that the origin of many 'marine-derived' isolates are unknown, and whether they play any ecological role(s) in the sea, or if they form stable relationships with other marine organisms are not known.

The fungal tree of life was traditionally classified as six major groups: *(a)* the subkingdoms Dikarya (includes the phyla Ascomycota, Basidiomycota and Entorrhizomycota), *(b)* Chytridiomyceta (which includes the phyla Chytridiomycota, Monoblepharidomycota, and Neocallimastigomycota), *(c)* the phyla Mucoromycota, Zoopagomycota, Blastocladiomycota and *(d)* Opisthosporidia (including the phyla Aphelidiomycota, Cryptomycota/Rozellomycota and Microsporidia) (Li et al., 2021). To date, the vast majority of fungi identified from marine environments belong to the Ascomycetes and Chytridiomycetes, while moderately less Basidiomycetes are reported (Grossart & Rojas-Jimenzes, 2016; Shearer et al., 2006). Depending on the marine habitats, different fungal phyla can grow on a wide variety of habitats such as wood, sediments, corals, algae, intertidal grasses and muds (Figure 2) (Hyde et al., 1998). Particularly notable are a large number of species belonging to chytrids which tend to dominate sediment samples and the littoral zone (Amend et al., 2019). For instance, molecular studies conducted in the Arctic have revealed that ascomycetes dwell mostly on sediments and driftwood, while chytrids dominated fungal communities in sediments (Blanchette et al., 2016; Hassett et al., 2017). Similarly, Comeau et al., (2016) found high dominance of novel Chytridiomycota like sequences from both arctic and temperate seawater, whereas studies examining marine sediments revealed new lineages of *Malassezia* (Basidiomycota). Conversely, Amend (2014) questions whether *Malassezia* species (Malasseziomycetes) recovered from deep-sea sediment are truly marine due to evidence from both large and small subunit loci of the ribosomal cistron. Examination of the evidence demonstrate a vast amount of phylogenetic novelty from various marine and terrestrial environments within and adjacent to the *Malassezia* lineage (Amend, 2014). *Malassezia* is a genus known primarily from human skin, however, the species are reported to occur in a great diversity of habitats and locations. For instance, host associated *Malassezia* can group with free-living taxa in various marine and terrestrial habitats (Amend, 2014). However, efforts to isolate marine *Malassezia*-like yeast remain unsuccessful, which might reflect the fact that marine *Malassezia*-like yeast are phylogenetically interdigitated amongst those from human hosts known as *Malassezia restricta* (Amend, 2014; Amend et al., 2019; Jones et al., 2015).

However, fungal communities are often being considered less important or given cursory analyses, despite their functional and taxonomic diversity in aquatic environments. Conversely, high-throughput sequencing methods have increased our capacity to assess microbial eukaryotic diversity (Comeau et al., 2016). Comeau et al., (2016) stresses the lesser attention to aquatic fungi perhaps being due to their overall low abundances in marine clone libraries. According to Richards et al., (2012), fungi accounts for ~1% of total eukaryotes, hence giving a perception that they may be of little ecological relevance. As a result, our understanding of the diversity and abundance of fungi in marine environments is still unclear (Richards et al., 2015). However, Richards et al., (2015) demonstrated how the use of a high-throughput diversity tag sequencing from DNA and RNA templates contributed to the evaluation of the diversity and relative abundance of fungi. The phylogenetic analysis by Richards et al., (2015) indicated that the marine environment encompasses a number of zoosporic fungi that are not previously described and new to taxonomic inventories. Though, a study done by Li et al., (2016) showed that the majority of the uncovered fungi found in the intertidal region in China are terrestrial fungi. As a result, one can argue that most of the fungi recovered from the ocean may derive from terrestrial environments via terrestrial runoffs from rivers (Li et al., 2016). Hence, the coastal ecosystem in China is influenced by human activities that may contribute to the intense disturbance from industrial wastes and pesticides. Still, some common fungi such as *Penicillium* had a relative high abundance in areas with aquaculture/farming activities (Li et al., 2016). In regard to temporal and spatial variation of fungal diversity, Li et al., (2016) reported that East China Sea differed from other regions in terms of species richness and community composition. The data suggest that due to sea coastal currents, sediments are pushed to the coasts of the East sea, forming offshore mud patches, hence favoring organism growth (Li et al., 2016). According to Hays (2017), heat and nutrient distribution by ocean have profound impact on the connectivity of marine populations and impacts on species. Currents are found at a range of scales from open ocean currents, tidal currents or surface water currents in fjords (Hays, 2017). Supplementary, due to the unique geologic history of fjords, currents in a fjord are generally forced both inside and outside the fjord. The influence of sea currents on the genetic structure in some species have been hypothesized as likely drivers of community structure in many species (Miller et al., 2013; Stigebrandt, 2012).

Following the characterization of fungal communities in sediments and driftwood using molecular methods, marine fungi also contain numerous undescribed species at relatively high taxonomic ranks (Amend et al., 2019; Richards et al., 2012). Specifically, the polymerase chain

reaction (PCR), amplification of phylogenetic gene markers from eDNA samples combined with clone library construction have introduced a much more complex microbial diversity than first expected. Fungi-specific environmental clone library analyses have generally used a range of approaches and sequences with sampled regions within the ribosomal RNA (rRNA) or Small subunit ribosomal DNA (SSU) rDNA sequence (Richards et al., 2012). Although the progress to document the abundance and diversity of fungal microbes in marine environments has increased, the diversity recovered is much lower than that of terrestrial environments. However, the emphasis to use environmental DNA-based analyses has contributed significantly to our understanding of marine fungal diversity (Richards et al., 2015). Richards et al., (2015) detected that PCR with primers that amplify the small subunit ribosomal RNA (SSU rRNA) gene had the potential to recover additional fungal diversity from marine sediment. Though, the proposed barcode for fungi is the Internal Transcribed Spacer (ITS) as it has a very broad range of fungi specific species resolution compared to other fungal marker genes (Banos et al., 2018; Schoch et al., 2012). However, unanticipated challenges have hampered the progress to assess fungal diversity in the marine environment. First, the amplicon sequencing based on the fungal ITS rDNA region also coamplifies other eukaryotes (invertebrate, plant hosts or gelatinous zooplankton) that dominate marine metagenomic sequence data, hence limiting the representation of marine fungi in the process (Amend et al., 2019; Gladfelter et al., 2019). Also problematic is the fact that many fungal taxa recovered by environmental ITS-sequencing can merely be identified to phylum or kingdom level due to lack of reference sequences in databases (Banos et al., 2018).

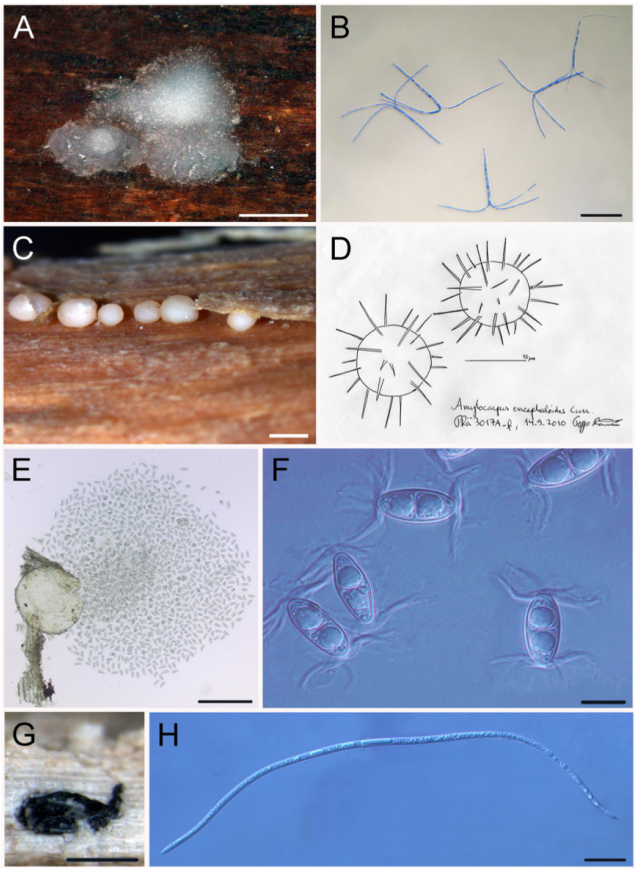

Figure 2. A collection of barcoded marine fungal species on wood residuals (A, C, E and G) and close up of spores (B, D, F and H) (Rämä et al., 2014).

1.5 Molecular markers in fungal identification

In animals, the mitochondrial cytochrome oxidase I (COI) appears to easily amplify a locus from most or all species in the target group using universal primers (Dentinger et al., 2011). However, the international fungal barcoding consortium recommended that the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster should be used as the primary fungal barcode (Dentinger et al., 2011; Schoch et al., 2012). In Addition, Dentinger et al., (2011) stresses that although COI and ITS perform similar as a barcode in a sampled set of closely related taxa, COI was found to be less divergent than ITS, failing to distinguish all terminal clades. Hence, the higher rate of variation within the ITS region gene compared to the COI gene provides for greater species identification and phylogenetic construction (Schoch et al., 2012).

According to Fajarningsih (2016) the use of ITS region has been designed as primary DNA barcode for fungal kingdom due to the ITS region being a highly polymorphic non-coding region with enough taxonomic units. As a result, the internal transcribed spacer region has the

highest probability of successful identification for the broadest range of fungi (Schoch et al., 2012). Though, ITS has been known as the primary DNA barcode for fungal species identification, there are still some drawbacks to the fungal marker. Disadvantages include the low species resolution power within *Saccharomycotina* and *ascomycete* genera that possess a short ITS sequence (ca. 400 bp), hence the lack of sequence divergence among well-known species including *Penicillium* and *Cladosporium* (Tekpinar & Kalmer, 2019; Reich et al., 2017; Xu, 2016). Although ITS can determine fungi to the genus level, identification to the species level is poor for ITS amplicons shorter than 500 bp (Tekpinar & Kalmer, 2019). As a result, the most prominent fungal phylogenetic markers are the 28S and the 18S ribosomal RNA (rRNA) gene sequences. rRNA has been by far the most used for taxonomic assignments of fungal species and to establish relatedness between fungal communities (Banos et al., 2018; Richards et al., 2012). Since Woese and Fox identified rRNAs and used them to establish a system comprised of three domains of life (eubacteria, archaebacteria and eukaryotes), the 18S gene has been the primary tool for phylogenetic analysis to identify new species of fungi (Woese & Fox, 1977).

1.6 eDNA metabarcoding

Environmental DNA (eDNA) is referred to as genetic material extracted directly from water, soil or sediment, including samples containing cellular DNA and extracellular DNA present after cell death or excretion (Thomsen & Willerslev, 2015; Peters et al., 2018). Environmental DNA analysis has become a useful tool for more accurate detection of species that are of low abundance or inaccessible (Peters et al., 2018). By using eDNA-based methods, DNA molecules from urine, skin, saliva and mucus can be captured through the amplification by the polymerase chain reaction (Bohmann et al., 2014; Collins et al., 2018). eDNA metabarcoding offers a great taxonomic resolution for species identification and composition in complex environmental samples. eDNA metabarcoding has the potential to supplement traditional taxonomic analysis of macrofauna from sediments for biomonitoring and environmental assessments (He et al., 2020). eDNA sampling is extensively used for monitoring aquatic species from both seawater and freshwater in order to detect species-specific DNA fragments in the water which allows for the detection of target species (Takahara et al., 2013).

However, the success of a metabarcoding analysis is dependent upon the primer set used and the target loci for determining the accuracy and efficiency of taxon identification and detection. In general, there is some inherent difficulty of designing universal primers as the versatile primers that amplify short fragments of the nuclear 18S and 28S ribosomal markers evolve

slowly and underrate diversity (Leray et al., 2013). Though the 28S rRNA gene resolve to a lower taxonomic level, the most prominent fungal phylogenetic marker is 18S rRNA gene sequence (Banos et al., 2018). For the method to be effective, the gene sequences must derive from a designated gene region that are highly conserved to ensure that the primers are able to bind to the gene and amplified for sequencing (Leray et al., 2013; Ratnasingham & Hebert, 2007).

Next generation sequencing (NGS) enables the use of universal primers to amplify DNA from many different organisms within one sample (Peters et al., 2018). The amplicon-seq technique enables parallel processing of multiple samples during the sequencing run, that can read several hundred samples in a single run (Guardiola et al., 2015; Peters et al., 2018). The number of DNA reads for a sequencing run can reach the order of 20 billion sequencing reads per flow cell, and by focusing on gathering short sequences (~100-300 bp), allows for a substantial resolution between microbial samples (Caporaso et al., 2011; Liu et al., 2007). In order to maximize the number of targeted reads during the sequencing process, the specific DNA sequence derived from the gene of interest must be amplified by PCR. During the PCR process, the separation of the complementary strands allows for designed primers to amplify and bind to the target DNA segments to proceed the production of nucleotide sequences, doubling the copied DNA molecules (Garibyan & Avashia, 2013; National Laboratory of Enteric Pathogens, 1991). The copied DNA fragments are further isolated for sequencing and designing robust assays for taxonomic group identifications (Angles D'Auriac, 2016; Garibyan & Avisha, 2013). On the contrary, Bellemain et al., (2010) used a more standardized method relying on an electronic PCR application to compare fungal ITS primers. The study showed that some ITS primers were hampered with a high proportion of mismatches relative to the target sequences, introducing bias during PCR amplification.

Although DNA metabarcoding provides an opportunity to easily produce large amounts of data on biodiversity and species detection, there are still some challenges and shortcomings to the application of this methodology. To start, as soon as an eDNA particle is released into the environment, abiotic environmental factors such as temperature, salinity, pH or UV exposure influence the degradation rate of DNA. Ultraviolet radiation is generally one of the most influential factors as exposure to high levels of ultraviolet B (UV-B) light alters the DNA amplification due to photochemically damaged DNA (Coissac et al., 2012; Hansen et al., 2018; Strickler et al., 2015). Another drawback with DNA metabarcoding approach is the presence

of artefactual sequences generated during PCR or sequencing. The artefactual sequences are often mistaken for rare MOTUs with the number deviating considerably from the number of taxa observed in the same environmental samples (Coissac et al., 2012; Pawlowski et al., 2018). The most significant biological factor for richness overestimation is single recognized species comprising a variety of genotypes that can cluster into different MOTUs within the same taxon (Pawlowski et al., 2018). However, there is still a controversial issue to whether eDNA metabarcoding can provide quantitative estimates in regard to community-level abundance. Due to differences in DNA shedding rates between communities and species, the interpretation of results of amplicon studies remains ambiguous, as it is argued that PCR products are not fully proportional to real abundances as a result of primer efficiency varying among species templates. In order to resolve the issue with closely related species, it will be essential to design alternative primers that are able to amplify a longer fragment of the gene region (Bakker et al., 2017). This is also emphasized by Ushio et al., (2017), advocating PCR and experimental conditions as potential causes of non-detection among individuals of the same species. By modifying the annealing temperature and primer concentrations of the PCR, the results showed that the number of detected species increased if a lower annealing temperature with a higher primer concentration (15 μM in total) were chosen (Ushio et al., 2017).

1.7 Objectives and hypotheses

The primary objective of this master thesis was to assess the impacts of salmon aquaculture on the fungal diversity present in water and sediments in Arctic Norway using metabarcoding of the 18S rRNA gene sequences. Based on previous findings from Li et al., (2016) on the temporal and spatial variation of fungal diversity, sea currents and discharge of nutrients have an important role in modifying fungal community diversity and composition by pushing nutrients and sediments in line with currents to different habitats. As a result, sediments carrying organic matter form mud patches at a new habitat, which likely favor organism growth (Li et al., 2016). Although the impact of aquaculture on fungal community is unknown, studies investigating the effects of effluents and biomass concentration, have revealed that aquaculture may cause disturbances on the benthic macrofaunal community. Studies show an increase in the natural biomass and nutrient concentration after the implementation of aquaculture and the effects can be observed at the seabed within 250 m from the fish farm (Kalantzi & Karakassis, 2006; Kutti et al., 2007). Nonetheless, the correlation between environmental impacts and abiotic factors such as temperature, hydrodynamic regime and sediment type have been excluded as potential factors from previous studies on environmental impact of aquaculture. In

addition to assessing the impact of aquaculture on the fungal diversity, this master thesis also investigated the importance of spatial (distance) and temporal (season) factors for the structuring of the fungal communities. Lastly, the study evaluates the comparisons of fungal diversity between different habitats (water and sediment).

This study was particularly designed to assess the spatial and temporal patterns of the impact of aquaculture on fungal community diversity in water and sediment. Based on previous studies on environmental impacts of aquaculture on benthic communities, I hypothesize that fungal diversity will decrease with increased geographical distance from the aquaculture cages. I further hypothesize the analysis of the variance of fungal diversity will show a higher spatial variation in water than in sediment. I also theorize the analysis will show that physical variables such as season will be the main factor affecting the temporal patterns in fungal community structure.

2 Methods

2.1 Description of the aquaculture site

Sampling took place at an inshore salmon farm located in close vicinity of Skogshamn, Dyrøya located at 69° latitude (Figure 3). The aquaculture site, referred to as Skogshamn, houses up to 5280 tons per production cycle of Atlantic Salmon and is composed of six net cages (Directorate of fisheries; retrieved from portal.fiskeridir.no). The salmon farm operates on a research farming permit, where Salmar AS is responsible for the everyday operation farm.

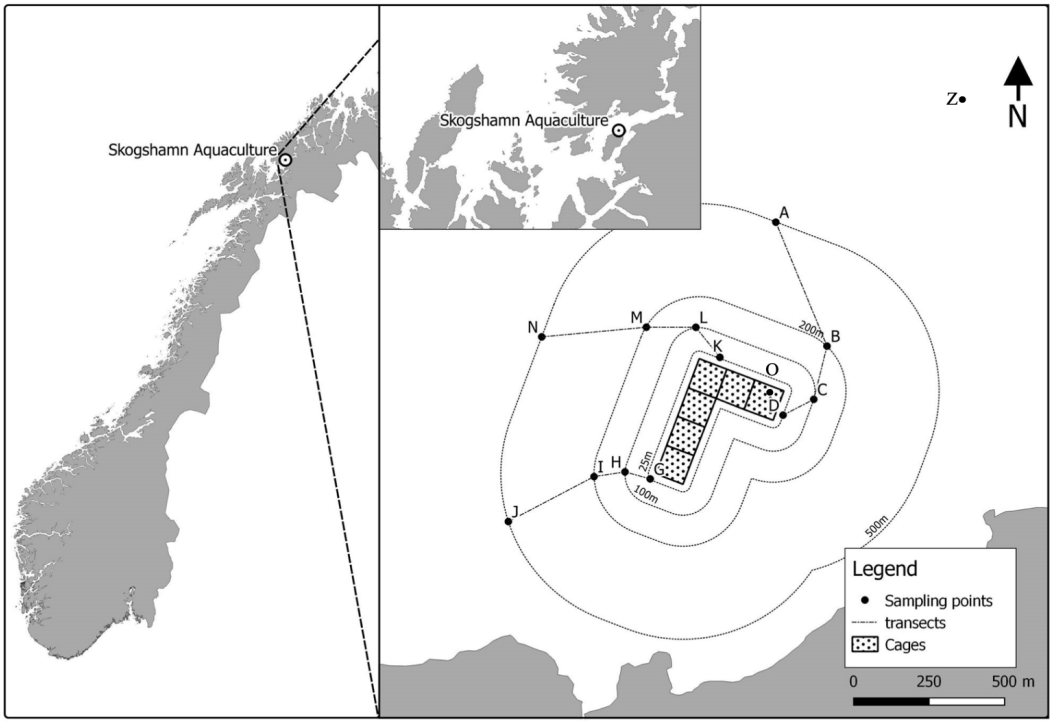

Figure 3. Location of Skogshamn aquaculture facility and sampling points. Transects and distances from the cage are displayed with sampling points radiating north (K-N), west (G-J) and east (D-A) from distances from 25m to 500m as indicated. Additionally, points include point O located southeast from point A and point Z approximately 1km northeast from the facility.

Moreover, the placement and the establishment of the facility is in line with the Norwegian authorization system (Directorate of Fisheries, 2005). The inshore salmon farm in Skogshamn is the largest commercial salmon farm among five other permitted aquaculture sites at the island of Dyrøya (Directorate of fisheries; retrieved from portal.fiskeridir.no). The Norwegian Aquaculture industry is highly influenced by weather conditions in the fjords along the Norwegian coast. The strong seasonality of Norwegian coastal areas is influenced by a higher frequency of extreme weather and changes in water temperatures are of great impact on the

aquaculture industry (Hermansen & Troell, 2012). Skogshamn is a good reflection on the conditions of most open net salmon farms in Northern regions of Norway with seasonally strong winds with clay dominated sea bottoms. The active underwater currents with the most frequent currents having a north east and south west flow, ranges from 5-15cm/s at the aquaculture site (Figure 4).

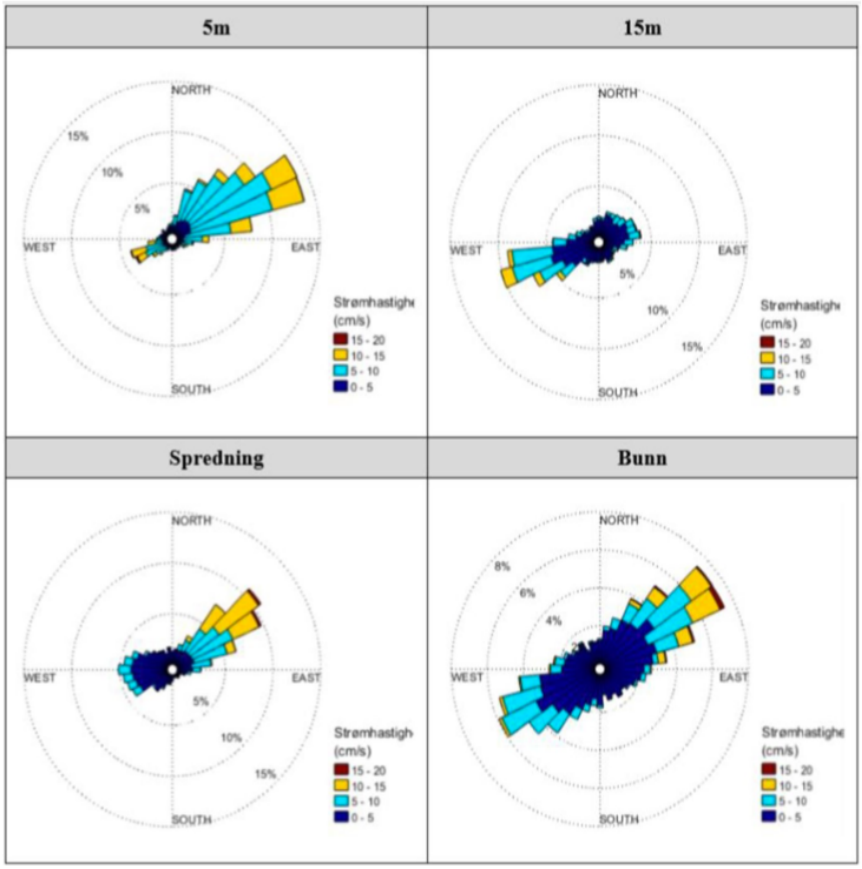

Figure 4. Barplot of current directions and intensity at four depths displayed (5m, 15m, sea bottom and average spreading. Current intensity is indicated by color (dark blue, light blue, yellow and red) with the occurence (%) at each intensity and speed is represented.

2.2 Sampling design

Twelve monitoring points were selected and divided into three transects: North, West and South, where each sampling transect follow the main currents of the area (Figure 4). Each transect was monitored at different distances from the farm, namely 25m, 100m, 200m and 500m. There are both temporal and spatial samplings with different spatial points starting right next to the farm to 500m distance. The distances and directions were chosen to establish temporal and spatial system for assessing the effect of the aquaculture and its impact on the surrounding fungal diversity. All twelve sampling stations were collected before and after the commercial operation of the aquaculture took place at the site in June 2019. This includes the time period before any commercial production with no fish in the cages, the time period after the fish was introduced and the aquaculture was fully operating in fall 2019. The samples collected at these sites included both seawater and sediment samples.

A temporal and spatial sampling strategy was designed to evaluate any impact on the fungal diversity. As part of a larger project between UiT and Havbruksstasjonen i Tromsø, bimonthly sampling was established together with a continuous site monitoring for these samples over the course of 15 months (June 2019 – September 2020 and ongoing). Water sampling were conducted twice a month for the 25m and 500m distance sampling points (Figure 3) in period from September 2019 to September 2020. The six 100m and 200m sampling points (Figure 3) were collected monthly during the same period. Additional samples were collected from a point Z, approximately 1000m from the farm to be used as a seasonal control and monitoring of anthropogenic pollution. For the purpose of this thesis, only water samples corresponding to the dates of sediment collections were analyzed.

As with the water samples, the sediment samples were collected from the twelve monitoring sampling points (Figure 3), approximately every 6 months. The sampling started before the start of the salmon production and continued throughout the operation of the aquaculture. Over the course of 15 months of sampling, 3 points were selected. This included samples from September 2019, March 2020 and September 2020. For the ease of reading these three samplings will hereafter be named as Sep19, Mar20 and Sep20, respectively, for both sediment and water samples (Table 1).

Table 1. Skogshamn sampling schedule, categorized by "before" and "after". Number of samples collected each day for water and sediment. Sampling points representing station ID and distances from the cage.

	Sampling date	Points	Sample name	Distance(m)	Sampling Points
Before				*Sediment*	
	september 2019	12	Sep19	0, 25, 100, 200, 500	A/B/C/D/G/H/I/J/K/L/M/N
				Water	
	september 2019	12	Sep19	0, 25, 100, 200, 500	A/B/C/D/G/H/I/J/K/L
After				*Sediment*	
	mars 2020	12	Mar20	0, 25, 100, 200, 500	A/B/C/D/G/H/I/J/K/L/M/N
	september 2020	3	Sep20	200	B/I/M
				Water	
	mars 2020	12	Mar20	0, 25, 100, 200, 500	A/B/C/D/G/H/I/J/K/L
	september 2020	12	Sep20	0, 25, 100, 200, 500	A/B/C/D/G/H/I/J/K/L

2.3 Sample collection

2.3.1 Seawater collection and filtration

Seawater samples were collected using a 2.5L Model 1010 Niskin Water Sampler (General Oceanics, Miami, FL, USA) lowered to the surface depth (~2m). The water was transferred directly from the Niskin bottle to prelabeled bags that were sealed and stored on board at the zodiac for a short time period while sampling. After collection of the 12 water samples, a static pump and filtering station was set up, where each sample bag containing seawater was filtered using a multichannel pump while following a strict cleanliness between each new sample. Between each replicate for the respective sample, the filtering station including pump tubes and nitrile gloves were sterilized using 5% bleach solution and MilliQ water to reduce contamination. In order to control contamination, a blank was run between each sampling day using the same procedure following the detailed protocol for the sampling and filtering process described above (Appendix A).

Each sample bag was filtered through three 0.22 μm Sterivex^{TM} filter units (Merck KGaA, Darmstadt, Germany). The same output volume from each filter was used (0.5L) to ensure a standard volume between each replicate for each sample. Upon drying of the filters, the filters were placed in prelabeled 50ml Falcon tubes and bags. After completion of filtration of all

samples, the samples were double bagged to be transported back to UiT and stored in a dedicated freezer only for filters only at -80°C.

2.3.2 Sediment collections

Sediments were collected from the sea bottom using a 250cm^3 Van Veen Grab (model 12.110, KC Denmark, Silkeborg, Denmark) with the guide of a petrol driven winch attached to the zodiac. From each sample site, the grab usually produced 100 cm^2 from depths between 300-400m. Three replicates were taken from each sampling station through the top surface of the Van Veen Grab using prelabeled Falcon tubes. The prelabeled 50 ml Falcon tubes were slowly inserted with the cap end down and twisting into the sediments. The samples were bagged and stored at -80 °C at UiT until DNA extraction. To reduce the risk of cross-contamination during sampling, sterile nitrile gloves were used when in contact with the sediment samples, and all sampling equipment was sterilized with bleach and rinsed with seawater between each sampling point.

2.3.3 Other samples

Four feed samples (feed pellets), used for feeding the cultured species, *S. salar* and the cleaner fish, *C. lumpus,* were collected over the period when sampling occurred. The schedules were assigned to account for any factors that can influence the fungal community.

2.4 Laboratory work

2.4.1 Water sample extractions

The sterivex filters used for the water sampling endured DNA extractions in highly controlled eDNA clean labs using eDNA extraction protocols (Appendix B), specifically designed to prevent airborne contaminants. These clean labs involve extensive care for cleanliness both within and outside of the eDNA laboratory. Personnel to be working in the eDNA labs were needed to follow strict entrance rules, and the lab users were restricted to certain areas in the NFH building on the days of eDNA extractions to prevent any airborne DNA present on the lab user's hair, skin or clothes. After endured strict entrance rules, eDNA extraction protocols were precisely followed for the use of DNEasy Blood and Tissue® (Qiagen, Hilden, Germany) kits. An extended incubation period (24h) was used to achieve complete lysis of the sterivex filters. Following the extraction kit protocol (Qiagen, 2006), the lysed solution was then centrifuged out of the filter and into a 2ml Eppendorf tube. Each sample containing DNA was eluted in 75 µl of elution buffer (buffer AE), of which 20 µl aliquot of the extracted DNA was transferred

from each sample for library preparation and sequencing. The remaining DNA product from each sample was stored with 55 µl of elution buffer solution in a prelabeled Eppendorf tube in a cryobox at -80°C for future sequencing runs. An extraction blank was also used during each extraction day to control any contamination that may have occurred during the DNA extraction process. In total 108 water samples were extracted and sequenced, together with 6 blanks.

2.4.2 Sediment and feed sample extractions

DNA from sediment samples were extracted using DNEasy Powersoil® (Qiagen, Hilden, Germany) kits (Appendix C). For each replicate, 0.3g of sampled soil were taken from each of the subsamples from the 12 sampling points. Prior to extraction to minimize the risk of sample cross-contamination, laboratory workspace was cleaned and rinsed with 5% bleach solution, MilliQ water, 70% ethanol and then exposed to UV light for 10 minutes. An extraction blank was also added for each extraction round to control for any residual contamination in the extraction room which may have been introduced during the DNA extraction. The final DNA was eluted with 100 µl of elution buffer solution (solution C6), of which 30 µl of the extracted DNA was transferred from each sample for sequencing. The remaining 70 µl DNA product from each subsample was stored in a prelabeled 2ml Eppendorf tube in a cryobox at -40°C. The same procedure and kits were used for the DNA extraction of the formulated pellets used as feed for salmon and cleaner fish during the sediment sampling events. In total, 99 sediment samples were extracted and sequenced, as well as two blanks.

2.4.3 PCR amplification, library preparation and sequencing

The following steps after DNA extraction, such as PCR amplification, library preparation, sequencing and bioinformatic pipeline workflow were conducted by RGG members; Marta Turon and Owen Wangensteen. Prior to sequencing, extraction aliquots for each sample were pipetted into PCR well plates for the amplification of the fungi-specific 18S rRNA gene sequence (Banos et al., 2018). PCR amplifications were conducted in 20 µl reaction mixtures containing 3 µl of DNA template, 10 µl of AmpliTaq Master mix, 0,16 µl of Bovine Serum Albumin (20µg/µl), 2 µl of each forward and reverse primer (5 µm), 2.84 µl of H_2O and 0.5 µl of each blocking primer targeting Alveolata (sequence: gtcgctcctaccgattga), Rhizaria (sequence: ttaacgaacgagacctcga), Stramenopiles (sequence: tcgcacctaccgattgaa) and Telenoma (sequence: gaccttaacctactaaatagtta), (Banos et al., 2018). PCR profile was as follows: 94ºC for 4 min; 35 cycles x (94ºC/30 s, 50ºC/60 s, 72ºC/90 s); 72ºC/10 min. Subsequent to the PCR amplifications, PCR products were added to single-clone sample pools. To concentrate the

amplified DNA, the pooled clone libraries were purified using MinElute columns for removing DNA fragments below 70bp. Library preparation was performed with the NEXTflex PCR-free library preparation kit (BIOO Scientific) and exact concentrations were measured by qPCR using the NEBNext Library Quant Kit (New England BioLabs). Finally, pools were sent for Novaseq sequencing at Novogene (China). Bioinformatics pipeline was conducted following the UiT MJOLNIR pipeline (https://github.com/uit-metabarcoding/ MJOLNIR /blob /main /README.md) in R environment, based on OBITools v. 0.1.0 (Boyer et al., 2016) software suite. Initially, paired-end reads were aligned using *illuminapairedend* and only sequences with alignment quality score > 40 were kept. Demultiplexing was done with *ngsfilter* that removed primer sequences. Aligned reads with length of 290-350 bp and without ambiguous positions were selected using *obigrep* and then dereplicated with *obiuniq.* Chimeric sequences were removed using the uchime-denovo algorithm implemented in vsearch v1.10.1 (Rognes et al., 2016). Clustering of sequences into MOTUs was performed using SWARM 2.0 algorithm (Mahé et al., 2014, 2015) with a *d* value of 2. Taxonomic assignment of the most abundant (representative) sequence of each MOTUs was done with the *ecotag* algorithm (Boyer et al., 2016), against a local reference database. Further manual refining of the dataset consisted of removing some MOTUs not identified as Fungal.

2.5 Statistical analysis

Data manipulation and statistical analysis took place in R software v4.0.4, starting with normalization of raw sequencing reads through base R functions (see appendix G for full script for the statistical analysis and generation of results figures). To minimize the inclusion of spurious sequences, a general threshold of a minimum 0.1% reads was established. MOTU rows were summed by their best ID, before relative read abundance was established, discarding MOTUs with a relative abundance lower than or equal to the threshold. Additionally, to account for potential cross-contamination, MOTUs present in blank samples with more than 10% of the total MOTU reads were removed. Similarly, MOTUs with low counts in the samples were removed to avoid bias. Since the 18S primer amplified additionally organisms other than fungi, sequences not containing fungi were removed for the initial analysis. Additionally, to investigate variation within eDNA sampling effort (total number of reads), rarefaction curves were generated to examine the quality of sequencing effort using *rarecurve()* function in vegan package in R (version 2.5-6, Oksanen et al., 2019).

To account for alpha diversity comparison, the samples were rarefied using *rrarefy()* function in *vegan* package in R. The following analyses were conducted in R *v.3.6.3*, and the mean values of rarefied fungal diversity per sample were assigned as alpha diversity indices (Oksanen, 2015). In this particular study, the Shannon diversity index (H) was used to characterize fungal diversity (Marcon & Herault, 2015). Additionally, to estimate the change of alpha diversity among time points and transects, analysis of variance (ANOVA) displayed approximations of degrees of freedom (df), F and p values through the *aov()* function. A one-way *anova* were performed to compare fungal diversity among time points and fungal diversity among distances. To create a set of confidence intervals on the differences within levels of each variable, Tukey's "Honest Significant Difference" method was performed. To investigate temporal and spatial patterns of changes in community composition, the dissimilarity indexes of the data were obtained through *vegdist* function in *vegan* package. In order to assess similarities between the samples, the *sqrt()* function was chosen to attain square-root transformed relative abundances. The dissimilarity indexes were visualized through non-metric multidimensional scaling (nMDS) ordination. using *metaMDS* in *vegan* package, with two dimensions (k=2) together with 200 random starts in search of a stable solution (version 2.5-7, Oksanen et al., 2020).

In order to compare the temporal and spatial changes of community changes, the analyses were conducted in R *0.1.0* through permutational analysis of variances (PERMANOVA) using *adonis* function in *vegan* package with 1000 permutations. Differing fixed variables were tested throughout the analysis where both time point and distances were treated as fixed factors respectively. A permutational pair-wise comparison were conducted via *betadisper()* function in *vegan* package together with *permutest()* to test if one or more factors are more variable than the others. The data values were then visualized using *ggplot()* with a combination of boxplot and point layers using *ggplot2* library. This analysis was conducted for each of the designed spatial and temporal variables (distance and date) for both water and sediment samples. The analysis described above was run on the whole fungi MOTU dataset.

3 Results

3.1 Sequences and eDNA collection effort

To investigate whether sufficient sequencing effort was applied to capture realistic MOTU richness for the sample types, the relation between sequencing depth and genetic diversity (MOTU richness) from different sample types were assessed using rarefaction curves. The sequencing yielded 161,881,462 raw reads. After pair-end merging, quality check, removal of chimeric sequences and singletons (Mjolnir pipeline), 28,555,065 reads were assigned to 8,852 final MOTUs. After the initial filtering, approximately 4,298 MOTUs could either not be assigned to any MOTU or identified as fungi. A total of 4,554 fungi MOTUs were identified in the dataset and the remaining result section and discussion will report only on those 4,554 MOTUs (see Figure 10, Appendix D for complete diversity for all sample types combined: water, feed and sediment). The saturation of sequencing effort was almost reached, where after ca. 180,480 reads per sample, the majority of samples approached a plateau (Figure 5).

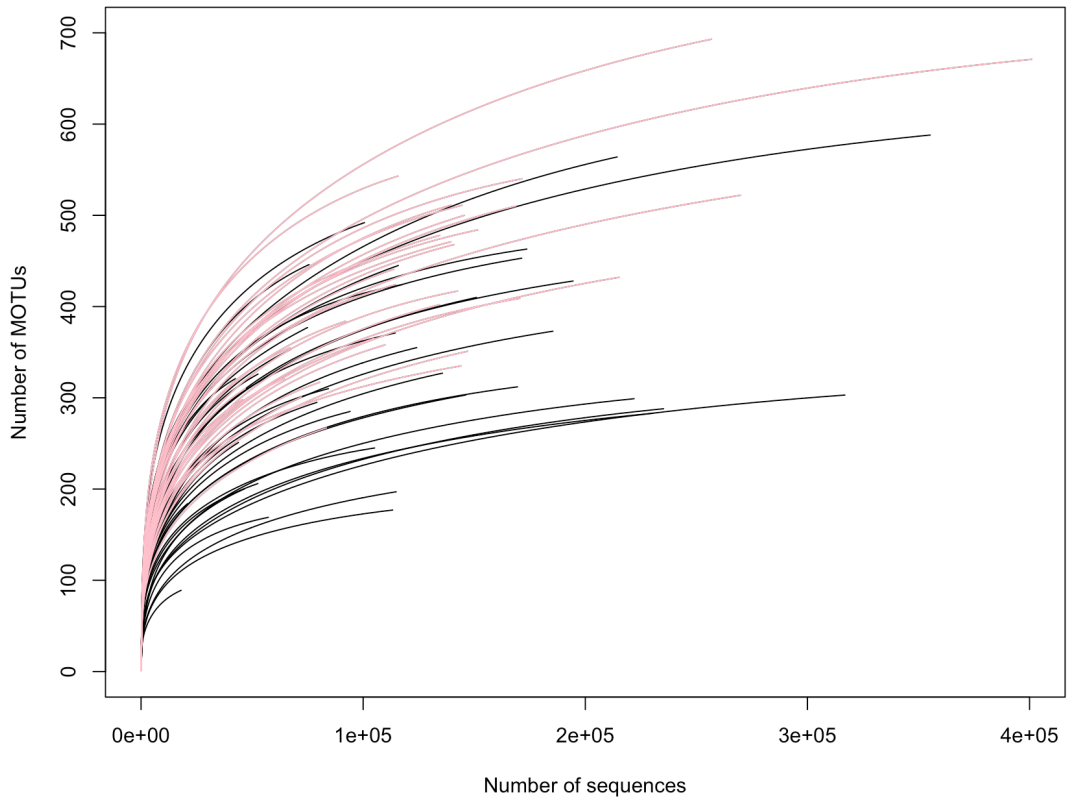

Figure 5. Rarefaction analysis on the number of MOTUs obtained for different samples and the sequence identity across each sample type: sediment (pink), water (black).

3.2 Variation of fungal diversity over time and space in sediments and water

The analysis on the effects of distance from the cages and time of sampling on community diversity was done separately for sediment and water samples. The fungal Shannon diversity index was in general lower for water samples compared to the values obtained for sediment samples (Figure 6a, 6b). Most of the Shannon diversity ranged between 2 and 3, whereas values of Shannon diversity for the sediments ranged from 3 and 4. Overall, effects of distance on fungal diversity in water samples were significant when looking across water samples taken at all distances (p-value < 0.05). However, differences between distances were not observed as statistically significant (TukeyHSD, p-value > 0.05) for distances further from the nets (100-200, 100-500 and 200-500m). Distance 25-200m and 25-500m were the only distance comparisons with a statistically significant distance effect in water samples (p-value < 0.05). Similarly, analysis of the temporal impact on alpha diversity in water had a statistically significant effect (p-value < 0.05). Significant differences between dates were observed for water samples for each of the different dates (TukeyHSD, p-value < 0.05).

The temporal impact on MOTU abundance in sediments had a statistically significant effect (p-value < 0.05). Significant differences between dates in sediment samples were observed across Sep19 – Mar20 and Sep19 – Sep20 (TukeyHSD, p-value < 0.05). However, Mar20 – Sep20 produced no statistically significant difference in sediments. Conversely, a significant difference between distances were not observed for sediment samples when looking across the entire sampling period (p-value > 0.05).

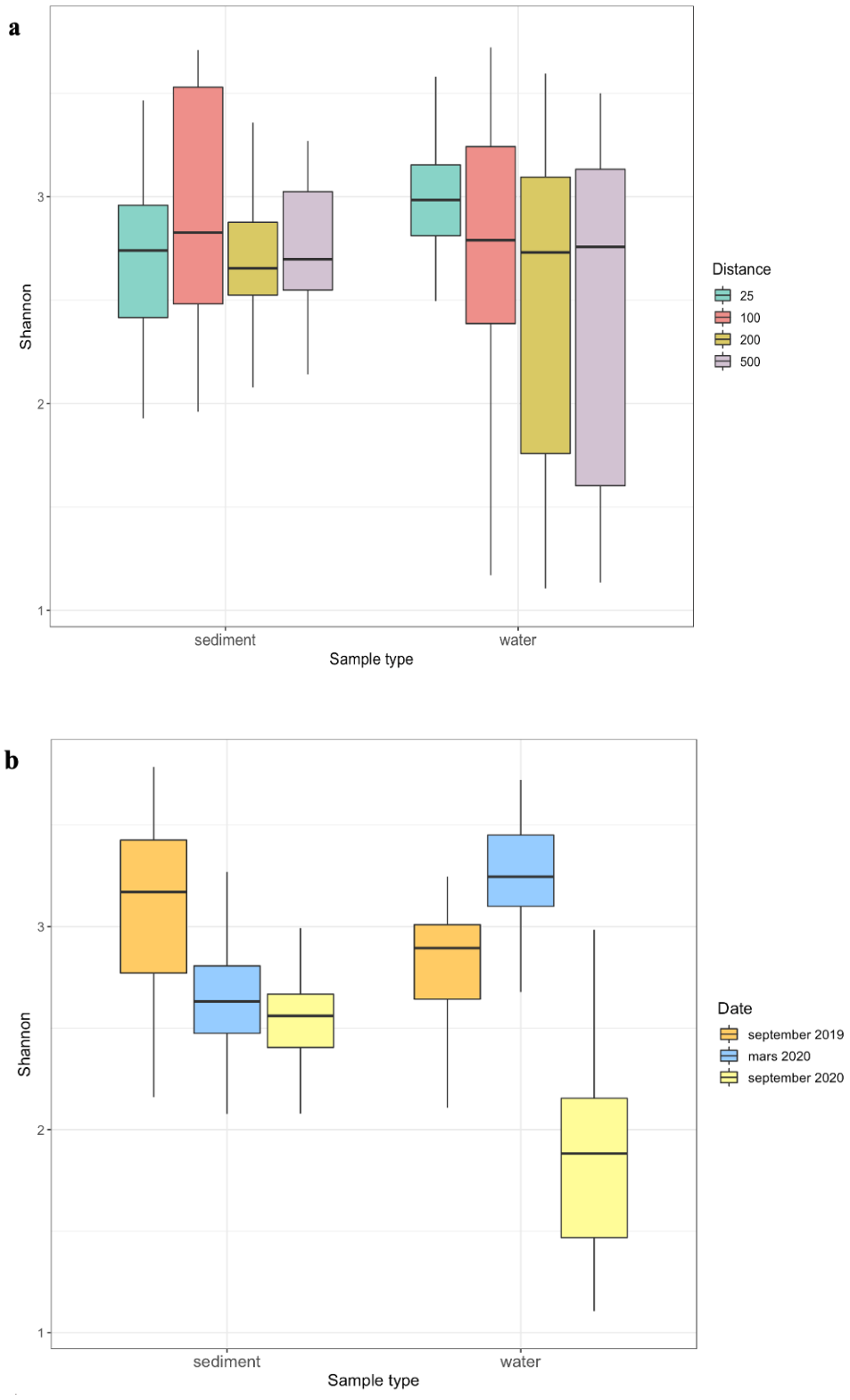

Figure 6. Alpha diversity (Shannon index) variation within water and sediment samples. (a) Box plot showing the diversity index of water and sediment samples across distances. (b) Box plot showing the corresponding time points for water and sediment samples against Shannon diversity index.

3.3 Distribution of (dis)similarities of fungal community composition in water and sediment samples

Non-metric multidimensional scaling (nMDS) ordination plot was generated to display dissimilarities in fungal community composition among sample types (water, sediment and feed). The ordination plot, based on Bray-Curtis dissimilarity index, indicated no overlap of ellipses between water and sediment samples, however an overlap between water and feed samples are indicated. Indeed, a great dissimilarity between water and sediment samples are depicted, with less variation in community composition within sediment samples compared to water samples (Figure 7).

The PERMANOVA analysis indicated a statistically significant difference in community composition between sample types (p-value < 0.05). Analysis of the homogeneity of dispersion among the different sample types produced a significant dispersion (permutest: p-value < 0.05). The nMDS plot showing the variation in community composition among sediment samples for the different distances from the cages and time of sampling are shown in Figure 11, Appendix E. The PERMANOVA analysis produced a significant difference in the composition between dates for sediment samples (p-value < 0.05). Additionally, analysis of dispersion among the different time points for sediment indicated a significant dispersion between time points (permutest: p-value < 0.05). However, the PERMANOVA test indicated a non-significant difference in the composition between distances for sediment samples (p-value > 0.05). Hence, the permutation test analysis produced a non-significant difference of dispersions within distances for sediment (permutest: p-value > 0.05).

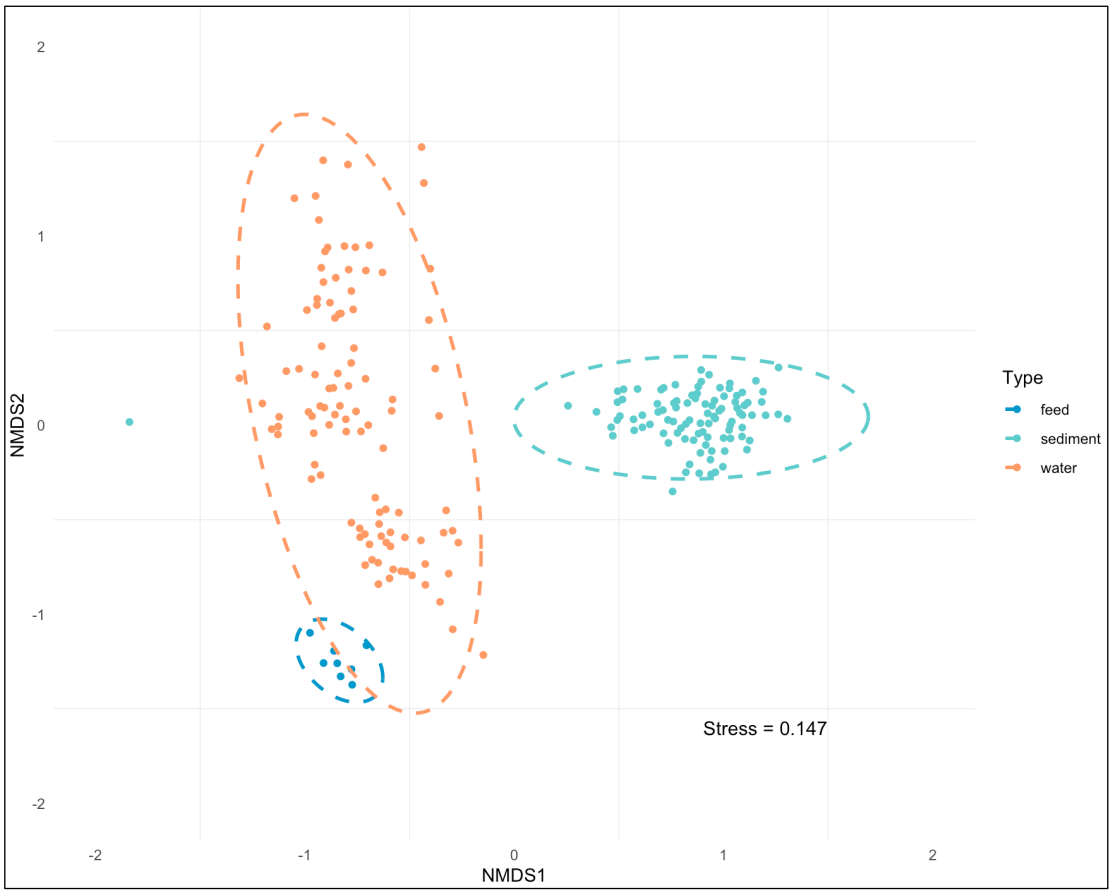

Figure 7. Non-metric multidimensional (nMDS) ordination plot illustrating differences among sample types obtained using Bray-Curtis dissimilarity index. The centroids for the sample types are indicated. Ellipses indicate 95% confidence interval of the group centroid dispersion.

3.4 Distribution of (dis)similarities of fungal community composition in water over time and space

An nMDS ordination plot was generated separately for water samples indicating dissimilarities of fungal community composition between distances and time points (dates) for sampling. The ordination plot, based on Bray-Curtis dissimilarity index, indicated an overlap of 95% confidence ellipses between the time points: Mar20 and Sep20 (Figure 8). The PERMANOVA analysis indicated a statistically significant difference in the composition between the two time points (p-value < 0.05). Analysis of the homogeneity of dispersion within the different time points produced a significant dispersion within time points (permutest: p-value < 0.05). Differences in the composition within distances were found non-significant (p-value > 0.05). The permutation analysis did not detect any significant dispersions within distances (permutest: p-value > 0.05).

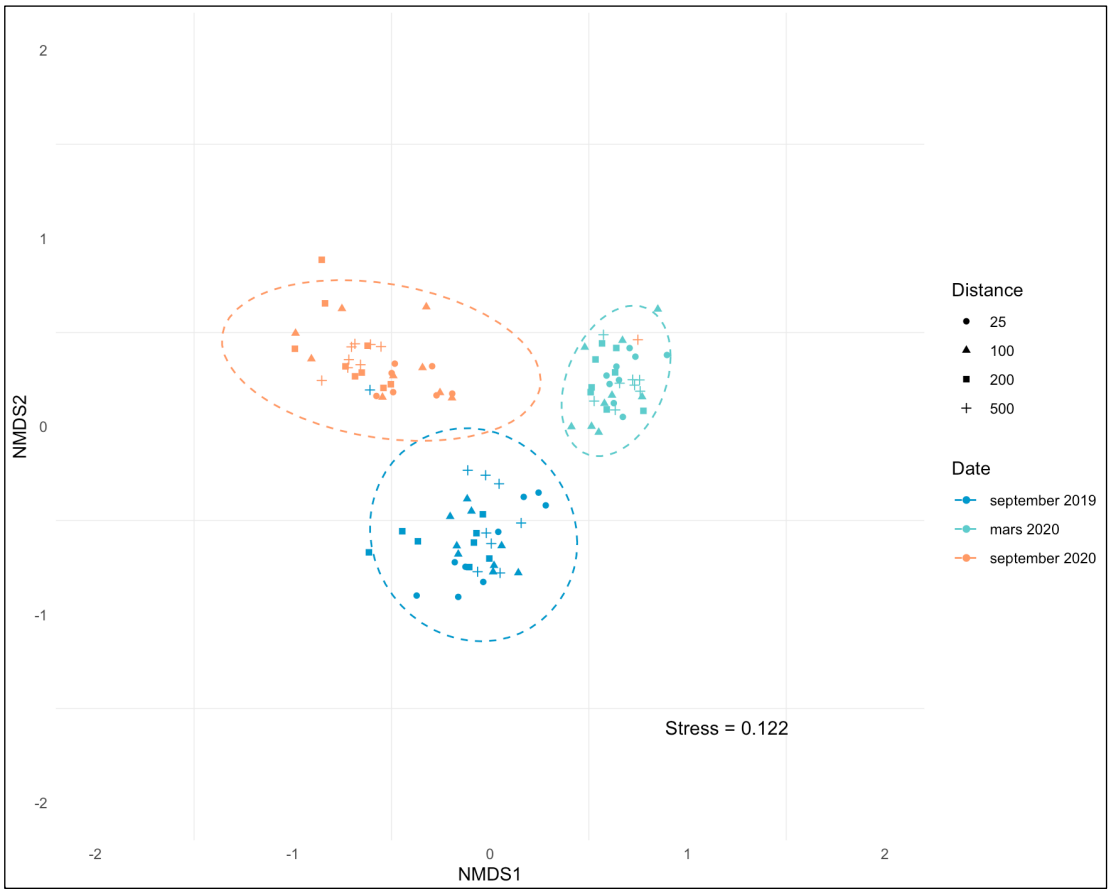

Figure 8. Non-metric multidimensional (nMDS) ordination plot of water samples obtained using Bray-Curtis dissimilarity index with 95 % confidence interval of the group centroid dispersion. The centroids for the distances and their corresponding time points are indicated.

3.5 Taxonomic composition across different sample types and time points

The taxonomic composition of the communities at phylum level was studied at different sample types and dates. In both sample types, unidentified fungi contributed as the most dominating group, followed by Basidiomycota, Ascomycota and Cryptomycota. In sediment samples, unidentified fungi were the dominating group with 89.5% of the relative sequence abundance, and only few MOTUs of Basidiomycota, Ascomycota, Chytridiomycota and Cryptomycota were detected. In contrast, the fungal community of water samples had a lower proportion of unidentified fungi (58.2%) compared to the sediment samples, but yet they represented the dominating group in the water samples. Basidiomycota (27.7%), and to a less extent Ascomycota and Cryptomycota, accounted for the major proportion of the identified groups of fungi (Figure 9a).

The fungal community at phylum level at different sampling time points (see figure 12, Appendix F for taxonomic summaries for sediment and water combined together) showed that Sep20 was dominated by unidentified fungi (66.0%) and Basidiomycota (19.8%) when looking at the total relative sequence abundance, and only a few MOTUs of Chytridiomycota, Cryptomycota and Ascomycota were accounted for. Similarly, Mar20 was dominated by unidentified fungi (69.8%), together with Ascomycota and Basidiomycota (22.7%), and only a few MOTUs of Chytridiomycota and Cryptomycota were detected. The cohorts of Sep19 and Mar20, all have roughly equal composition amounts of the same taxa identified. Conversely, Sep20 was mainly structured by taxa of unidentified fungi (86.9%) and to a less extent Ascomycota and Basidiomycota compared to Sep20 and Mar20 (Figure 9b).

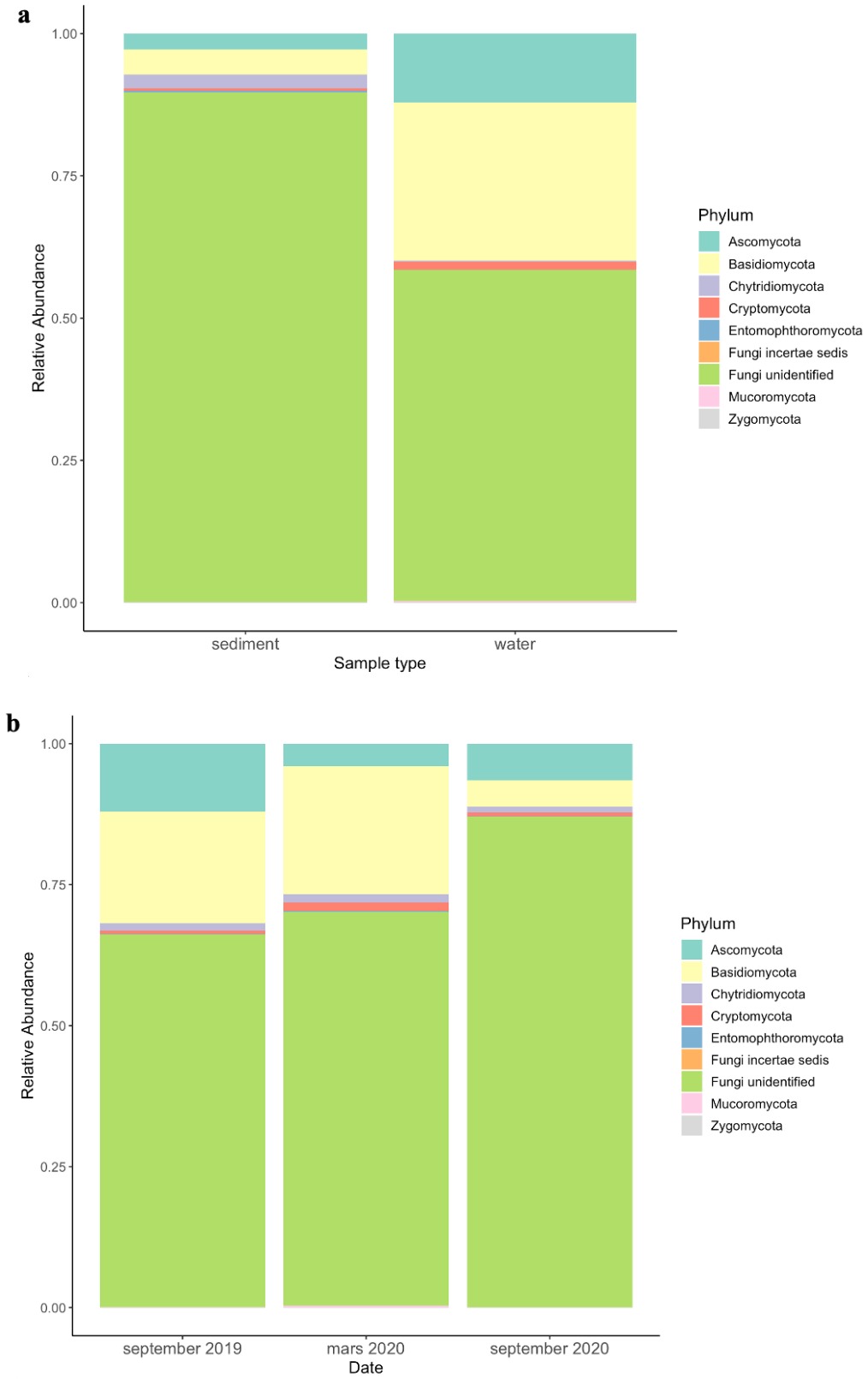

Figure 9. Taxonomic composition of the fungal communities in different sample types and in different time points. (a) Color-coded bar plot showing the relative abundance in different phyla in distribution different sample types. (b) Color-coded bar plot showing the phylum distribution at different sampling time points.

4 Discussion

This study had the objectives to assess the impacts of salmon aquaculture on fungal diversity present in water and sediments at one aquaculture site in Northern Norway. The results showed that the aquaculture only affected the alpha diversity, assessed by Shannon diversity index at the nearest 250m in water samples. Although statistically significant differences between distances were observed between 25-500m in water samples, there was no significant difference between distances detected in sediments. Beta diversity indicated a higher spatial variation in fungal diversity in water samples compared to sediments. Additional patterns in spatial and temporal variances in fungal community structure were discovered, relating to changes in season and aquaculture activities. The detected change in fungal community diversity and composition discovered herein, associated with the temporal factors (season) and activities from the farm (organic discharge), provide further insight on impacts from aquaculture on the fungal diversity. Rarefaction curves showed that many samples nearly reached a plateau and could describe the fungal community of each sample or group of samples and provide accurate fungal diversity values.

4.1 Comparison of fungal MOTU community composition in sediment and water

In this study, alpha diversity reflects the within-habitat diversity and the fungal diversity within a single ecosystem and habitat, whereas beta diversity is the component of total diversity that is produced by measure of similarity or dissimilarity in species composition among the sampling units (Marcon et al., 2012; Marcon et al., 2014). The PERMANOVA analysis indicated a statistically significant difference in the fungal community composition between water and sediment samples. Processes that cause changes in fungal composition include nutrient recycling, organism recruitment and structural stabilization of sediments (Austen et al., 2002). The latter being the significant factor contributing to the differences in MOTU composition between sediments and water. Although sediment stabilization is most relevant in littoral sediment, vegetation such as seagrasses, algal and diatom mats together with large epifaunal organisms can act as structuring agents, creating a new interface between the sediment and the overlying water (Austen et al., 2002). As a result, these biogenic structures can stabilize sediments and trap nutrients that may explain why this study indicated less variance in the fungal community composition in sediments, compared to water. The varying temperature and light intensities in subsurface water further increases the assumption of light as the potential factor, explaining the significantly greater variation in community composition in water samples. Hence, light is an important factor, and since optimal adaptation to the

beneficial effects of light significantly enhances fitness of an organism, it can be considered crucial for successful vegetative growth. It is likely to expect that the higher variation in light and other parameters such as temperature and nutrients, in the surface waters than in the sediments have influenced the fungal diversity at Skogshamn. These expectations are supported by Akbari et al., (2017), that reports that temperature is an important factor influencing the sea surface temperature, as well as differing light penetrations in the water layers. Visible light, such as violet light consists of various wavelengths that are able to penetrate deeper in the water column than other wavelengths. Penetration of the visible portion of the spectrum in the upper layers plays a crucial role in heat transfer and varying surface temperature. However, while light can penetrate easily in the upper water surfaces, the light intensity decreases with increasing depth. As a result, the epipelagic zone from the surface to approximately 200m is where most of the light penetrates and causes an increase in temperature. Conversely, in the mesopelagic zone from 200m to 1000m, the light penetrating to this depth is limited and causes lower water temperatures (Akbari et al., 2017). Specifically, fungi use light as a source of induction or inhibition of sexual development as well as spore release (Corrochano, 2007; Tisch & Schmoll, 2010). As a result, our findings on enhanced diversity of fungi in water corroborate the theory by Li et al., (2019) that the surface sea layer is influenced by light available for photosynthesis. As a result, photosynthesis becomes an important ecological phenomenon, enhancing the accessibility of organic compounds available to heterotrophs such as fungi.

According to a study by Li et al., (2019), the epipelagic zone harbors a higher Operational Taxonomic Unit (OTU) richness compared to meso-, bathy-, and abyssopelagic zones. As a result, depth-stratified distribution trends were observed, highlighting a higher OTU richness and distinct fungal communities in the upper water layers than in the deeper ones (Li et al., 2019). Likewise, it is well-known that fish farming releases a substantial amount of nutrients into the marine environment, and therefore it would be reasonable to expect that this might induce eutrophication of the waterbody (Neofitou & Klaoudatos, 2008). However, the homogeneity of nutrient distribution across water surface cannot be assumed, due to the effects of wind and turbulent surface currents which may aggregate nutrients both vertically and horizontally (Sarà et al., 2004). Despite the need of further analysis to assess the nutrient distribution across water surface, the patterns of fungal diversity displayed in water samples support the second hypothesis that the fungal diversity will show a higher spatial variation in water than in sediment samples. The discrepancy in community composition in water and sediments could also be due to the physical barrier between different water layers due to

samples taken at a continuous depth of 2m. Water masses are circulated through the ocean, driving changes in community structures and composition due to mixing of microbial communities (Hamilton et al., 2008). As a result, the physical barrier between the water layers is influenced by density and pressure changes, making it reasonable to think that the mixing of water layers could affect the fungal community, hence the fluctuating differences seen in water (Hamilton et al., 2008; Husum et al., 2019). However, we cannot be confident in the accuracy of weather as a factor, and if Sep19 and Sep20 had a drastically change in temperature or wind-driven mixing of water surfaces.

4.2 Potential factors influencing spatial variation of fungal diversity

The alpha diversity did not vary across the four different distances in the sediment samples, as the ANOVA found no significant distance effect. The PERMANOVA showed that the community composition did not differ within the different distances from the farm. Moreover, the permutest failed to show significant differences in dispersion of the community composition between distances, indicating that beta diversity remained uniform. Moreover, the variance in the community composition was sustained by the non-metric multidimensional scaling plot (nMDS), where findings suggested no systematic change in fungal community diversity and composition structure among distances for the sediment samples. In addition, the rarefaction curves for several sediment samples did not reach a plateau.

However, a significant ANOVA distance effect was found in water samples, hence suggesting that the fungal diversity in the water had a more distinct community composition that was structured by distance. Significant differences between distances were observed only between the water samples taken from close proximity to and furthest away from the nets (25-200m and 25-500m). The other distances (100-200m, 100-500m and 200-500m did not have a statistically significant difference between distances, providing further evidence that large-scale effects on fungi by organic effluents from aquaculture are restricted to the nearest 250m of the farm (Kutti et al., 2007). Patterns in the spatial distribution of fungal communities in water are in agreement with the spatial analysis in infauna community composition (Kutti et al., 2007). Considering the findings in Kutti et al., (2007), the alpha diversity in this particular study was lowest in the samples taken at an intermediate distance from the farm, while the greatest diversity was observed at close proximity to the farm. While these results can be due to the specific habitat preferences by the different fungi (Figure 9a), it is also likely that they are due to differential sedimentation rates. Sedimentation rates in fjords are strongly influenced by the strong current velocities in the upper water column due to the geological and physical landform of fjords

(Stigebrandt, 2012). Particles are transported by water currents and the resulting dispersion and sedimentation at various distances from the cages reflects site specific hydrodynamic features (Holmer et al., 2005). As a result, it is reasonable to expect that high settling rates lead to increased particle deposition near farms where accumulated organic matter is deposited. Hence the significant increase in alpha diversity in water between 25-500m from the farm in this study. Thus, the hypothesis of that fungal diversity will decrease with increased geographical distance from the aquaculture cages was confirmed for water samples. However, this runs contrary to the results observed for sediment samples, hence rejecting the hypothesis for sediment samples. Yet, it must be noted that in the Arctic there are regions that differ in the strength of seasonal processes (e.g., light conditions, air temperature and precipitation). Consequently, degradation of organic matter in water column is less efficient in the Arctic due to drastic variations in strength of seasonal variations such as ice-cover and shorter vegetation period (Lalande et al., 2020). However, fjords in Norway are influenced by the inflow of nutrient-rich Atlantic water into the fjords, and temporal changes of the level of organic matter are likely the result of changes in erosion controlled by precipitation and temperature variability (Faust et al., 2014; Lalande et al., 2020). However, it is reasonable to expect that the salmon farm located at the inner fjord site of Dyrøya leads to enhanced export of particulate matter in the surface water due to strong current velocities in the upper water column (Lalande et al., 2020; Stigebrandt, 2012). Hence, further studies of the effects of seasonal variations in downward particle fluxes in Norwegian fjords as a potential explanatory factor for variances in fungal community diversity in different habitats are necessary.

4.3 Effects dictating temporal variation of fungal communities

By analyzing community similarity and variability at seasonal intervals, the PERMANOVA showed that the community composition changed significantly during the different time points. As a result, a heterogeneity in dispersion of community composition between time points were indicated, with considerable variability in community structure and significant differences in fungal composition across sediment and water samples. Moreover, the physical dynamics can play important roles in community function and shifting in fauna, mostly as a result of different dominance structures (Baumgärtner et al., 2008; Kędra et al., 2013). The seasonality in pelagic processes and organic matter supply to the sea bottom at Skogshamn can be seen in significant variability of fungal community composition between the seasons in both water and sediment. However, stations in present study were located in the central and outer parts of

Solbergsfjorden, near Dyrøya, where the sediments are stable, further reasoning why we did not observe as great seasonal changes in sediments compared to water.

Statistically significant temporal effect was observed for the fungal alpha diversity in both water and sediment samples, with the exception of Mar20 for the sediment samples. Conversely, the highest fungal diversity was observed in the water samples from Mar20 compared to the lowest fungal diversity in the Sep20 water samples (Figure 6b). According to Sanz-Lázaro et al., (2008), along an organic enrichment gradient, macrofaunal abundance and species richness peak at intermediate levels of organic enrichment, before a sharp decrease at higher levels of organic enrichment until a threshold point is reached. This supports the influence by seasonal variation and aquaculture in fungal diversity of Mar20 samples, approximately 6 months into the production cycle and that late winter/spring community is richer than autumn community. A study done by Sun et al., (2017) revealed that fungal population structure is significantly influenced by temperate and nutrient availability. During a marine dinoflagellate (*Noctiluca scintillans*) bloom, there were more fungal patterns in terms of more fungal OTUs leading to more fungal taxa associated with Ascomycota, Chytridiomycota and Basidiomycota at the initial bloom stage (Sun et al., 2017). However, it is not clear whether this reflects similar bloom patterns occurring at Skogshamn during the spring bloom (April). In this particular study at phylum level, fungal communities were predominated by unidentified fungi and Basidiomycota during the suspected initial bloom stage, while unidentified fungi, Basidiomycota and Ascomycota were more abundant during onset and peak-bloom stages. Ascomycota gradually became more abundant and, in the decline stage, contributed still a significant amount (6.5%) compared to 6 months prior (1.2%). In the terminal stage of the suspected bloom for this particular time point and study, unidentified fungi increased significantly to approximately 86.9%. Overall, the peak in fungal communities suggest that symbiotrophic fungi dominated in the onset stage, but the reasoning of unidentified fungi dominating the terminal stage cannot be assumed due to lack of resolution in taxonomic assignment.

The fungal diversity peak drops significantly Sep20, approximately a year in the production cycle, indicating a relatively lower diversity compared to the year before (Sep19). However, it is not clear whether the decline in fungal diversity from Mar20 – Sep20 reflects technical limitations in the study or simply pointing to the effect of aquaculture as the plausible cause of decrease in fungal diversity. However, it is likely that such drop in fungal diversity is due to seasonal community shift between September and March. Conversely, the reduced diversity in Sep20 might be due to a negative effect of the spring bloom resulting in higher fungal mortality.

Hence, further studies are needed to verify whether this is due to seasonal community dynamics or simply a technical limitation due to insufficient sequencing depth.

In this study, there was a significant nutrient overload from the aquaculture, further enhancing speculation in that enrichment of nutrients contributes to the increasing abundance of some MOTUs. The seasonal factors such as weather conditions at Skogshamn during the initial period of study, provide further evidence for speculation of wind driven mixing as a seasonal source of sediment resuspension. Such activities have the potential to resuspend a significant amount of nutrients from the sediments into the surface waters and reintroduce nutrients. However, the onset of storms does not usually start before October in the Northern part of Norway, hence why wind mixing as a seasonal source of nutrient resuspension is not to be expected to have occurred at Skogshamn. In most cases, the organic matter tends to accumulate at the bottom beneath the cages, hence leading to a localized distribution of nutrient impacts (Kalantzi et al., 2013). This further support the evidence for increased diversity of fungi in the following months after initial production cycle started, as well as supporting the hypothesis that seasonal variation plays an important role affecting the temporal patterns in fungal community structure.

4.4 Fungal community composition patterns across different sample types and time points

By comparing fungal dynamics between the different habitats described in this study, consistent patterns begin to emerge. For instance, fungal communities show more seasonal variation in warmer and illuminated surface waters than in dark sediments, which is consistent with the seasonal variation in sunlight and the fact that there is more seasonal variation in the physical properties of surface waters. Additionally, dynamic microbial systems enable us to examine how different organisms change in relation to one another and to the environmental conditions. Hence, according to Fuhrman et al., (2015), microbial systems emphasize that insights into the processes that operate in complex microbial ecosystems are of importance to assess marine microbial community dynamics. Correlations between fungi may have direct causes, such as cross-feeding or indirectly causes such as overlapping preference for similar environmental conditions (Fuhrman et al., 2015). For example, the results in this study showed that Ascomycota and Basidiomycota were highlighted as the phyla detected in the water column (Figure 9a), which appear consistent with the current knowledge on marine fungi as revealed by the accepted names and classification (Jones et al., 2015). However, whether inferred relationships among organisms happen at Skogshamn cannot be assumed due to the need of

higher taxonomic resolution in marine systems. For instance, Horner-Devine et al., (2007), indicated that nonrandom co-occurrence patterns may be due to competitive interactions or occur through other mechanisms, such as non-overlapping habitats. However, assuming co-occurring MOTUs at Skogshamn, indicates important community variances, but do not identify the causal mechanisms responsible for such patterning. As a result, to explore the co-occurrence patterns, a more detailed analysis of the environmental parameters would be a prerequisite to establishing evidence of the co-occurring networks between fungi.

4.5 Perspectives

This thesis project was of limited duration but was successful in conforming and establishing evidence of impacts by salmon farms and their surrounding environment. However, the process of sequencing more samples for a better characterizing of the fungal community, continued studies and improved methods for capture of the fungal community would need to be combined to better understand the impact of salmon farms on fungi. Additionally, observing fungal community over a longer period, investigating several farm sites, and locations with no farming, would be of preference. This could allow for a more in-depth analysis on the seasonal fluctuations in marine fungal communities and give a better understanding of these organisms' behavior in the aquaculture environment. Moreover, metabarcoding approaches are paving the way to a better understanding of distribution patterns, diversity and abundance of fungi. Besides, incorporating metabarcoding analysis, one of the main technical limitations in this study was that most of the fungi were unidentified. Based on results of the many unidentified fungi in this study, it is obvious that there is a need for more accurate and better reference databases as a prerequisite for improving the taxonomic assignation of fungal MOTUs in the future.

Detection of spatial and temporal variations of fungal diversity in Northern Norway emphasizes the need for further investigations of 18S metabarcoding. However, due to time limitations, the method and pipeline used in this particular study gives an insight into the challenges in characterizing marine mycobiomes. The need for an adaptation of a new design taking into account the fact that amplicon sequencing based on 18S, readily coamplified other eukaryotes or invertebrates. These non-fungal eukaryotes tend to dominate the marine environmental metagenomic sequence data that results in limited representation by marine fungi. Also, problematic which is also discussed by Amend et al., (2019), is the fact that metagenome sequencing and amplicon-based methods are unable to distinguish between metabolically inactive fungi (spores and other dormant propagules) and active true marine fungi. As a result,

the results may fail to represent a realistic interpretation of how marine fungi contribute and participate in ecosystem processes.

In terms of establishing more well-designed marine fungal model systems, this work outlines several trends (e.g., season, light intensities, particle fluxes) suggesting the need for more studies on the potential links between environmental changes and microbial communities. In order to observe such changes, there is a need for carefully monitoring techniques of metabarcoding methods. It is enticing to imagine the possibilities that eDNA open up, if advances in molecular ecology, sequence technologies and bioinformatics continue to facilitate and accelerate the understanding of fungi in marine environments.

5 Conclusion

The present study suggest that the establishment of the aquaculture farm affected the alpha diversity in both water and sediments. The findings of this study do not support the hypothesis that fungal diversity will decrease with increased geographical distance from the aquaculture cages for sediment samples. However, significant differences between distances were observed for water samples, hence accepted the hypothesis that the fungal diversity decreases with increasing distance from the aquaculture cages. The beta diversity indicated a higher spatial variation in fungal diversity in water samples, supporting the hypothesis that higher spatial variation in water samples will be observed compared to sediments. Variances in fungal diversity and composition were also determined to be linked to environmental factors such as seasonal variability, further conforming that season is an important factor affecting the temporal patterns in fungal community structure.

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Appendix A – Sampling Protocols

Skogshamn eDNA project sampling guidelines

Water sample collection:

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

Once at the intended sampling point:

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

Static pump setup, water sample filtration, and storage protocols

Filtration station setup and pump assembly:

Bring container of water samples from boat to pumping area.

Set up folding table.

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

Table surface

Outside of tubing

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

Any other materials or surfaces used during the pumping process

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

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

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

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

Pumping procedures:

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

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

Freezer guidelines:

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

Benthic sediment collection:

Benthic sediment collection should always take place after water collection. The grab and other sampling equipment should remain on the float until water sampling is completed and sterile water bags have been removed from the vessel.

Before navigating to a sampling point, clean and rinse the sediment grabber, attached line, and sediment trays in the black storage bucket and make them ready for sampling. Clean and rinse the surfaces of the boat where sampling will occur.

Once at the intended sampling point

1. Rinse the sediment grabber and trays thoroughly with saltwater before each use.
2. Mount the sediment grabber on the winch.
3. Quickly lower the sediment grabber to the ocean bottom through the winch pullie but maintain contact with the line to prevent tangles.
4. When the grabber hits bottom and tension on the line is relieved, pull upwards to activate closing mechanism. Note: consider redropping the grab 2 or 3 times from ~5m off the bottom to ensure closing mechanism is activated.
5. Once closed, raise grabber with winch at agreed upon maximum speed. Coil line from the winch directly into the black bucket to prevent tangles on next deployment. Note: watch for the 15m indicator (black tape) on the line as you coil
6. Carefully bring the full sediment grabber onto the vessel by hand and place in a clean tray.
7. Open the doors on top of the grabber to check for adequate sediment, then pour off any excess water.
8. Take 3 replicate samples in pre-labeled falcon tubes. Note: by drilling a hole in the end of the falcon tubes, air can be released, and sediment easily enters the tube as a core sample.
9. Clean and dry surfaces of the falcon tubes, close air hole with plastic wrap, then place falcon tubes into a small Ziplock bag pre-labeled with the sampling location and date.
10. Place bag into a secure storage container.
11. Repeat from step 1 at the next sample station until all sediment samples are collected. Once sampling is complete, place all sample bags into a large ziplock bag labeled with the sampling date and place that bag into the specimen freezer on arrival at the shoreside facility.

Before leaving the float:

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

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

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

Appendix B – Water Extraction Protocol

UiT
THE ARCTIC
UNIVERSITY
OF NORWAY

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

EXTRACTION PROTOCOL FOR STERIVEX FILTERS

EDNA EXTRACTION BASED ON QIAGEN DNEASY BLOOD & TISSUE KIT

IMPORTANT NOTES

- Make sure that the incubator is set to 56°C before starting the work. The equipment you are going to use for the extraction protocol should always be cleaned.
- Always shake Eppendorf tubes out of the bag, don't put your hand inside of it. Discard any excess tubes.
- Only open the bags containing Eppendorf tubes, or other tubes inside the flowhood.
- Only use pipette tips with barriers/filters and only open the boxes inside the flowhood.
- Always follow the workflow or any precautions given for the eDNA clean lab working routines.
- Always discard tips/tubes/gloves if you have the slightest suspicion about contamination (e.g. if the tip touches the table before entering a tube or buffer bottle).
- Always work with at least one extraction blanks per extraction round (i.e. 24 samples). However, if you are working with 22 samples to extract, then to complete the number to 24, you work with two blanks.
- Always start with the lowest concentration i.e. air blanks and water blanks (if any) except extraction blanks, which should be treated as any regular sample.
- However, the extraction blanks
- If extracting samples from several species/locations, sterilize everything between samples.
- Do not touch the ends of the Sterivex filters or the inside of the tube caps with hands or tweezers.
- Always be careful when you open the Eppendorf tubes not to touch the inside of the cap. Hold them in your hand and flick them open with the tip of your thumb.
- MAKE SURE YOU HAVE ENOUGH TIPS! You will mainly use 1000µl tips but also stock up on 20µl and 200µl ones. You also need Eppendorf tubes (both 1.5ml and 2.0ml), 50ml falcon tubes. Always have enough of these things before you start working.

DAY 0:

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

DAY 1:

2. Follow the descriptions in the 'Clean Lab Routines' of how to enter the labs.
3. Clean the outside of the 50ml falcon tubes containing the filters with bleach. Alternatively, if your filters are in ziplock bags, clean the outside of the bag. Do not use ethanol if there is any labelling on the tube/bag.
4. To remove excess water inside the filters, place the inlet of the filter (narrow end) in a 1.5 ml Eppendorf tube and gently slide filter and tube into the 50 ml falcon tube that contained the filter (or in a new 50 ml tube if samples were stored in ziplock bags). If more than one filter is in the tube, label a new tube for the second filter. When done with filters from one species/station, clean everything again (forceps, gloves, working surface) with bleach, MilliQ water and ethanol, before proceeding to the next species/station.
5. Centrifuge the tubes at 1500 x g for 3 minutes to remove the remaining seawater from the filters.
6. Make extraction buffer solution for adding 2.5X the recommended volume = 500µl per filter.
   - Recommended volume is 20µl Proteinase K + 180µl Buffer ATL per sample:
     - 2.5 * 20µl ProK = 50µl
     - 2.5 * 180µl ATL = 450 ul
     - Total amount of extraction buffer per sample = 500µl
     - E.g. for 20 samples: 1000µl ProK, 9000µl ATL. First, pipette the 9ml with a sterile glass pipette into a clean 50ml or 15ml tube (if you have 24 or less samples the smaller tube is enough). Then pipette 1 ml of ProK into the same tube. Close with lid and invert solution, avoiding foaming.
7. Add 500µl of the extraction solution to each filter, starting with blanks, by pushing the 1000µl tip tight **into the outlet** end of the filter and gently aspirating the solution into the filter. Take care that all the solution goes into the filter. If the filter is clogged, then aspirate from the inlet end of the filter.
8. Cap the filters with sterile caps. Make sure that its **completely sealed**.
9. MAKE SURE YOU LABEL ALL THE FILTERS CORRESPONDING TO THE TUBES, by writing the label and the replicate letter (A, B, C etc.) on the filter and **cover with tape**.
10. Place the filters in rotator and fasten them with the elastic band.
11. When done with all filters, move the rotator to the incubator oven (56°C). Make sure that the rotator is moving at 6 rpm and not hitting the oven. Check the filters after a couple of hours and leave them overnight for the 2nd day of extractions. Minimum 8-12 hours incubation.
12. Note: Always use similar incubation time for all filters within a project. Note the time for when incubation in the incubator oven started.

DAY 2

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

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

19. "Measure" the approximate volume of 2-3 samples using a pipette with NEW tips for each sample. Round the mean volume to nearest 50µl.
20. Add an equal volume of the Buffer AL as the one determined above (7.) and ensure to mix it with the pipette immediately using new tips for each sample.
21. Add an equal volume of 100% EtOH as the one determined above (7.) and ensure to mix it with the pipette immediately using new tips for each sample.
22. Vortex and spin down the samples to make sure it is mixed and liquid from the cap is removed.
23. Place the spin columns in front of the samples in the rack.
24. Transfer 630µl of the sample into corresponding spin column. Be careful not to make any bubbles but at the same time try not to leave liquid in the tip because it is precious DNA.
25. Centrifuge the columns at 15.000 x g for 2 mins.
26. Discard the collection tube with the flow-through and transfer the spin column to a new collection tube. Make sure the flow-through has not spilled back to the column when you removed it from the centrifuge.
27. Transfer the rest of the sample to the corresponding spin column. If more than 630µl, three rounds of spinning are required.
28. Centrifuge the columns at 15.000 x g for 2 mins.
29. Discard the collection tube with the flow-through and transfer the spin column to a new collection tube. Make sure the flow-through has not spilled back to the column when you removed it from the centrifuge.
30. Add 500µl Buffer AW1 (check EtOH has been added to buffer) using new tips for each tube.
31. Centrifuge at 15000 x g for 2 mins.
32. Discard the collection tube with the flow-through and transfer the spin column to a new collection tube. Make sure the flow-through has not spilled back to the column when you removed it from the centrifuge.
33. Add 500µl Buffer AW2 and centrifuge for 4 mins at 20.000 x g.
34. While centrifuging, clean flowhood, pipettes, and pens with bleach, MilliQ and ethanol.
35. TAKE GREAT CARE that no flow-through is present on the sides of the spin columns. If so, spin the columns again in a new collection tube at 20.000 x g for 2 mins. Note what samples that have been centrifuged twice.
36. Transfer the spin-columns to the corresponding Eppendorf tubes. Make sure that the lid/tap of the spin column does not touch the cap of the Eppendorf tube to avoid contamination.
37. Add 75µl of Buffer AE to each spin columns. Make sure to add the buffer at the center of the membrane without touching the membrane. Incubate for 1 min, then spin the samples at 20.000 x g for 2 mins.
38. Discard the spin columns and transfer a 12µl aliquot of the extracted DNA from each sample to a PCR plate or PCR strips. It is very important the plate/strip is labeled properly with all necessary information (if using strips, use empty pipette tip boxes as racks). Wrap aliquots in two bags before temporary storage. Place the aliquot in the fridge at 4°C if you are certain it will be processed within the next 2-3 weeks or in the aliquot freezer if longer.
39. Store the rest of the DNA as stock in the freezer located in the extraction lab. Store the 1.5ml tubes in a cryobox that you have purchased at the store and brought with you. Make sure to label the box properly. Put the cryobox in two bags before storing it and **ONLY** thaw the stock if absolutely necessary.
40. Clean flowhood and all equipment according to the guidelines.

Appendix C – Sediment Extraction Protocol

DNeasy PowerSoil Kit Protocol (Qiagen)

Modified version by Research Group for Genetics

Before you start, place Solution C1 in the heating cabinet. Should be at around 60°C.

1. Add 0.3g of sample to the PowerBead Tube provided in the kit. Remember to change gloves between each sample (not replicates).
2. Add 60µl of Solution C1.
3. Secure the PowerBead Tubes with samples to the vortex adapter horizontally.
4. Vortex at speed 3 for 2 hours.
5. Centrifuge tubes at 10,000 x g for 1 min.
6. Transfer the supernatant to a clean, new 2 ml Collection Tube provided in the kit. Try to get all the liquid, avoid pellet. Expect between 400-500µl of supernatant.
7. Add 250µl of Solution C2 and shake/vortex the tube briefly.
8. Centrifuge tubes at 10,000 x g for 1 min.
9. Avoiding the pellet, transfer up to 600µl of supernatant to a clean, new 2 ml Collection Tube.
10. Add 200µl of Solution C3 and shake/vortex the tube briefly.
11. Centrifuge tubes at 10,000 x g for 1 min.
12. Avoiding the pellet, transfer up to 700µl of supernatant to a larger 2 ml tube, bought at the store.
13. Shake to mix Solution C4 and add 1200µl (2x600µl) to the supernatant in the larger tube. Shake/vortex the tube briefly.
14. Carefully open the tubes and transfer 630µl into a MB Spin Column. Change gloves before you and centrifuge at 10,000 x g for 1 min. Discard flow-through (collect the waste). Change gloves after discarding.
15. Repeat step 14 twice.
16. Add 500µl of Solution C5. Centrifuge at 10,000 x g for 1 min.
17. Discard the flow-through (collect the waste). Change gloves after discarding.
18. Carefully place MB Spin Column into a clean, new 2 ml Collection Tube. Avoid getting any of the C5 Solution on the column. Discard the flow-through (collect the waste). Change gloves after discarding.
19. Add 100µl of Solution C6 to the centre of the white filter membrane. Do not touch the filter.
20. Centrifuge at 10,000 x g for 1 min. Keep the flow-through! Discard the MB Spin Columns.

21. Transfer 30µl of your DNA as an aliquot into a PCR-plate. This eases the downstream lab work of PCR, library preparation etc. Freeze the remaining 70µl as stock in the freezer.

Appendix D – Supplementary material: Rarefaction curves of all sample types

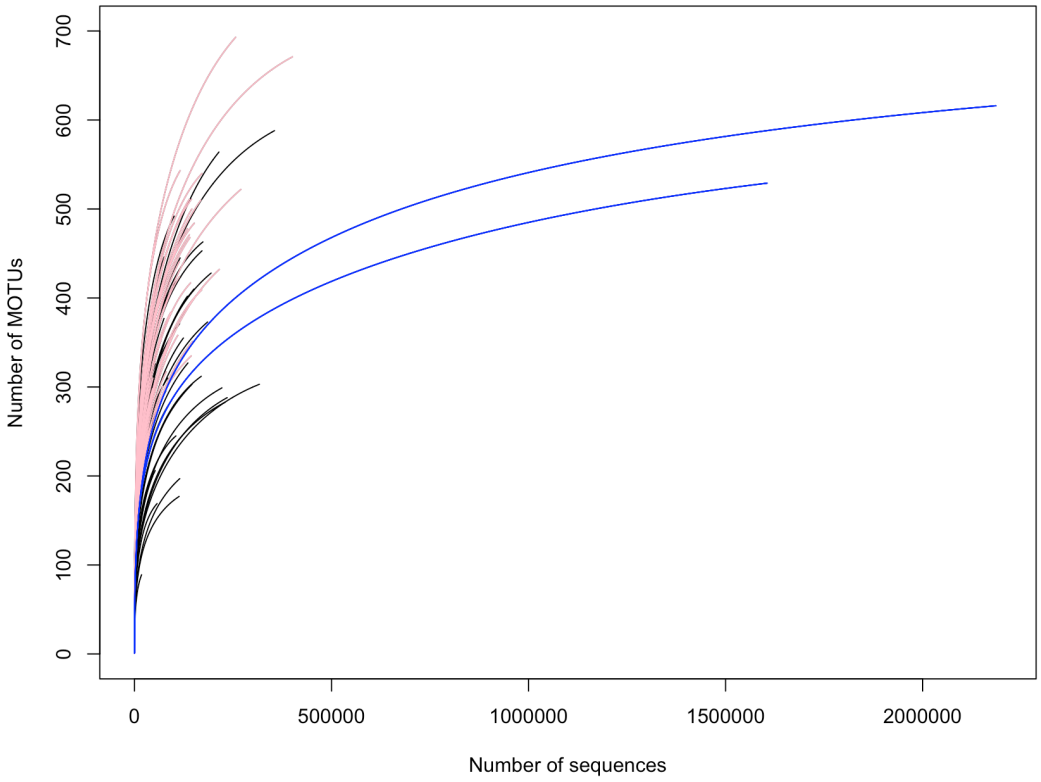


Figure 10. Rarefaction analysis on the number of MOTUs obtained for all sample types (sediment, water and feed) and their corresponding sequence identity: sediment (pink), water (black) and feed (blue).

Appendix E – Supplementary material: Distribution of dis(similarities) of fungal community composition in sediment over time and space

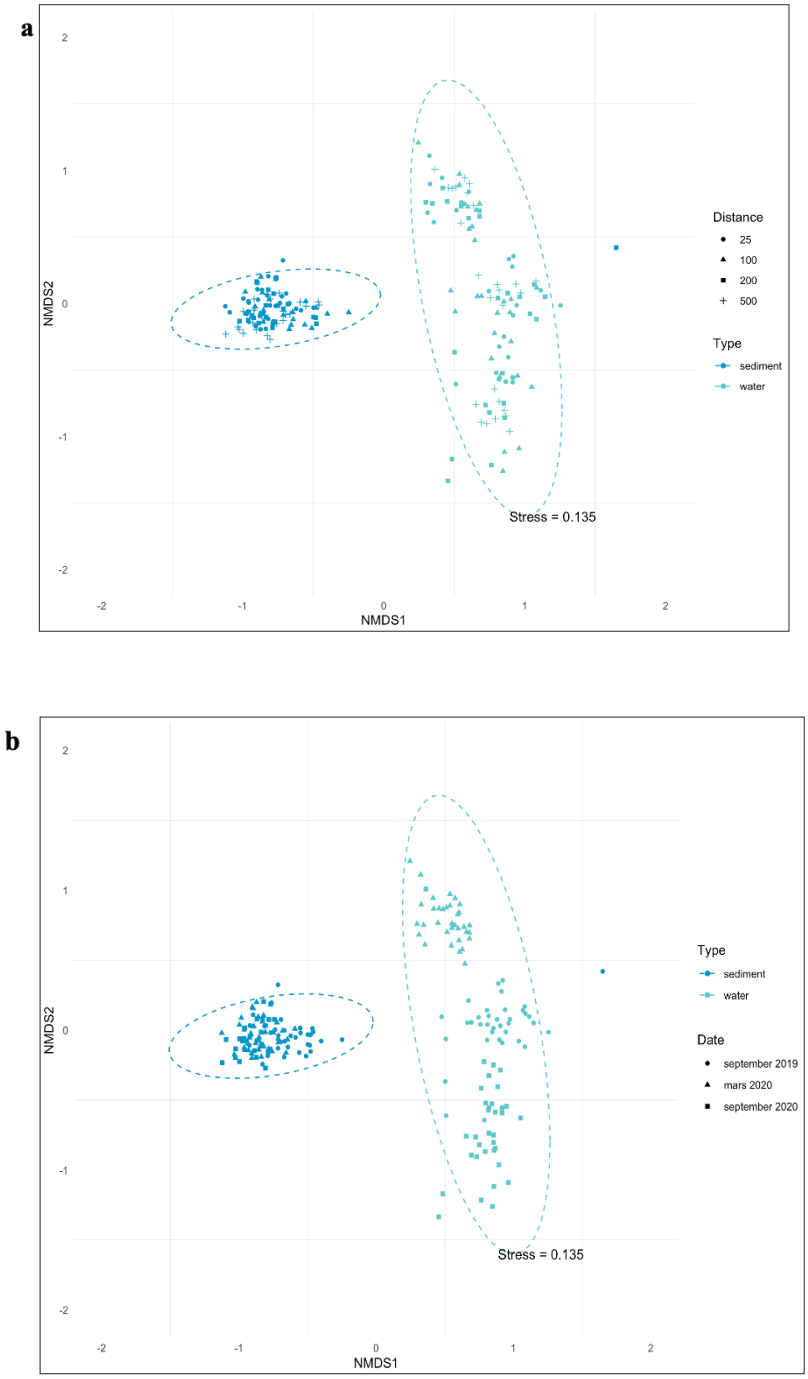


Figure 11. Nonmetric multidimensional (nMDS) ordination plot of sediment samples obtained using Bray-Curtis dissimilarity index with 95% confidence interval of the group centroid dispersion. (a) The centroids for the distances. (b) The centroids for time points.

Appendix F – Supplementary material: Taxonomic composition across different sample types and time points

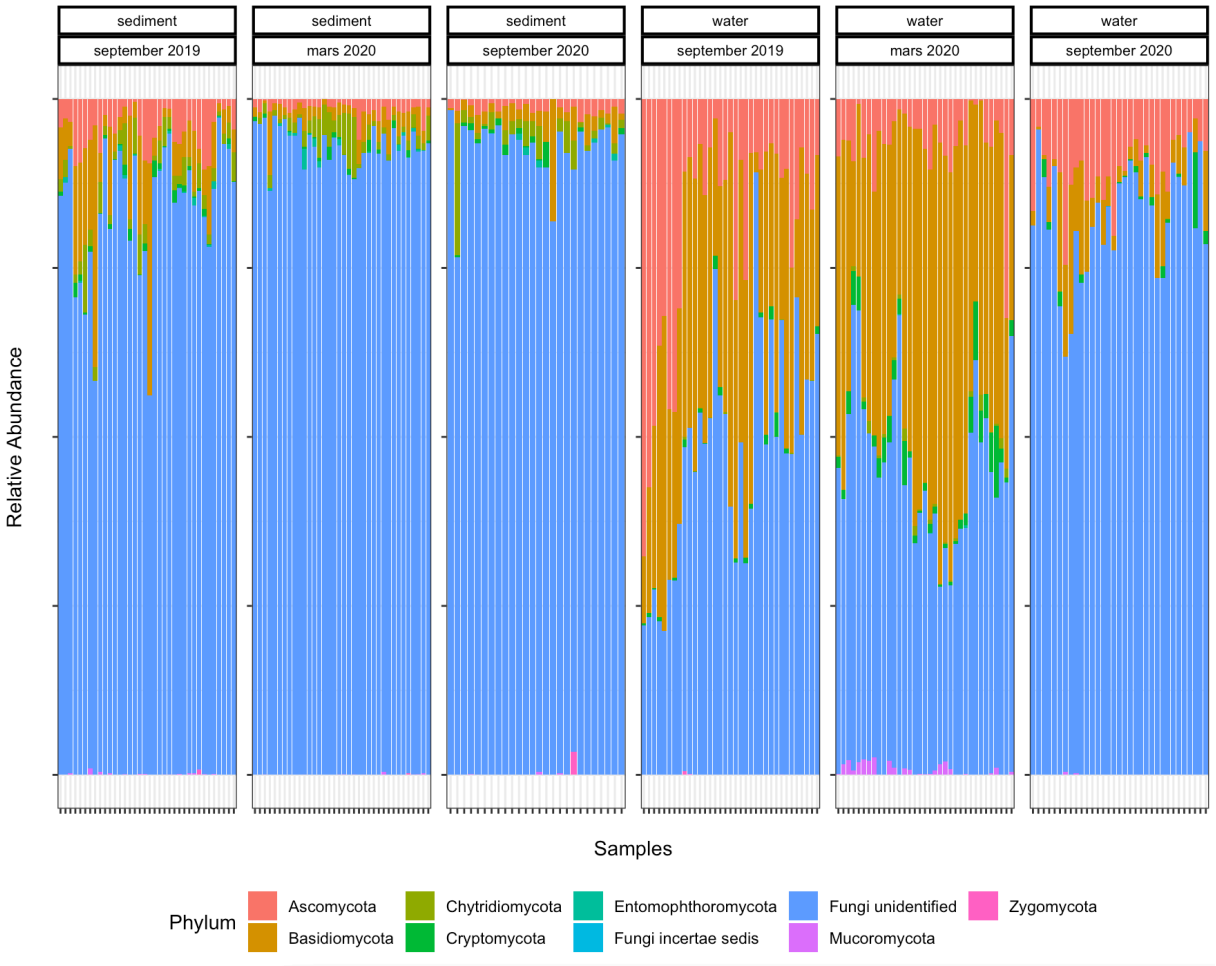


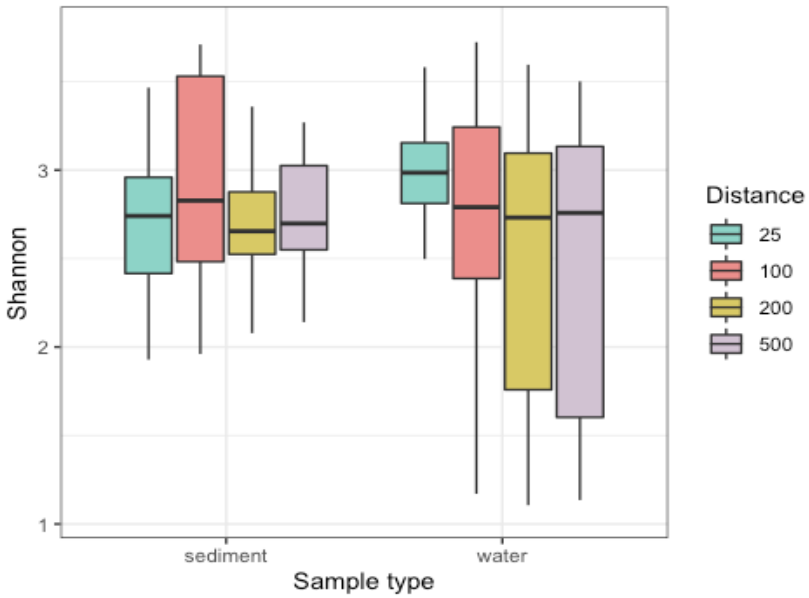
Figure 12. Taxonomic summaries of the fungal communities in different sample types and in different time points, showing the average relative abundance of each taxa at phylum level. Different colored bars represent different phyla (indicated by the key).

Appendix G – Programming script for RStudio

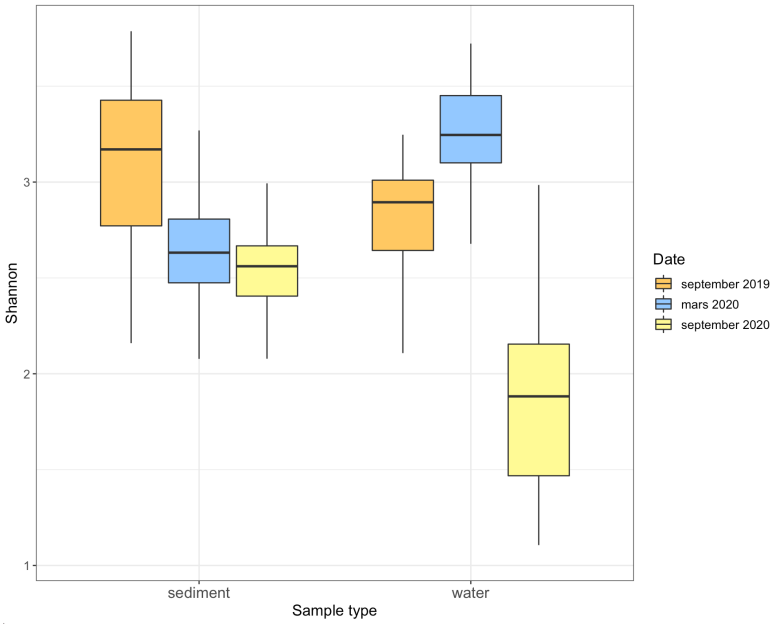
```
# Load libraries
library(dplyr)
library(ggplot2)
library(vegan)
library(RColorBrewer)
library(reshape2)

# Read data files
data <- read.csv2("SKOF_final_filtered.csv", stringsAsFactors = FALSE, row
.names = 1)
metadata<-read.csv2("metadata_filtered_fungi.csv", sep = ";", dec= ".", st
ringsAsFactor = FALSE, row.names = 1)

# Filtering considering blanks
Blanks <- data[,17:31]
Blanks <- as.data.frame(rowSums(Blanks))
colnames(Blanks) <- "Sum_Blanks"

data$Blanks <- Blanks
data$Blanks_perc <- data$Blanks/data$total_reads*100

data_Blanks <- data[data$Blanks_perc < 10, ] # discarding samples with mor
e than 10% of the toal reads in the Blank samples

Samples <- data[,32:261]
Samples_sum <- as.data.frame(colSums(Samples)) # calculate the num of read
s per MOTU in the samples
colnames(Samples_sum) <-"Sum_Samples"

data_Blanks <- data_Blanks[(1:261)]

df <- subset(data_Blanks, select = -c(196, 254))

# Look at the reads distribution, min. number of reads
otu_table_final <- df[,17:259] #without taxonomy and extras
otu_table_final_without_ex <- subset(otu_table_final, select = -c(1:15))
OTU_table <- otu_table_final_without_ex
otu_table_final_fil <- OTU_table[, colSums(OTU_table) > 1000] # Min. 1000
reads per sample
otu_table_final<- otu_table_final[rowSums(otu_table_final) > 0,] # Remove
OTUs that are present in discarded samples
otu_table <- subset(otu_table_final_fil, select = -c(9:15)) # remove NB sa
mples to avoid bias

write.csv(otu_table, "OTU_table_filt.csv")

# Create a relab table
otu_relab_filt <- apply(otu_table, 2, function(x){x/sum(x)})
otu_relab_filt [is.na(otu_relab_filt)] <- 0
otu_relab_filt <- as.data.frame(otu_relab_filt)

write.csv(otu_relab_filt, "OTU_relab_filt_fungi.csv")
```

```r
# Creating the final table
data <- read.csv2("SKOF_final_filtered.csv", stringsAsFactors = FALSE, row
.names = 1)
data_filt <- data[row.names(otu_relab_filt), c(1:16,262)] # To filter the
number of MOTUs and keep metadata info
data_filt_samples <- data[row.names(otu_relab_filt), colnames(otu_relab_fi
lt)]
data_final <- merge(data_filt, data_filt_samples, by= "row.names")
row.names(data_final) <- data_final[,1]
data_final <- data_final[ ,-1 ]

write.csv2(data_final, "SKOF_final_filtered_fungi.csv")

# Create the final relab table
otu_relab_filt_t <- as.data.frame(t(otu_relab_filt))

# Creating taxonomic df
tax<-data_final[1:13] # create taxa df
tax[which(tax$phylum_name == ""),7:8]<- tax$kingdom_name[which(tax$phylum_
name == "")]
tax[which(tax$phylum_name == "Fungi"), 8]<- "Fungi unidentified"

# Define blanks, feed, sediment and water samples
stations<-colnames(data_final[26:ncol(data_final)], ) # Without Feed sampl
es
water_stations<-colnames(data_final[124:226])
sediment_stations <-colnames(data_final[26:123])
feed_stations<-colnames(data_final[18:25])

# Calculate Shannon Index

### Water + Sediment ###
otusamples <- otu_table[,stations]
es <- vector(length = ncol(otusamples))
for (i in 1:ncol(otusamples)){
  es[i]<-rarefy(otusamples[,i],100) # richness
}
es<-as.data.frame(es)
rownames(es)<-colnames(otusamples)

es$Shannon<-diversity(t(otu_table[,stations]))

meta_point_samples <- metadata[c(9:209), 4]
meta_type_samples <- metadata[c(9:209), 2]
meta_date_samples <- metadata[c(9:209), 17]
meta_distance_samples <- metadata[c(9:209), 11]

es$Date <- meta_date_samples
es$type <- meta_type_samples
es$type <- meta_type_samples
es$unit<-paste0(substr(es$date,0,1),"-",substr(es$type,1,2))
es$Distance <- as.factor(as.numeric(meta_distance_samples))
es$Date <- factor(es$Date, levels = unique(es$Date))
```

```
# Creating boxplots
Colors2 <- colorRampPalette(brewer.pal(12,"Set3"))(5)
Colors3 <- (c("#8DD3C7","#EB8E8B","#D8C965","#D1C2D2","#FFED6F","#CC99FF")
)
Colors <- c("#8DD3C7","#EB8E8B","#D1C2D2")
Colors4 <- c("#FFCCFF","#66CCFF","#FFFF99")

ggplot(es, aes(x=type, y=Shannon, fill=Distance)) +
  geom_boxplot(outlier.shape = NA) +
  scale_fill_manual(values=Colors2)+
  theme_bw()  + ylab ('Shannon') + xlab('Sample type')
```




```
ggplot(es, aes(x=type, y=Shannon, fill=Date)) +
  geom_boxplot(outlier.shape = NA) +
  scale_fill_manual(values=Colors4)+
  theme_bw()  + ylab ('Shannon') + xlab('Sample type')
```



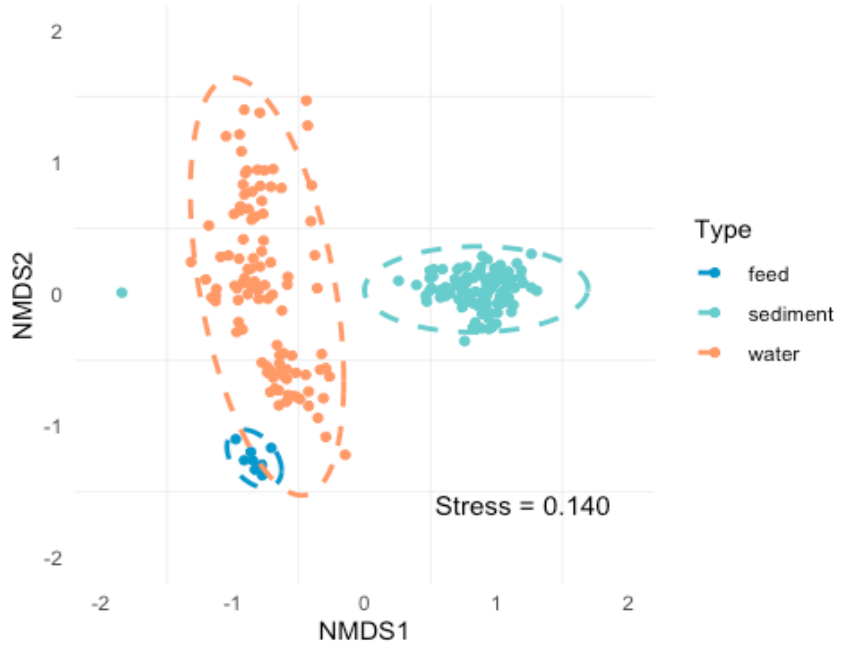
```r
# ANOVA

### WATER ###
otusamples <- otu_table[,stations]
richness <- vector(length = ncol(otusamples)) # this is the same as above
(es factor) - but made a new one only for richness and shannon separately
for (i in 1:ncol(otusamples)){
  richness[i]<-rarefy(otusamples[,i],100)
}

richness <- vector(length = ncol(otusamples))
for (i in 1:ncol(otusamples)){
  richness[i]<-rarefy(otusamples[,i],100) # richness
}

richness<-as.data.frame(richness)
rownames(richness)<-colnames(otusamples)

metadata_filt_alpha <- metadata[c(9:209), ]
metadata_filt_alpha$richness <- richness

richness$shanon <- diversity(t(otu_table[,stations]))
shanon <- as.vector(richness[,2]) # must be numeric
metadata_filt_alpha$H <- shanon

metadata_filt_alpha$Distance <- as.factor(as.numeric(metadata_filt_alpha$D
istance))

metadata_filt_water_alpha <- metadata_filt_alpha[c(99:201), ]

res.aov_water_distance <- aov(metadata_filt_water_alpha$H~metadata_filt_wa
ter_alpha$Distance, data = metadata_filt_water_alpha)
summary(res.aov_water_distance)

##                                   Df Sum Sq Mean Sq F value Pr(>F)
## metadata_filt_water_alpha$Distance  3   5.06  1.6862   3.497 0.0184 *
## Residuals                          99  47.74  0.4822
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

TukeyHSD(res.aov_water_distance)

##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = metadata_filt_water_alpha$H ~ metadata_filt_water_al
pha$Distance, data = metadata_filt_water_alpha)
##
## $`metadata_filt_water_alpha$Distance`
##                 diff        lwr         upr     p adj
## 100-25  -0.265119150 -0.7687736  0.23853534 0.5175792
## 200-25  -0.540002862 -1.0532516 -0.02675409 0.0351955
## 500-25  -0.537931205 -1.0462209 -0.02964148 0.0337212
## 200-100 -0.274883713 -0.7785382  0.22877078 0.4861429
```

```
## 500-100 -0.272812055 -0.7714121  0.22578798 0.4839278
## 500-200  0.002071658 -0.5062181  0.51036139 0.9999996

res.aov_water_date <- aov(metadata_filt_water_alpha$H~metadata_filt_water_
alpha$date, data = metadata_filt_water_alpha)
summary(res.aov_water_date)

##                                Df Sum Sq Mean Sq F value Pr(>F)
## metadata_filt_water_alpha$date   2  32.93  16.467   82.92 <2e-16 ***
## Residuals                      100  19.86   0.199
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

TukeyHSD(res.aov_water_date)

##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = metadata_filt_water_alpha$H ~ metadata_filt_water_al
pha$date, data = metadata_filt_water_alpha)
##
## $`metadata_filt_water_alpha$date`
##                            diff        lwr        upr   p adj
## 12.sep.19-11.mar.20 -0.5269752 -0.7804247 -0.2735258 9.1e-06
## 29.sep.20-11.mar.20 -1.3823407 -1.6396016 -1.1250798 0.0e+00
## 29.sep.20-12.sep.19 -0.8553654 -1.1126264 -0.5981045 0.0e+00

### SEDIMENT ###
metadata_filt_sediment_alpha <- metadata[c(9:106), ]

metadata_filt_sediment_alpha$richness <- es[c(1:98),1]
metadata_filt_sediment_alpha$H <- es[c(1:98),2]
alpha_distance_sediment <- metadata[c(9:106), ]

res.aov_sediment_distance <- aov(metadata_filt_sediment_alpha$H~as.factor(
metadata_filt_sediment_alpha$Distance), data = metadata_filt_sediment_alph
a)
summary(res.aov_sediment_distance)

##                                               Df Sum Sq Mean Sq F value
## as.factor(metadata_filt_sediment_alpha$Distance) 3  0.984  0.3281   1.662
## Residuals                                       94 18.557  0.1974
##                                                 Pr(>F)
## as.factor(metadata_filt_sediment_alpha$Distance)   0.18
## Residuals

TukeyHSD(res.aov_sediment_distance)

##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = metadata_filt_sediment_alpha$H ~ as.factor(metadata_
filt_sediment_alpha$Distance), data = metadata_filt_sediment_alpha)
##
## $`as.factor(metadata_filt_sediment_alpha$Distance)`
##                 diff         lwr        upr     p adj
```

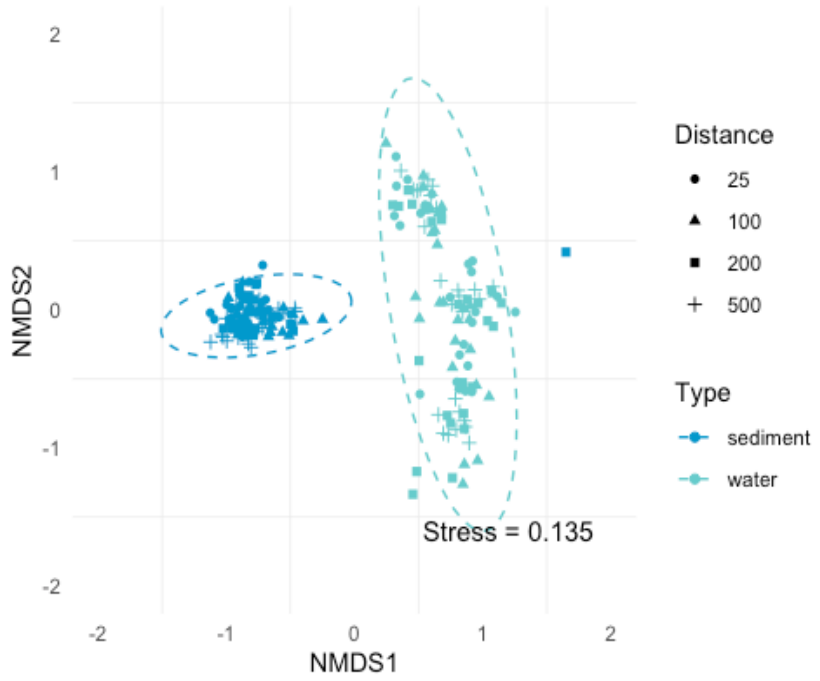
```
## 100-25   0.262060365 -0.09156587 0.61568660 0.2192463
## 200-25  -0.005890559 -0.32218348 0.31040236 0.9999582
## 500-25   0.106580571 -0.21273915 0.42590029 0.8187805
## 200-100 -0.267950925 -0.62157716 0.08567531 0.2021652
## 500-100 -0.155479794 -0.51181585 0.20085626 0.6650524
## 500-200  0.112471131 -0.20684859 0.43179085 0.7935511

res.aov_sediment_date <- aov(metadata_filt_sediment_alpha$H~metadata_filt_
sediment_alpha$date, data = metadata_filt_sediment_alpha)
summary(res.aov_sediment_date)

##                                 Df Sum Sq Mean Sq F value   Pr(>F)

## metadata_filt_sediment_alpha$date  2  4.692  2.3460   15.01 2.17e-06 **
*
## Residuals                         95 14.849  0.1563

## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

TukeyHSD(res.aov_sediment_date)

##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = metadata_filt_sediment_alpha$H ~ metadata_filt_sedim
ent_alpha$date, data = metadata_filt_sediment_alpha)
##
## $`metadata_filt_sediment_alpha$date`
##                             diff        lwr        upr     p adj
## 12.sep.19-11.mar.20  0.41345384  0.1915774  0.6353303 0.0000725
## 29.sep.20-11.mar.20 -0.08403798 -0.3263112  0.1582353 0.6879425
## 29.sep.20-12.sep.19 -0.49749182 -0.7397651 -0.2552186 0.0000122

# Prepare the data for ordination plot

### RUN NMDS ###
OTU_table_tax <- merge(tax, otu_relab_filt, by="row.names")
df <- OTU_table_tax[!(is.na(OTU_table_tax$phylum_name) | OTU_table_tax$phy
lum_name==""), ]

row.names(df) <- df[,1]
df <- df [,-1]

df_table <- df[,14:ncol(df)]
df_relab <- apply(df_table,2, function(x){x/sum(x)})
df_relab [is.na(df_relab)] <- 0
df_relab <- as.data.frame(df_relab)
df_relab_t <- as.data.frame(t(df_relab))
samples <- df_relab_t[c(9:209), ]
merge_table_metadata <- merge(metadata, df_relab_t, by = "row.names")

scores <- merge_table_metadata[,c(20:ncol(merge_table_metadata))]

score_sqrt <- sqrt(scores)
```

```r
MDS_scores_test <- metaMDS(score_sqrt, trymax = 500, try = 200, k=2)

stress <- MDS_scores_test$stress

# Convert scores to a data.frame
MDS_points <- data.frame(MDS_scores_test$points)

# Define groups
Date <- merge_table_metadata$Date_new
Time <- merge_table_metadata$Time
Point <- merge_table_metadata$point
Type <- merge_table_metadata$sample_type
Distance <- merge_table_metadata$Distance

MDS_points <- cbind(MDS_points, Date, Point, Time, Type, Distance)

# Plot NMDS
p<-as.data.frame(MDS_points[,1:2])
p$Time<-as.factor(merge_table_metadata$Time)
p$Distance<-as.factor(as.numeric(merge_table_metadata$Distance))
p$Type <- as.factor(merge_table_metadata$sample_type)
p$date <- as.factor(merge_table_metadata$Date_new)

Colors2 <- c("#0099CC", "#66CCCC", "#FF9966")

ggplot(p, aes(x=MDS1, y=MDS2, col=Type)) +
  geom_point ()+
  scale_color_manual(values=Colors2)+
  stat_ellipse(level = 0.75) +
  xlim(-2, 2)+
  ylim(-2, 2)+
  theme_minimal() + theme(panel.grid.major = element_blank()) +
  annotate(geom="text", cex=4, x=1.2, y=-1.6, label=paste0("Stress = ", le
vel = 0.137))

ggplot(p, aes(x=MDS1, y=MDS2, col=Type)) +
  geom_point()+
  scale_color_manual(values=Colors2)+
  stat_ellipse(level = 0.75) +
  xlim(-2, 2)+
  ylim(-2, 2)+
  theme_minimal() + theme(panel.grid.major = element_blank()) +
  annotate(geom="text", cex=4, x=1.2, y=-1.6, label=paste0("Stress = ", le
vel = 0.137))
```

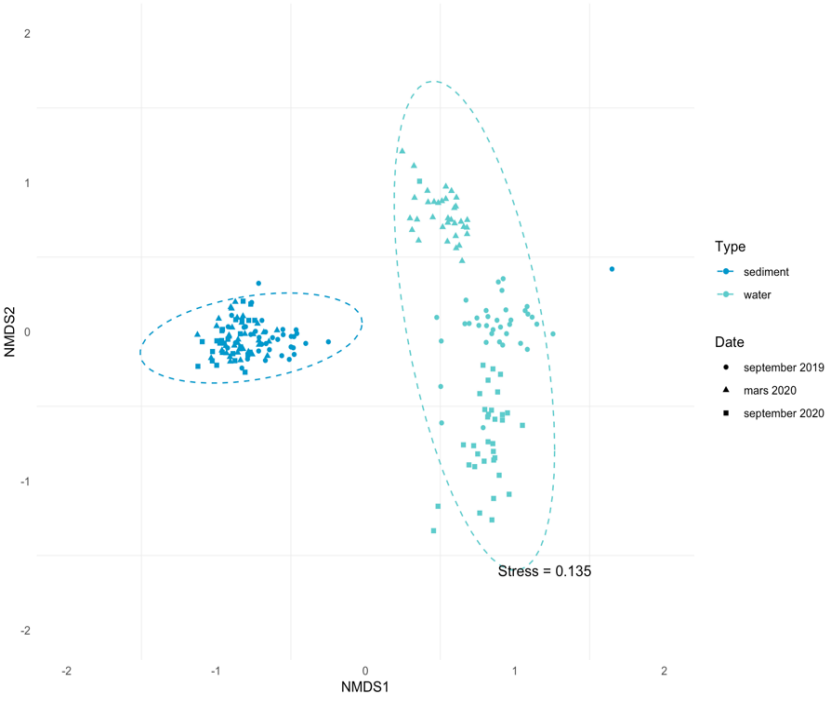
```
# Calculate ellipse

veganCovEllipse<-function (cov, center = c(0, 0), scale = 1, npoints = 100
)
{
  theta <- (0:npoints) * 2 * pi/npoints
  Circle <- cbind(cos(theta), sin(theta))
  t(center + scale * t(Circle %*% chol(cov)))
}

plot.new()
ord<-ordiellipse(MDS_scores_test, as.factor(merge_table_metadata$sample_ty
pe), display = "sites", kind = "sd", conf = 0.95, label=T)

ellipse<-data.frame()
for (g in levels(p$Type)){
  if(g!="" && (g %in% names(ord))){
    ellipse<-rbind(ellipse, cbind(as.data.frame(with(p[p$Type==g,],
                                                     veganCovEllipse(ord[[
g]]$cov, ord[[g]]$center, ord[[g]]$scale))), Type=g))
  }
}

# Plot NMDS with ellipses
ggplot(p, aes(x=MDS1, y=MDS2, col=Type)) +
  geom_point () + geom_path(data=ellipse, aes(x=NMDS1, y=NMDS2), size=1, l
inetype=2) +
  scale_color_manual(values=Colors2)+
  xlim(-2, 2)+
  ylim(-2, 2)+
  theme_minimal() + theme(panel.grid.major = element_blank()) + labs(x = "
NMDS1", y = "NMDS2")+
  annotate(geom="text", cex=4, x=1.2, y=-1.6, label=paste0("Stress = ", le
vel = 0.140, digits = 0))
```



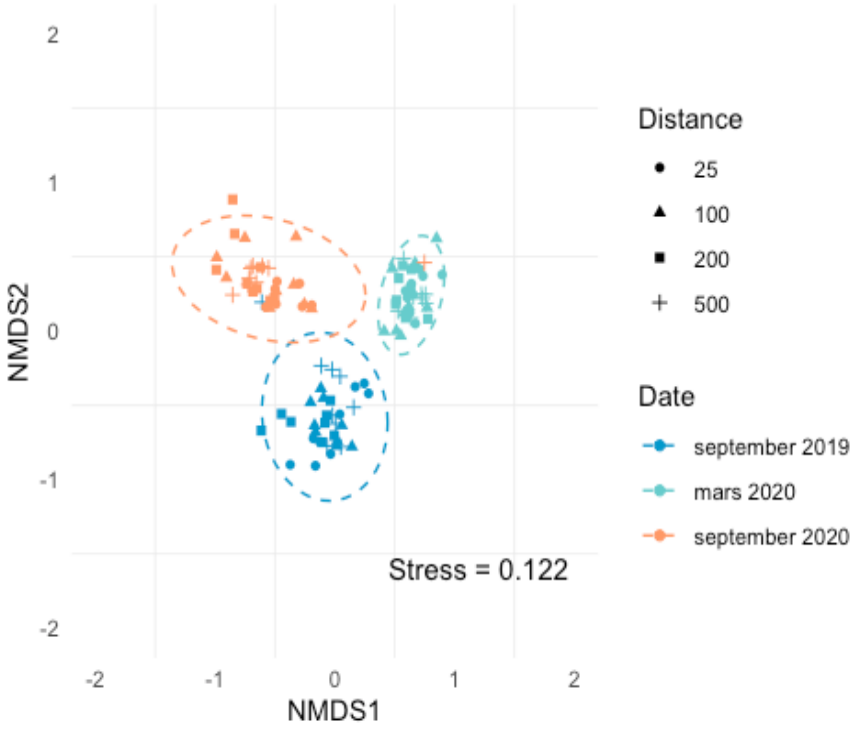
```r
# NMDS without feed

metadata_samples <- metadata[c(9:209), ]

merge_table_metadata_exfeed <- merge(metadata_samples, samples, by = "row.
names")

scores_nofeed <- merge_table_metadata_exfeed[,c(20:ncol(merge_table_metada
ta_exfeed))]

score_nofeed_sqrt <- sqrt(scores_nofeed)

MDS_nofeed_scores <- metaMDS(score_nofeed_sqrt, trymax = 500, try = 200, k
=2)

stress_nofeed <- MDS_nofeed_scores$stress

# Convert scores to a data.frame
MDS_nofeed_points <- data.frame(MDS_nofeed_scores$points)

# Define groups
Date_samples <- merge_table_metadata_exfeed$Date_new
Time_samples <- merge_table_metadata_exfeed$Time
Point_samples <- merge_table_metadata_exfeed$point
Type_samples <- merge_table_metadata_exfeed$sample_type
Distance_samples <- merge_table_metadata_exfeed$Distance

MDS_nofeed_points <- cbind(MDS_nofeed_points, Date_samples, Point_samples,
 Time_samples, Type_samples, Distance_samples)

# Plot NMDS
p_samples<-as.data.frame(MDS_nofeed_points[,1:2])
p_samples$Time<-as.factor(merge_table_metadata_exfeed$Time)
p_samples$Distance<-as.factor(as.numeric(merge_table_metadata_exfeed$Dista
nce))
p_samples$Type <- as.factor(merge_table_metadata_exfeed$sample_type)
p_samples$Date <- as.factor(merge_table_metadata_exfeed$Date_new)
p_samples$Date <- factor(p_samples$Date, levels = unique(p_samples$Date))

ggplot(p_samples, aes(x=MDS1, y=MDS2, col=Type)) +
  geom_point(aes(shape=Distance))+
  scale_color_manual(values=Colors2)+
  stat_ellipse(type = "norm", linetype = 2) +
  xlim(-2, 2)+
  ylim(-2, 2)+
  theme_minimal() + theme_minimal() + theme(panel.grid.major = element_bla
nk()) + labs(x = "NMDS1", y = "NMDS2") +
  annotate(geom="text", cex=4, x=1.2, y=-1.6, label=paste0("Stress = ", le
vel = 0.135))
```




```
ggplot(p_samples, aes(x=MDS1, y=MDS2, col=Type)) +
  geom_point(aes(shape=Date))+
  scale_color_manual(values=Colors2)+
  stat_ellipse(type = "norm", linetype = 2) +
  xlim(-2, 2)+
  ylim(-2, 2)+
  theme_minimal() + theme_minimal() + theme(panel.grid.major = element_bla
nk()) + labs(x = "NMDS1", y = "NMDS2") +
  annotate(geom="text", cex=4, x=1.2, y=-1.6, label=paste0("Stress = ", le
vel = 0.135))
```



```r
# plot NMDS only for water

### WATER ###

water_samples <- df_relab_t[c(107:209), ]

metadata_water <- metadata[c(107:209), ]

merge_table_water <- merge(metadata_water, water_samples, by = "row.names"
)

scores_water <- merge_table_water[,c(20:ncol(merge_table_water))]

score_water_sqrt <- sqrt(scores_water)

score_water_4sqrt <- sqrt(sqrt(scores_water))

MDS_water_scores <- metaMDS(score_water_sqrt, trymax = 500, try = 200)

stress_water <- MDS_water_scores$stress

# Convert scores to a data.frame
MDS_points_water <- data.frame(MDS_water_scores$points)

# Define groups
Date_water <- merge_table_water$Date_new
Time_water <- merge_table_water$Time
Point_water <- merge_table_water$point
Type_water <- merge_table_water$sample_type
Distance_water <- merge_table_water$Distance

MDS_points_water <- cbind(MDS_points_water, Date_water, Time_water, Type_w
ater, Distance_water)

p_water <- as.data.frame(MDS_points_water[,1:2])
p_water$Date <- as.factor (merge_table_water$Date_new)
p_water$Distance <- as.factor(as.numeric(merge_table_water$Distance))
p_water$Date <- factor(p_water$Date, levels = unique(p_water$Date))

colors3 <- c("#0099CC", "#006666", "#00CC99")

ggplot(p_water, aes(x=MDS1, y=MDS2, col=Date)) +
  geom_point(aes(shape=Distance))+
  scale_color_manual(values=Colors2)+
  stat_ellipse(type = "norm", linetype = 2) +
  xlim(-2, 2)+
  ylim(-2, 2)+
  theme_minimal() + theme_minimal() + theme(panel.grid.major = element_bla
nk()) + labs(x = "NMDS1", y = "NMDS2") +
  annotate(geom="text", cex=4, x=1.2, y=-1.6, label=paste0("Stress = ", le
vel = 0.122 ))
```




```
# PERMANOVA

# Sample type
adonis(df_relab_t~Type, data = metadata, perm=1000, method = "bray")

##
## Call:
## adonis(formula = df_relab_t ~ Type, data = metadata, permutations = 100
0,      method = "bray")
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##            Df SumsOfSqs MeanSqs F.Model      R2   Pr(>F)
## Type        2    27.345  13.673   67.34 0.39533 0.000999 ***
## Residuals 206    41.826   0.203         0.60467
## Total     208    69.171                 1.00000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

BC <- vegdist(df_relab_t)
disp<-betadisper(BC,metadata$sample_type)
permutest(disp)

##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 999
##
## Response: Distances
```

```
##            Df Sum Sq Mean Sq      F N.Perm Pr(>F)
## Groups      2 2.7327 1.36637 186.25    999  0.001 ***
## Residuals 206 1.5113 0.00734
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
### WATER ###
codisframe <- data.frame(id = row.names(metadata),
                         WATER = c(metadata$sample_type == "water"),
                         SEDIMENT = c(metadata$sample_type == "sediment"))
BCwater <- vegdist(df_relab_t[codisframe$WATER,])

# Date
water_relab <- df_relab_t[107:209]
water_relab_filt <- as.data.frame(t(water_relab))

adonis(water_relab_filt ~ date, data = metadata_filt_water_alpha, perm = 1
000, method = "bray")
```

```
##
## Call:
## adonis(formula = water_relab_filt ~ date, data = metadata_filt_water_al
pha,      permutations = 1000, method = "bray")
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##            Df SumsOfSqs MeanSqs F.Model      R2   Pr(>F)
## date        2     5.324 2.66183   6.457 0.11437 0.000999 ***
## Residuals 100    41.224 0.41224         0.88563
## Total     102    46.548                 1.00000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
disp_water_date <- betadisper(BCwater, metadata$date[codisframe$WATER])
permutest(disp_water_date)
```

```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 999
##
## Response: Distances
##            Df  Sum Sq Mean Sq      F N.Perm Pr(>F)
## Groups      2 0.71851 0.35926 39.066    999  0.001 ***
## Residuals 100 0.91962 0.00920
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
# Distance
water_relab <- df_relab_t[107:209]
water_relab_filt <- as.data.frame(t(water_relab))
```

```r
adonis(water_relab_filt ~ Distance, data = metadata_filt_water_alpha, perm
 = 1000, method = "bray")
```

```
##
## Call:
## adonis(formula = water_relab_filt ~ Distance, data = metadata_filt_wate
r_alpha,      permutations = 1000, method = "bray")
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##            Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
## Distance    3     1.246 0.41547 0.90796 0.02678 0.6813
## Residuals  99    45.301 0.45759         0.97322
## Total     102    46.548                 1.00000
```

```r
disp_water_distance <- betadisper(BCwater, metadata_filt_water_alpha$Dista
nce)
permutest(disp_water_distance)
```

```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 999
##
## Response: Distances
##           Df   Sum Sq   Mean Sq      F N.Perm Pr(>F)
## Groups     3 0.011472 0.0038241 1.2724    999  0.297
## Residuals 99 0.297537 0.0030054
```

```r
### SEDIMENT ###
BCsediment <- vegdist(df_relab_t[codisframe$SEDIMENT,])

sediment_relab <- df_relab_t[9:106]
sediment_relab_filt <- as.data.frame(t(sediment_relab))

# Date
adonis(sediment_relab_filt ~ date, data = metadata[codisframe$SEDIMENT,],
perm = 1000, method = "bray")
```

```
##
## Call:
## adonis(formula = sediment_relab_filt ~ date, data = metadata[codisframe
$SEDIMENT,      ], permutations = 1000, method = "bray")
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##           Df SumsOfSqs MeanSqs F.Model      R2   Pr(>F)
## date       2     4.535 2.26757  5.3269 0.10084 0.000999 ***
## Residuals 95    40.440 0.42568         0.89916
```

```
## Total     97    44.975                 1.00000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
disp_sediment_date <- betadisper(BCsediment, metadata$date[codisframe$SEDI
MENT])
permutest(disp_sediment_date)
```

```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 999
##
## Response: Distances
##           Df  Sum Sq  Mean Sq      F N.Perm Pr(>F)
## Groups     2 0.12328 0.061639 6.9428    999  0.002 **
## Residuals 95 0.84342 0.008878
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

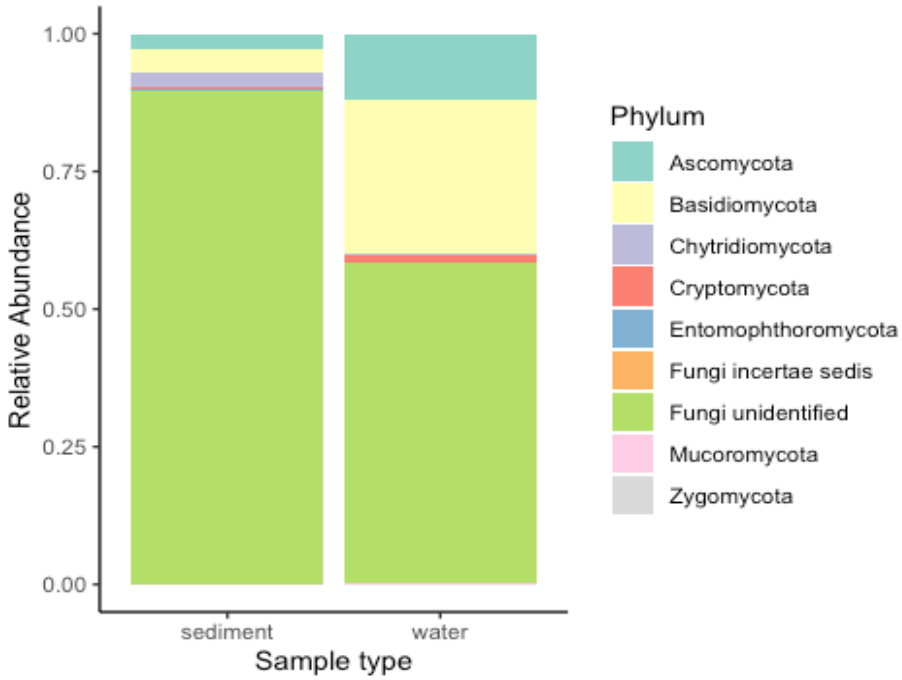
```
# Distance
adonis(sediment_relab_filt ~ Distance, data = metadata_filt_sediment_alpha
, perm = 1000, method = "bray")
```

```
##
## Call:
## adonis(formula = sediment_relab_filt ~ Distance, data = metadata_filt_s
ediment_alpha,      permutations = 1000, method = "bray")
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##           Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
## Distance   1     0.542 0.54224  1.1715 0.01206 0.2138
## Residuals 96    44.433 0.46284         0.98794
## Total     97    44.975                 1.00000
```

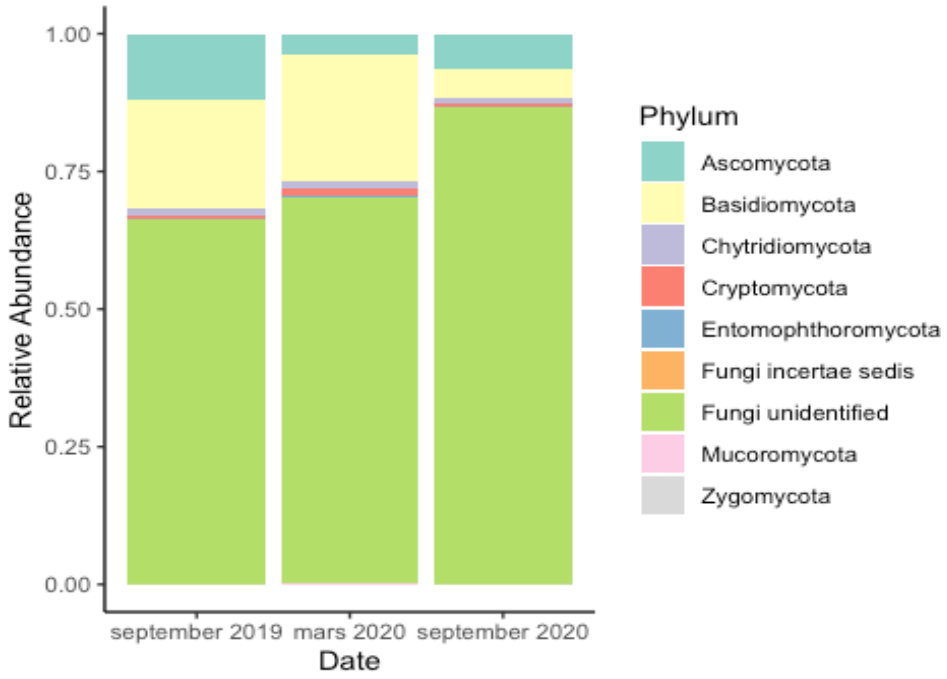
```
disp_sediment_distance <- betadisper(BCwater, metadata_filt_water_alpha$Di
stance)
permutest(disp_sediment_distance)
```

```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 999
##
## Response: Distances
##           Df   Sum Sq   Mean Sq      F N.Perm Pr(>F)
## Groups     3 0.011472 0.0038241 1.2724    999  0.304
## Residuals 99 0.297537 0.0030054
```

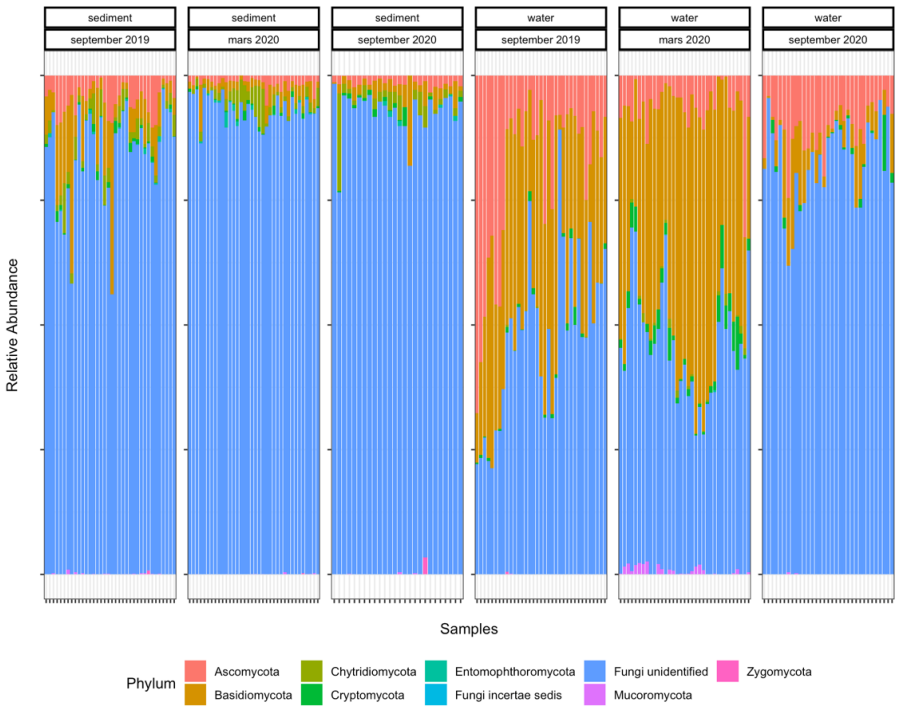
```r
# Composition barplots

### SAMPLES ###

# Now filter out the blank rows in phylum
df_relab_tax <- merge(tax, df_relab, by="row.names")

aggregate_phylum <- aggregate(df_relab_tax[,23:ncol(df_relab_tax)], by=lis
t(df_relab_tax$phylum_name),FUN=sum)

row.names(aggregate_phylum) <- aggregate_phylum[,1]
aggregate_phylum <- aggregate_phylum[,-1]

aggregate_phylum_t <- as.data.frame(t(aggregate_phylum))
aggregate_phylum_t$Sample <- row.names(aggregate_phylum_t)

melt <- melt(aggregate_phylum_t, id.vars = "Sample")
colnames(melt) <- c("Sample", "Phylum", "Rel.Ab.")

merge_table_aggregate <- merge(metadata, aggregate_phylum_t, by="row.names
")

merge_table_aggregate <- merge(metadata, aggregate_phylum_t, by="row.names
")

# Type
aggregate_type <- aggregate(merge_table_aggregate[,19:ncol(merge_table_agg
regate)], by=list(merge_table_aggregate$sample_type), FUN = mean)

melt2 <- melt(aggregate_type)

## Using Group.1 as id variables

colnames(melt2) <-c("type","Phylum","value")

Colors4 <- colorRampPalette(brewer.pal(9,"Set3"))(9)

ggplot(melt2, aes(x=type, y=value, fill=Phylum))+
  geom_bar(stat="identity") +
  scale_fill_manual(values = Colors4)+
  xlab("Sample type")+
  ylab("Relative Abundance")+ theme_classic()
```



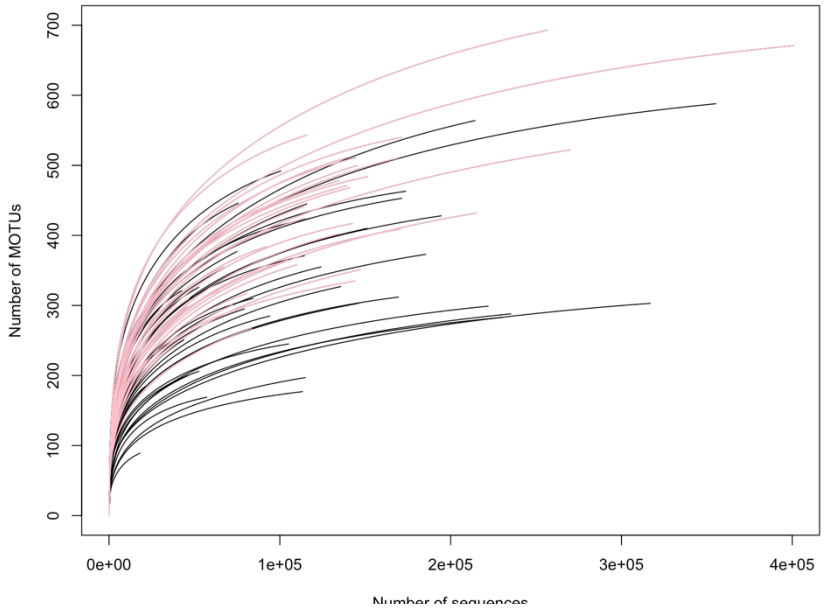

```
# Date
aggregate_date <- aggregate(merge_table_aggregate[,19:ncol(merge_table_agg
regate)], by=list(merge_table_aggregate$Date_new), FUN = mean)

melt3 <- melt(aggregate_date)

colnames(melt3) <-c("date","Phylum","value")

melt3$date <- factor(melt3$date, levels = c("september 2019", "mars 2020",
 "september 2020"))

Colors5 <- colorRampPalette(brewer.pal(9,"Set3"))(9)

ggplot(melt3, aes(x=date, y=value, fill=Phylum))+
  geom_bar(stat="identity") +
  scale_fill_manual(values = Colors5)+
  xlab("Date")+
  ylab("Relative Abundance")+ theme_classic()
```




```
# Date + sample type
aggregate_date <- merge_table_aggregate[,c(1,3,18,19,20,21,22,23,24,25,26,
27:ncol(merge_table_aggregate))] #To select Sample, Date and Type

melt <- melt(aggregate_date, id.vars = c("Row.names","Date_new","sample_ty
pe"))
colnames(melt) <-c("Sample","date","type","Phylum","value")
melt$Sample <- as.factor(melt$Sample)

melt$date <- factor(melt$date, levels = c("september 2019","mars 2020","se
ptember 2020"))

p <- ggplot(melt, aes(x=Sample, y=value, fill=Phylum)) +
  #scale_fill_manual(values=col3)+
  geom_bar(stat="identity") +
  xlab("\nSamples") +
  ylab("Relative Abundance\n") +
  theme_bw() +
  theme(axis.text=element_blank())+
  theme (legend.position="bottom")
p

p + facet_wrap(type~ date, scales = "free", ncol = 6)+
  theme(strip.background = element_rect(colour="black", fill="white",
                                        size=1.5, linetype="solid"))+
  theme(strip.text.x = element_text(size=8, color="black"))
```




```
# RARECURVE

### Without feed samples ###

otu_table_rare_exfeed <- otu_rare_samples[3:71] #Merged replicates

otu_table_rare_exfeed_t = (t(otu_table_rare_exfeed))

out <- rarecurve(otu_table_rare_exfeed_t, step = 500, xlab = "Number of se
quences", ylab = "Number of MOTUs", label= FALSE, col=, lty = 1, xlim=c(0,
400000), ylim=c(0,700))

Nmax <- sapply(out, function(x) max(attr (x, "Subsample")))

Smax <- sapply(out, max)

col <- c("pink")

set.seed(3)

grp <-factor(sample(seq_along(col), nrow(otu_table_rare_exfeed_t), replace
 = TRUE))

cols <-col[grp[1:33]]

raremax <-min(rowSums(otu_table_rare_exfeed_t)) #18048

for (i in seq_along (out)) {

  N <- attr(out[[i]], "Subsample")

  lines(N, out[[i]], col = cols[i])

}
```




```
### With feed samples ###

otu_rare_samples_t = (t(otu_rare_samples))

out_all <- rarecurve(otu_rare_samples_t, step = 500, xlab = "Number of seq
uences", ylab = "Number of MOTUs", label=FALSE, col=, lty = 1, xlim=c(0,22
00000), ylim=c(0,700))

col <- c("blue")

col <- c("pink")

set.seed(3)

grp2 <- factor(sample(seq_along (col), nrow(otu_rare_samples_t), replace =
 TRUE))

cols <- col[grp2[1:33]]

cols1 <- col[grp2[1:2]]

raremax2 <- min(rowSums(otu_rare_samples_t))

# Sediment

col <-c("pink")

cols <-col[grp2[1:33]]


for (i in seq_along (out_all)) {

  N <- attr (out_all[[i]], "Subsample")

  lines(N, out_all[[i]], col = cols[i])

}
```

```r
# Feed
col <- c("blue")
cols1 <- col[grp2[1:2]]
for (i in seq_along(out_all)) {
  N <- attr (out_all[[i]], "Subsample")
  lines(N, out_all[[i]], col = cols1[i])
}
```